Stem Cell Biology and Regenerative Medicine

Nagwa El-Badri *Editor*

Advances in Stem Cell Therapy

Bench to Bedside

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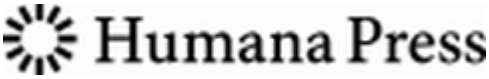

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Foreword

It is a pleasure to write a foreword to this book on stem cell therapy, edited by Nagwa El-Badri, at a very opportune time when cautious clinical trials are under way by responsible clinicians. Unfortunately, hyped-up claims for stem cells of miraculous cures are recorded almost daily by the media. This undermines serious science, since it raises hopes and expectations in vulnerable people, fearful and often in pain. They may spend their whole life savings in a useless treatment that could even harm them.

The chapters in this book span the whole field of current stem cell biology, both experimental and clinical. There is a chapter on bone marrow transplantation where hematopoietic stem cell transplants have been used for many years with increasing success in the treatment of leukemia and lymphoid malignancies. The results have been particularly good overall in the treatment of children. It is against this background that more recent work in a variety of different stem cell procedures has been pursued, especially since the dramatic experiments were described of nuclear transfer with cloning of Dolly the sheep and the potential of embryonic stem cells. Recently, induced pluripotent stem cells (iPSCs), pioneered by Yamanaka in Kyoto, showed that skin and fibrous tissue cells can be dedifferentiated into cells that have many characteristics of embryonic stem cells and have the theoretical advantage that the iPSCs could be used as autografts and avoid the ethical worries of destroying a fertilized egg.

Adult stem cells have been identified in most tissues, and the most available source is adipose tissue. Fat stem cells will differentiate in culture and have been used often uncritically in the treatment of more than 20 diseases, varying from osteoarthritis to spinal cord injury. Unfortunately, few studies have provided credible evidence of efficacy, good results often being recorded by mere anecdote. Concerns would apply to the use of adult stem cells in neurological diseases, and unfortunately some published clinical trials were later

retracted. Experiments were reported in a mouse model of Alzheimer's disease treated with stem cells in which the mice appeared to develop memory improvement after treatment, learning how to navigate a water maze.

Umbilical cord blood banks have been established in many countries and provide human leukocyte antigen (HLA) typed blood with useful properties. Cord blood can be used to generate the iPSCs. Cells from the dental pulp grow well in culture and differentiate into a variety of types, especially neural cells. There have been great expectations on the possibility of using stem cells in the treatment of liver disease in view of the ability of the liver to regenerate after damage. It would seem likely that some of the functions of isolated liver cells could be of therapeutic value, but we are still a long way from being able to grow the full structural anatomy of a liver in the laboratory or in vivo from stem cells. Stem cell treatment for vascular disorders and autoimmune disease has been disappointing, although anecdotal reports of good results have been published. Bone marrow stem cells have also been differentiated into insulin-producing cells, which proliferate after transplantation into immune-incompetent diabetic mice.

There is considerable hope that micro- and nanotechnologies will be used in stem cell therapy. These new techniques are still at an early stage, but they do open the possibility of novel approaches. This book describes a number of different types of scaffold on which to seed stem cells, including a bio-scaffold produced from amnion.

This volume is an important contribution to a rapidly expanding field that clearly has great therapeutic promise, especially in the context of bone marrow transplantation. Bone marrow is special in that transplantation of the stem cells into the bloodstream home naturally to bone marrow niches, where they assume a normal physiological role. It is important that studies with stem cells be done in a scientific manner with appropriate controls and unbiased, long-term assessment.

Roy Calne

Preface

Over the past century, the human life span has almost doubled owing to technological advancements. Thanks to modern medications, sterile techniques, antibiotics, and preventive health care, people are living longer, and critical health issues have changed from infectious epidemics to diseases associated with aging. Today, chronic illnesses top the list of the causes of morbidity and mortality in almost every country: cardiovascular diseases, diabetes, cancer, and neurodegenerative disorders. This paradigm shift has created new challenges and calls for new treatments. Symptomatic therapies and temporary relief for chronic illness can no longer accommodate the expanding needs of an aging population and the extended life span that older people now enjoy without providing similarly enjoyable healthy living. This has created a need for new therapies that aim at curing chronic diseases and not just palliating them. It was thus inevitable that stem cell research would gain substantial momentum since it was first discovered that stem cells could save lives following lethal irradiation.

The first modern bone marrow transplant was performed in the 1950s. Unlike blood transfusions, which failed to save victims of nuclear accidents, cells in marrow transplants seem to have the capacity to sustain the production of blood cells. These cells were dubbed *hematopoietic stem cells* (HSCs). To date, HSCs are the most and best researched and characterized cells. A vast array of specialized equipment is now available on the market that can be used to purify HSCs to a clinical grade for direct infusion into patients. Diseases now routinely curable by HSC transplantation include leukemia, lymphomas, multiple myeloma, and many disorders of the blood and lymphoid tissue. Many clinical trials have also shown promising therapy for solid organ diseases, such as breast cancer and autoimmune diseases. However, HSC transplantation is a complex and expensive procedure that puts patients, who must endure long absences from work and prolonged recovery issues, under significant physical and financial stress. Furthermore, HSC transplantation entails a

long search for a matched donor and significant perioperative immune suppression, causing significant perioperative issues and higher morbidity and mortality. These challenges regarding the consistency and safety of treatment begged for alternative cells that would bring better therapeutic outcomes.

Within the bone marrow lies another population of stem cells, characterized by multilineage differentiation into stromal cells such as fibroblasts, bone, fat, and cartilage cells. These are called *mesenchymal stromal cells* (MSCs) (or, more commonly and less accurately, mesenchymal stem cells). MSCs have quickly gained popularity over HSCs and became the preferred cells for the treatment of nonhematopoietic disorders, for several reasons. Mainly, MSCs are easy to culture and relatively safe and offer a low-cost transplantation procedure. MSCs were first obtained from the bone marrow and then from other tissues, including umbilical cord, placenta, and adipose tissue. Today, we can collect MSCs from almost any tissue in the body. They can be expanded with relative ease, as plastic adherent cells, to large numbers, based on patient needs. They can be autologous or allogeneic, but because of their low immunogenicity and favorable immune functions, especially their immune suppressive qualities, they have some advantages in terms of transplant procedures. In this regard, they are considered safer than HSC transplantation, which necessitates the vigorous immune suppression of often already debilitated patients. Because of the ease of their culture and expansion, they are also the preferred candidates for scaffolds, for purposes of tissue and organ engineering. Plausibly, there has been a surge of clinical trials that have disproportionately favored MSCs.

Surveying the several thousand publications and clinical trials using stem cell therapy—hematopoietic disorders aside—MSCs appear to be the most utilized stem cells in experimental transplantation. In many of these experiments and trials, MSCs are transplanted without prior differentiation into the desired cell population. Nevertheless, reports on improvements in the symptoms and signs of recovery are consistent. Many scientists argue that the efficacy of MSCs is not attributable to their multipotency or contribution to the

reconstitution of the damaged tissue by replacing diseased cells with healthy ones. Rather, they work because of paracrine or immune-modulating effects, which improve the microenvironment of the affected organ and promote growth factors and endogenous stem cells. New technologies and tracking methodologies will help provide a better understanding of the mechanism of restructuring a diseased tissue following stem cell treatment. Whether infused stem cells contribute to restructuring damaged tissue, which was initially thought to be the ultimate target of stem cell therapy, remains to be verified.

Recently, sporadic clinical trials using embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) started in centers mainly in Asia and the USA. Although these efforts are not covered in this book, much hope rests on such trials. ESCs are considered “de facto” stem cells. They can robustly differentiate into cells of multiple lineages both in vitro and in vivo. When injected into animal models, they form teratomas, composed of a mixture of tissues that correspond to all lineages. They have longer telomeres and express embryonic genes. Among all types of stem cells, ESCs have a unique flexibility and unmatched capacity for differentiation into the desired cell populations. However, many issues need to be overcome before ESC use becomes a reality in routine clinical settings. For example, the reliability and sustainability of the differentiated cell type remain issues as does the safety of transplanted cells. Ethical considerations have also hampered ESC research, although restrictions are being relaxed in several countries in response to public awareness and strict regulations. Much anxiety over ESC research has been relieved with the introduction of iPSCs.

Indeed, since the first cloning of frogs by Gurdon and colleagues in the fifties, and later, the famous sheep Dolly in the nineties, it has become apparent that our understanding of biology, embryology, and organ formation has been severely deficient. It also became apparent that cell manipulations by chemicals or additives could change cells in fundamental ways not considered previously. Yamanaka’s work, which won him the 2012 Nobel Prize in Physiology or Medicine, achieved

what many had only dreamed of: changing somatic cells into ESC-like cells. Using four transcription factors, Yamanaka's group induced pluripotency in an adult fibroblast. No longer should the scientific community endure the controversies of using ESCs. This technique, however, is still far from perfect. Some of the transcription factors used to induce pluripotency were oncogenic and could stimulate tumorigenesis. Many ongoing efforts are improving the safety of iPSCs; however, issues with the reliability of differentiation and long-term safety remain unresolved.

Where do we stand now, and is the use of stem cells for the treatment of non-hematopoietic disorders close to being a reality? The answer to this question varies depending on many factors. Our group has recently performed meta-analyses to evaluate the use of stem cells in the treatment of diabetes mellitus. It was interesting to find that among the 4000+ studies that appeared in response to the key words "diabetes" and "stem cells," only 22 trials were eligible for inclusion in our study on using stem cell transplantation for the treatment of uncomplicated diabetes. The discrepancy between the benchtop and bedside is indeed significant. This analysis led to several conclusions, most importantly, that systematic, well-controlled clinical trials are severely lacking in the area of treatment of diabetes using adult stem cells. It is not unreasonable to generalize this finding and extend it to other applications of stem cell therapy for cardiovascular diseases, neurodegenerative disorders, and urogenital diseases. Our study showed that the type of stem cell, the source of the cells, the route of administration, and dose all contribute to the outcome of stem cell therapy. Patient-related factors that supported a more favorable outcome included earlier intervention, lack of complications, and overall health of the treated patient. Universal conclusions from our study and others reveal the critical need for fine-tuning of stem cell therapy in a much better and more systematic approach than the current practice. This fine-tuning, which encompasses factors related to the diseases, stem cell transplant ation, conditioning protocol, and patient will all ultimately determine the success or failure of the transplant.

The prevalence of diseases of aging, the lack of satisfactory therapy for today's many intractable illnesses, and the anxiety experienced by patients and their families over finding a cure have all driven stem cell research onto a fast, not well-controlled track. As a result, much hype has diluted efforts to systematically design clinical trials and critically evaluate outcomes. Embarking on writing this book at this time is thus an attempt to provide an overview of a work in early progress. Some of the clinical trials covered here are mature, and data are available in large, reproducible outcomes to be recommended for patients on a routine basis. On the other hand, many tissue and organ engineering efforts, as well as utilization of ESCs and iPSCs, are still almost exclusively experimental, and results are too preliminary to recommend for routine practice. Technological advances in the fields of nanotechnology and material science should, however, accelerate stem cell therapy at unprecedented rates. These technologies should allow for advances in studying the biology of stem cells and enhancing their application in vitro, for both diagnostic and therapeutic purposes. The book covers some of those promising technologies and how they impact the study of biology in general and stem cells in particular. We expect that next-generation stem cells will be those which have been studied and manipulated using technologies that are just being developed and will revolutionize their applications in the very near future.

Nagwa El-Badri

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Part I
The Use of Adult Stem Cell Therapy for Selected Diseases

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1. Hematopoietic Stem Cell Transplantation in Pediatric Diseases

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Hematopoietic stem cell transplantation (HSCT) is currently the only stem cell treatment modality with well-accepted clinical efficacy. Similar to adults, HSCT is commonly used for the treatment of hematological malignancies, solid tumors, and acquired aplastic anemias; but in childhood there are additional indications that patients may benefit from HSCT these include metabolic diseases, immunodeficiency

syndromes, osteopetrosis, inherited bone marrow failure syndromes, and hemoglobinopathies.

1.1 Pediatric HSCT Applications

1.1.1 In Malignancies

1.1.1.1 Pediatric Acute Myeloid Leukemia

In patients with high-risk acute myeloid leukemia (AML), survival rates of patients who receive HSCT from human leukocyte antigen (HLA) matched related donors at complete remission (CR)-1 are better (55–72 %) compared to those who receive chemotherapy alone. Therefore, HSCT at CR-1 is indicated in high-risk AML patients. However, almost 60 % of patients do not have an HLA-matched related donor, and in these patients the treatment is planned according to indications determined by the European Group for Blood and Marrow Transplantation (EBMT) (Table 1.1) [1]. In the pediatric population, response to initial induction therapy and cytogenetic findings are consistent prognostic markers. The low-risk group includes patients with favorable karyotypes, including t(8;21), inv(16), t(15;17), and, in some studies, t(9;11), t(16;16), and FAB-type M3. The poor-risk group includes patients with adverse karyotypic abnormalities, including −5, −7, del(5q), abn(3q), and complex karyotypes and patients with poor treatment response. The patients who have neither favorable nor high-risk features are considered those with intermediate risk [2]. It is recommended that patients with low-risk features be treated with standard chemotherapy; on the other hand, patients with intermediate features should be considered for HSCT in the presence of a related donor. Relapse of a patient at any time with AML is considered an indication for HSCT from the best available donor [1]. In the pediatric age group, the addition of total body irradiation (TBI) to the conditioning of patients who undergo HSCT at CR-1 showed no additional benefit to transplant outcome, and considering the long-term potential deleterious effects of TBI, its use was not suggested for AML patients. In most cases, patients were conditioned with busulfan and

cyclophosphamide; however, some regimens add melphalan to athe busulfan, cyclophosphamide regimen [3]. On the other hand, conditioning regimens including treosulfan and fludarabine have been reported to be associated with lower transplantation-related mortality (TRM) [4].

Table 1.1 Indications for hematopoietic stem cell transplantation in pediatric patients with AML

Disease status	**Sibling donor**	**Matched unrelated/1 Ag-mismatched related**	**Mismatched unrelated/>1 Ag-mismatched related**	**Autologous**
AML CR-1—low risk	HSCT not recommended	HSCT not recommended	HSCT not recommended	HSCT not recommended
AML CR-1—high risk	HSCT	Decision of clinician	HSCT not recommended	HSCT
AML CR-1—very high risk	HSCT	HSCT	Decision of clinician	HSCT not recommended
CR-2	HSCT	HSCT	HSCT	HSCT
>CR-2	Decision of clinician	Inadequate evidence	Inadequate evidence	HSCT not recommended

CR complete remission, *Ag* antigen, *HSCT* hematopoietic stem cell transplantation

Autologous HSCT has been studied as a consolidation therapy for patients without allogeneic-related donors; however, the results suggest the noninferiority of chemotherapy alone or alternative donor HSCT to autologous HSCT [5]. Currently autologous HSCT is not suggested for patients with AML at CR-1 [5].

Patients with infant AML or those with FAB-type M7, who have no related donor, should be considered for HSCT from an unrelated donor. Additionally, the patients who do not achieve remission with the initial induction treatment should be considered for HSCT as soon as possible since the extended use of chemotherapeutics in these patients may add to transplant toxicities without beneficial effect [1].

The targeting of donor T cells against leukemic cells of the patient is called graft versus leukemia (GVL), and GVL accompanies graft-versus-host disease (GVHD). The advantages of GVL development on the long-term outcome and eradication of AML is unclear. T-cell depletion from a harvest is not related to the relapse of AML following HSCT. However, patients who develop chronic GVHD have been reported to relapse less frequently [6]. Moreover, AML patients who develop mixed chimerism following HSCT are considered candidates to receive donor lymphocyte infusions (DLIs) to prevent relapses [7].

1.1.1.2 Pediatric Acute Lymphoblastic Leukemia (ALL)

Improvements in current chemotherapy protocols have led to survival rates of around 85 %. High-risk ALL patients and relapsed ones are being preferentially treated with HSCT. Because of the poor prognosis of patients with t(9;22) with chemotherapy, it is recommended that these patients receive transplants from a matched sibling donor at CR-1. Currently, most patients with Philadelphia positive (Ph+) ALL undergo HSCT at CR-1 using the best available donor [8]. Five-year event-free survival (EFS) rates using mismatched family donors or unrelated donors are almost 50 % in these patients. On the other hand, the use of intensive chemotherapy plus tyrosine kinase inhibitors has increased the survival rates of these patients, and there is emerging evidence for reserving the HSCT option for those Ph+ ALL patients who do not achieve molecular response, although this approach is still not a well-accepted standard of care in these patients.

On the other hand, patients with extreme hypodiploidy (fewer than 44 chromosomes) have been reported to have EFS rates of around 20 ± 10.3 % with standard chemotherapy [9], and such patients have also been proposed to be indicated for HSCT at CR-1.

Since the outcome of patients with T-cell ALL with very high white blood cell count at diagnosis has been reported to have less promising outcomes, these patients have been

recommended to undergo HSCT at CR-1, although this approach is not definitive.

Infants with t(4;11) Mixed-lineage *leukemia* (*MLL*) rearrangement have a worse prognosis compared to those who do not have this karyotype, and these patients and infants with higher white blood cell count at diagnosis or those with poor prednisolone response also have worse outcomes. Infants with t(4;11) with the aforementioned higher-risk features have been recommended to undergo HSCT; however, a recent report found no difference in the disease-free survival rates of these patients when compared to standard chemotherapy [10, 11].

Furthermore, the assessment of early response to treatment is a good indicator of prognosis. The minimal residual disease (MRD) follow-ups and bone marrow blast percentages by the end of induction may have an effect on the decision to do HSCT. The MRD positivity just prior to HSCT also has a negative impact on HSCT outcome [12]. Patients with pediatric ALL may also see beneficial effects of GVL after HSCT. Patients with ALL who develop early (defined as relapse occurring during therapy or within 18–36 months from diagnosis) bone marrow relapse are indicated for HSCT using related or unrelated donors at CR-2.

The long-standing conditioning regimen preferentially used for ALL patients has been TBI and cyclophosphamide. The lesser toxicity of fractionated TBI compared to single-dose TBI has prompted the use of fractionated TBI for conditioning. Additionally, TBI/etoposide has replaced the classical TBI/cyclophosphamide [8, 13]. In the case of a contraindication for TBI use, such as high doses of previous irradiation or in infant patients, TBI is replaced with intravenous busulfan. On the other hand, pediatric ALL patients with t(4;11) to be transplanted after conditioning with busulfan, cyclophosphamide, and melphalan should have better outcomes [8].

1.1.1.3 Pediatric Chronic Myelogenous Leukemia

The only curative treatment option for pediatric Ph+ Chronic Myelogenous Leukemia (CML) is HSCT. Especially in the presence of a matched sibling donor, survival rates are around 87 %, and the success rate of HSCT is good, especially in the chronic phase of CML, but much lower in other phases of the disease [14]. The long-term adverse effects of tyrosine kinase inhibitors are unknown, and currently the suggestion of lifelong use of tyrosine kinase inhibitors makes HSCT a good curative therapeutic option. However, careful monitoring of the response to tyrosine kinase inhibitors presents a safer alternative, and patients with poor response to tyrosine kinase inhibitors are considered HSCT candidates. Patients who fail to respond tyrosine kinase inhibitors are strongly suggested to undergo HSCT from a matched donor, related or unrelated. In the absence of an available donor, second-generation tyrosine kinase inhibitors might be considered [15].

Although cytogenetic and molecular remission could be achieved with tyrosine kinase inhibitors, the longer life expectancy of children may put HSCT still as a first-line curative therapeutic option, especially when made within 1 year of diagnosis.

Monitoring for *bcr/abl* transcripts in peripheral blood with polymerase chain reaction (PCR) is strongly recommended 1, 3, 6, 9, and 12 months post-HSCT and then every 6 months. Positive bcr/abl transcripts after HSCT are not uncommon (20 %) and can be successfully treated with donor lymphocyte infusions (DLIs). The induction of GVL with DLIs is most efficiently achieved with patients with CML. Donor lymphocyte infusions may induce molecular remission and prevent relapses in such patients [16]. The potential risks of DLI are GVHD development or bone marrow aplasia. The efficacy of DLI at chronic phase is 75 % and between 13.5 and 33 % when made at accelerated and blastic phases of CML.

1.1.1.4 Hodgkin and Non-Hodgkin Lymphoma

HSCT is not the first-line treatment of pediatric and adolescent patients with Hodgkin and non-Hodgkin lymphoma. In both

discases, HSCT is indicated in resistant patients or in recurrence of the discase. Besides autologous HSCT applications, allogeneic HSCT is also increasingly being used for the treatment of patients with lymphoma. Autologous HSCT should be considered for chemosensitive relapse of mature B-cell lymphoma. The addition of rituximab (anti-CD20 monoclonal antibody) before or after HSCT for B-cell lymphoma may further improve the outcome of HSCT [17]. Refractory or relapsed T-cell lymphoblastic lymphoma is treated similarly to T-cell ALL, and allogeneic HSCT from the best available donor is the standard of care after the achievement of remission with salvage therapy. In patients with non-Hodgkin lymphoma, allogeneic HSCT is usually considered for relapsed and refractory lymphoblastic lymphoma and anaplastic large cell lymphoma cases or following recurrence subsequent to autologous HSCT. Patients with Hodgkin lymphoma who are responsive to chemotherapy may benefit from autologous HSCT [18, 19].

1.1.1.5 Neuroblastoma and Other Solid Tumors

In patients with high-risk solid tumors, mainly autologous HSCT and less commonly allogeneic HSCT might be applied; allogeneic HSCT has been associated with better outcomes. The most commonly used conditioning regimen is busulfan/melphalan. The most extensive data in pediatric solid tumors are in patients with advanced-stage neuroblastoma [20]. The conditioning with busulfan/melphalan as compared with a carboplatin approach has ended up with lower rates of relapse in patients with advanced-stage neuroblastoma. Treatment of MRD during the post-HSCT period with cis-retinoic acid as a differentiating agent has a favorable impact on relapse rates with minimal toxicity [21]. On the other hand, anti-GD2 antibody treatment also has been shown to have a favorable impact on survival rates when used after HSCT [22, 23]. Of the common complications during the post-HSCT period, short stature, gonadal failure, and hearing loss must be closely monitored.

There is an experience with autologous HSCT subsequent to high-dose chemotherapy in patients with medulloblastoma, primitive neuroectodermal tumor, and germ-cell tumor, mostly applied in patients with recurrent disease and, to a lesser extent, those with unfavorable features at the first remission.

1.1.2 In Nonmalignant Diseases

1.1.2.1 Immunodeficiency Disorders

HSCT is the only curative treatment modality in most primary immunodeficiency syndromes, primarily for patients with severe combined immunodeficiency (SCID). In patients with SCID, HSCT from sibling donors and HSCT within 6 months of diagnosis have better outcomes. On the other hand, patients with T-B-SCID have worse outcomes, as do patients who have comorbidities prior to HSCT, including pneumonia, sepsis, viral infections, or malnutrition [24]. In HSCT from a matched sibling donor in patients with SCID, survival rates are as high as 90 %. Related to the underlying cellular immunological defect, SCID patients with a matched related donor do not require conditioning or GVHD prophylaxis. This special situation causes T cells of the patient to be of donor origin, whereas myeloid and erythroid cells to be derived from host cells during post-HSCT period [25]. The outcome of HSCT from phenotypically identical relatives or HLA-matched unrelated donors is less favorable compared to matched sibling donors and a conditioning regimen, and GVHD prophylaxes are required in transplants from such donors [25]. Intravenous busulfan/fludarabine or treosulfan/fludarabine are the most preferred conditioning regimens [26]. In patients with SCID who lack a matched sibling donor, the use of cord blood as stem cell source may have an advantage of producing lesser rates of GVHD. On the other hand, the slower engraftment of cord blood HSCT, in addition to the absence of viral-specific cytotoxic T cells in such transplants, should be considered [27]. Patients who lack a matched sibling donor may undergo T-cell-depleted haploidentical transplantation. These patients may have high rates of graft failure and opportunistic infections. However, the advantages are the high donor

availability related to the use of parents as donors and less cytoreductive conditioning. T-cell functions appear several months after haploidentical transplantation in grafted patients [28]. The graft rejection rates in such haploidentical transplantations may be as high as 20 %. These risks of haploidentical HSCT increase the use of matched unrelated donors for SCID patients who lack a matched sibling donor. However, patients who undergo unrelated HSCT will mandate the use of conditioning regimens. Survival rates as high as 97 % have been reported with matched sibling donors, 79 % in HSCT using T-cell-depleted grafts from mismatched related donors without conditioning, 66 % using mismatched related donors with conditioning, and 58 % after cord blood HSCT [29]. T-B- and radiosensitive patients, including those with DNA ligase 4 deficiency and cernunnos deficiency are suggested to be conditioned with lesser intensity regimens such as fludarabine/cyclophosphamide [26, 27].

Other immunodeficiency disorders excluding SCID should be conditioned prior to HSCT. In patients with Wiskott-Aldrich syndrome, unless HSCT has been done, patients are usually lost due to bleeding, infections, or the development of lymphoproliferative diseases. In a series of 194 patients with Wiskott-Aldrich syndrome, the overall survival rate with HSCT has been reported to be as 84 %.

Patients who were splenectomized prior to or subsequent to HSCT have been reported to have increased risk for developing fatal sepsis. Additionally, patients who underwent HSCT before 2 years of age were found to have better outcomes compared to those who underwent HSCT after 5 years of age [30].

Among patients with severe chronic neutropenia (Kostmann syndrome), more than 90 % respond to recombinant human granulocyte-colony stimulating factor (G-CSF) treatment. HSCT is reserved for severe chronic neutropenia patients who are unresponsive to G-CSF treatment. Patients who are unresponsive to G-CSF treatment are those with G-CSF receptor mutations who have 40 % risk of myelodysplastic syndrome/AML and 14 % of sepsis-related mortality within 10 years of follow-up [27].

Among the other immunodeficiencies that benefit from HSCT are leukocyte adhesion defects, Omenn syndrome, chronic granulomatous disease, and Chédiak-Higashi syndrome.

Familial hemophagocytic lymphohistiocytosis may develop in the setting of an underlying immunodeficiency, including X-linked lymphoproliferative syndrome and Griscelli or Chédiak-Higashi syndrome or related to a primary genetic defect in *PRF1*, *UNC13D*, *STX11*, or *STXBP2* genes [31]. Three-year survival rates after HSCT conditioned with busulfan and cyclophosphamide ± etoposide have been reported to be 64 %. The survival rates increase to 71 % in transplants from matched related donors, 70 % using matched unrelated donors, and 54 % using haploidentical family donors or mismatched unrelated donors [27]. One of the major determinants of HSCT success is the remission in the disease criteria just prior to HSCT with the preceding treatment regimens. Reduced intensity conditioning for transplantation of patients with hemophagocytic lymphohistiocytosis is under investigation, but the initial results indicate that mixed chimerism and graft failure are major post-HSCT issues. Thus, the use of reduced intensity conditioning should not be preferred, particularly when the source of the stem cell is cord blood.

1.2 Inherited Metabolic Disorders and Osteopetrosis

Allogeneic HSCT is a therapeutic option for select cases of lysosomal and peroxisomal disorders. The basic criteria for the selection of indicated patients are based on clinical findings, the rate of progression, and the specific diagnosis. Established neurological findings are usually not restored after HSCT; therefore, advanced cases do not benefit from the transplant. In these metabolic disorders, one of the determinants of HSCT success is the post-HSCT enzyme levels of patients. Thus, the preference for noncarrier HLA-matched members will further improve the transplant outcome in these patients. In lysosomal diseases the correction of the disorder results from the donor

leukocytes that engraft in the host tissue and secrete the deficient enzyme. The secreted enzymes are endocytosed by the neighboring cells through mannose-6-phosphate-mediated receptors or direct transfer [32].

Peroxisomal diseases benefit from HSCT not only by the replacement of the lacking enzyme but also with the immunosuppression and the inhibition of the perivascular inflammation following HSCT. The transfer of metabolically normal host-derived cells to the tissues of the host, including the central nervous system, is a very crucial determinant of HSCT success. Microglial cells are mononuclear cells of the central nervous system that have a phagocytic function and are derived from hematopoietic stem cells [33].

Among lysosomal diseases, allogeneic HSCT has the best outcome in mucopolysaccharidosis type I (Hurler syndrome) and is considered investigational for other diseases. Upper airway obstruction, hepatosplenomegaly, and corneal clouding usually benefit from HSCT in patients with Hurler syndrome [34]. Hydrocephalus, growth, and developmental abnormalities may also improve, but to a lesser extent. Skeletal abnormalities of patients usually do not improve following HSCT. In a recent study of 258 children with Hurler syndrome, enzyme levels were superior with HLA-matched umbilical cord blood transplantation, compared to other donor sources. However, carrier siblings were included in the study, and this may have contributed to the lower outcome in the matched sibling group [35].

Sphingolipidoses are a group of lipid storage disorders and include Tay-Sachs, Niemann-Pick, Gaucher, Fabry, Krabbe, and metachromatic leukodystrophy. Enzyme replacement therapy is not available currently for Krabbe and metachromatic leukodystrophy, and HSCT is the only potential curative treatment option in these patients. However, patients with advanced neurological findings usually do not benefit from the treatment. The early onset/infantile forms of both diseases benefit most from HSCT if transplantation was made before symptoms appeared [36].

X-linked adrenoleukodystrophy (ALD) is a peroxisomal disorder, and cerebral ALD is the most severe phenotype. Patients with ALD are asymptomatic in early life and usually become symptomatic by the end of the first decade. Additionally, some patients are asymptomatic throughout their lives. Patients have typical MRI changes that are scored using the Loes severity scoring system, and those with lower scores have better outcomes, related to a less severe disease. Allogeneic HSCT is currently the only curative treatment option for patients with cerebral ALD. Patients with lesser neurological deterioration and lower Loes scores benefit more from HSCT [37].

Malignant infantile osteopetrosis is a disorder of osteoclast dysfunction. The natural course of autosomal recessive malignant infantile osteopetrosis often comprises severe complications and death during childhood. Currently, the only curative therapy is HSCT [38]. An increasing number of genetic defects are being described in osteopetrosis. Among these *TCIRG1, CLCN7, OSTM1*, and *RANK* are known as intrinsic defects, and the *RANKL* defect is defined as an extrinsic defect. In the *RANKL* defect the bone biopsy exhibits the absence of osteoclasts, and these patients do not respond to HSCT since *RANKL* is produced by osteoblasts [38, 39]. Of the intrinsic defects, patients with *OSTM1* have severe progressive neurological problems, and HSCT is contraindicated in this subset. Therefore, *OSTM1* and *RANKL* defects should be ruled out prior to HSCT [38, 39].

The absolute indications for HSCT in malignant infantile osteopetrosis include hematological failure and imminent visual loss including the nystagmus and narrowing of the optic nerve foramina. The relative indications include multiple fractures and severe bone malformations.

If the donor is HLA matched, the bone marrow is preferred as the stem cell source, and no graft manipulation is required. Osteopetrosis patients are prone to veno-occlusive disease (VOD) development (as high as 63.6 %) [40]. The risk decreases with the use of intravenous forms of busulfan and fludarabine, instead of oral busulfan and cyclophosphamide, and with prophylactic use of defibrotide [41]. One third of

children develop pulmonary arterial hypertension during 90 days post-HSCT and may easily be misdiagnosed as having pneumonia. These patients may benefit from prostacyclin and nitric oxide [42]. Another complication during the post-HSCT period is severe hypercalcemia that is common after engraftment. Recently two patients with *RANK* mutation who developed severe hypercalcemia were successfully treated with a monoclonal *RANKL* antibody, namely, denosumab [43].

For patients with a *RANKL* mutation, since they do not benefit from HSCT, mesenchymal stem cell transplantation is being considered as an alternative treatment approach.

The indications for HSCT in inherited metabolic disorders are summarized in Table 1.2 [36].

Table 1.2 Indications for HSCT in inherited metabolic disorders (Adapted from Ref. [36])

Disease	**Indication for HSCT**	**Comment**
Mucopolysaccharidoses		
Hurler (MPS IH)	Standard	–
Hurler/Scheie (MPS IH/S)	Option[a]	ERT first-line therapy
Scheie (MPS IS)	Option[a]	ERT first-line therapy
Hunter, severe (MPS IIA)	Investigational[b]	Only early or asymptomatic
Hunter, attenuated (MPS IIB)	Investigational[b]	Only early or asymptomatic
Sanfilippo (MPS IIIA)	Investigational[b]	Only early or asymptomatic
Sanfilippo (MPS IIIB)	Investigational[b]	Only early or asymptomatic
Sanfilippo (MPS IIIC)	Investigational[b]	Only early or asymptomatic
Sanfilippo (MPS IIID)	Investigational[b]	Only early or asymptomatic
Maroteaux-Lamy (MPS VI)	Option[a]	ERT first-line therapy
Sly (MPS VII)	Option[a]	

Disease	Indication for HSCT	Comment
Leukodystrophies		
X-ALD, cerebral	Standard	–
MLD, infantile	Unknown[c]	–
MLD, juvenile	Option[a]	Only early or asymptomatic
MLD, late onset	Standard	Only early or asymptomatic
GLD, early onset	Option[a]	Neonate, screening diagnosis, or second case in known family, not for advanced disease
GLD, late onset	Option[a]	Not for advanced disease
Others		
Fucosidosis	Option[a]	
Alpha-mannosidosis	Standard	
Aspartylglucosaminuria	Option[a]	
Farber	Option[a]	
Tay-Sachs, early onset	Unknown[c]	
Tay-Sachs, juvenile	Investigational[b]	Neonate, screening diagnosis, or second case in known family
Sandhoff, early onset	Unknown[c]	
Sandhoff, juvenile	Investigational[b]	Neonate, screening diagnosis, or second case in known family
Gaucher I (non-neuronopathic)	Option[a]	
Gaucher II (acute neuronopathic)	Unknown[c]	ERT first-line therapy
Gaucher III (subacute neuronopathic)	Unknown[c]	
Pompe	Investigational[b]	Limited benefit of ERT

Disease	Indication for HSCT	Comment
Niemann-Pick type A	Unknown[c]	ERT available
Niemann-Pick type B	Option[a]	ERT first-line therapy
Niemann-Pick type C	Option[a] in type C2	Only early or asymptomatic
Mucolipidosis type II (I cell)	Investigational[b]	
Wolman syndrome	Option[a]	
Multiple sulfatase deficiency	Investigational[b]	
MNGIE (mitochondrial neurogastrointestinal encephalomyopathy)	Option[a]	Not in advanced disease

MPS mucopolysaccharidoses, *ERT* enzyme replacement therapy, *ALD* adrenoleukodystrophy, *MLD* metachromatic leukodystrophy, *GLD* globoid cell leukodystrophy

[a]Option: HSCT is effective, but another therapy is increasingly considered the first choice or insufficient published evidence for HSCT to be considered standard

[b]Investigational: possible a priori reason for HSCT

[c]Unknown: no published evidence that HSCT is beneficial

1.3 Acquired and Inherited Bone Marrow Failure Syndromes

In both acquired and inherited bone marrow failure syndromes, HSCT is a widely accepted therapeutic option. The differential diagnosis of acquired and inherited forms of bone marrow failure is crucial in order to make a decision regarding the conditioning regimen.

Bone marrow failure, clonal cytogenetic abnormalities, and MDS/AML are indications for HSCT in patients with Fanconi anemia. In Fanconi anemia patients who develop bone

marrow failure, HSCT is recommended prior to transfusions (×20) or before the initiation of androgen treatment [44].

The best outcome in HSCT is achieved using matched sibling donors in Fanconi anemia. On the other hand, despite the absence of phenotypical findings of Fanconi anemia, all sibling donors should be tested with DEB- or MMC-induced chromosomal breakages in order to rule out the presence of Fanconi anemia, since 20 % of Fanconi anemia patients do not have the physical marks of the disease. In the case of the absence of a sibling donor, matched related donors are the second choice. In the initial series, 5-year survival rates of around 85 % have been reported in matched sibling donor transplants after conditioning with low-dose cyclophosphamide and 4Gy thoracoabdominal irradiation [45]. However, in recent years, to decrease the early- and late-term consequences of irradiation (GVHD and malignancies), irradiation is replaced with fludarabine-based regimens. Because of the underlying DNA repair defect, Fanconi anemia patients are very sensitive to conventional conditioning regimens. Fludarabine-based conditioning regimens, capable of intense T-cell immunosuppression, have been reported to lead to early, stable engraftment with minimal toxicity in patients with Fanconi anemia [46]. Hematopoietic stem cell transplantation still represents the only option able to definitively cure the bone marrow failure associated with this disease, as well as to prevent/treat myeloid malignancies, although it does not prevent the occurrence of solid tumors, mostly head and neck squamous cell carcinoma. In a recent EBMT report of 795 patients with Fanconi anemia, being older than 10 years of age at the time of HSCT, the use of peripheral blood stem cells, and the history of chronic GVHD were found as significant risk factors for the development of post-HSCT secondary malignancies [47].

There is no consensus guideline for the conditioning or even the pre-HSCT treatment of patients with Fanconi anemia who developed advanced MDS or acute leukemia. In a recent study of 21 patients with advanced MDS or acute leukemia, pre-HSCT remission induction chemotherapy offered no

additional benefit, and the overall 5-year survival rate was reported as 33 % [48].

The only therapeutic modality for patients with dyskeratosis congenita after the commencement of bone marrow failure or leukemia development is HSCT. However, in these patients HSCT has potential early- and late-term complications with high mortality rates. Infections and fatal pulmonary complications are the primary early complications, whereas diffuse vasculitis and pulmonary fibrosis are the potential late-term complications [49]. The development of post-HSCT secondary malignancies is less common than in Fanconi anemia [49].

Among Diamond-Blackfan anemia patients, almost two thirds of patients are responsive to medical treatment with steroids; however, some patients who are initially responsive may lose response over time. Patients who are unresponsive to steroids or those who have additional cytopenias not restricted to anemia are potential candidates for HSCT [50]. One of the major determinants of transplant outcome in patients with Diamond-Blackfan anemia is a preceding iron accumulation prior to HSCT, which is reflected by the unfavorable outcome in patients who underwent HSCT after 10 years of age [50, 51]. The absence of erythroid precursors in these patients related to disease biology itself puts additional iron loading risk on the transfusional iron burden, and patients with Diamond-Blackfan anemia accumulate iron much earlier than those with other transfusion-dependent anemias. The initial reports of allogeneic transplants from unrelated donors have been associated with poor outcomes; however, a recent series of 13 patients with Diamond-Blackfan anemia, which included the bone marrow as the stem cell source from both sibling and unrelated donors, reported a 5-year survival of 100 %, although three of the patients developed graft failure [52]. Related donors should be genetically tested for disease related to the incomplete penetrance characteristic of the disease, even if the donor is asymptomatic.

1.4 Hemoglobinopathies

Risk classification for HSCT in patients with thalassemia major has been suggested by Lucarelli et al. and is known as the Pesaro classification [53]. According to the Pesaro classification, hepatomegaly, hepatic fibrosis evaluated with liver biopsy, and history of inadequate iron chelation are used to classify patients into three risk classes. Accordingly, patients who do not possess any of the aforementioned risk factors are classified as class I, those who have one or two are classified as class II, and patients with all of the risk factors are classified as class III Pesaro criteria. The overall and thalassemia-free survival rates in class I patients were reported to be 94 % and 87 %, whereas in class II patients they were 84 % and 81 %, respectively. However, in class III patients, thalassemia-free survival was reported to be as low as 58 %, related to high graft rejection rates and increased transplant-related mortality [54]. Ineffective erythropoiesis and chronic transfusions cause iron burden in thalassemic patients. The choice of conditioning regimen should suppress the bone marrow hyperactivity related to ineffective erythropoiesis, but it also should not add much to the organ toxicities related to pre-HSCT iron loading. The most preferred regimen for class I and II patients is intravenous busulfan and cyclophosphamide (200 mg/kg/total dose). In some centers, thiotepa is added to this regimen for patients under 4 years of age, and some other centers add antithymocyte globulin for all ages. In class III patients, a regimen called Protocol 26 is widely used, based on increasing the immunosuppression. Protocol 26 includes pre-HSCT hypertransfusion of patients to maintain hemoglobin levels above 14 g/dL and intravenous deferoxamine, hydroxyurea, azathioprine, fludarabine, busulfan, and cyclophosphamide (160 mg/kg/total dose) [55].

Although most data on HSCT outcomes in thalassemic patients are from matched sibling donors, there are emerging data of transplant outcomes using alternative donors. In a recent Italian study of 60 patients, two thirds of the patients received transplants from unrelated donors after conditioning with a treosulfan-based regimen and were reported to have a 5-year thalassemia-free survival rate of 84 % [56].

Since there is no excretory mechanism for iron in the body, iron-decreasing management strategies are considered after HSCT. Phlebotomy is a simple and cheap way of decreasing iron load during the post-HSCT period, but it should be reserved for patients with acceptable hemoglobin levels. Patients who are inappropriate for phlebotomy may be chelated with iron chelators [57].

Allogeneic HSCT is a curative therapeutic option for patients with sickle cell anemia. Compared to thalassemia major, there are much restricted data on the HSCT outcome of patients with sickle cell anemia related to the clinical heterogeneity of patients, causing some patients to have milder phenotypes and the advent of pneumococcal prophylaxis, which decreases the disease-related mortality. The potential indications for HSCT in patients with sickle cell anemia are the history of stroke, recurrent painful crises or recurrent acute chest syndrome despite the use of hydroxyurea, and alloimmunization in patients who should be under a chronic transfusion program [58].

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2. Stem Cell Therapy in Diabetes Mellitus

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2.1 Introduction

In 2014, the global prevalence of diabetes mellitus (DM) was estimated to be 9 % among adults aged 18 years and older [1]. Type 1 DM (T1DM) accounts for 5–10 % of diabetic patients. Its pathogenesis involves autoimmune-mediated destruction of the pancreatic islets. Maintenance of appropriate glycemic control is possible using exogenous insulin for life, which imposes a burden on these patients. Transplantation of pancreatic islets or an intact pancreas is an ideal alternative for lifelong treatment. However, the shortage of cadaveric organs and the need for immunosuppression are limiting factors for

pancreatic transplantation. Type 2 DM (T2DM) accounts for the majority of diabetic patients, with the highest prevalence in the Eastern Mediterranean region and the Americas. The disease can be initially treated by dietary modifications and oral medication. Eventually, some 27 % of diabetic patients become insulin dependent. Of these, less than half achieve the recommended hemoglobin A1c (HbA1c) level for therapeutic efficacy since exogenous insulin cannot provide the tight glycemic control exerted by pancreas-derived insulin [2].

Recent progress in the field of regenerative therapies provides the potential for the generation of surrogate ß-cells, and efforts to engineer insulin-producing cells (IPCs) from stem cells are gaining momentum. Recent studies on IPCs from three sources, namely, embryonic stem cells (ESCs), induced pluripotent stem cells (iPS cells), and mesenchymal stem cells (MSCs), derived from a variety of adult tissues will be reviewed in this chapter.

2.2 Embryonic Stem Cells

Cells of embryonic origin have the capacity for rapid replication and the ability to differentiate into cells of all three germ layers (trilineage differentiation). These two characteristics make them an attractive source for the generation of IPCs. Lumelsky and associates reported successful differentiation of mouse ESCs using a five-step protocol [3]. Segev et al. modified the Lumelsky protocol by adding a step of suspension culture at the end of the differentiation protocol [4]. These early reports were challenged by Rajagopal and colleagues, who provided evidence that both the presence of insulin inside the cells and its apparent release are the result of insulin absorbed from the culture medium [5]. Paek and coworkers suggested that insulin release is the result of sequestration of insulin from the culture mediim as well as from de novo synthesis [6]. In a series of studies, Baetge and colleagues provided a proof of principle and refined a protocol for the efficient differentiation of human ESCs into insulin-secreting cells. Their differentiation scheme mimicked the in vivo pancreatic development. This

was achieved by directing the cells through successive stages toward definitive endoderm, gut-tube endoderm, pancreatic endoderm, and finally pancreatic endocrine lineage [7–10]. The strategy of this group of investigators is to transplant the resulting pancreatic progenitors within an encapsulation device to prevent immunorejection. The grafted cells would undergo further maturation into IPCs under the influence of the in vivo milieu, a process that can take 3–4 months [11]. Using an undifferentiated human ESC line, successful generation of putative IPCs was reported by Pagliuca and associates [12]. These cells share significant functional features within normal human beta cells. Their reported method of differentiation involved a complicated multistep protocol that lasts up to 6 weeks. These authors suggest that using their differentiation protocol, hundreds of millions of glucose-responsive ß-cells from human pluripotent stem cells can be produced. It is clear that important progress in differentiating ESCs into IPCs has been achieved. However, the use of embryonic cells suffers from two drawbacks: their teratogenicity and immunogenicity. These two problems could be contained if such cells are transplanted within an encapsulation device.

2.3 Induced Pluripotent Stem Cells

Yamanaka and his groups were the first to prove that by forcing the expression of a small number of factors, terminally differentiated cells could revert back to a pluripotent state [13, 14] and were termed *induced pluripotent stem cells* (iPS cells). Initial derivation of iPS cells utilized retroviral-mediated introduction of Oct3/4, Sox2, Klf4, and c-Myc (Yamanaka factors). Later, nonviral methodologies were introduced. Repeated transfection of plasmids containing the Yamanaka factors resulted in the production of iPS cells without evidence of plasmid integration [15, 16]. The iPS cells generated from somatic cells are expected to resolve problems that pertain to embryonic cells. The use of embryonic cells has been limited to certain established clones; accordingly, immunorejection is considered a major obstacle for cell therapy. In contrast, patient-derived iPS cells would theoretically not suffer

immunorejection since they are autologous. However, the efficiency of their generation remains low [17]. Moreover, the formed iPS cells show unlimited proliferative activity and form teratomas upon transplantation [18]. They also carry epigenetic memory characteristic of the somatic cell of their origin. This favors differentiation along lineages related to the donor cells [19].

The pluripotency of generated iPS cells provides a potential for their differentiation to IPCs. Tateishi et al. were probably the first to report the possibility of generating insulin-secreting isletlike clusters from iPS cells derived from human skin fibroblasts [20]. Using a three-step differentiation protocol, iPS cells derived from mice fibroblasts were differentiated into IPCs by Alipio and associates [21]. These cells were able to reverse hyperglycemia in diabetic mouse models. Zhu and colleagues developed a four-stage protocol to generate IPCs from rhesus monkey iPS cells. The resulting cells could secrete insulin in response to glucose stimulation, and when they were transplanted into diabetic mice, the blood glucose levels were reduced in 50 % of the treated animals [22]. Jeon et al. generated iPS cells from Non obese diabetic (NOD) mouse embryonic fibroblasts and from NOD mouse pancreatic epithelial cells. They applied a directed differentiation protocol to induce the formation of functional pancreatic beta cells. They found that the iPS cells derived from NOD mouse pancreatic epithelial cells differentiated more readily into IPCs. Transplantation of these cells in diabetic mice could normalize their blood glucose levels [23]. In a more recent report, human iPS cells derived from both fetal and adult human tissues were differentiated in vitro into pancreas-committed cells. At the end of in vitro differentiation, approximately 5 % of cells became insulin positive. When transplanted into immunodeficient mice, the transplanted cells lost their insulin secretion capacity in response to glucose stimulation. Histology of the graft demonstrated a mixed population of cells containing pluripotent, neuronal, and mature pancreatic cells [24].

It is abundantly clear that the utilization of iPS cells to form IPCs requires further refinements and optimization

before their application can be clinically meaningful.

2.4 Mesenchymal Stem Cells

Earlier studies by Friedenstein and colleagues reported that bone marrow stroma could generate bone, fat cells, and cartilage following heterotropic transplantation in mice [25]. This suggested the existence of non hematopoietic bone marrow precursor cells with skeletal and adipogenic potential. The notion of a stromal stem cell was proposed subsequently by Owen [26]. The term *mesenchymal stem cells*(MSCs) was popularized by Caplan to refer to plastic-adherent cell preparations isolated from a variety of tissues [27]. Recently, leading investigators of mesenchymal cell therapy concluded that convincing data to support the "stemness" of these unfractionated plastic-adherent cells are lacking [28]. Therefore, the term *mesenchymal stromal cells* has been suggested, allowing the abbreviation "MSCs" to be maintained. Several independent studies have demonstrated that MSCs can differentiate not only into mesodermal but also ectodermal and endodermal lineages [29]. Based on these findings, the term *multipotent mesenchymal stromal cells* appears to be the most scientifically accurate descriptor of this plastic-adherent population. The term *mesenchymal* is maintained to imply the origin, but not the differentiation potential, of these cells [30]. The International Society for Cellular Therapy proposed three criteria to define MSCs [30]. First, MSCs must be plastic adherent when maintained in standard culture conditions using tissue culture flasks. Second, 95 % of the MSC population must express CD105, CD73, and CD90 as measured by flow cytometry. In addition, these cells must lack expression (≤2 %) of CD45, CD34, CD14, and HLA class II. Third, the cells must be able to differentiate into osteoblasts, adipocytes, and chondrocytes under standard culture in vitro differentiating conditions.

MSCs can be derived from a variety of human tissues and have a high capacity to replicate. They are easy to cultivate and expand and can maintain their multilineage potential following prolonged culture conditions [31]. In addition they

are nonteratogenic and their utilization is free of any ethical consideration. All of these reasons have rendered them a good tool for use in regenerative medicine, including in potential therapeutic use for DM.

MSCs derived from different sources were coaxed using different approaches to differentiate into IPCs. The bone marrow [32–34], adipose tissue [35], umbilical cord, umbilical cord blood [36, 37], fibroblasts [20], endometrium [38], and liver cells [39] are among several tissues that are rich in MSCs. Of these, the bone marrow and adipose tissue offer distinct advantages in view of their availability, abundance, and the extent of their documentation in the literature. To this end, two approaches were used for their differentiation into IPCs: genetic manipulation or directed differentiation. Transfection with genes important in pancreatic development was reported by several investigators. Karnielli et al. transfected human bone marrow stem cells with a virus vector carrying a rat PDX-1 gene. The extent of differentiation of these cells toward the β-cell phenotype was evaluated. The authors reported that the treated cells expressed all four islet hormones but lacked the expression of NeuroD-1. Cell transplantation into streptozotocin (STZ)-induced diabetic immunodeficient mice resulted in their further differentiation, including the induction of NeuroD-1 and reduction of hyperglycemia [34]. Porcine bone marrow stromal cells were electroporated with an insulin-expressing plasmid vector. When these cells were engrafted in the liver of STZ-induced diabetic pigs, partial but significant improvement in hyperglycemia was observed [40]. For directed differentiation, many protocols were evaluated using culture media rich in glucose [41, 42]. Initial experiments used cells of murine origin [43–45]. Subsequently, MSCs derived from human tissues were tried [23, 35, 46]. The early reports demonstrated variable degrees of success but were met with skepticism since it was argued that MSCs should not differentiate toward an endocrine pancreatic lineage.

In our laboratory [32], bone marrow cells were obtained from three adult diabetic and three nondiabetic volunteers. MSCs were isolated, expanded, and differentiated using a

three-stage protocol. Cells were cultured in a glucose-rich medium containing several activation and growth factors. Initially, mercaptoethanol was used to induce the cells toward a pancreatic endocrine lineage. Subsequently, nonessential amino acids, basic fibroblast growth factor, epidermal growth factor, and B27 supplement were added. Finally, activin A and nicotinamide were supplemented. At the end of differentiation, approximately 5 % of cells were positive for insulin and c-peptide by immunofluorescence. Insulin and c-peptide were coexpressed by the same cells **(**Fig. 2.1 **)**. Electron microscopy with nanogold immunolabeling demonstrated the presence of c-peptide granules in the rough endoplasmic reticulum. The differentiated cells expressed transcription factors and genes of pancreatic hormones similar to those of pancreatic islets. Furthermore, there was a stepwise increase in human insulin and c-peptide release in response to increasing glucose concentrations. Transplantation of these cells into diabetic nude mice resulted in control of their diabetes. The sera of the treated mice contained human insulin and c-peptide with negligible levels of mouse insulin. When the kidneys bearing the transplanted cells were removed, rapid return of diabetes was noted. In summary, evidence was provided that MSCs can indeed be differentiated into IPCs. Nevertheless, two observations remained to be addressed. First, improvement of the yield of IPCs following directed differentiation of human bone marrow-derived mesenchymal stem cells (HBM-MSCs) is needed. Second, an explanation of the ability of transplanted cells to cure diabetic nude mice in spite of the modest yield of IPCs in vitro is also required.

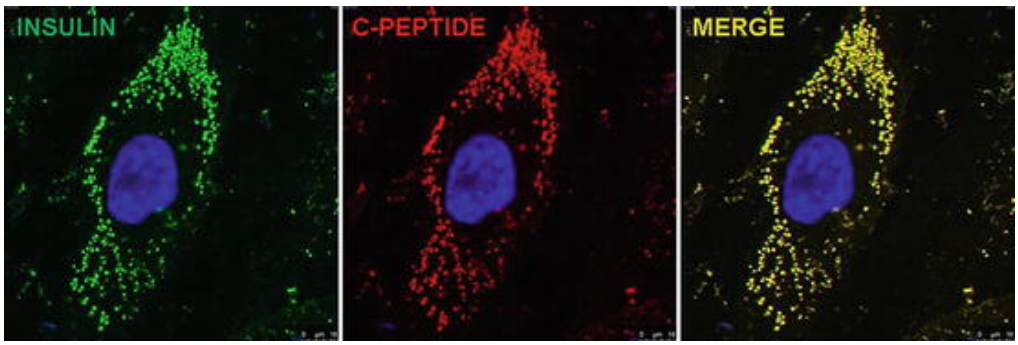

Fig. 2.1 Immunofluorescence staining of differentiated HBM-MSCs (a selected field). (**a**) Positive staining for intracytoplasmic insulin granules (*green*) with counterstaining for DAPI (*blue*). Positive staining for c-peptide (*red*) with

counterstaining for DAPI (*blue*). Electronic merge of insulin and c-peptide staining. The coexpression of insulin and c-peptide (*yellow*) was detected in the same cells

In a subsequent study, we compared the efficiency of the original protocol in which mercaptoethanol was used for the induction of differentiation of HBM-MSCs into IPCs with two other agents: conophylline and trichostatin [47]. The yield of functional IPCs was again modest and comparable among the three protocols (≃3 %). This is in agreement with the *data* of other investigators who reported that the proportion of IPCs at the end of in vitro differentiation was small irrespective of the employed protocol. In view of its simplicity and the short period required for its completion, only 10 days, the trichostatin-based protocol is currently our traditional method for directed differentiation of HBM-MSCs into IPCs. Several laboratories have also reported that, although the proportion of IPCs generated in vitro from MSCs was meager, they could induce euglycemia when the cells were transplanted into diabetic nude mice [48, 49]. Without providing clear evidence, it was suggested that this was the result of further maturation of the implanted cells in vivo. To confirm this finding, we carried out a series of experiments in our laboratory [50]. HBM-MSCs were obtained from three insulin-requiring type 2 diabetic patients. Following expansion, cells were differentiated according to a trichostatin-A/GLP protocol. One million cells were transplanted under the renal capsule of 29 STZ-induced diabetic mice. Mice were euthanized 1, 2, 4, and 12 weeks after transplantation. The IPC-bearing kidneys were immunolabeled, the number of IPCs counted, and the expression of relevant genes determined. The diabetic animals became euglycemic 8 ± 3 days after transplantation. The percentage of IPCs from the harvested kidneys increased gradually to reach a peak of ≃18 % at 4 weeks after transplantation without a substantial change thereafter (Fig. 2.2). Relative gene expression of insulin, glucagon, and somatostatin showed a similar increase. We concluded that the ability of the transplanted cells to induce euglycemia was due to an increase in the numbers of IPCs. It is reasonable to assume that the in vivo milieu contains factors that promote the maturation of the transplanted cells. It was reported that the source of these factors can be from the regenerating

pancreas after it had sustained a toxic or traumatic injury [51]. It was shown that cytosolic extracts from the regenerating pancreas have the potential to initiate neogenesis in STZ-induced diabetic animals [52]. An extract obtained from a regenerating pancreas 2 days after 60 % pancreatectomy was utilized with success for differentiation of rat mesenchymal cells into IPCs [53]. Further studies to identify the factor(s) secreted during pancreatic regeneration can provide an important tool for achieving the efficient differentiation of HBM-MSCs into IPCs.

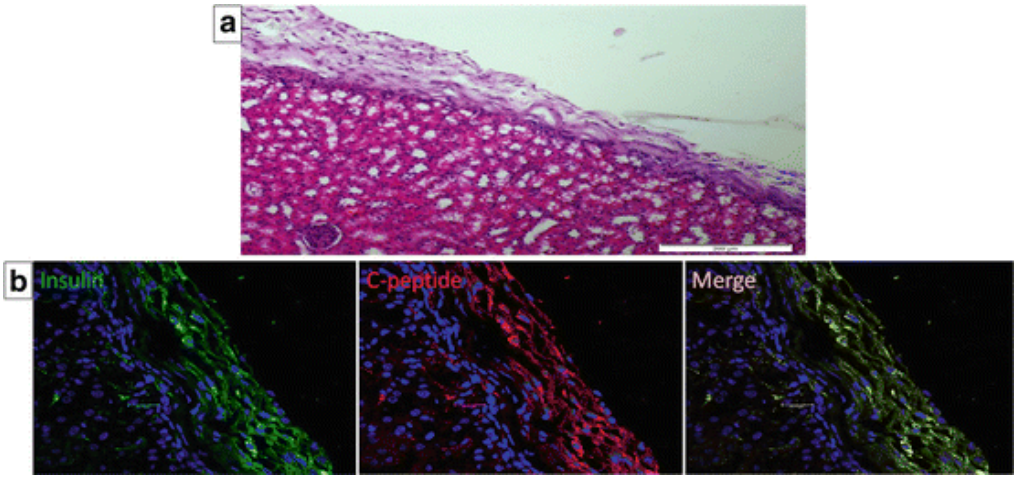

Fig. 2.2 Histology of IPC-bearing kidneys harvested from mice 4 weeks after transplantation. (**a**) Hematoxylin and eosin staining revealed the implanted cells beneath the renal capsule. (**b**) Positive immunofluorescence staining for insulin (*green*) and c-peptide (*red*). Electronic merge (*yellow*) reveals the coexpression of insulin and c-peptide in the same cells

It is clear that only a subset of MSCs is capable of trilineage differentiation [54]. The identification, sorting, expansion, and subsequent differentiation of this subpopulation can result in the production of sufficient IPCs with adequate functional capacity. Previous reports by Catherine Verfaillie's group described a culture system for MSCs that favors the selection of a subpopulation of primitive cells referred to as *multipotent adult progenitor cells* (MAPCs) [55]. A variety of other cells derived from postnatal tissues that demonstrated pluripotency were more recently reported: unrestricted somatic stem cells (USSCs) [56], marrow-isolated adult multilineage-inducible cells (MIAMIs) [57], and very small embryonic-like (VSEL) stem cells [58]. However, all of them were associated with controversies regarding lack of reproducibility and skepticism. The intermediate filament

protein nestin has been detected in several cellular phenotypes during embryonic and adult life. It was proposed that the expression of nestin may reflect the multipotential and regenerative abilities of cells [59]. Kabos et al. described a method for isolating nestin-positive cells from adult bone marrow [60]. Using this method, successful differentiation of nestin-positive subset of bone marrow-derived pancreatic endocrine cells was achieved by Milanesi and colleagues [61]. However, superiority of this method over the use of unfractionated cells in terms of the number or functionality of the generated IPCs was not shown. Recently, Kuroda and colleagues isolated what they defined as multilineage-differentiating stress-enduring (Muse) cells cultured from skin fibroblasts or bone marrow stromal cells [62]. These cells were positive for both CD105, a mesenchymal cell marker, and stage-specific embryonic antigen-3 (SSEA-3), a human pluripotency marker. Muse cells were indistinguishable from other MSCs in adherent culture, but when they are transferred to suspension culture, they form characteristic cell clusters that are capable of self-renewal as well as differentiation into all three germ layers. To our knowledge, the differentiation of these cells into IPCs has not been reported yet.

2.5 Mesenchymal Stem Cells and Diabetic Complications

Uncontrolled or poorly controlled DM promotes the development of serious complications. These result essentially from vascular pathologies. Microvascular affections manifest as retinopathy, nephropathy, and debilitating neuropathies. Macrovascular involvements lead to accelerated cardiac disease, sexual dysfunctions, and diabetic foot ulcers.

MSCs have the ability to migrate and home in injured tissues, where they act by secreting trophic factors and paracrine mediators, leading to their regeneration. As a result, research efforts are now directed not only to generating IPCs but also to use unmodified MSCs in the management of serious diabetic complications. Experimental evidence shows that MSCs can reverse the manifestations of diabetic

neuropathy [63] and retinopathy [64]. MSCs were utilized with success in the treatment of rabbit ulcer model [65]. It was also reported that MSCs ameliorated podocyte injury and proteinuria in a rat model with type 1 diabetic nephropathy [66]. A possible role for MSCs in the regeneration of intervertebral disk was suggested by Huang et al. [67]. Given the capacity of MSCs to home to damaged tissues, their possible role in the management of infertility or reproductive disorders was also reported [68]. Out of 86 diabetic patients, treatment with bone marrow-derived MSCs promoted ankle nonunion healing in 70 [69].

2.6 Concluding Remarks

To establish persuasive proof that a certain type of stem cell has been successfully differentiated into IPCs, Calne and Ghoneim [70] defined the following criteria: (1) coexpression of insulin and c-peptide by the same cells, (2) demonstration of insulin storage granules, (3) identification of specific gene expression similar to those of pancreatic ß-cells, (4) stepwise increase in insulin and c-peptide release as a function of increasing glucose concentration in vitro, (5) cure of hyperglycemia following cell transplantation in diabetic animals, and (6) prompt return of diabetes when these cells are removed. It is abundantly clear that stem cells, embryonic cells, and induced pluripotent or mesenchymal stem cells met these criteria at the experimental level. For the translation of these research findings to a clinical application, additional questions need to be answered: How many functioning cells are needed per kilogram body weight? How long will these cells remain functioning? What is the optimal site for their transplantation?

In August 2014, the US Food and Drug Administration (FDA) approved an Investigational New Drug (IND) application for the treatment of patients with type I DM to be carried out by a biotechnology company in San Diego, California (ViaCyte). Pancreatic progenitor cells derived from a human embryonic cell line will be transplanted within a device to prevent allogeneic rejection. Their strategy depends

on spontaneous maturation of the grafted progenitor cells into IPCs in the body. It is a first step and will not be the last. Medical history indicates that innovations are always incremental. The potentials of Muse cells are great owing to their pluripotency, the lack of teratogenicity, and the possibility of their use in an autologous fashion. The caveat in using these cells is that, with expansion, they lose their pluripotency marker and tend to differentiate into cells of their tissue of origin. Meanwhile, the potential applications of unmodified MSCs for the treatment of diabetic complications are ever growing.

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3. Stem Cell Therapies in Neurological Disorders

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3.1 Introduction

Stem cells are divided into three main groups: embryonic stem cells (ESCs), fetal stem cells, and adult stem cells. They vary in terms of their accessibility, ease of culture, and potency to give rise to a multitude of cell types.

ESCs, derived from the inner cell mass of human blastocysts, can potentially proliferate indefinitely and are capable of giving rise to all types of cells in the human body. But the use of human embryos raised ethical debate within and outside the scientific community, hampering their clinical use.

For therapeutic purposes, somatic pluripotent stem cells are the most extensively investigated group. They include (1) **neural and progenitor stem cells** residing in the central nervous system (CNS), (2) **mesenchymal stem cells** found in the bone marrow and other regions of the human body, and (3) **induced pluripotent stem cells (iPSCs), among others**. These cells hold the potential to act on a multitude of target sites of injury in the nervous system to alleviate the devastating disease symptoms where single-molecule-based pharmaceutical drugs did not bring many benefits [1, 2]. This chapter discusses these three types and others that have been investigated in clinical trials for the treatment of neurological diseases.

Mechanism of action: Stem cells share a similar therapeutic mechanism of action owing to their ability to replace or regenerate damaged tissue either (1) **directly** by differentiating into the different neuronal subtypes depending on the environmental cues in the damaged area or, most importantly, (2) **indirectly** through their numerous paracrine effects, including the secretion of growth factors and axon guidance molecules, which stimulate neuronal and axonal regeneration. They also produce numerous immune-modulating cytokines and chemokines that alleviate the inflammatory processes found in many neurological diseases, thereby contributing to the support, remodeling, and metabolism of the toxic macromolecules in the affected area.

Neurotrophic factors (*neurotrophins*): These are a family of large protein molecules secreted by cells of the developing nervous system and supporting glial cells. They are responsible for the survival, repair, and differentiation of neurons of the peripheral nervous system (PNS) as well as the CNS. This family of proteins includes nerve growth factor (NGF), neurotrophin-3 (NT-3), basic fibroblast growth factor (bFGF), brain-derived neurotrophic factor (BDNF), insulinlike growth factor (IGF), neurotrophic factor-4/5 (NT-4/5), and ciliary neurotrophic factor (CNTF).

Neurotrophins have been the target of extensive research and clinical trials for the treatment of neurological conditions. For example, the cognitive function of Alzheimer's disease animal models

was improved without altering Aß or tau pathology via the potent effects of BDNF secreted by stem cells [3].

3.2 Types of Stem Cells Available for Neurological Disease Modeling and Treatment

In this section, we will go over the main characteristics of the different types of stem cells used in clinical trials in terms of plasticity, proliferation, and expression of specific molecular markers and, finally, their therapeutic significance.

3.2.1 Neural Stem and Progenitor Cells

Neural stem cells (NSCs) were first described by Altman in 1960 [4, 5], contradicting the established notion at that time that the nervous system had no regeneration capabilities. A wealth of data has now proven that neurogenesis exists in the adult human spinal cord and in two main areas of the brain [6], one being the subventricular zone (SVZ) along the wall of lateral ventricles, where NSC-derived neurons migrate to the olfactory bulb and the striatum [7], the second being the subgranular zone (SGZ) of the hippocampus whose NSCs integrate the dentate gyrus.

NSCs and neural progenitor cells (NPCs) are both unspecialized, self-renewing cells with a differentiation capacity within the neural lineage. Progenitors have less self-renewal capacity and are more committed to neural differentiation.

3.2.1.1 Plasticity

A NSC must have the differentiation potential to give rise to the three neuronal components of the nervous system, namely: neurons, astrocytes, and oligodendrocytes. Environmental cues or the "niche," including growth factors and CNS region-specific intrinsic factors, influence the fate of adult NSCs. Understanding these elements is important in therapy where unipotency or replacing a specific neuronal subtype is the key in repair and regeneration (Fig. 3.1).

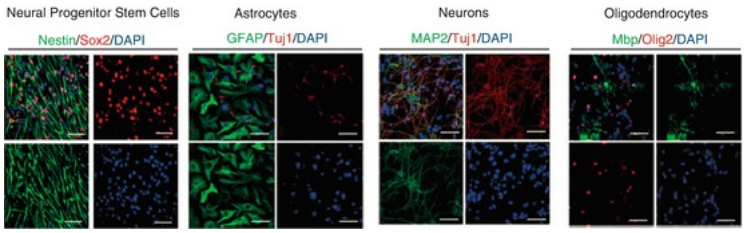

Fig. 3.1 Multipotency of induced neural progenitor stem cells (iNPSCs). Expression of marker genes: nestin and Sox2 by iNPCs, GFAP by astrocytes, Tuj1 and MAP2 by neurons, Olig2 and Mbp by oligodendrocytes. DAPI stained all nuclei. Fluorescent microscopy. *Cheng L, Hu W, Qiu B, Zhao J, Yu Y, Guan W,* et al. *Generation of neural progenitor cells by chemical cocktails and hypoxia. Cell Research. 2014;24(6):665–79*

3.2.1.2 Proliferation

The self-renewal ability is a unique feature of stem cells. NSCs undertake symmetrical and asymmetrical types of divisions in vivo [8]. NPSCs have a lesser self-renewal capability than NSCs and are more readily differentiated. Numerous protocols have been developed for the in vitro growth and large-scale proliferation of NSCs/NPSCs isolated from the human CNS [2]. Two-dimensional (2D) or three-dimensional (3D) tissue culture settings are used. The fibroblast growth factor (FGF) and the epidermal growth factor (EGF)—for late-emerging neural stem cells—are the main needed factors. In addition, telomerases are commonly used to prevent senescence related to a prolonged in vitro expansion time.

3.2.1.3 Expression of Molecular Markers

Intracellular as well as surface markers are available for the immunocytometric and hystocytometric detection of neural stem cells with flow cytometry and fluorescent microscopy, respectively. Those markers include nestin (an intermediate filament found in neuroepithelial stem cells), PSA-NCAM (a polysialylated neural cell adhesion molecule), and *Sox2*. Sox2 is highly expressed in adult NSCs, is one of the earliest functional markers of neuroectodermal specification in the embryo, and plays a

key role in neural lineage specification. Many other neural markers exist, such as TUJ-1, Mash1, Nkx2.2, Sox1, Pax6, Otx1, Otx2, NeuroD1, and Doublecortin (Dcx).

3.2.1.4 Therapeutic Effects

NSCs are capable of producing neurotransmitters that accelerate remodeling and healing in addition to all the shared mechanisms of action with other stem cells. Their differentiation potential is restricted to the neuronal lineage, eliminating the risk of cartilage or bone formation post-transplantation. On the other hand, adult NSCs are not readily accessible, and the use of fetal-derived or embryonic stem cell-derived NSC therapies is not ethically accepted in general. A new approach has recently emerged, with FDA approval, using NSCs/NPCs differentiated from other types of stem cells in clinical trials [9, 10] (Table 3.1).

Table 3.1 Examples of clinical trials using neural stem/precursor cells to treat neurological disorders

Sponsor and place	Disease	Trial phase	Patients (number)	Age at enrollment (y)	Follow-up (months)	Transplant features					Pr in
						Cell type	Cell no./patient	Route	Time after disease/injury	Immune suppression	
StemCells, Inc. at University Hospital Balgrist-Uniklinik Zurich (Switzerland)	Thoracic spinal cord injuries (SCIs)	I/II	12	18–60	12	HuCNS-SC® (fetal, brain-derived, allogeneic, single donor)	2×10^7	Multiple injections, single dose, intramedullar	≥3 months	Y (9 months pt)	Ar Cu
ReNeuron, Ltd. at Glasgow Southern General Hospital, Glasgow (UK)	Stable ischemic stroke (PISCES)	I	12	60–85	24	CTX0E303 (fetal, brain-derived, c-Myc immortalized, allogeneic, single donor)	$2–20 \times 10^6$	Single injection, four ascending doses, intracerebral (putamen)	0.5–5 years	NA	Ke M
Neuralstem, Inc. at Emory University, Atlanta (USA)	Amyotrophic lateral sclerosis (ALS)	I	18	>18	48	NSI-566RSC (fetal, spinal cord-derived, allogeneic, single donor)	$0.5–1 \times 10^6$	Multiple injections, intraspinal	≥ 1.5 years	Y (≥4 months pt)	Ev Fe M Ph
Azienda Ospedaliera Santa Maria, Terni (Italy)	ALS	I	18	20–75	36	Fetal, brain-derived, allogeneic, single donor	NA	Multiple injections, single dose, intraspinal	> 6 months	NA	Ar Ve Ph
StemCells, Inc. at University of California, San Francisco (USA)	Pelizaeus–Merzbacher disease (PMD)	I	4	0.5–5	12	HuCNS-SC®	3×10^8	Multiple injections, single dose, intracerebral	NA	Y (9 months pt)	St Hu
StemCells, Inc. at Oregon Health and Science University, Portland (USA)	Neuronal ceroid lipofuscinosis (NCL)	I	6	1.5–12	13	HuCNS-SC®	$0.5–1 \times 10^9$	Multiple injections, single dose, intracerebral	NA	Y (12 months pt)	Re St M
StemCells, Inc. Retina Foundation of the Southwest, Dallas (USA)	Age-related macular degeneration (AMD)	I/II	16	>50	12	HuCNS-SC®	$0.2–1 \times 10^6$	Single injection, single dose, subretinal	NA	Y (3 months pt)	Da Bi Ph

pt post-transplant, *NA* information not available. *Giusto E, Donega M, Cossetti C, Pluchino S. Neuro-immune interactions of neural stem cell transplants: from animal disease models to human trials. Exp Neurol. 2014;260:19–32*. External link. Please review our privacy policy

3.2.2 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are adult multipotent stem cells found in many tissues including fat, muscle, and umbilical cord, with the bone marrow being the first and most studied source. MSCs are a heterogeneous population of cells. Fate mapping studies have shown that bone marrow (BM) MSCs originate from the paraxial mesoderm, but not in an exclusive manner. Another subpopulation of MSCs originating from the neural crest (NC), NC-derived progenitors, was proven to exist [8, 11]. This helps explain MSCs' ability to differentiate into neural progenitor and neuronal cells. It also reinforces the link between the nervous and the hematopoietic systems. It was demonstrated that nestin + MSCs are NC progenitor cells that persist in the adult BM, skin, dental pulp, and other tissues. The developmental origin of MSCs could be even more diverse, which explains their heterogeneity, the need for subpopulation characterization, and the diverse therapeutic potential [12].

3.2.2.1 Plasticity

MSCs must differentiate into osteoblasts, adipocytes, and chondroblasts in vitro using the appropriate induction medium. The differentiation is verified histologically using special stains or using the appropriate gene primers with real time polymerase chaine reaction (RT PCR). In addition, MSCs have been found to differentiate into many types of cells, including myocytes, neurons, and glia cells, using favorable culturing conditions.

3.2.2.2 Proliferation

MSCs must be plastic adherent when maintained in standard culture conditions of DMEM or alpha-MEM supplied with fetal bovine serum or its human substitutes. This feature is shared with fibroblasts in addition to the morphological similarities between them (Fig. 3.2). Accordingly, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy added in 2006 two more criteria regarding the plasticity and expression of markers to define MSCs.

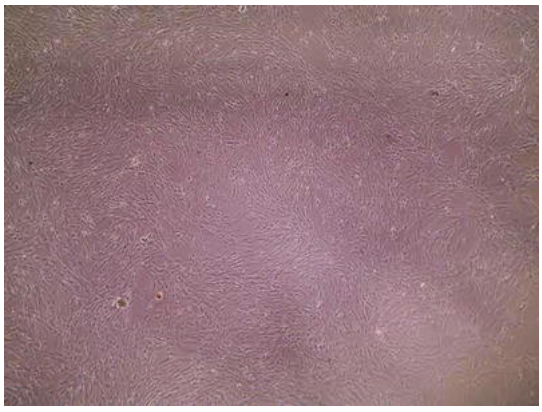

Fig. 3.2 Mesenchymal stem cells (MSCs). Light microscopy 5× magnification. Courtesy of Dr. Dana Hattab, Cell Therapy Center, Jordan University

3.2.2.3 Expression of Molecular Markers

The minimal surface marker expression criteria to define human MSCs are as follows: MSCs must express CD105, CD73, and CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR surface molecules [13]. In addition, other markers have been used to characterize and isolate the different MSC subpopulations, including CD271, CD146, CD56 (NCAM), CD166, CD106 (VCAM), CD14, HLA-ABC, CD29, CD44 (HCAM), and GD2. It is worth noting that none of these markers is unique to MSCs and that the percentages of expression of the positive markers differ depending on the source of MSCs, among other factors.

3.2.2.4 Therapeutic Effects

Unlike induced pluripotent stem cells (iPSCs) or induced neural stems cells (iNSCs), MSCs do not contain any risks related to viral transfection. This explains the relatively high number of clinical trials investigating their therapeutic benefits. These include two complementary lines: the immunomodulatory approach and the regenerative approach.

Immunomodulatory approach: MSCs have been found by numerous groups to have potent immunomodulatory effects, which can be relevant in treating neuroinflammatory diseases. MSCs suppress T cells, B cells, natural killer (NK), and dendritic cell activation and proliferation and are able to promote FoxP3 regulatory T cells [14–16]. Grafted MSCs are able to modify the inflammatory environment by shifting the macrophage phenotype from M1 to M2 and by reducing the levels of tumor necrosis factor-α and other inflammatory cytokines [17, 18]. They are thought to do so as a result of the variety of cytokines and chemokines they produce, both continuously and as a result of activation [19]. The influence of these cytokines on the milieu of injury is favorable and promotes inherent regeneration.

Neuroregenerative approach: In vitro and in vivo animal studies on human MSCs revealed the ability to promote post-injury neuronal functional recovery. This is achieved through their production of neurotrophic factors that induce the survival and regeneration of host neurons such as brain-derived neurotrophic factor (BDNF) and nerve growth factor-β (β-NGF) [19, 20]. Neuroectodermal differentiation has also been confirmed by several studies. MSCs may differentiate into neurons directly or give rise to intermediate NPSCs/NSCs capable of generating neurons, oligodendrocytes, and astroglial cells [21, 22]. The differentiated neurons show electrophysiological functional properties concomitant with the upregulation of neural-specific genes. Assuming the in vivo conditions of the transplanted MSCs are favorable, this effect can be expected to be reproduced in patients. To increase the therapeutic potential of MSCs, there is a focus now on their proper characterization and on determining more specific molecular markers of the different subtypes forming heterogeneous populations.

3.2.3 Induced Pluripotent Stem Cells

IPSCs are differentiated adult cells that return to their pluripotent state by in vitro manipulation, which results in the production of dedifferentiated and multipotent cells. In 2007, Yamanaka and his team published their work on the first successful induction of pluripotent stem cells from human skin fibroblasts by retroviral transfection of four transcription factors: Oct3/4, Sox2, c-Myc, and Klf4 [23]. These cells had a high telomerase activity resembling that of ESCs and were able to differentiate into cells of neuronal and cardiac lineages. This work was reproduced by different groups using a variety of somatic cells and including the usually discarded disc-derived cells [24].

Although not identical to ESCs in terms of their gene expression profile, there are many similarities between ESCs and iPSCs in terms of morphology, surface marker expression, feeder dependence, and in vivo teratoma formation capacity [24]. This makes iPSCs an appealing and less controversial source of stem cells for regenerative medicine. iPSCs are also readily differentiated into many cell types including neurons and myocytes. For neuronal differentiation, induction media, though expensive, are used in a variety of protocols. Small molecule signaling pathway inhibitors including DMH, a BMP inhibitor, are a cheaper substitute for the growth factors used in commercial media and were found to produce less heterogeneous cultures. These small molecule inducers of neuronal differentiation can be used in different combinations and concentrations to produce any of the four main neuronal sublineages: dopaminergic, GABAergic, serotonergic, and cholinergic/motor neurons.

3.2.3.1 Plasticity

iPSCs can be induced to differentiate in vitro into cells of the three germ layers. This is accomplished by cultivating iPSCs in suspension for about a week to form embryoid bodies, which are subsequently transferred to gelatin-coated plates for further culturing. The attached cells differentiate freely into cells expressing molecular markers of ectodermal, endodermal, and mesodermal cells. Immunocytochemistry is used to detect, for example, the glial fibrillary acidic protein (GFAP) and bIII-tubulin as ectodermal markers; α-smooth muscle actin (α-SMA), desmin, and vimentin as mesodermal markers; and a-fetoprotein (AFP) as endodermal marker of iPSC differentiation [23].

3.2.3.2 Proliferation

Following transfection with the dedifferentiating growth factors, the self-renewing ability of iPSCs is maintained for months. For that, conditions similar to ESC cultures are required. These include fibroblast feeder cells, ESC-conditioned media, and basic fibroblast factor to obtain differentiated neurons from iPSCs (Fig. 3.3).

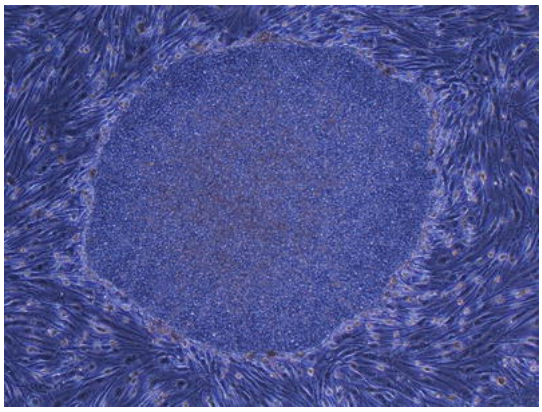

Fig. 3.3 Induced pluripotent stem cells (iPSCs). Light microscopy 5× magnification. Courtesy of Nidaa Ababneh. Pathology Department, Oxford University

3.2.3.3 Expression of Molecular Markers

Human iPSCs do not express stage-specific embryonic antigen (SSEA), but they do express known human embryonic stem cell (hESC)-specific surface antigens, including SSEA-3, SSEA-4, tumor-related antigen (TRA)-1-60, TRA-1-81, and TRA-2-49/6E (alkaline phosphatase), and NANOG protein.

In addition, iPSCs express many undifferentiated ESC marker genes at levels similar to those found in hESC lines. These markers include T3/4, NANOG, SOX2, growth and differentiation factor 3 (GDF3), reduced expression 1 (REX1), fibroblast growth factor 4 (FGF4), embryonic cell-specific gene 1 (ESG1), developmental pluripotency-associated 2 (DPPA2), DPPA4, and telomerase reverse transcriptase (hTERT) [23].

3.2.3.4 Therapeutic Effects

The therapeutic potential of iPSCs in neurological disease treatment is high (Fig. 3.4). Yet concerns remain over epigenetic and transcriptome aberrations as well as tumor formation related to this technology [5]. Extensive molecular studies of iPSC cultures to understand and eliminate any harmful risks will pave the way to many clinical applications. Because the importance of iPSCs relies on their ability to differentiate into any neuronal cell type and even subtype, they are of significance for many neurodegenerative diseases characterized by the dysfunction or loss of a specific neuronal sublineage. iPSCs' ability to give rise to mitotically active NSCs/NPSCs is also promising in cases of spinal cord injury (SCI) and other neurological conditions.

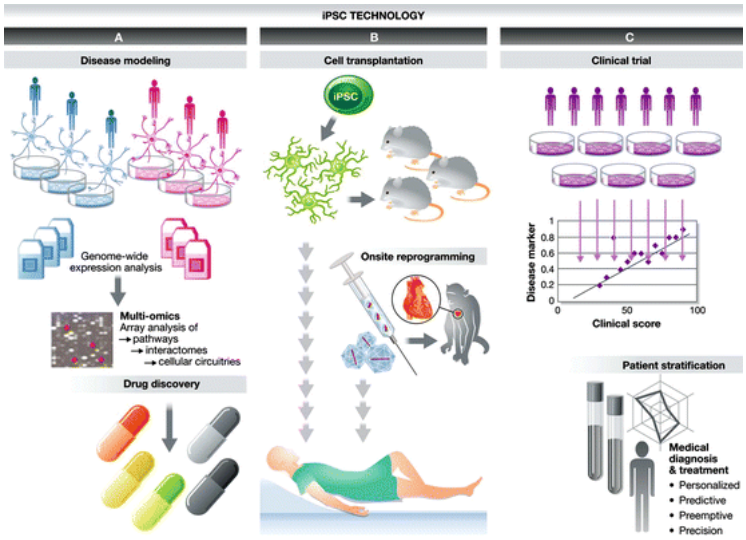

Fig. 3.4 Multipurpose use of iPSCs: (**a**) Disease modeling using patient iPSCs for "multi-omics" and "drug discovery." (**b**) Onsite reprogramming instead of "cell transplantation." (**c**) iPSCs from a large cohort of patients can be used in clinical trials and for patient stratification. *Inoue H, Nagata N, Kurokawa H, Yamanaka S. iPS cells: a game changer for future medicine. The EMBO Journal. 2014;33(5):409–17*

Cell transplantation: Although tested on many neurological animal models for years, it was not until 2013 that the Japanese government approved the first clinical study using human iPSCs. Autologous retinal pigment epithelium (RPE) cells derived from human iPSCs were to be used to treat six patients with age-related macular degeneration [25]. The proof of safety and efficacy of the iPSC technology in the treatment will encourage the initiation of many more trials around the world. The transplant ation of safe human leukocyte antigen (HLA)-matched iPSC-derived NSCs, neurons, and glial cells is promising for the treatment of many neurological diseases [26]. This technology should resolve issues of rejection and at the same time eliminate risks of transplanting autologous cells with inherent aberrations.

Disease modeling and drug screening: Neuronal subtypes derived from a patient's iPSCs are considered a recapitulation of the individual's cellular phenotype and genotype. This approach has been used in clinical trials to stratify patients into responders versus nonresponders to a therapeutic drug. It can be used either for screening patients for a clinical trial or to explain results of clinical trials where the patients' response is variable [24]. One of the most significant issues that still need to be addressed in disease modeling is that iPSC-derived neuronal cells are not as mature as adult patients'. Much research is ongoing to improve the maturity status of generated cells to better reflect the patients' own "neuronal cells."

Gene correction: The heterogeneity of iPSC clones resulting from somatic trans-differentiation can be an advantage when searching for autologous regenerative cures for genetic disorders. For instance, Hong Ma et al. isolated wild-type mitochondrial DNA (mtDNA) iPSCs from heteroplasmic fibroblasts of patients with the most common neurological mitochondrial DNA mutation syndromes [27]. These wild-type, mutation-free iPSCs produced cells with normal metabolic functions. Transplanting these rescued cells would alleviate symptoms affected by the heteroplasmy or the relative levels of wild-type to mutant mtDNA within each cell.

3.2.4 Induced Neural Stem Cells/Induced Neural Progenitor Cells

This is a novel approach that could be developed via direct lineage reprogramming of nonneuronal cell types, bypassing the need for pluripotent stem cells. Human fibroblasts and cord blood stem cells, as well as peripheral blood cells, have been reprogrammed into self-renewing NSCs, capable of giving rise to neurons and glial cells. This was achieved via the ectopic expression of all predefined transcription factors such as Sox2 or Oct4, Klf4, and c-Myc [10, 28, 29]. Direct conversion into postmitotic functional neurons has also been achieved by many groups, with the potential of use for modeling and treating neurodegenerative disorders [30, 31] (Table 3.2).

Table 3.2 Induced neural stem cell (iNSC) versus induced pluripotent stem cell (iPSC) technologies

Cell type	Advantages

Cell type	Advantages
Induced neural stem cell (iNSC) technology	Multi sample analysis, cost and time effective, and cellular maturation of neural progenitors
Induced pluripotent stem cell (iPSC) technology	Gene editing, unlimited resource, can differentiate into a large variety of cells

3.2.4.1 Plasticity

In contrast to iPSC technology, which can produce a multitude of cell types, iNSCs and iNPCs can be used to generate neural stem/progenitor cells and neurons specifically.

3.2.4.2 Proliferation

iNSCs achieve a stable neuronal state independent of viral transgene expression and can be expanded for more than 20 passages. Human neonatal foreskin fibroblasts are used as feeder layer for the culturing and expansion of iNSCs after transfection in the presence of hESC culture medium and the addition of bFGF. For neural differentiation, separated cells are grown on coated culture plates with or without astrocytes as a feeder layer. The human astrocyte layer seems more effective in directing the maturation of neurons [26].

3.2.4.3 Markers of the Generated Neurons

Similar markers for NSCs are used to characterize iNSCs, including Sox2 and nestin, while neuronal markers and neuronal sublineage markers are used to characterize the resulting neurons and glia. Examples of these markers include b-Tubulin, Map2, and the inhibitory GABA marker.

It is thus suggested that "a combination of the induced neural stem cell (iNSC) technology and the induced pluripotent stem cell (iPSC) technology has been used to produce functional neurons. These neurons have a superior neural culture purity and are generated by the transfection of only one transcription factor, either neurogenin-2 (Ngn2) or NeuroD1" [32].

3.2.5 Glial-Restricted Progenitor Cells

As their name indicates, glial-restricted progenitor (GRP) cells are self-renewing with a limited differentiation potential. They can give rise to oligodendrocytes and astrocytes but not neurons. They can be found in the developing mammalian brain and spinal cord. Most studies involving glial progenitor cells investigated their effect on the developing nervous system of model animals. Later, it was found that these cells have promising regenerative and remyelinating effects on the adult nervous system in animal models of multiple sclerosis (MS) and transverse myelitis. This would be possible by replenishing the demyelinated regions by oligodendrocytes, the myelinating cells of the CNS [33]. The protocol of their isolation and expansion was adopted by Q Company, after which these cells are sometimes called Q-cells. This company secured the approval to use these cells for the treatment of amyotrophic lateral sclerosis (ALS) [34].

According to the Q-cell protocol, human GRP cells are isolated from aborted fetal brain tissue, followed by manual and enzymatic dissociation to yield a single cell suspension. GRP cells are then purified by magnetic beads coated with anti-A2B5 antibody, which is the selective surface marker for GRP cells [35]. Subsequently, purified GRP cells are cultured and expanded for three passages in neural media supplemented with bFGF.

The estimated number of oligodendrocytes needed for transplantation is between 4–5 × 10^3 cells/mm^3 of lesion. Because the source of these cells is not abundant, they are made immortal by viral transduction with the myc-oncogene with a reporter marker for analysis. The astrocytic differentiation of GRP cells at the site of the lesion is also beneficial owing to the neurotrophic factors they produce, which counteract the inhibitory environment in the lesion [33].

3.2.6 Glial Cells

Although glial cells are terminally differentiated cells, olfactory ensheathing cells (OECs) and Schwann cells (SCs) have been used in clinical trials in protocols similar to stem cells for the treatment of neurological diseases. Therefore, they are discussed briefly in what follows.

3.2.6.1 Olfactory Ensheathing Cells

OECs are glial cells that are responsible for the continuous regeneration of olfactory axons throughout the adult life in mammals. OECs are isolated from aborted human fetal olfactory bulb, cultured for three passages, and characterized by immunohistochemistry with antibodies targeting an OEC-specific surface marker, neurotrophin receptor p75 [36]. Alternatively, OECs are isolated from the adult nasal mucosa under local or general anesthesia and cultured and expanded for about 4 weeks for autologous transplantation procedures [37].

OECs ensheathe axons spanning both the CNS and PNS parts of the primary olfactory pathway. In vitro coculture data demonstrated their ability to support the sprouting of new nonolfactory bulb axons. Their transplantation into animal models proved they could support axonal regeneration in a multitude of sites in the nervous system. This phenomenon was attributed in part to their diverse secretome consisting of glial cell line-derived neurotrophic factor (GDNF), NGF, BDNF, and neuregulins, which are well-known neurotrophic factors.

The wealth of in vitro and in vivo data in favor of OECs led to the initiation of clinical trials exploring their safety and efficacy in treating patients with SCI, amyotrophic lateral sclerosis, and other conditions [34, 37]. It is worth mentioning that there is a debate over the nomenclature of these cells and their role; some groups call them nasal stem cells, while others call them olfactory ectomesenchymal stem cells capable of neurogenesis [38].

3.2.6.2 Schwann Cells

SCs are PNS glial cells equivalent to CNS oligodendrocytes. They are mainly responsible for the myelination of axons of peripheral nerves. In addition, SCs allow damaged PNS axons to regrow and myelinate after damage. Interestingly, SCs have been found in the spinal cord at injury sites. They dedifferentiate and secrete growth factors, leading to schwannosis in humans and neuromatous structures. For years, in vitro and in vivo studies investigated their effects on CNS axons. SCs were isolated from both fetal and adult peripheral nerves, cultured, and expanded. Viral oncogene transfection is usually performed to obtain large cell numbers, except in one protocol that used autologous serum deprivation and addition to obtain safer cells for clinical trials [39]. Over the years, the safety of SCs has been established, and the potential of SCs in promoting the survival and regeneration of CNS axons was demonstrated in several clinical trials for the treatment of SCIs and ALS. The first FDA-approved clinical trial for treating paralysis is ongoing, whereas the safety of the treatment has been demonstrated in the first patient [40].

3.2.7 Hematopoietic Stem Cells and Mononuclear Cells

Hematopoietic stem cells (HSCs) give rise to all cellular blood components, including mononuclear and multinuclear cells, in addition to platelets and red blood cells. They are easily isolated from marrow, cord blood, and peripheral blood using antibody-coated beads against CD34 and CD45 specific markers. ESCs and iPSCs are good experimental sources of HSCs (Fig. 3.5). Immunocompatible, HLA-matched allogeneic bone marrow and peripheral blood transplants were the first type of stem cells successfully used in treating hematopoietic diseases. Recent advancements in HLA-matching technology have increased the success rates of these myoablative therapies. The role of HSCs, referred to as a mononuclear subset, in the treatment of neurological disorders has been investigated. In the first human trial, umbilical cord blood mononuclear cells were administered to an infant with cerebral palsy via a peripheral injection. Functional neuroregeneration was observed to proliferate via the stimulation of endogenous NSCs. A CD133-expressing subset of HSCs has been found to have superior homing capabilities to neural sites of injury. On the other hand, HSCs had lower survival rates in the brain unless combined with immunosuppressants [41].

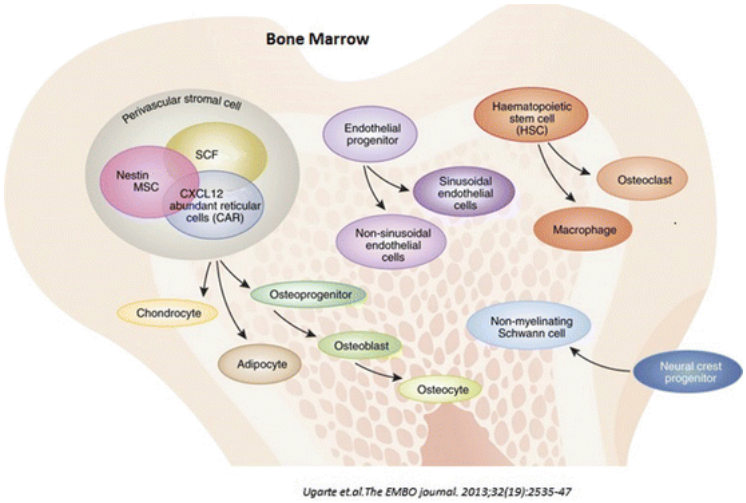

Fig. 3.5 Lineage hierarchy of bone marrow perivascular stromal cells (including MSCs) and hematopoietic stem cells [SCF and CAR are rare populations of mesenchymal stem/progenitor cells (MSPCs) highly enriched for factors implicated in HSC maintenance, including CXCL12 and SCF]. *Ugarte F, Forsberg EC. Haematopoietic stem cell niches: new insights inspire new questions. The EMBO journal. 2013;32(19):2535–47*

In a clinical trial for the treatment of MS, patients were subjected to intense immunosuppression followed by autologous HSC transplantation. The results were compared to those with patients receiving mitoxantrone (MTX), which is traditionally used in the treatment of MS. After a 4-year follow-up period, the group that had received HSCs showed a significant reduction in disease activity measured by magnetic resonance imaging (MRI) [42].

A debate exists on whether the beneficial effects of mononuclear cell-derived HSCs should be attributed to a MSC subpopulation of mononuclear cells. This highlights the need to properly differentiate between the two neighboring and complementary types of cells found in the bone marrow.

3.3 Induction of Neural Subtype Differentiation as a Tool for Neurorestorative Therapy

The development of efficient protocols to induce specific types of neurons from different stem cells and later characterize them has been an active area of research. This successful differentiation using either growth factors or small molecules has helped scientists explore therapeutic options in several diseases.

The benefits of this approach can be by direct restoration of lost damaged neurons or the use of these neurons in the characterization of the diseases, as well as pharmacological screening of these cells.

For Parkinson's disease, in vitro differentiation of dopaminergic neurons is an important tool. Cholinergic neurons are known to be affected in Alzheimer's patients. In the same manner, the degeneration of a specific subset of GABAergic neurons, the medium-sized spiny neurons (MSNs), is well characterized in Huntington's disease, while in amyotrophic lateral sclerosis, cholinergic motor neurons are the ones affected [24].

3.4 Routes of Stem Cell Administration, Homing, and In Vivo Tracking

The route of stem cell administration is an important issue to consider when planning a clinical trial for CNS diseases in order to obtain the best outcome. The choice of local or systemic transplantation mode depends on the neurological disease and on the presence of focal or multifocal lesions on the target [1]. Also, the ability of stem cells to home to the target site of injury needs to be considered when choosing among intravenous, subcutaneous, and intrathecal routes.

The **intravenous** route of stem cell injection is the least invasive but may not be the most efficient. Stem cells have been shown to reach the CNS via this route using the necessary machinery. This includes the extension of podia, followed by rolling and adhesion and firm

adhesion to the endothelial cells, allowing their extravasation to different body tissues [43]. Cells transplanted via this route have been found to accumulate in the lungs and spleen [44]. Methods to overcome this drawback are being investigated by different groups, while others have suggested immunomodulatory benefits in the CNS due to splenocyte stem cell interactions [45].

On the other hand, **intrathecal** transplantation of stem cells results in higher engraftment in the CNS site of injury. Many molecules have been found to direct stem cell homing into the site of injury, which is improved by their in vitro prior exposure to inflammatory cytokines [46].

MSCs, for instance, have been found to highly express matrix metalloproteinases (MMPs), which allow them to cross the basement membrane barrier and home to the desired site. Stem cell **homing** ability could also be related to their expression of different chemokine receptors responsive to chemokines released at the site of injury.

Our knowledge of the homing and precise effects of stem cell transplantation on patients is being hindered by in vivo **tracking** issues. These include finding safe and effective molecules, of long life span, to label the stem cells, in combination with the appropriate imaging technologies that localize stem cells with minimum artifacts. There are also issues related to the choice of controls in such tracking experiments. Most of the knowledge in this field resulted from in vitro or animal tracking experiments using contrast agents that cannot be reproduced in humans, such as 5-bromo-2′-deoxyuridine (BrdU), green fluorescent protein (GFP), and enhanced (eGFP) labeling. Nanotechnology has potential in this regard, where nanoparticles bound to different contrast molecules such as iron oxides are being used along with MRI. Few tracking studies have been conducted on human subjects, while outcome assessment has relied on clinical findings in most trials [47, 48]. The tracking durations in those studies was no more than 2 months, which is sufficient for tracking homing only. Thus, advances in tracking techniques are necessary to provide conclusive evidence of the therapeutic efficacy of MSC transplantation. Table 3.3 summarizes tracking options that have been used in clinical trials treating neurological diseases with stem cell transplantation.

Table 3.3 Stem cell tracking tools used in different clinical trials to locate stem cells after injection

	First author/year	Number of patients	Disease	Number of cells/type	Delivery method	Imaging modality	Labeling technique	Cell retention/survival (%)	Study observations
Studies based on nuclear medicine techniques (SPECT, PET, GC)	Hofmann et al. [59]	9	STEMI	2–4.5 × 10^9 BMC	ICA ICV + ICA	PET	^{18}F-FDG BMC or ^{18}F-FDG $CD34^+$ fraction of BMC	2 (ICA, 1.5 h); 3.8 (ICV + ICA, 1.5 h); 25 (ICA, $CD34^+$, 1.5 h)	Homing only to perfused area of delivery artery, no retention after ICV, higher retention for CD34+ cells
	Karpov et al. [60]	44 9	Transmural MI	9×10^7 BMMNC	ICA	SPECT	99 m$_{Tc}$-HMPAO	6.8 (2.5 h); 3.2 (24 h)	No differences in cardiac function between control and treatment groups
	Kang et al. [61]	20	STEMI	PBMNC	ICA ICV	PET	^{18}F-FDG	1.5 (ICA, 2 h); 0 (ICV, 2 h)	Cell retentions in old and new infarcts were not different
	Correa et al. [62]	1	Ischemic stroke	3×10^7 BMMNC	OTW LCMA	SPECT	99 m$_{Tc}$-HMPAO	n/a	Substantial amount of delivered cells in brain
	Schächinger et al. [63]	19	Acute–chronic MI	1.5×10^7 PBMNC	OTW ICA	GC	^{111}In-oxine	6.9 (1 h); 2 (3–4 days)	Reduced retention in chronic compared to acute MI

	First author/year	Number of patients	Disease	Number of cells/type	Delivery method	Imaging modality	Labeling technique	Cell retention/survival (%)	Study observations
	Silva et al. [64]	30	STEMI	1×10^8 BMMNC	OTW: ICA or ICV	SPECT	99 m_{Tc}-HMPAO	10.3 (ICA, 24 h); 3.1 (ICV, 24 h)	Six-month LVEF improvement is correlated with cell retention and higher in ICA group
	Barbosa da Fonseca et al. [65]	6	Ischemic stroke	$1–5 \times 10^8$ BMMNC	MCA	SPECT WB GC	99 m_{Tc}	1.7 (2 h)	Uptake primarily in hemisphere with stroke lesion
	Barbosa da Fonseca et al. [65]	6	Chagasic cardiomyopathy	$1–10 \times 10^8$ BMMNC	ICA	SPECT WB GC	99 m_{Tc}	5.4 (1 h); 4.3 (3 h); 2.3 (24 h)	Homing not correlated with number of cells administered; poor uptake in areas with perfusion deficit
	Vrtovec et al. [66]	40	DCM	1×10^8 $CD34^+$ from PBMNC	ICA, IM	GC	99 m_{Tc}-HMPAO	4.4 (IC, 18 h); 19.2 (IM, 18 h)	Six-month LVEF improvement is correlated with cell retention and higher in IM group
Studies based on magnetic resonance imaging (MRI)	Zhu et al. [67]	2	Brain trauma	NPC	Stereo-tactical injection	3 T MRI	SPIO	n/a	Migration of cells from injection site to border of lesion, signal persistent for 7 weeks
	Callera and de Melo [47]	16	Spinal cord injury	0.7×10^6 $CD34^+$ from PBMNC	Lumbar puncture	1 T MRI	Antibody: SPIO	n/a	Cell migration 35 days after injection, cells were not detected in 50% of patients

Nguyen, Patricia K., Johannes Riegler, and Joseph C. Wu. “Stem cell imaging: from bench to bedside.” Cell stem cell 14.4 (2014): 431–444

STEMI ST elevation myocardial infarction, *BMC* bone marrow cells, *ICA* intracoronary artery infusion, *ICV* intracoronary vein infusion, *IM* intramyocardial delivery, *PET* positron emission tomography, *^{18}F-FDG* ^{18}F-fluorodeoxyglucose, *MI* myocardial infarction, *BMMNC* bone marrow mononuclear cells, *SPECT* single-photon emission computed tomography, *^{99m}Tc-HMPAO* ^{99m}Tc-hexamethylpropyleneamine oxime, *PBMNC* peripheral blood mononuclear cells, *OTW* over the wire balloon infusion, *LCMA* left cerebral middle artery, *GC* gamma scintillation camera, *LVEF* left ventricular ejection fraction, *MCA* middle cerebral artery, *WB GC* whole body gamma scintillation camera, *DCM* dilated cardiomyopathy, *SPIO* superparamagnetic iron oxide nanoparticle, *NPC* neuronal progenitor cells

3.5 Animal Studies: The Transition Between Preclinical and Clinical Trials

Animal studies are still a prerequisite for any clinical trial, mainly to prove safety and to provide evidence for the efficacy of the therapeutic agent for neurological diseases, although it has been recognized that there are significant differences between humans and animal nervous system cells.

Hippocampal neurogenesis, for example, is more active in humans than in mice and declines in a less dramatic manner with age [49]. Also, many promising therapeutic results in rodent models could not be reproduced in humans. The number of registered clinical trials using human stem cells is far less than in vitro and animal studies. Both will be discussed subsequently for the main neurological diseases that remain without satisfactory treatments to date either because of the low efficacy or very high cost.

Safety issues related to tumor formation and viral transfection have hampered the use of iPSC technology in clinical trials so far in all diseases, although encouraging results from animal studies have been demonstrated by many groups for most neurological ailments.

3.6 The Case of Spinal Cord Injury: The Need for Bioscaffolds

SCI is a result of a complete or incomplete cut in any of the thoracic, lumbar, or sacral regions of the spinal cord, the main cause being vehicle or sports accidents or violence-related incidents. The available treatments are primarily surgical decompression or pharmacologically by administering high doses of methylprednisolone, which do not save patients from the debilitating results of the injury. SCI patients generally suffer from quadriplegia or paraplegia, loss of sensation, severe pain, and often urinary tract and other infections [50]. These symptoms are due to immediate posttraumatic axonal and blood vessel damage and inflammatory response to phagocytose debris. At a later stage, a scar of the extracellular matrix (ECM) is formed, surrounded by astrocytes that secrete several axonal growth inhibitors such as chondroitin sulfate proteoglycans [50], thereby forming a physical and chemical barrier against the healing and regeneration processes.

3.6.1 Animal Studies

Numerous studies have been reported using human iPSC-derived NSCs for SCI treatment in rat and mice induced spinal cord injuries. In most studies, the transplanted cells engrafted the host, differentiated into neural lineages, and showed functional locomotor improvement [51]. In addition, SCI was induced in nonhuman primates, and iPSC-derived NS/PCs were transplanted. The results were encouraging; there was neuronal differentiation, remyelination of axons due to differentiated oligodendrocytes, and gain of motor function with no tumors [52]. Similar results were observed when human NSCs were used in primate as well as chronic rodent SCI models with the addition of neurotrophic agents at the site of injury [50]. MSCs were also extensively studied using different tracking methods to understand the precise mechanism of action with respect to regeneration and attenuation of the posttraumatic inflammatory response.

3.6.2 Clinical Trials

As a result of the encouraging data related to the use of NSCs, a clinical trial was launched in 2011 by a Swiss company using fetal-derived NSCs to treat patients suffering from complete and incomplete SCI. Positive results were demonstrated with a gain of sensation in 6 patients out of 12 recruited and 8 analyzed.

Many sources of MSCs have been used in clinical trials, and they all proved to be safe (Fig. 3.6). Because SCI affects mainly healthy individuals, the use of autologous MSCs raises no questions regarding the inherent properties of the isolated stem cells. Consequently, both allogeneic and autologous MSCs have been administered to SCI patients via many routes, with encouraging results in most trials. A difference in results was observed in one study between acute cervical injury patients who improved significantly, while only a mild improvement was observed in chronic SCI patients.

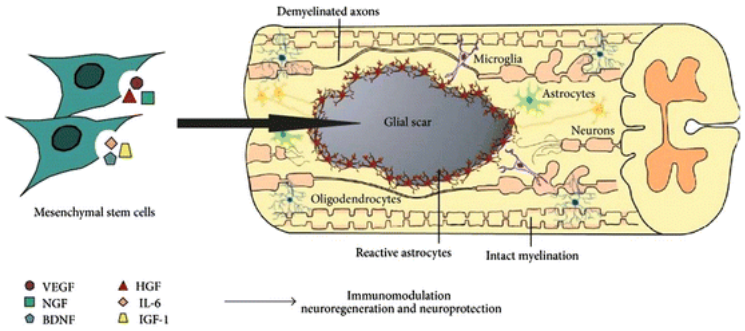

Fig. 3.6 Application of MSCs as a treatment for SCI. The MSC secretome is believed to be a key player in the promotion of neuroregeneration and neuroprotection, as well as the modulation of the inflammatory response. *Assuncao-Silva RC, Gomes ED, Sousa N, Silva NA, Salgado AJ. Hydrogels and Cell Based Therapies in Spinal Cord Injury Regeneration. Stem Cells Int. 2015;2015:948040*

Hematopoietic CD34+ bone marrow cells were injected intrathecally into chronic SCI patients. Their magnetic labeling proved their homing to the site of injury, but no clinical improvements were observed [47].

Another phase I/II clinical trial of autologous BM-derived mononuclear cell therapy for SCI patients showed neurological status improvement in one-third of patients [53].

OECs were also transplanted in two clinical trials; the first proved safe with no clinical improvement, while the other proved safe and showed locomotor improvement in 11 of 20 chronic SCI patients with tissue replacement at the site of injury [37].

3.6.2.1 Recommendations for Future Studies

With time, SCI becomes inevitably chronic and results in a multifactorial inhibitory environment with scar formation, hence the need for a combinatorial therapy approach. In addition to stem cells, bioscaffolds and neurotrophic factors can be incorporated for the best therapeutic outcome. The use of bioscaffolds along with stem cells is promising because they can physically mimic the CNS tissue to be replaced. Many types of biomaterials have been tested, with natural-based hydrogels being the most appealing for neuronal regeneration. These hydrogels are composed of macromolecular-based components with a high water content that can act as a filler at the site of injury. Examples of natural hydrogels are fibrin, collagen, agarose, gellan gum, and chitosan. They meet certain required criteria in terms of biocompatibility, biodegradability, and porosity. They are also permeable to nutrients, waste products, and ions [50]. Encouraging animal studies resulted from using different stem cell types in combination with a variety of hydrogels. It is thus highly recommended for future trials to incorporate the use of hydrogels in their attempt to treat SCI with stem cells. In addition, a closer look at stem cell secretome would enable the identification of potent neurotrophic factors, which would then be simultaneously and repetitively injected into the site of SCI to accelerate the healing process.

3.7 Amyotrophic Lateral Sclerosis: A Case of No Prevention and No Effective Conventional Treatment

ALS is another neurological disease for which patients and physicians are eager to see effective stem cell therapy. This is reflected by the wealth of animal studies followed by numerous clinical trials that have used almost all the existing stem cell types as well as their progenitors. This was encouraged by the absence of effective pharmaceutical drugs, pushing some companies to adopt stem cell types for clinical research.

ALS is a devastating neurodegenerative disorder that leads to paralysis and respiratory insufficiency with a life expectancy of 2–5 years from onset. It is characterized by the progressive degeneration of motor neurons in the spinal cord, cortex, and brain stem. While 5% of ALS is familial due to genetic alterations, 95% of the cases are sporadic with unknown etiology. It has been proposed that hyperexcitability and neurodegeneration of the corticospinal motor neuron precede the symptoms [54, 55]. Accordingly, the intraspinal route is used to deliver stem cells to the dying neurons, while intrathecal injections help the injected stem cells reach the brain. Systemic injections of stem cells into the blood stream as well as intramuscular administration have been used. The regeneration of nerves in the affected areas is the ultimate goal of all experimental and clinical

studies on ALS treatment. One approach explored by many groups is the replacement of damaged neurons by new transplanted ones. The challenge in this disease is in the long distance the axons of the transplanted neurons need to travel in order to reach the muscle they innervate. The distance is much shorter during the developmental stages when the axon first makes contact with the muscle in response to specific environmental cues. It would then be more realistic to expect stem cells to counteract the inhibitory, inflammatory environment that is consequent to degeneration. A more permissive environment would promote the survival of existing neurons through the release of stem cell secretome.

Table 3.4 compares the design and outcome of many published clinical trials in this field. The first trial was performed in 2003, where Mazzini and his group aimed to assess the safety of autologous MSC injection in ALS patients [34]. Autologous SCs were also used by a few groups and proved to be safe and promising [56]. Scientists continue to use experimental animal models to explore new treatment approaches. In a recent study, gene therapy was combined with stem cell transplantation in a murine ALS model. Umbilical cord mononuclear cells were transfected with epidermal growth factor protein (EGFP). The results were encouraging, and the mice showed better survival, motor and explorative activities, and grip strength [41]. Such combinatorial approaches will be needed in future clinical studies to improve the efficacy of transplanted stem cells.

Table 3.4 Reported clinical trials using different types of stem cells for the treatment of ALS

	Type of cell	Method of delivery	Study type Sample size	Results	Study
1	OECs	Intraspinal	Efficacy	Delayed progression + beneficial effects using ALSFRS-R	[68]
			First: 15—OECs, 20—control	0.07 vs. 6.12 score deterioration over 4 months between treated sample and control	[36]
			Second: 42 OECs		
2	Autologous MSC	Intraspinal	Phase I	Safe and feasible	[69–72]
		Intrathecal	Safety + efficacy	Safe. **Trend toward disease stability using** ALSFRS-R	[73, 74]
		Motor cortex		Safe + extension of median survival time	[75, 76]
4	T cell + MSC + NPC therapy	Intravenous	Sample size 7	Improved median survival	[77]
			Safety and preliminary survival results		
5	Endogenous MSC mobilization with G-CSF stimulation	–	Pilot study	Safe but no significant effect found on disease progression using ALSFRS-R	[78]
7	NPC	Intraspinal	Phase I only	Phase I: safe and feasible	[57, 58, 79]
			Sample sizes 6 and 12		

There is definitely room for innovation in this area to make results stemming from clinical trials using stem cells more reliable and ethical at the same time.

3.8 Control Group Selection and Ethics

Although the use of controls in clinical trials usually provides more confidence in the results and conclusions, the use of placebo in stem cell transplantation is debatable in devastating neurological diseases, both from an ethical and scientific point of view. From an ethical point of view, the potential treatment in stem cell transplantation cannot be exchanged with placebo in patients with short life expectancies owing to the severity of the diseases. This is especially true when the follow-up periods are no less than a year, when time means aggravation of the clinical status.

In addition, institutional review boards (IRBs) are unlikely to approve putting patients through invasive procedures related to the harvest of stem cells or their transplantation without any expected benefits. On the other hand, from a clinical and scientific point of view, there are no true controls in many neurological disorders. Neurologists agree that in many conditions, it is very hard to find

matching patients presenting the same clinical features, and thus the patients cannot be accurately compared.

While many clinical trials include sham patient groups, some innovative ideas have been used, including injecting the control group at a delayed period of 6 months. Other groups have considered patient history as the control comparing the post- to the preinjection time period. In this way, every patient is his/her own control, which saves the control group the delay period. Also, in cases where the affected regions are bilateral such as muscle dystrophies and ALS, unilateral injection is a good alternative, where only the neurosurgeon would know the treated side while the rest of the team would be blinded when assessing the patient's progress.

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4. Stem Cells in Diseases of Aging

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Keywords Stem cells – Aging-related diseases – Cardiovascular diseases – Neurodegenerative diseases – Diabetes – Osteoporosis – Cancer

Abbreviations

AD Alzheimer's disease

ASCT Allogeneic stem cell transplantation

BMT Bone marrow transplantation

EPCs Endothelial progenitor cells

ESCs Embryonic stem cells

GVHD Graft-versus-host disease

HSCs Hematopoietic stem cells

IBM-BMT Intrabone marrow-bone marrow transplantation

iPSCs Induced pluripotent stem cells

MHC Major histocompatibility complex

MSCs Mesenchymal stem cells

PD Parkinson's disease

SAMP Senescence-accelerated mouse prone

T2DM Type 2 diabetes mellitus

TECs Thymic epithelial cells

TT Thymus transplantation

UCB Umbilical cord blood

4.1 Introduction

Aging is associated with cellular or molecular damage that ultimately induces the development of various chronic diseases such as cardiovascular diseases (CVDs), neurodegenerative diseases, type 2 diabetes mellitus (T2DM), and cancer [16]. These diseases have been shown to be associated with an aging immune system. The immune system cells include lymphoid and myeloid lineage cells; lymphoid cells, including T and B cells, which play an important role in adaptive immunity; and myeloid cells, including megakaryocytes, erythrocytes, monocytes, and polymorphonuclear leukocytes. One report has indicated that aging is associated with alterations in the T-cell and B-cell compartments and with a decline in immune functions [69]. Moreover, hematopoietic stem cells (HSCs) show aging-related changes, including an increase in myeloid cells and a decrease in lymphoid cells [74]. One report has shown that the number of T- and B-cell progenitors decreased with aging, resulting from higher rates of apoptosis, while the number of memory T cells increased with aging [68].

The thymus is a lymphoid organ, and aging-related changes result in a reduction in thymic lymphopoiesis and a disruption of the thymic architecture [55]. The thymus mainly consists of T-cell precursors, thymocytes, and thymic epithelial cells (TECs). T-cell progenitors from the bone marrow enter the thymus and differentiate into mature cells by negative and positive selection, and the mature T cells then enter the peripheral blood [65]. Subsets of T cells include

helper, cytotoxic, memory, regulatory, and natural killer T cells. Memory T cells are antigen-experienced T cells and are classified into central memory and effector memory T cells. Phenotypic and functional changes in the effector memory T cells are features of immunosenescence. Naïve T cells respond to pathogens that enter the body, but in the elderly, there is a reduction in immune responses to new pathogens, suggesting that the number of naïve T cells also diminishes with age. Such reductions are associated with decreased numbers of signal joint T-cell receptors [27].

Moreover, thymic cellularity decreases with age even when there is no change in the major thymocyte subsets. As well, thymocyte development in the older thymus shows more resistance to apoptosis than in the young [8]. TECs provide signals during thymopoiesis and are thus important for thymic involution, but one report indicated that adipocytes infiltrate the aged thymus, affecting the TECs [23]. Moreover, accelerated thymic aging is primarily a function of stromal cells, and stromal gene expression changes in the aged thymus [32]. Thus, preventing aging-related changes in the immune system and any loss of function in the thymus should help ameliorate aging-related diseases.

Stem cells include embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and tissue-derived stem cells such as bone marrow (BM), umbilical cord blood (UCB), and adipose tissue-derived stem cells, which are used to treat a range of diseases [60]. Stem cell treatments involve the use of stem cells to replace damaged tissues and help heal diseases. In this chapter, we focus on stem cell treatments for aging-related diseases such as CVDs, neurodegenerative diseases, T2DM, osteoporosis, and cancer.

4.2 Stem Cell Biology

ESCs are derived from 5-day preimplantation embryos and are able to differentiate into endoderm, mesoderm, and ectoderm cells. Somatic stem cells are found in many organs but can only differentiate into all cell types of the organ. Somatic cells can be induced into iPSCs by inserting transcription factors

[64], and these iPSCs can directly differentiate into other cell types. Both these stem cells have self-renewal and differentiation capacity. Some reports have indicated that mouse fibroblasts can directly reprogram themselves to become neural stem cells [34], cardiomyocytes [39], bipotential hepatic stem cells [106], and endothelial cells [54]. However, the restricted proliferative and lineage potential of the resulting cells limits the scope of their potential applications.

Stem cell aging is related to reactive oxygen species (ROS), DNA damage, and mitochondrial dysfunction with aging [71]. ROS has been shown to affect stem cell functions in aged human mesenchymal stem cells (MSCs) [92], and one report showed DNA damage in stem cells and that DNA damage affects stem cell functions with aging [9]. Aged HSCs showed age-related nontelomeric DNA damage in these stem cells in aged humans. Moreover, aging reduces the capacity for the repair of double-strand breaks, contributing to the age-associated accumulation of DNA damage [79]. Sirt1 is a class III histone deacetylase within the sirtuin family of related proteins that is uniquely dependent on NAD^+ for catalysis. Sirt1 has been implicated in processes as varied as metabolism, differentiation, cancer, stress response, and aging [14]. One report showed that Sirt1 function decreases in human MSCs with aging [107]. In contrast, mitochondria regulate different metabolic and signaling pathways, but it has been reported that mitochondrial function decreases with aging [13].

Age-related hematologic changes are reflected by a decline in bone marrow cellularity and a declining adaptive immunity [33, 61]. Aging is associated with profound alterations in the innate immune system, as exemplified by alterations in the T-cell and B-cell compartments, a functional decline in monocytes and macrophages, low expression of Toll-like receptors from activated splenic and peritoneal macrophages, and an altered secretion of several chemokines and cytokines [59]. Stem cell transplantation has been used to replenish stem cells in case of degenerative diseases such as osteoporosis, diabetes, and neurological diseases [46, 82]. Intrabone

marrow-bone marrow transplantation (IBM-BMT) can replace both HSCs and MSCs, meaning that hematopoietic recovery is rapid even in donor-recipient combinations across major histocompatibility complex (MHC) barriers. Thus IBM-BMT has been proven to be the best method for allogeneic BMT [51]. In our experience, IBM-BMT has succeeded in experimental animals in the treatment of aging-related diseases such as osteoporosis, AD, T2DM, and cancer (Fig. 4.1). These results suggest that the transplantation of stem cells may improve immune dysfunction and slow the aging process, which has been borne out in experimental studies [40].

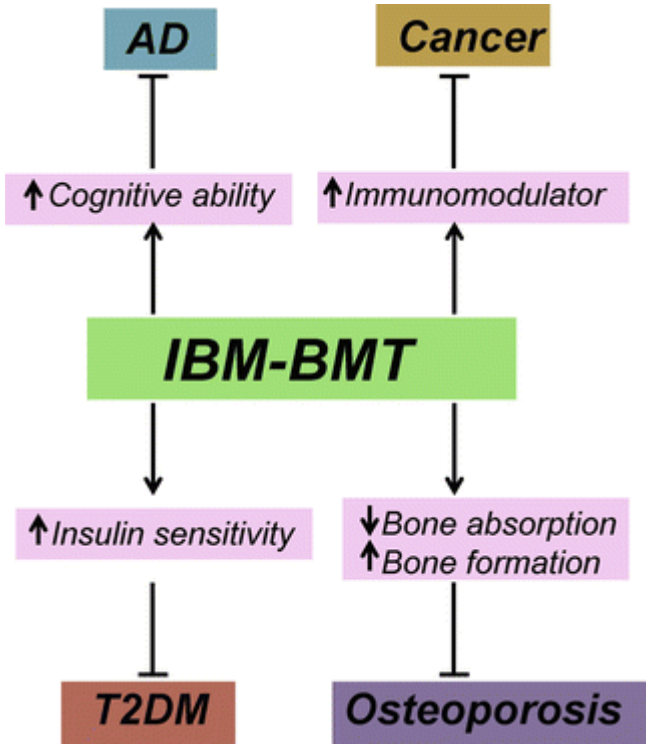

Fig. 4.1 IBM-BMT improves aging-related diseases

4.3 Stem Cell Treatments for Cardiovascular Diseases

CVDs are a major cause of death worldwide. Cell-based therapies, such as those based on BM-derived stem cells, skeletal myoblasts, resident cardiac stem cells, ESCs, and iPSCs, have reportedly been used for cardiomyocyte regeneration. Moreover, the transplantation of BM-derived stem cells, MSCs, and cardiac stem cells has been reported to be useful in the treatment of CVDs [21]. MSCs, which are able

to differentiate into myocardiocytes in vitro and in vivo and are able to repair a damaged myocardium, are mainly isolated from BM, adipose tissue, and UCB [6, 15]. MSCs strongly inhibit the maturation and functioning of monocyte-derived dendritic cells, alter the natural killer cell phenotype, and suppress the proliferation and cytokine secretion of natural killer cells [80, 91]. One report has shown that, in animals, treatment with MSCs is probably safe and effective following acute myocardial infarction [77]. Furthermore, the differentiation of human MSCs into myocardiocytes was reported in the healthy heart [96], and transplanted MSCs were able to improve myocardiocyte damage and regeneration in the ischemic area [88]. MSCs have been used to treat ischemia-related heart diseases in phase I/II clinical trials, suggesting that MSC treatment is a potential therapy for CVDs [20]. A very recent review summarizes the use of MSCs to treat CVDs, from basic to clinical studies [43]. $CD34^+$ BM stem cells have also been used to treat cardiac diseases clinically [30].

One report showed that ESC-derived myocardiocytes improved infarcted heart function in a rat model [52]. And yet another report showed that human ESC-derived myocardiocytes suppressed arrhythmias when the differentiated cells were transplanted into the injured heart in a guinea pig model [89]. Moreover, human ESC-derived cardiomyocytes can generate large-scale myocardiocytes in the infarcted monkey heart, although there was no improvement in arrhythmic complications [19]. Human iPSCs were generated from human fibroblasts using transcription factors OCT4, SOX2, NANOG, and LIN28. Functional myocardiocytes were induced from iPSCs, showing responsiveness to β-adrenergic stimulation [108]. One review suggested that iPSCs might be a source of cells for repairing damaged myocardiocytes [103].

4.4 Stem Cell Treatment for Neurodegenerative Diseases

Aging-related neurodegenerative diseases mainly include Alzheimer's disease (AD) and Parkinson's disease (PD). AD

and PD are the most common forms of dementia in the elderly, with β-amyloid plaques, neurofibrillary tangles, and neurodegeneration being hallmarks of AD and β-amyloid fibrils having been shown to lead to calcium influx and neuronal death [22]. The pathologic features of PD show that dopaminergic neurons decrease in the midbrain, resulting from the activation of microglia [42]. Both AD and PD have been shown to be associated with oxidative stress that was induced by an imbalance between oxidant and antioxidant agents. Oxidative stress induces damage to macromolecules and disrupts the reduction/oxidation (redox) signaling. Moreover, reports have shown that mitochondria contain many redox enzymes and generate ROS, resulting in mitochondrial dysfunction in AD and PD [26, 38].

Pharmacological therapies for AD and PD are mainly aimed at relieving symptoms, but stem cell therapy has the potential to not only regenerate new neurons and replace damaged neurons but also to modulate the immune system. Stem cells have been shown to secrete neurotrophic factors that modulate neuroplasticity and generate new neurons [25]. One report has shown in an animal model that stem cell transplantation improves cognition and memory by increasing acetylcholine levels [75]. Moreover, BM-derived MSCs were able to remove β-amyloid plaques from the hippocampus and to reduce β-amyloid deposits in an AD mouse model [83]. Induced neurons from BM-derived MSCs improved learning and memory in Abeta injured mice [101].

BM cells have also been shown to increase the number of activated microglia and to reduce amyloid deposits via phagocytosis of Abeta and to thereby prevent the progression of AD [90]. We used allogeneic IBM-BMT to transfer normal BM stem cells to senescence-accelerated mouse prone (SAMP) 8, an AD model mouse, which we used for examining the effects of IBM-BMT on spatial learning and memory ability. Analyses of water maze tests showed the impairment of spatial memory in SAMP8 to have been ameliorated [58]. Human neuron-like cells differentiated from UCB-derived MSCs decreased Abeta deposition and improved memory deficits in this AD mouse model [104]. Furthermore, adipose-

derived MSCs were injected into the cerebrum, and these cells activated microglia and ameliorated the neuropathological defects in AD mice [66]. There is a report showing that neuron progenitor cells differentiate from ESCs and help ameliorate AD when transplanted into an animal model of AD. This report also showed that ESCs could differentiate into astrocytic and neuron-like cells that ameliorated memory impairment [95]. Human ESCs differentiated into ganglionic eminence-like cells, and learning and memory were improved when these induced cells were transplanted into AD mice [63].

In contrast, one report showed that neuron-like cells could differentiate from human BM-derived MSCs and improve PD in an experimental animal model [102], while another showed that human UCB-derived MSCs could improve damaged neurons in PD animal models [62]. Human neural crest-derived stem cells differentiated into dopaminergic neurons in vitro and improved rotational behavior functions when these differentiated cells were transplanted into PD rats [70]. Undifferentiated mouse ESCs can secrete dopamine after mitomycin treatment, suggesting it would be beneficial for treating PD [1]. iPSC-derived neuron-like cells benefited PD when these cells were transplanted into a PD rat model [35]. One review has suggested the clinical use of stem cell-based therapies for PD, although this potential is limited by ethical and practical considerations [31]. One report showed that dopaminergic neurons could be induced from human ESCs, iPSCs, and nonhuman primate iPSCs and are useful for PD therapy [93]. Thus, advanced stem cell therapy seems to be a potential clinical approach to the treatment of neurodegenerative diseases.

4.5 Stem Cell Treatment for T2DM

T2DM, in which there is impaired insulin sensitivity and loss of beta cells, is associated with obesity as well as lifestyle and nutritional factors, while there are also genetic factors related to its development [4]. General therapies for T2DM, such as those using diets and drugs, fail to maintain normal blood glucose levels all the time, and islet or pancreas

transplantation is limited by a lack of donors. Thus, stem cells have been the focus of studies into alternative treatments in experimental animals and clinical applications. One report showed that BM-derived MSCs can differentiate into insulin-producing cells and that these induced insulin-producing cells improved hyperglycemia when transplanted into diabetic mice [28]. Moreover, multiple intravenous BM-derived MSC injections normalized hyperglycemia in rats in which T2DM had been induced by a high-fat diet and streptozotocin [36]. When human BM-derived MSCs were cocultured with human islets and proinflammatory cytokines such as interferon-γ and TNFα were added, the BM-derived MSCs were shown to protect human islets from cytokines [105]. Autologous BMT was shown to decrease insulin requirements, which correlates with stimulation of C-peptide in T2DM patients [11]. Furthermore, one report suggested that the implantation of autologous BM mononuclear cells for the treatment of T2DM not only is safe and effective but also partially restores the function of islet beta cells and helps maintain blood glucose homeostasis [37]. Another report showed that functional beta cells could be generated from human pluripotent stem cells in vitro and that these differentiated cells expressed markers of mature beta cells and secreted insulin in response to glucose [73]. We previously described how BMT could reduce high blood glucose levels in KK-Ay mice, a T2DM mouse model [57]. We also used a combination of IBM-BMT and thymus transplantation (TT) to treat the db/db mouse, another T2DM mouse model, because this mouse exhibits a marked reduction in the size and cellularity of the thymus [48]. Our results showed that IBM-BMT + TT increased insulin sensitivity and decreased blood glucose levels from a normalization of the ratio of CD4:CD8 in the peripheral blood, an increase in adiponectin levels, and enhanced insulin receptor sensitivity [56].

ESC-derived insulin-producing cells have been shown to reverse hyperglycemia in streptozotocin-treated diabetic mice when transplanted into these mice [78]. Human ESCs effectively differentiated into islet-like cells, and these insulin-producing cells ameliorated hyperglycemia in NOD/SCID diabetic mice when transplanted into these mice [12]. On the

other hand, iPSCs can differentiate into insulin-producing cells responding to glucose stimulation, thereby improving the hyperglycemia in T2DM mouse models [5]. In conclusion, stem cells are potentially invaluable agents for the treatment of T2DM.

4.6 Stem Cell Treatment for Osteoporosis

Osteoporosis is a degenerative bone disease characterized by decreased bone mass and microarchitectural alterations. Osteoporosis is defined as a bone mineral density below the mean peak bone mass of young healthy adults. MSCs may differentiate into osteoblasts, but the capacity to do so decreases with age [87]. Therapeutic strategies include nonpharmacological treatments such as vitamin D supplementation and physical activity and pharmacological treatments to decrease bone resorption or directly stimulate increases in bone mass. Drugs such as bisphosphonates and denosumab, as well as hormone replacement therapy, have been used to treat osteoporosis [10]. Basic experiments have shown that cytokines such as IL-6, TNFα, and TGFβ play an important role in the regulation of osteoblastogenesis and osteoclastogenesis. One report indicated that strontium ranelate acts on lineage allocation of MSCs by antagonizing the age-related switch in osteoblasts to adipocyte differentiation via mechanisms involving the nuclear factor of activated T-cell and Wnt signaling, resulting in increased bone formation and an attenuation of bone loss in senescent osteopenic mice [81]. Importantly, MSC differentiation into osteoblasts or adipocytes is regulated by transcription factor activity, Wnt signaling, hedgehog signaling, and BMP signaling in the BM [41]. The senescence-accelerated mouse prone (SAMP) 6 is a mouse that shows accelerated senescence and that spontaneously develops osteoporosis [17]. When such mice were treated with IBM-BMT, the BM microenvironment was normalized, the IL-11, IL-6, and RANKL levels increased, and the imbalance between bone absorption and formation was ameliorated, resulting in the prevention of osteoporosis in these mice [94, 98]. Human UCB-derived cells improved the balance between osteoblastic and osteoclastic

activity, resulting in decreased osteoporosis in these mice [2]. Moreover, human adipose tissue-derived stroma cells increased serum osteocalcin levels and enhanced osteogenic differentiation in ovariectomized nude mice [18]. Autologous cultured osteoblasts were used to treat bone fractures and showed significantly accelerated fracture healing without any complications. This suggested that autologous cultured osteoblast injection may be a useful therapy for bone fractures [45]. Granulocyte colony stimulating factor (G-CSF)-mobilized $CD34^+$ peripheral blood cells were used successfully to clinically treat tibial nonunion [50]. Human ESCs may generate osteogenic cells, and human ESC-derived MSCs may secrete bioactive factors to improve osteogenesis [7, 47]. iPSCs from mice were induced to differentiate into MSCs, and these induced MSCs were able to differentiate into osteoblasts [53].

4.7 Stem Cell Treatment for Cancer

Cancer is strongly related to aging and can be classified into five major categories according to the histology: carcinoma, sarcoma, myeloma, lymphoma, and mixed types. Cancer treatment mainly includes surgery, radiotherapy, chemotherapy, and immunotherapy. Although radiotherapy and chemotherapy slow the development of cancer, these interventions are prone to side effects. Immunotherapy is an effective method in which immune cells such as T cells are able to recognize aberrant proteins from tumor cells and either destroy the tumor cells or inhibit their growth [67, 85]. Allogeneic stem cell transplantation (ASCT) has been proven to be a useful method of treating hematological malignancies via donor-derived lymphocytes, which are used to eradicate residual tumor cells [76]. ASCT has also been shown to be an effective immunotherapy for leukemia [72]. Leukemia is a group of hematologic malignancies that includes acute and chronic myeloid leukemia. Some leukemia cells express CD80 and CD86, and some leukemia cells may express MHC class I and II, which can be recognized and eradicated by T cells [100]. We performed experiments to treat leukemia by allogeneic BMT using leukemia-bearing mice induced by EL-

4 cells. EL-4 cells are derived from the thymoma of mice and can induce the mimicking of leukemia in mice. Our results showed that IBM-BMT + TT prevented the growth of leukemia by improving mitogen responses to both T and B cells and significantly increased IL-2 production, with IL-2 having been reported to protect against allogeneic BMT-induced GVHD [84, 109]. Thus, IBM-BMT has proven to be useful for the treatment of aging-related diseases.

HSC transplantation is a useful therapy for hematological malignancies, but this intervention also induces graft-versus-host disease (GVHD), which injures the thymus, including inducing the apoptosis of TECs and delaying T-cell recovery and thereby injuring the immune system [97]. Allogeneic HSC transplantation is a general therapy for cancer and immunodeficiency disorders and helps by reconstituting the immune system. Human leukocyte antigen, which is the human version of MHC, allows the immune system to distinguish between self- and non-self-derived proteins or cells. Specifically, T-cell reconstitution is affected not only by aging but also by human leukocyte antigen, which is mismatched after allogeneic HSC transplantation [86]. One report showed that allogeneic HSC transplantation was a potential treatment for non-Hodgkin's lymphoma [3]. Naïve stem cells enhanced host antitumor immune responses to decrease the proliferation of tumor cells [44]. Moreover, one report showed that human MSCs, which express IL-12, have antitumor effects in mice [29]. ESCs had an antitumor effect when injected with a lung cancer cell line into mice [24]. iPSCs were generated from mature $CD8^+$ T cells, and these iPSCs were able to generate $CD4^+CD8^+$ double-positive cells, which then generated an abundance of $CD8^+$T cells, specific to human tumor antigen, suggesting this approach may be a therapy for cancer [99]. Human natural killer cells, a kind of lymphocyte that prevents tumor growth, have been used for anticancer therapy. The use of induced natural killer cells from iPSCs should thus be a useful therapy for cancer [49].

4.8 Conclusion and Future Direction

Immune dysfunction, including defective T cells and B cells, may accelerate the aging process in experimental animals, and stem cell treatments have been shown to be a valuable strategy for the treatment of aging-related disorders in such animals (Fig. 4.2). Future studies will focus on related mechanisms through which stem cells improve or ameliorate aging-related diseases. Additionally, we will attempt to determine whether stem cells can be used to prevent or treat other aging-related diseases

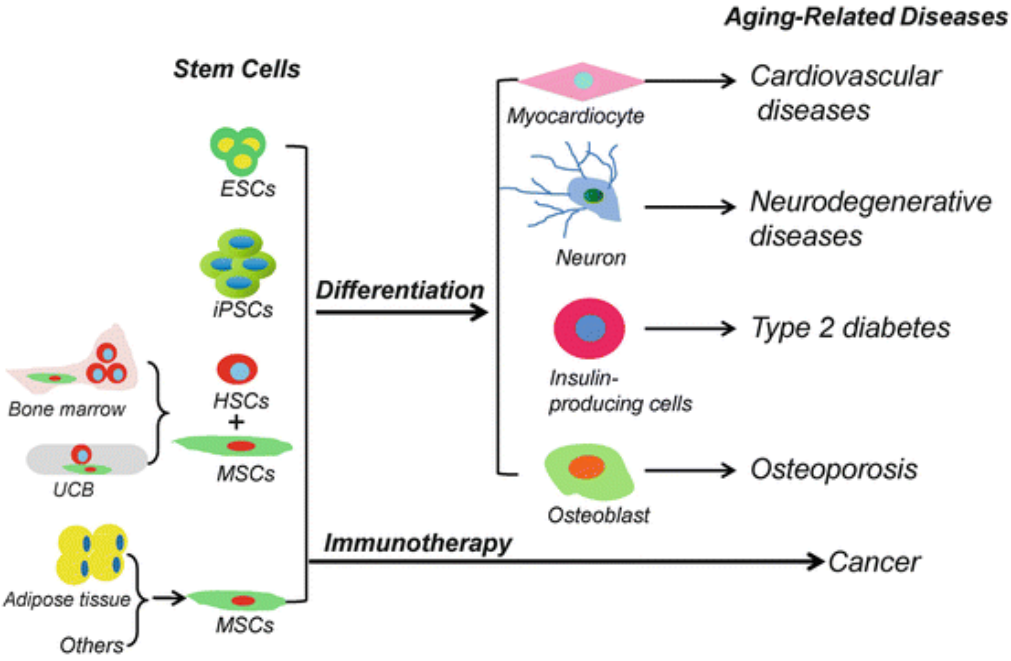

Fig. 4.2 Stem cell treatment for aging-related diseases

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5. Therapeutic Prospects of Stem Cells in Benign Urological Conditions

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5.1 Introduction

Stem cells (SCs) have the potential to divide indefinitely and differentiate into different specialized tissues and organs. This self-renewal and differentiation potential makes them valuable for tissue repair [1]. There are several types of SCs, based on their differentiation potential: totipotent, pluripotent, multipotent, unipotent, and induced pluripotent [2].

Totipotent SCs have the highest differentiation potential, with the ability to differentiate into any tissue cell type. Examples of this SC type are the zygote and morula. Pluripotent SCs can differentiate into cells from the three different germ cell layers but not extraembryonic tissues. Embryonic SC (ESC), a derivative of the inner cell mass of the blastocyst, is an example. Multipotent SCs are capable of differentiating into organ-specific cell types. Examples include hematopoietic, mesenchymal stem cells, and neural stem cells. In contrast, unipotent SCs can give rise to only one defined cell type, such as epithelial cells.

Induced pluripotent stem cells (iPSCs), on the other hand, are reprogrammed cells, where differentiated cells are induced to express ESC genes, thereby behaving like one. They are capable of differentiating into cells of any tissue [2].

Under normal conditions, SCs are in a quiescent state, and they become stimulated to divide and regenerate tissues when tissue damage ensues. The niche, which is the microenvironment for SCs, is crucial for this process through its unique characteristics such as proximity to the blood stream, presence of certain cytokines and growth factors, and low oxygen tension. This allows optimal cell-cell interaction and interaction with surrounding molecules [3].

5.2 Erectile Dysfunction

Erectile dysfunction (ED) is the inability to achieve or maintain penile erection satisfactory for sexual intercourse [2]. It negatively impacts the quality of life and relationships of patients with their partners [4]. ED affects 52 % of men aged 40–70 years, with growing prevalence with increased age [5]. Several treatment options are available for ED, including lifestyle modifications (such as exercise and weight loss), oral phosphodiesterase 5 inhibitors (PDE5Is), intraurethral alprostadil (MUSE®), intracorporal (IC) injections, vacuum erection devices, and surgery (including penile revascularization and penile implants). Despite the success of many of these options, several factors limit their use. For instance, there are contraindications for the use of PDE5Is in

patients taking nitrates (due to serious hypotension). Some patients become intolerant to side effects, in addition to the high cost, and the fact that not all patients achieve a satisfactory outcome [6]. These options are not directed at curing ED but rather providing symptomatic management, thereby encouraging the development of a curative therapy for ED, such as SC treatment [7].

Several conditions are associated with ED through different mechanisms. Aging causes diminished response to cavernosal nerve stimulation [8] and decreases nitric oxide (NO) levels through increasing levels of reactive oxygen species (ROS), resulting in endothelial dysfunction [9]. In addition, structural changes also occur with aging, including degenerative changes in elastic fibers and reduction of smooth muscles with the abundance of collagen fibers [10]. Diabetes mellitus (DM) is associated with reduced smooth muscle and endothelial cells and reduced cavernosal NO [11]. Hyperlipidemia results in endothelial and neuronal dysfunction by reducing levels of cavernosal NO [12].

Despite the development of nerve-sparing radical prostatectomy (RP) to reduce the incidence of post-RP ED, cavernosal nerve (CN) may occur in nearly 20 % of patients at 2 years after nerve-sparing RP [13]. This is likely due to smooth muscle apoptosis and fibrosis, neurapraxia, and reduced NO production [14]. Radiation-based therapies cause ED through similar mechanisms [15].

5.2.1 Types of SC Therapy Used in ED

Several SC types have been studied in the treatment in ED. ESCs improved erectile function in a CN injury ED model in the first study investigating the use of SC therapy in ED [16]. Because of ethical concerns, however, no further studies were done using these cells. In one study, vascular endothelial growth factor (VEGF)-transfected endothelial progenitor stem cells (EPSCs) demonstrated improvement in erectile function in DM rat models [17]. Several preclinical studies have shown the beneficial effect of bone marrow-derived stem cells (BMSCs) on erectile function in aging, CN injury, and DM rat

models [18–20]. Skeletal muscle-derived stem cells (SKMSC), which are easily obtained through muscle biopsy, have been shown to improve erectile function in a CN injury and aging ED rat models [21, 22]. Neural crest SCs showed the potential to differentiate into smooth muscle cells (SMCs) and endothelial cells in the rat penis [23]. Adipose tissue-derived stem cells (ADSCs) are the most widely used type of SCs in ED [7]. They improve erectile function by promoting angiogenesis and through direct transformation to neurons, smooth muscle cells, and endothelial cells and also through the release of stimulatory cytokines such as VEGF and fibroblast growth factor (FGF) [24–26]. Testicular and human urine SCs have also been studied [27].

5.2.2 Methods of SC Delivery

SC performance may be potentiated by modifying their characteristics via manipulation of their genes or by incubating them with scaffolds, growth factors, or other substances. The therapeutic effect of SC injection may be via migration of these cells to the injury site [28]. Intravenous injection of adipose stem cells (ADSC) showed improvement of erectile function [28]. Moreover, IC SC delivery for ED treatment is by far the most popular route for SC administration in ED. SCs achieve their regenerative effect by either secreting growth factors into the blood stream or migrating to major pelvic ganglia (MPG) [7]. Technical difficulties in the direct injection of SCs into the MPG limit the utilization of this route [16, 29]. Periprostatic injection with or without simultaneous IC injection has also been tried [30–32]. Intraperitoneal injection of SCs was less effective than IC injection in restoring erectile function in CN injury mouse models [33]. Table 5.1 summarizes the preclinical SC studies performed on ED.

Table 5.1 Preclinical stem cell studies for erectile dysfunction

Trial year	First author	Animal model	Stem cell type	Method of transplantation	Reference
2004	Bochinski	CN injury rat	Allogeneic ESC	IC or Intra-MPG	[16]

Trial year	First author	Animal model	Stem cell type	Method of transplantation	Reference
2006	Kim	CN injury rat	Allogeneic SKMSC	IC	[22]
2007	Bivalacqua	Aging rat	Allogeneic BMSC	IC	[20]
2008	Nolazco	Aging rat	Mouse SKMSC	IC	[87]
2009	Fall	CN injury rat	Allogeneic BMSC	IC	[19]
2010	Garcia	DM rat	Autologous ADSC	IC	[88]
2010	Huang	Hyperlipidemia rat	Autologous ADSC	IC	[12]
2010	Albersen	CN injury rat	Autologous ADSC	IC	[89]
2010	Kendirci	CN injury rat	Allogeneic BMSC	IC	[90]
2010	Abdel Aziz	Aging rat	Allogeneic BMSC	IC	[91]
2011	Lin	CN injury rat	Autologous ADSC	Nerve graft	[92]
2011	Woo	CN injury rat	Allogeneic SKMSC	IC	[93]
2011	Gou	DM rat	Allogeneic EPC	IC	[17]
2011	Qiu	DM rat	Allogeneic BMSC	IC	[18]
2012	Qiu	DM rat	Allogeneic BMSC	IC	[94]
2012	Qiu	Radiation injury rat	Allogeneic ADSC	Intra-MPG	[28]
2012	Qiu	CN injury rat	Autologous SVF	IC	[24]
2012	Kovanecz	CN injury rat	Mouse SKMSC	IC	[95]
2012	Kim	CN injury rat	Allogeneic BMSC	CN scaffold	[29]

Trial year	First author	Animal model	Stem cell type	Method of transplantation	Reference
2012	Sun	DM rat	Allogeneic BMSC	IC	[96]
2012	Fandel	CN injury rat	Autologous ADSC	IC	[97]
2012	Nishimatsu	DM rat	Allogeneic ADSC	IC	[98]
2012	Ryu	DM mouse	Syngeneic SVF	IC	[25]
2012	Piao	CN injury rat	Human ADSC	CN scaffold	[99]
2012	Ma	TA injury rat	Autologous ADSC	SIS graft	[39]
2013	Castiglione	TA injury rat	Human ADSC	Intratunical	[40]
2013	Jeong	CN injury rat	Human ADSC	CN scaffold	[100]
2013	Kim	CN injury rat	Human ADSC	CN scaffold	[101]
2013	You	CN injury rat	Human BMSC	IC + periprostatic	[31]
2013	You	CN injury rat	Human ADSC	IC + periprostatic	[32]
2013	Choi	CN injury rat	Human testis SC	Periprostatic	[30]
2013	Ying	CN injury rat	Autologous ADSC	IC	[102]
2013	He	DM rat	Allogeneic BMSC	IC	[103]
2013	Liu	DM rat	Human ADSC	IC	[104]
2014	Ryu	CN injury mouse	Allogeneic clonal BMSC	IC + IP	[33]
2014	Ying	CN injury rat	Autologous ADSC	Vein graft	[105]
2014	Das	DM mouse	Human SVF	IC	[106]
2014	Gokce	TA injury rat	Autologous ADSC	Intratunical	[107]

Trial year	First author	Animal model	Stem cell type	Method of transplantation	Reference
2014	Ouyang	DM rat	Human USC	IC	[27]
2014	Lee	CN injury rat	Human ADSC	IC	[108]
2014	Song	CN injury mouse	Allogeneic SVF	IC	[109]
2014	Mangir	CN injury rat	Autologous vs. allogeneic ADSC	IC	[110]
2014	Bae	CN injury rat	Human ADSC	IC + hydrogel	[111]
2015	You	CN injury rat	Autologous ADSC vs. SVF	IC	[112]
2015	Gokce	TA injury rat	Allogeneic ADSC	Intratunical	[113]

IC intracavernosal, *MPG* major pelvic ganglia, *ESC* embryonic stem cell, *SKMSC* skeletal muscle-derived stem cell, *BMSC* bone marrow-derived stem cell, *ADSC* adipose-derived stem cell, *CN* cavernosal nerve, *EPC* endothelial progenitor cell, *DM* diabetes mellitus, *SVF* stromal vascular fraction, *TA* tunica albuginea, *SIS* small intestinal submucosa, *IP* intraperitoneal, *USC* urine-derived stem cells

5.3 Peyronie's Disease

Peyronie's disease (PD) affects 3 % of men, resulting in pain and penile deformities such as curvature, indentation, and shortening [34]. While the exact pathogenesis of PD is not completely understood, the most likely mechanism for its etiology is repeated microtrauma to the penis during sexual intercourse in genetically susceptible individuals, leading to inflammation and fibrous plaque formation in the tunica albuginea and corpus cavernosum [35]. Transforming growth factor (TGF)-β1 and other local inflammatory mediators are thought to play an important role in abnormal fibrogenesis and scar formation [35]. PD is thought to cause ED directly, most likely through veno-occlusive dysfunction [35, 36]. Incising or excising the plaque followed by patch grafting the tunica is

also done in severe PD cases to maintain penile length, which can also increase the risk of ED [37, 38]. Advances in SC research have allowed the development of porcine small intestinal submucosa grafts seeded with SCs to reduce the risk of ED [39]. ADSCs inhibited the development of ED in TGF-β1-induced rat PD model, with a reduction of elastin tissues and disordered collagen type III [40]. Further research using SCs in PD is needed to reduce the risk of ED or ameliorate the disease.

5.4 Infertility

Treatment of cancer can affect the fertility of the individual, whether it is a surgical treatment, chemotherapy, novel target therapy, or radiation. These treatment modalities can damage germ cells, Sertoli cells (critical for germ cell development), or Leydig cells (responsible for testosterone production). The degree of damage depends on the type of cancer, the age of the patient, and the modality of treatment. Cytotoxic therapy would particularly affect spermatogonial stem cells (SSCs) [41].

Through the isolation by cryopreservation of SSCs from the prepubertal testes prior to cytotoxic therapy commencement, hope is given to patients who are potentially undergoing a sterilizing treatment. The procedure is done through a testicular biopsy followed by cryopreservation. SSCs would be utilized then for in vitro spermatogenesis or autologous transplantation into the patient's testicles. Several animal studies have shown the reproducibility of this technique [42].

5.4.1 Urinary Incontinence

Urinary incontinence (UI), defined as the involuntary loss of urine, affects 200 million individuals around the world. It affects women two to three times more than men until age 80 years, after which the prevalence becomes equal between the two sexes. It has been reported that 50 % of women experience UI after the age of 20, and 50 % of those develop stress urinary incontinence (SUI). There are other types of UI,

including urge UI and mixed UI. Oral pharmacotherapy fails to ameliorate SUI, paving the way for surgical modalities such as mid-urethral sling insertion. However, surgical therapy, though effective, is invasive, and studies have examined the utilization of less invasive treatment options for SUI, such as SC therapy [43, 44].

The urethra is a structure composed of epithelium and connective tissue, which includes striated and smooth muscles and small blood vessels [45]. Striated and smooth muscle cells were found to be reduced in humans and animal models of SUI [45]. SCs can differentiate into any muscle type, in addition to secreting musculogenic and angiogenic growth factors, enhancing their regenerative effect. These properties prompted the utilization of SCs in SUI [46]. ADSCs were found to improve the urethral connective tissue, likely through the production and processing of collagen and elastin [47].

5.4.1.1 Preclinical Studies

Initially, the concept of cell-based therapy for SUI included the use of skeletal myoblasts to replace the deficient urethra [48]. SCs were used subsequently instead of myoblasts. Yiou et al. in 2002 reported the first preclinical study utilizing SKMSCs in SUI treatment [49]. Autologous SKMSCs were harvested from limb skeletal muscle and injected into the injured skeletal urethral sphincter. SKMSCs were found to accelerate sphincter muscle repair. After that, SKMSC was utilized exclusively in all preclinical studies on SC therapy of SUI until 2010; from there six preclinical studies were done using BMSCs [50–55]. One of those studies involved seeding a degradable silk scaffold to be used as a sling as well [53]. Umbilical cord blood stem cells (CBSCs) were also used in one preclinical trial [56]. More recently, seven studies utilized ADSCs in SUI [47, 57–62], including one study where fibroin microspheres were used as a bulking agent [62]. Furthermore, amniotic fluid stem cells (AFSCs) were used in four preclinical studies [63–66]. Of note, all these studies produced improvements in SUI.

Rat models are the most common animal models utilized in SUI preclinical studies. Mice have been utilized in several studies [63–66], including the first study by Yiou et al. [49]. Only one study utilized monkeys [67], and one study utilized dogs [68]. Several techniques have been used to establish animal models of SUI. A sphincteric injury model using electrocoaugulation, muscle resection, cauterization, or injection of myotoxin has been used. Sciatic and pudendal nerve injury models using crush injury or transection have also been developed. The delivery, vaginal distension, and ovariectomy (DVDO) is the most widely used animal model for birth injury [43, 69–71]. All these models suffer from their short durability of 2–3 weeks [72–74]. Periurethral injection of SCs is the most widely used method of administration in preclinical studies; however, Lin et al. compared intravenous and periurethral injection of SCs and identified improvements in SUI in both routes [47].

Functional and histological assessments are used to assess outcomes of SC use in SUI. Functional assessment is typically achieved by either measuring leak point pressure (LPP) using Crede or vertical tilt table methods or through electrical stimulation of the urethral sphincter neurovascular bundle [43]. The purpose of histological assessment is to locate SCs, identify SC differentiation, and assess for tissue improvement. It is typically done by sacrificing the animal and harvesting the urethral tissue, followed by staining with H&E or trichrome. To identify possible differentiation of transplanted SCs, immunohistochemical and immunoelectron microscopy was performed in several studies [43].

5.4.1.2 Clinical Studies

Five clinical trials, done by the same group of researchers, have examined the effect of injected SKMSCs in male and female UI between 2007 and 2008. They reported 80–90 % improvement in UI [58, 75–79]. However, two of those trials were retracted later citing ethical concerns [78, 79]. Carr et al. showed that five out of eight women with SUI achieved total continence using SKMSCs [80]. Lee et al. demonstrated 70–80 % improvement in continence in 39 female patients with

SUI using CBSC [56]. A small case series utilizing ADSCs for SUI was later retracted for unknown reasons [81]. Using SKMSCs in 12 female patients with SUI, Sebe et al. showed improvement in 10 out of 12 women but worsening of SUI in 2 patients [82]. The typical injection method in clinical trials has been transurethrally, although Carr et al. utilized both transurethral and periurethral routes and showed improvement in incontinence using both routes [80]. In a small pilot study of three male patients with SUI, Yamamoto et al. showed an improvement in SUI using ADSC at 6 months [83]. Another study using ADSCs showed 60 % improvement in SUI in 8 out of 11 male patients at 1 year [84]. A Polish study with a longer follow-up of 2 years reported 75 % improvement in 16 female patients with SUI using SKMSCs, with 50 % of patients achieving complete continence [85]. Most recently, Kuismanen et al. showed improvement in SUI in three of five female patients at 1-year follow-up using ADSCs with collagen gel as a bulking agent [86]. Functional assessment in clinical trials has been through measuring pad weights, bladder diaries, and quality of life (QoL) assessment, in addition to urodynamic (UDS) findings such as peak flow rate, postvoid residuals, and maximal urethral closing pressure [43]. Table 5.2 summarizes the SC studies performed on SUI.

Table 5.2 Stem cell studies for stress urinary incontinence

First author	Year of publication	Animal model/patients	Stem cell type	Injection method
Yiou [49]	2002	Sphincter injury mice	Autologous	Periurethral
			SKMSC	
Lee [114]	2003	Sciatic nerve transection rats	Allogeneic	Periurethral
			SKMSC	
Yiou [115]	2003	Sphincter injury rats	Autologous	Periurethral
			SKMSC	
Cannon [116]	2003	Sciatic nerve transection rats	Allogeneic	Periurethral
			SKMSC	

First author	Year of publication	Animal model/patients	Stem cell type	Injection method
Chermansky [117]	2004	Sphincter cauterization rats	Allogeneic	Periurethral
			SKMSC	
Lee [118]	2004	Pudendal nerve transection rats	Allogeneic	Periurethral
			SKMSC	
Yiou [119]	2005	Sphincter injury rats	Autologous	Periurethral
			SKMSC	
Kwon [120]	2006	Sciatic nerve transection rats	Allogeneic	Periurethral
			SKMSC	
Kim [121]	2007	Sciatic nerve transection nude rats	Human	Periurethral
			SKMSC	
Mitterberger [75]	2007	123 female patients	Autologous	Transurethral
			SKMSC	
Mitterberger [76]	2008	63 male patients	Autologous	Transurethral
			SKMSC	
Mitterberger [77]	2008	20 female patients	Autologous	Transurethral
			SKMSC	
Carr [80]	2008	8 female patients	Autologous	Transurethral/periurethral
			SKMSC	
Hoshi [122]	2008	Periurethral injury rats	Allogeneic and xenogeneic rodent	Periurethral
			SKMSC	
Furuta [123]	2008	Pudendal nerve transection nude rats	Human	Periurethral
			SKMSC	
Lin [47]	2010	Vagina	Autologous	Periurethral and IV

First author	Year of publication	Animal model/patients	Stem cell type	Injection method
		distension rats	ADSC	
Fu [57]	2010	Vagina distension rats	Allogeneic	Periurethral
			ADSC	
Kinebuchi [51]	2010	Sphincter injury rats	Autologous	Periurethral
			BMSC	
Lim [124]	2010	Sphincter injury rats	Human CBSC	Periurethral
Lee [56]	2010	39 female patients	Allogeneic	Periurethral
			CBSC	
Zou [53]	2010	Sciatic nerve transection rats	BMSC on scaffold	Sling surgery
Xu [125]	2010	Pudendal nerve transection rats	Allogeneic	Periurethral
			SKMSC	
Zhao [60]	2011	Pudendal nerve transection rats	Autologous	Periurethral
			ADSC	
Kim [52]	2011	Pudendal nerve transection rats	Allogeneic	Periurethral
			BMSC	
Corcos [50]	2011	Pudendal nerve transection rats	Allogeneic	Periurethral
			BMSC	
Wu [59]	2011	Pudendal nerve transection rats	Allogeneic	Periurethral
			ADSC	
Watanabe [58]	2011	Pelvic nerve transection rats	Allogeneic	Periurethral
			ADSC	
Sebe [82]	2011	12 female patients	Autologous	Endourethral
			SKMSC	

First author	Year of publication	Animal model/patients	Stem cell type	Injection method
Yamamoto [83]	2012	3 male patients	Autologous ADSC	Transurethral
Kim [63]	2012	Pudendal nerve transection mice	Human AFSC	Periurethral
Li [61]	2012	Vagina distension rats	Autologous ADSC	Periurethral
Chun [64]	2012	Pudendal nerve transection mice	Human AFSC	Periurethral
Badra [67]	2013	Pudendal nerve transection monkeys	Autologous	Periurethral
			SKMSC	
Stangel-Wojcikiewicz [85]	2014	16 female patients	Autologous SKMSC	Transurethral
Dissaranan [54]	2014	Vagina distension rats	Allogeneic	Periurethral
			BMSC	
Gotoh [84]	2014	11 male patients	Autologous ADSC	Transurethral
Shi [62]	2014	Pudendal nerve transection rats	Autologous ADSC with silk fibroin microspheres	Periurethral
Chun [65]	2014	Pudendal nerve transection mice	Human AFSC	Periurethral
Kuismanen [86]	2014	5 female patients	Autologous ADSC with collagen gel	Transurethral

First author	Year of publication	Animal model/patients	Stem cell type	Injection method
Deng [55]	2015	Pudendal nerve crush + vagina distension rats	Autologous BMSC	IV and IP
Williams [68]	2015	Sphincter injury dogs	Autologous SKMSC	Periurethral
Choi [66]	2015	Pudendal nerve transection mice	Human AFSC	Periurethral

SKMSC skeletal muscle-derived stem cells, *BMSC* bone marrow-derived stem cells, *ADSC* adipose-derived stem cells, *CBSC* umbilical cord blood stem cells, *AFSC* amniotic fluid stem cells, *IV* intravenous, *IP* intraperitoneal

5.5 Future Directions

ADSCs represent an easier SC type to obtain given the availability of adipose tissue and ease of acquisition. Therefore, future use of SCs would probably utilize ADSCs more than other SC types. Current SUI animal models have the disadvantage of short durability. The development of more durable chronic type of SUI animal models is important to accurately determine SC therapeutic effects. The method of SC administration is still an area of active research as IV administration has been shown to be equivalent to direct injection into the damaged area. Further research is also needed to improve the understanding of SC therapeutic mechanisms, as cellular differentiation is not the only mechanism and paracrine effects are likely involved. The development of iPSCs represents a milestone in SC research, and utilization of this technology in urology should be a future goal. More clinical trials recruiting larger numbers of patients are needed, and they should adhere to the highest standards of ethical considerations.

Author Disclosure Statement

No competing financial interests exist.

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6. Clinical Applications of Stem Cells in Women's Reproductive Health

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Tissues of reproductive origin represent a rich source of important types of stem cells such as embryonic stem cells and umbilical cord blood stem cells. A recent interest has also developed in the heterogenic stem cell populations isolated from the amniotic fluid, amniotic membrane, umbilical cord Wharton's jelly, and placenta. Furthermore, gynecologic tissues, including uterine and vaginal tissues, are increasingly being recognized as sources of autologous adult stem cells. Each of these tissues has its unique biological and technical features that make it an attractive option in one or more clinical applications. For a broader outlook, several benign and malignant disorders of the reproductive tract are now acknowledged as a system disorder initiated at the stem cell level. Female hormones, including sex steroids and their metabolites, have a central role in controlling both physiology and disease in women. As a result, and unlike other body systems, the hormonal interplay with stem cells should be a cornerstone of our understanding of the role of stem cells in women's health.

In this chapter, we would like to provide an overview of two distinct directions relevant to the clinical applications of stem cell therapy in this field: The first (A) includes clinical applications that emerge from understanding the role of stem cells in women's reproductive system disorders, which should be indirectly translated into therapeutics through targeting the relevant stem cells. The second (B) includes the potential applications of stem cell therapies in maternal and reproductive health problems.

6.1 Targeting Stem Cells as Precursors of Reproductive Pathologies

In physiological situations, stem cell expansion and differentiation are strictly controlled by interconnected microenvironmental factors, so that these functions are only exerted for the repair of damaged tissues or within the scope of a specialized regenerative function such as the remarkable

regeneration of the endometrium during the menstrual cycle or pregnancy. However, it seems that this balanced control of stem cell fate is occasionally disturbed, so that the most common pathologies of the female reproductive system are of a proliferative nature because of the uncontrolled proliferation or differentiation of stem cells. Uterine fibroid, endometriosis, endometrial polyps, and ovarian cysts are examples of such proliferative conditions.

6.1.1 Uterine Leiomyoma

Uterine leiomyoma, commonly referred to as fibroid, is the most common gynecologic tumor and one of the most common benign tumors affecting women; it affects around 70–80% of women by the age of 50 [1]. They often present as multiple tumors, and some attain very large sizes. Although leiomyomas are known to arise from myometrial smooth muscle cells, their exact clonal origin is debated [2]. However, leiomyomas have been sex steroid hormone-dependent. Accordingly, most medical treatment options are based on reducing the effect of sex hormones [3]. A defined stem cell-enriched population, known as a myoma-derived side population, has been suggested to contribute to the pathogenesis of uterine leiomyoma [4]. This population of undifferentiated cells within the leiomyoma appears to represent tumor-initiating cells that are possibly prompted by paracrine mechanisms delivered from differentiated receptor-sensitive co-cells. The paracrine signals may activate the Wnt/β-catenin pathway and the transforming growth factor β3 (TGF-β3), which together stimulate stem cell proliferation and differentiation toward abnormal leiomyomatous progeny [5, 6]. However, several other factors are involved in this process that need to be interpreted together, such as chromosomal abnormalities and the disproportionate expansion of the extracellular matrix characteristic for fibroid, for a better understanding of the pathogenesis of leiomyomas [7]. Few clinical trials are ongoing to isolate leiomyoma stem cells from patients undergoing myomectomy aiming to achieve further characterization of these cells and to use them as therapeutic targets [8].

6.1.2 Endometriosis

Endometriosis is a prevalent benign pathology that affects up to 15% of women, mostly during their reproductive years. It is a common cause of infertility, chronic pelvic pain, and related sexual dysfunction [9]. Retrograde menstruation has been accepted for a long time as an explanation of endometriosis, where endometrial cells get access to the peritoneal cavity through the fallopian tubes. However, controversy about its pathogenesis has never settled, perhaps because the old theory, proposed in 1925, did not explain the presence of endometriosis in sites that are remote from the peritoneal cavity. Accordingly, diverse etiologies were suggested, including genetic, hormonal, immunogenic, and infective factors [10]. Eventually, stem cell-related functions were added to the list of hypotheses. Despite the recognized oversell of stem cells as a cause and solution to almost every human disease [11], the monoclonal origin of the glandular lesions and the proliferative and multiple phenotypic nature of endometriotic implants seem to favor the stem cell contribution to this pathology [12, 13]. Moreover, some of the old hypotheses, such as retrograde menstruation, the embryonal rest theory, and the coelomic metaplasia theory, share similar concepts and can be put in context with the new stem cell theory of endometriosis. Despite common traits between fibroid and endometriosis, including their hormonal dependency and proliferative nature, and unlike fibroid, the origin of lesion-initiating stem cells in endometriosis remains difficult to locate, and its tendency to invade locally and distantly adds undefined malignant-like behavior. For instance, the expression of stem cell factors such as Musashi-1 was found to significantly increase in both endometriosis and endometrial carcinoma [14].

The suggested origins of endometriosis-initiating stem cells include the endometrial epithelial or mesenchymal stem cells (MSCs) [15], peritoneal cells, and bone marrow MSCs. Because of the circulating nature of bone marrow-derived stem cells, this theory may explain how endometriosis develops in remote sites [16] (Fig. 6.1).

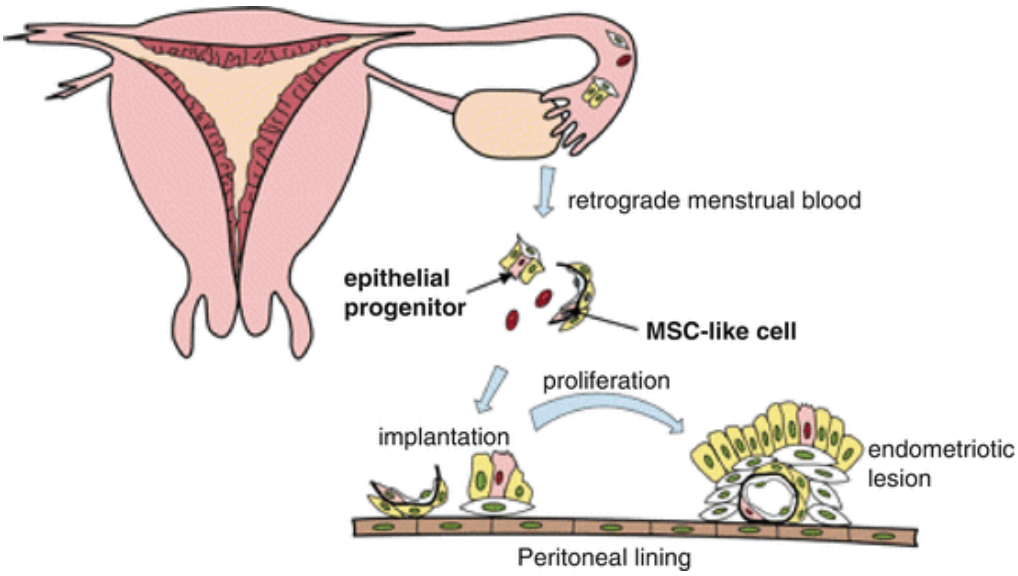

Fig. 6.1 The stem cell theory is one of many theories postulated for the pathogenesis of endometriosis. It is believed that the stem or progenitor cells come through the fallopian tubes during menstruation. Obtained with permission from Oxford University Press [66]

6.1.3 Preeclampsia

Preeclampsia is a multisystem disorder that affects about 5–10% of pregnant women and causes 15–20% of maternal mortality in developed countries and around 70,000 death per year worldwide [17]. Preeclampsia is associated with acute grave fetal and maternal complications. With the exception of pregnancy termination, there is currently no definitive treatment for this disorder. Various etiologies have been investigated for the pathogenesis of preeclampsia that support its multifactorial origins. However, poor placental perfusion and defective invasion of the trophoblast into the uterine blood vessels seem to be central mechanisms [17, 18]. Trophoblastic stem cells are pluripotent cells derived from the trophectoderm and can differentiate into all types of cells that make extraembryonic tissues [19]. The role of trophoblast stem cells in the development of preeclampsia is supported by a number of studies that demonstrated a difference in the expression levels of stem cell markers in the placenta and umbilical cord of preeclamptic women compared to normal women [20]. Similarly, the expression of miRNA in placental MSCs has been found to be aberrant in cases of preeclampsia causing impaired angiogenesis and cell migration [21, 22].

Additionally, preeclampsia disease models found that trophoblastic stem cell defective differentiation may contribute to the impaired placentation and vascular insufficiency associated with preeclampsia [23–25].

On the other hand, the immunomodulatory and angiogenic effect attributed to MSCs in vascular limb ischemia, where placental stem cells significantly improved blood flow, was extrapolated to preeclampsia [26], leading to recent applications for FDA approval to use these cells in preeclampsia patients [27]. It is, however, not clear how safe such therapy would be, or how to justify its complexity, in pregnant women.

6.1.4 Cancers Specific to Women

Like other solid cancers, cancer-initiating cells or cancer stem cells (CSCs) have been isolated from women-specific cancers, including breast [28], uterine [29], ovarian [30], and tubal [31] cancers. The common feature of these findings was the isolation of cells that are capable of self-renewal in vitro and inducing histopathologically identical tumors in immunocompromised mice. Uncommon cancers, such as vaginal, vulval, and trophoblastic tumors, have not been fully investigated, although indirect evidence of the role of CSCs in these cancers exists [32, 33]. The isolation methods of CSCs, however, have not been standardized and are becoming more variable as more specific markers are identified. Although all cancers share a universal malignant behavior, women's cancers encompass a wider tumor behavioral span. Breast cancer lies at the top of the prognosis list, with a 5-year survival rate of more than 90–95%, whereas ovarian cancer lies at the bottom, with a very poor prognosis (5-year survival rate is only around 40–45%), owing to its silent clinical course and late diagnosis and treatment [34]. Therefore, the CSC hypothesis is of utmost significance to ovarian cancer since it may help explain its heterogeneous phenotypes, considerable resistance to traditional chemotherapies, and fast recurrence [35]. While most chemotherapies are directed against the metabolically active well-differentiated cancer cells, quiescent CSC populations could remain unaffected by chemotherapy.

Accordingly, innovations related to CSCs in fatal cancers are highly endorsed by funding agencies. One of the rising promising markers of CSCs is ALDH1A1, a cellular detoxifying enzyme associated with an aggressive course of cancer [36–38]. Its activity was distinctly linked to poor clinical outcomes and resistance to chemotherapy in epithelial ovarian cancer, which makes it a potential clinical diagnostic and prognostic marker [39]. Ovarian CSCs have also been identified by their ability to retain DNA dyes such as BrdU for long times compared to rapidly dividing daughter cells and progenitors [40]. This marker has helped the localization of fimbrial stem cells that could be a source of cancer-initiating cells in ovarian tumors [41]. This new understanding could change the way ovarian cancer prevention is approached; for example, in high-risk women, the fallopian tubes may be surgically removed, compared to the current practice of simply performing an oophorectomy.

6.2 Stem Cell-Based Therapies for Women's Reproductive Disorders

6.2.1 Vaginal Agenesis and Vaginoplasty

Mayer–Rokitansky–Küster–Hauser syndrome is a classic example of vaginal agenesis. It complicates 1 in 4500 female births [42]. The syndrome, which is characterized by an absence of the vagina and the uterus, is traditionally managed by vaginal reconstruction using various surgical techniques. Although Abbè-McIndoe vaginoplasty is considered the standard surgical procedure for the creation of neovaginas in women with vaginal agenesis, the type of tissue lining used in this procedure has been debatable. Because a skin lining was associated with healing problems, surgeons have tried using various other tissues, especially those that are thinner, including the peritoneum, intestine, buccal mucosa, amnion, and epidermal sheets. In 2007, the first case of vaginoplasty with autologous vaginal tissue transplantation in a young woman was reported. A full-thickness mucosal biopsy from the patient's vaginal vestibule was obtained and cultured for 1 week. The authors of this case reported preliminary

improvement measured by patient satisfaction and an absence of surgical complications such as scarring and infection [43]. A few years later, a multilayered neovagina was constructed using autologous muscle and skin-derived and expanded stem cells that were further seeded on a biodegradable scaffold molded into a tube-like structure before transplantation in four women with this syndrome. This was a milestone study that demonstrated the long-term success and safety of a stem cell-based bioengineered organ. The women were able to have normal sexual relations afterward without significant complications [44]. One of the next-generation developments of this model may possibly include the use of 3-D printing methods to add more complex biomimetic details to the design of the scaffolds on which stem cells grow [45].

6.2.2 Obstetric Fistulae

An obstetric fistula is a complication of prolonged and neglected labor, suffered by women in poorly developed healthcare settings [46]. It can lead to devastating outcomes in terms of women's morbidity and mortality. In this disorder, an abnormal tract is created between the female reproductive organs (either the uterus or vagina) and the urinary tract (bladder, ureters, or urethra) or the lower gastrointestinal tract (most commonly the rectum). Patients usually have total urinary or fecal incontinence, resulting in an enormous impact on the quality of physical, psychological, and social life. Traditional surgical options require special expertise and the reappearance of fistulae after primary surgery remains a challenge. Furthermore, intractable fistulae could lead to repeat surgeries (up to 12 repeat fistula surgeries in one patient were reported) [47]. The repair failure is attributed to factors such as poor healing due to tissue necrosis and inadequate vascularization. Accordingly, stem cell-based therapies are being investigated owing to their inherent regenerative capacity to create new tissue to close the fistulous defect. In addition, stem cells can revitalize the necrotic tissue around the fistulous tract by introducing vital cells to provide essential paracrine factors and cell–cell interactions. In real practice, very few studies have investigated the effectiveness of stem

cell therapies in the treatment of reproductive tract fistulae in humans [48]. Most notably, autologous or allogeneic adipose-derived stem cells were used to treat intractable rectovaginal fistulae, with varying success [47]. More recent attempts showed that surgical closure was primarily required to close the internal side of the defect, whereas stem cells were injected in between the communicating ends with a long-term success rate of 60% [49]. One of the factors that would enable faster progress in achieving effective stem cell therapies in this hard-to-treat pathology would be to provide fistula animal models, such as a recently reported pig model of vesicovaginal fistulaa [50]. Such models overcome the ethical barriers associated with attempting unproven stem cell therapies in patients and enable an objective comparison between various types and protocols of stem cells without technical limitations.

6.2.3 Female Urogynecologic Disorders

In the last two decades, stem cells have been intensively investigated as a potential treatment for a variety of urogynecologic conditions. Stem cell applications in the treatment of overactive bladder and stress urinary incontinence are covered in detail in a dedicated chapter in this book.

6.2.4 Pelvic Organ Prolapse

Pelvic organ prolapse is one of the most common indications for surgery in gynecologic practice [51]. Women with this disorder could have one or more parts of their reproductive organs descending below the normal anatomical level, causing a multitude of symptoms and complications. Despite being relevant to lifestyle and obstetric factors, a genetic element is strongly suggested to produce defective connective tissue cells and extracellular matrix composition [52]. Several surgeries and forms of synthetic mesh, slings, and tapes have been used to support the prolapsed part and revert it to its correct location. However, the synthetic nature of these products has resulted in serious complications, with the rate of vaginal mesh erosion estimated to be 15.6% [53]. Accordingly, interest in finding less invasive materials invited the consideration of

stem cell therapies as a biological alternative. Thus far, researchers have proposed in vitro models or animal models of stem cell-based biomaterials for the treatment of pelvic organ prolapse. Although these bioscaffolds were suggested as an alternative to the synthetic vaginal mesh, they were mostly generic and could be used in other general surgical applications [54–56].

One of the questions raised by this application relates to the route of stem cell therapy in treating pelvic organ prolapse. The intravenous route is quite questionable since adequate homing and engraftment remain a challenge; however, some evidence supports the idea that stem cells will home toward inflamed and injured sites. For instance, tracking adipose-derived stem cells showed their homing to the injured pelvic organs and pelvic floor muscles [57].

6.2.5 Female Factor Infertility

6.2.5.1 Ovarian Failure

The term *oogonial stem cells* has been used to describe germline cells that can be isolated from adult ovaries and differentiated in vitro into oocytes. In 2012, oogonial stem cells were isolated from both adult rat and human ovaries [58]. The cells, which are mitotically active, are isolated from adult ovarian cortex and cultured to produce mature oocytes. During culture, they were transfected with green fluorescent protein (GFP). Oogonial stem cells were then injected again into fragments of ovarian cortex, where they acquired a somatic cell coverage of the produced oocytes and gave rise to primordial follicles [58].

The success of this strategy will determine its use in the treatment of incurable ovarian failure; one particular issue related to this treatment is the fact that oocyte donation has long-term social and psychological implications and is not accepted in many communities [59, 60]. Oogonial stem cells could also be helpful in young women with cancers who are exposed to gonadotoxic therapies. However, this may require a multistep culture technique that develops a stem cell into an

oocyte, primordial follicle (using ovarian cortical strips), preantral follicle, and eventually antral follicles [60]. At this stage, stem cell-derived immature follicles can be managed in a manner similar to autologous immature oocytes that are currently obtained from the ovaries of children and adolescents for fertility preservation [61].

Induced pluripotent stem cells (iPSCs) have also been used in mice for the generation of haploid cells that act as functional oocytes and were fertilized in vitro to produce embryos [62]. This represents a substantial advancement in the field of reproductive science because it could allow future treatment of infertility even in the absence of ovarian tissue; somatic cells can be manipulated to produce iPSCs and, subsequently, oocyte-like cells [63].

6.2.5.2 Asherman Syndrome

Asherman syndrome, a disease characterized by partial or complete uterine cavity obliteration and damage of the basal endometrium, is an excellent indication for the application of these discoveries. Endometrial tissue regeneration is one of the anticipated stem cell clinical applications. Endometrial cells have been successfully generated from both human embryonic stem cells (hESCs) and iPSCs [64]. hESCs were differentiated into Mullerian duct epithelium to serve as a model for studying the development of the female genital tract [65].

An endometrial side population of cells was characterized as endometrial stem/progenitor cells in both the functionalis and basalis layers of the endometrium. The work of Gargett and colleagues in this field is particularly significant. Both mesenchymal and epithelial stem cells were isolated, with the MSCs believed to be pericytes that reside around blood vessels. Both of these cell types can be shed into the menstrual blood with the functionalis layer. Therefore, menstrual blood could serve as a noninvasive source of autologous stem cells [66–68]. Endometrial biopsy, using minimally invasive procedures, can also be used to obtain stem cell-rich tissue from the basalis layer in cases such as Asherman syndrome. In addition, bone marrow-derived stem cells were found to

increase their trafficking to the injured endometrium, while they seem to make less of a contribution to normal cyclic endometrial buildup during the menstrual cycle [69]. This may explain the improved fertility observed in murine models of Asherman syndrome following injection with bone marrow-derived stem cells [70, 71]. A similar outcome of increased endometrial thickness occurred in rat models of induced endometrial thinning following direct uterine perfusion with bone marrow stem cells [72]. In 2011, a published case report described the success of this strategy in one patient with resistant Asherman syndrome who had received intrauterine infusion of a single-dose therapy containing 39 million characterized bone marrow MSCs. The patient grew to a good thickness a functional endometrium that enabled her to conceive in the next few cycles using in vitro fertilization [73]. This was followed by reports of a similar cases who responded to bone marrow stem cell treatment by an increased endometrial thickness and resumption of menstruation [74].

As outlined, several promising options for endometrial regeneration and treatment of Asherman syndrome exist, including iPSCs, endometrial stem cells, and bone marrow MSCs. Direct comparison between the efficiency and long-term function of these different stem cell types should be the next step to establish reproducible clinical protocols.

6.2.6 Intrauterine Fetal Stem Cell Therapy

Intrauterine stem cell therapy (IUSCT) refers to the treatment of a variety of fetal genetic disorders through the transplantation of either allogeneic or genetically modified autologous stem cells. Ideally, stem cells are expected to be engrafted into the recipient tissue, proliferate, and differentiate into healthy specialized cells that make up for a genetically defective function. It is hoped that this will solve incurable fetal genetic disorders, which are considered to be perinatally lethal or associated with significant disability and morbidity if intervention is delayed to the postnatal period [75].

Recent advances in prenatal diagnostic techniques have enabled early prenatal diagnosis of a wide variety of genetic

disorders. IUSCT has the advantage over postnatal therapy that the immune system of the fetus at early gestation is still immature, which is the basis for the unique immunologic tolerance phenomenon described decades ago [76]. Such tolerance allows for the engraftment of donor stem cells without the need for myeloablation or the use of immunosuppressive medications. Also, the sterile environment inside the uterus facilitates remodeling of the fetal immune system [77]. Another major advantage of IUSCT is the small size of the fetus at early gestation compared to the postnatal size, allowing the transfusion of higher concentrations of stem cells [78]. These factors contribute to the promising potential of IUSCT in managing a wide variety of genetic disorders. Two main routes have been described for the delivery of intrauterine stem cells: the intravascular route and the intraperitoneal route; both are used under ultrasound guidance. In contrast to the intraperitoneal route, it is believed that the intravascular route is associated with a better cell uptake; however, it is technically more challenging and associated with higher complications [79].

IUSCT has been experimentally evaluated in a variety of conditions that will be outlined in the following sections.

6.2.6.1 Hematological and Lymphatic Disorders

Many congenital hematological disorders that have been considered chronic with high morbidity and mortality (such as thalassemia and sickle cell anemia) are now considered potentially curable through hematopoietic stem cell (HSC) transplantation. However, postnatal stem cell transplantation has well-known problems related to donor matching and the need for immunosuppression [75, 78]. The distinctive fetal immunologic features combined with recent advances in early prenatal diagnosis of many genetic disorders could help overcome these difficulties and make intrauterine hematopoietic stem cell transplantation (IUHSCT) a potentially promising alternative. IUHSCT has been extensively investigated in animal models and has shown

successful results [80–87]. However, in humans, IUHSCT results have been conflicting. *HSCs* can be isolated from the bone marrow, fetal cord blood, fetal liver [88], or, less commonly, from mobilized peripheral blood [89].

Immunodeficiency Syndromes

The first successful IUHSCT was reported by Touraine et al., following prenatal diagnosis of bare lymphocyte syndrome, which is one of the most severe immunodeficiency syndromes. Although this case needed further postnatal stem cell transplantation and sterile isolation, an improved success rate and low rejection rate were reported with IUHSCT [90]. Sequentially, trials on patients with X-linked severe combined immunodeficiency syndromes also demonstrated good outcomes following IUHSCT [91–93]. However, despite these successful reports, IUHSCT has not gained sufficient popularity in the treatment of X-linked severe combined immunodeficiency syndromes, owing to the fact that recipients demonstrate split chimerism with selective engraftment of T cells only as result of donor T cells' advantage and competitiveness [78], a finding that carries no actual advantage compared to postnatal transplantation [94]. Moreover, trials of IUHSCT for other immunodeficiency syndromes, such as chronic granulomatous disease and Chediak–Higashi syndrome, yielded disappointing results [95, 96].

Hemoglobinopathies

IUHSCTs have been investigated in a variety of hemoglobinopathies, including sickle cell anemia and alpha- and beta-thalassemias, as well as some cases of Rh isoimmunization:

1. *Alpha-thalassemia:*

   Homozygous alpha-thalassemia major (Hb Bart syndrome) is an autosomal recessive disease. Approximately 4.5% of the world's population carry a hemoglobinopathy gene. If both parents are carriers, the fetus may be affected by the disease, which often leads to severe fetal anemia and hydrops fetalis. Most fetuses with

this condition end as stillbirth or die in the early neonatal period [97] unless supported by early intrauterine exchange transfusions [97–101]. As this condition is now diagnosed early in pregnancy through antenatal screening programs available in several developed settings, it became possible to identify pregnant women who are potential candidates for IUSCT, who otherwise often opt to terminate their pregnancy [102]. In practice, only three cases of IUHSCT for the treatment of alpha-thalassemias have been reported. Although one case demonstrated microchimerism and tolerance, following IUHSCT by paternal bone marrow cells, as evidenced by the persistence of alpha-globin DNA signal on autopsy, the donor stem cells did not show a survival advantage compared with host cells [103]. Other cases showed no engraftment of the donor cells [88, 103, 104].

2. *Beta-thalassemia:*

Beta-thalassemia is a common autosomal recessive disorder with higher prevalence in certain ethnic groups, for example, those of Mediterranean, Middle Eastern, East Asian, and African descent [105]. Several cases with prenatal diagnosis of beta-thalassemia were treated by IUHSCT using different types of donor cells, for example, paternal, maternal, and sibling bone marrow, fetal liver and thymic cells, fetal blood, and paternal circulating hematopoietic progenitor cells. While some of these cases showed evidence of engraftment [106–108], all cases that continued to survival became transfusion-dependent. The successful chimerism in combined immunodeficiency syndromes and poor results in thalassemias led Renda et al. to try immunosuppression using low-dose dexamethasone followed by IUHSCT using paternal circulating hematopoietic progenitor cells. Although microchimerism was demonstrated in one case by identifying the ABO allele, donor DNA in peripheral blood, and a hemoglobin value of 14.4 g/dL at 2 months of age, both infants subsequently required repeated transfusions [108].

3. *Sickle cell anemia:*

Sickle cell anemia is a single gene disorder caused by the substitution of Hb-S for Hb-A in the beta subunit of the hemoglobin molecule owing to mutation in the HBB gene [109]. There is only one published case of IUHSCT for sickle cell anemia, which was diagnosed prenatally as homozygous sickle cell disease by chorionic villus sampling at 10 weeks. IUHSCT with fetal liver cells was carried out at the 13th week of gestation, with no evidence of engraftment at birth [104].

4. *Rh isoimmunization:*

IUHSCT was investigated in pregnancies with recurrent gestational failure due to Rh isoimmunization to decrease disease severity and delay the need for intrauterine blood transfusion until it could be safely performed [110, 111]. Nonetheless, both reported cases demonstrated failure of engraftment at the time of cordocentesis and required repeated intrauterine blood transfusions.

Possible Barriers to Successful Intrauterine Hematopoietic Stem Cell Transplantation

There are a number of obstacles that warrant further investigations before IUHSCT becomes a routine clinical practice, most importantly:

– The strong functional competition between the already present host hematopoietic cells and the donor cells. Such competition is usually absent in postnatal transplantation as a result of induced myeloablation [77]. This could also be explained by the relative excess of hematopoietic stem cells during fetal life compared to postnatal life, leaving a limited intrauterine hematopoietic niche for donor cell engraftment [112].

– The timing and presentation of foreign donor stem cell antigens determine the successful occurrence of the tolerance phenomenon documented in fetuses at an early gestational age [77].

6.2.6.2 Osteogenesis Imperfecta

Osteogenesis imperfecta (OI) is a rare genetic disease with an incidence of 1 out of every 20,000 births. It affects type I collagen synthesis and is characterized by recurrent low trauma fractures and deformities. Other features include short stature, deafness, blue sclera, and dental changes [113, 114]. Most cases are inherited as an autosomal dominant disease. The disorder has a spectrum of severity ranging from mild cases to most severe cases that are perinatally lethal [115]. MSCs can be obtained from various accessible sources, such as bone marrow, umbilical cord blood, amniotic fluid, periosteum, trabecular bone, adipose tissue, dermis, muscles, synovial membrane, and lungs [116]. Owing to their ability to expand in vitro under normal culture conditions, small amounts of MSCs can be used for transplantation purposes. In addition, MSCs have a favorable low immunologic profile and an ability to suppress T-cell activities, which makes allogeneic transplantation possible. They also rely on chemotactic forces to guide their homing to injured sites [79]. These features suggest a promising role of MSCs in the treatment of genetic disorders of mesenchymal origin, such as OI and muscular dystrophies, and expanding the possibility for IUSCT by overcoming immunologic barriers.

Animal studies of IUSCT for OI demonstrated evidence of donor cell engraftment [117–119]. These studies also showed increased survival [118]; decreased incidence of fractures [117, 119]; increased bone length, strength [117], and thickness [117, 118]; and improvement of matrix quality, stiffness [118, 119], and bone mechanics [118].

With regard to human studies, one unpublished case demonstrated no fractures or events during an infant's first year of life, and a bone biopsy showed 5% of the osteoblasts were of donor origin [120]. The second case demonstrated a

higher level of engraftment (7.4%) and received bisphosphonate therapy at the age of 4 months. However, three fractures were reported by the age of 2 [121]. In conclusion, although studies are limited, results are conflicting, and long-term follow-up is usually absent, the use of MSCs in the intrauterine treatment of OI represents a promising approach for the treatment of such a lethal and deforming disease.

6.2.6.3 Storage Diseases

There are several reported cases of attempted IUSCT for storage diseases. Of these, only one case with prenatal diagnosis of globoid cell leukodystrophy showed evidence of engraftment of donor cells; however, the fetus died at 20 weeks of gestation [122]. Other cases with globoid cell leukodystrophy, Hurler's syndrome, Niemann–Pick disease, and metachromatic leukodystrophy were unsuccessful, with no evidence of engraftment [96, 123–125].

As seen in the highlighted examples, IUSCT is a promising alternative to postnatal stem cell therapy; it has the potential benefit of avoiding immunosuppression and its associated complications and providing a timely management for disorders with high perinatal mortality and morbidity. To date, patients who have been successfully treated by IUSCT are those with immunodeficiency syndromes. Despite the paucity of evidence based on human studies in most cases of intrauterine fetal stem cell therapy, the most recent consensus from experts in this field came in 2014 and confirmed that this therapy is viable and its obstacles should receive adequate research attention [126] (Table 6.1, reproduced with permission from [127]).

Table 6.1 Potential applications of intrauterine fetal stem cell therapy

Immunodeficiency disorders	**Hemoglobinopathies and Rh disease**	**Enzyme storage diseases**	**Other genetic disorders**
Bare lymphocyte syndrome	Congenital erythropoietic porphyria (Gunther's disease)	– Mannosidosis	Dyskeratosis congenita

Immunodeficiency disorders	Hemoglobinopathies and Rh disease	Enzyme storage diseases	Other genetic disorders
Cartilage-hair hypoplasia	– Thalassemia	Adrenoleukodystrophy	Familial hemophagocytic lymphohistiocytosis
Chediak–Higashi syndrome	– Thalassemia	Fabry disease	Hemophilia A
Chronic granulomatous disease (CGD)	Sickle cell disease	Farber's disease	Infantile osteopetrosis
Kostmann's syndrome	Erythrocyte alloimmunization (Rh isoimmunization)	Fucosidosis	Osteogenesis imperfecta
Leukocyte adhesion deficiency		Gaucher disease	Shwachman–Diamond syndrome
Omenn syndrome		Globoid cell leukodystrophy (Krabbe's disease)	
Severe combined immunodeficiency syndrome (SCID)			
Wiskott–Aldrich syndrome		Metachromatic leukodystrophy	
X-linked immunodeficiency with hyperimmunoglobulin M		Mucopolysaccharidoses (MPS) I-H (Hurler's syndrome)	
X-linked Bruton agammaglobulinemia		MPS II (Hunter syndrome)	
		MPS IIIB (Sanfilippo B syndrome)	
		MPS IV-A/B (Morquio syndrome)	
		MPS VI (Maroteaux–Lamy syndrome)	
		MPS VII (Sly syndrome)	
		Niemann–Pick disease (types A and B)	

Immunodeficiency disorders	Hemoglobinopathies and Rh disease	Enzyme storage diseases	Other genetic disorders
		Wolman's disease	

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Part II
Novel Applications of Special Types of Stem Cells

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7. Adipose-Derived Stem Cell-Based Therapies in Regenerative Medicine

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7.1 Introduction

While adult stem cells have a lower differentiation capacity than embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) [1–7], the use of adult stem cells bypasses many of the ethical and safety issues that limit clinical applications of stem cell therapy. Since the identification of multipotent stem cells in the bone marrow 40 years ago [8], bone marrow stem cells (BMSCs) have become the gold standard in regenerative medicine because of their accessibility, availability in both autologous and the allogeneic settings, multipotency, and relative safety [1, 9, 10]. However, the harvesting of BMSCs requires bone marrow aspiration, which is still an invasive, relatively painful procedure that yields low to moderate numbers of cells [1]. Recently, adipose

tissue has been reported by many groups to be a safe, easy, and efficient source of mesenchymal stromal cells (MSCs). Compared to bone marrow, adipose tissue is abundant, superficial, and easy to access, enabling the collection of relatively large quantities of fat and harvesting large numbers of cells. Furthermore, adipose tissue harvest contains larger numbers of mesenchymal cells compared to bone marrow. One gram of adipose tissue can yield roughly 2×10^6 cells, with 10 % of these cells believed to be adipose-derived stem cells (ASCs) [11–13]. Since the ability to obtain and infuse large numbers of cells is frequently considered a limiting factor in cell transplantation therapy, adipose tissue provides a significant advantage as a promising alternative source for marrow stem cells [14–16].

ASCs can be expanded in vitro, and differentiate into cells of different lineages, including adipogenic, osteogenic, chondrogenic, myogenic and neurogenic lineages [14–20]. Despite this apparent multipotency, the suitability of ASCs to differentiate in vivo into the desired cell populations is still under investigation, and clinical trials utilizing ASCs are still considerably few compared to in vitro studies, and to animal experimentation.

In addition to multilineage differentiation potential, colony forming unit fibroblast (CFU-F) is another pluripotency assay that characterizes the “stemness” of ASCs. Compared to marrow MSCs, ASCs have been shown to form tenfold more CFU-F units [21]. Moreover, ASCs exhibit immunomodulatory properties [22, 23] and seem to be more genetically stable during long-term culture procedures [24] compared to BMSCs [25]. Despite these advantages that make ASCs an excellent candidate for clinical transplantation settings, clinical trials using ASCS have not been similarly promising. Comprehensive characterization of ASCS, and in vivo tracking studies to confirm their origins and differentiation potential are still lacking.

7.2 Isolation, Characterization and Immunophenotype of ASCs

Unlike marrow MSCs, ASCs are significantly heterogeneous populations of cells and can be collected from variety of sources. In rodents, ASCs can be obtained from the fat pads of rats, as first described by Rodbell and colleagues in the 1960s [26–28]. In his protocol, the rat fat pads were minced into small fragments, digested at 37 °C with type I collagenase. After centrifugation, the supernatant which consisted of mature floating adipocytes, was separated from the pellet that consisted of the stromal vascular fraction (SVF). The latter fraction contained adipocyte progenitors as well as cells of the hematopoietic lineage.

In humans, ASCs can be isolated from several types of adipose tissue, including visceral fat, subcutaneous fat, and organ fat. Convenient sources of human fat include adipose tissue waste that results from plastic reconstructive surgeries and liposuction aspirates [29]. Liposuction material is an especially convenient source of ASCs as the procedure provides homogeneous, finely minced adipose fragments, which can be efficiently subjected to enzymatic digestion. Compared to whole adipose tissue fragments, where the tissues are minced manually, liposuction products require less time and effort for sufficient enzymatic digestion [30]. While many reports show that ASCs can be collected in large numbers compared to cells from bone marrow harvest, conflicting data on the effect of the site and methods of harvesting on the overall viability of the ASCs have been reported. For example, in a study by Fraser et al. [31] the data demonstrated that neither the harvesting technique nor the site of harvest affected the number of collected ASCs. Oedayrajsingh-Varma and colleagues investigated three harvesting techniques (tumescent liposuction, fat resection and ultrasound-assisted liposuction), and the results showed that the harvesting procedure affected the recovery of ASCs, whereas ultrasound-assisted liposuction resulted in the lowest number of proliferating ASCs [12]. Since then, many groups working independently have generated and refined methods of isolation and characterization of ASCs [32–35]. However, because of the small number of published studies and the differences in the separation protocols, there is no consensus

on the optimal site of harvest, harvesting technique or purification strategy.

Like bone marrow mesenchymal stromal cells (BM-MSCs), ASCs are characterized based on adherence to plastic, expression of phenotypic surface markers in their undifferentiated state, and differentiation into cells of chondrogenic, adipogenic, and osteogenic lineages when cultured with the appropriate growth factors. Unlike ESCs, undifferentiated ASCs cannot be characterized using well-defined markers but by a combination of markers that are not exclusive to ASCs but also shared by marrow MSCs, such as CD271 [36], STRO-1 [37], STRO-3 [38] and MSCA-$1^{+}CD56^{+}$ [39]. BMSCs and ASCs thus demonstrate very similar cell surface marker expression patterns [40], and both express the same cell surface marker characteristic for MSCs, meeting the criteria set by the International Society for Cellular Therapy (ISCT) [41]. Marrow MSCs, however, lack the expression of CD49d, which is substantially expressed by ASCs, while the latter lacks expression of CD106, which is highly expressed on marrow MSCs [42]. This divergent expression pattern is intriguing because CD106 is the receptor for CD49d and the two molecules are important for hematopoietic stem cell (HSC) homing to, and mobilization from the bone marrow [43, 44]. CD29, CD73, CD13, CD90, CD133, and MHC I surface molecules have been identified with highly coherent patterns of expression on the cell surface of ASCs (Table 7.1). Markers that are consistently and strongly expressed by ASCs are CD73, CD44, CD29, CD13, CD90, CD105, CD166 and MHC I, while markers of the angiogenic and hematopoietic lineages, such as CD133, CD45 and CD31, were demonstrated to be lacking or modestly expressed. As such, there is currently no consensus on uniform markers that could be detected on ASCs and characterize their phenotype consistently and reproducibly. The lack of consistency may be in part due to our lack of knowledge on the optimum type of fat source of ASCs, the heterogeneity of donors, the inconsistent culture conditions, and the sensitivity of the detection methods.

Table 7.1 Phenotype of freshly isolated human SVF and ASCs

CD Antigen	SVF	ASCs	Ref.
CD10	+	+	[34, 132]
CD13	+	+	[34, 41, 132]
CD19	−	−	[132]
CD24	+	−	[32, 132, 133]
CD29	+	+	[32, 34, 41, 134]
CD31	−	−	[132, 134, 135]
CD34	+	−	[32, 34, 41, 132, 134, 135]
CD44	+	+	[32, 34, 41, 132]
CD45	−	−	[34, 136]
CD71	+	+	[32, 41]
CD73	+	+	[32, 34, 41, 132, 134–136]
CD90	+	+	[32, 34, 41, 132]
CD105	+	+	[41, 134–136]
CD106	−	−	[132, 137]
CD146	−	−	[132, 133]
CD166	+	+	[32, 34, 41, 133, 135]
CD271	+	+	[32, 137]
MHC II	−	−	[41, 133, 135]
STRO-1	+	+	[132, 133]

7.3 Immunomodulatory Properties of ASCS

The unique immune profile of ASCs and MSCs in general makes them attractive source for cell transplantation purposes. The functional characterization of both marrow MSCs and ASCs shows both cell types lack expression of MHC II molecule, which renders them less immunogenic than other

cell types [41, 45]. In particular, ASCs were shown to inhibit the production of proinflammatory cytokines and augment the production of anti-inflammatory cytokines and antigen-specific Treg cells [46]. Unlike marrow MSCs, however, full characterization of the immune profile, and specifically, the immunosuppressive properties of ASCs have not been achieved yet. The immunosuppressive potential of ASCs has been observed in experimental transplantation, where they provided profound immunomodulatory properties and protective effects against acute graft-versus-host disease (GvHD) [47]. ASCs have been shown be immuneprivileged [48], in addition to exerting an immunosuppressive effect and inhibiting the proliferation of activated allogeneic lymphocytes [48–50]. Moreover, ASCs have been demonstrated to ameliorate the production of proinflammatory cytokines by both CD4 T helper cells and CD8 cells, stimulate the production of the anti-inflammatory cytokine IL-10 by T lymphocytes and monocytes, and prompt the generation of antigen-specific regulatory T cells [46]. In vivo, ASCs were shown to promote engraftment of allogeneic stem cells and treat or prevent severe GvHD in allogeneic transplantation settings [47, 50]. Compared to marrow MSCs, ASCs were shown to have more potent immunomodulatory effects, as lower numbers of ASCs triggered the same level of immunomodulation elicited by marrow MSCs [51], indicating that ASCs present a viable, effective and easily accessible alternative to marrow MSCs for immunomodulatory therapy (Fig. 7.1).

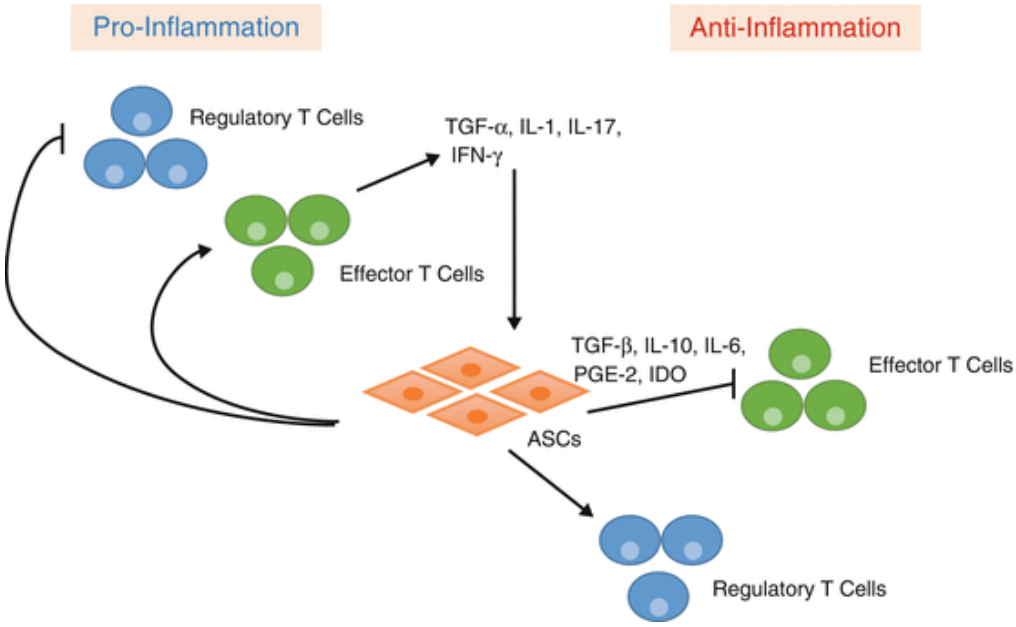

Fig. 7.1 The immunomodulatory properties of ASCs are related to the inflammatory status. Inflammatory mediators such as TNF-α, IL-1 and IL-17 produced by effector T cell in response to inflammation cause ASCs to migrate to the inflammatory site. At the site of inflammation, these mediators stimulate the production of immunosuppressive cytokines, such as TGF-β, IL-10, IL-6 and PGE-2, which in turn inhibit effector T cells and stimulate regulatory T cells. Subsidence of inflammation causes T cell activation and promotes cell-mediated immune response

When considering the immunosuppressive effects of ASCs for the treatment of GvHD and autoimmune diseases, caution should be taken to avoid some of the undesirable effects observed in experimental transplantation. Muehlberg and colleagues showed that upon intravenous administration of ASCs in mice, they home to the tumor site and support tumor growth [52]. Yu and colleagues reported that cotransplantation of human ASCs together with cancerous cells into BALB/c mice, either subcutaneously or intracranially, supported tumor growth [53]. Conversely, Kucerova and colleagues reported that cytosine deaminase-expressing ASCs carry the cytosine deaminase transgene to the site of tumor formation and mediate a strong antitumor effect in vivo [54]. Similarly, Grisendi and colleagues showed that ASCs are good candidates for cellular vectors in TNF-related apoptosis-inducing ligand (TRAIL)-based cancer therapy [55]. Cousin and colleagues showed that ASCs strongly hinder the proliferation of pancreatic ductal adenocarcinoma cells, both in vivo and in vitro, by interfering with the proliferation of cancerous cells and altering cell cycle progression [56]. These

conflicting reports may be explained in part by varieties in the used protocols, both in vitro and in vivo. Although an experimental model is never totally consistent with the complex mechanisms in nature, the discrepancies in these reports demonstrate that full biological characterization of ASCs is still for the most part lacking. In absence of well-characterized, pure populations of ASCs, the conflicting data on their tumor-enhancing effect cannot be attributed to specific cell population and defined mechanisms. The ASC-rich vascular fraction contains several other cells, especially vascular cells with high angiogenic functions that may contribute to the observed effects on tumor formation

7.4 Preclinical Studies Using ASCs

There is an abundance of published studies in animal models assessing the safety and efficacy of ASCs (reviewed in [57]). The majority involves the use of rodents, owing to their low cost, size, availability of antibody probes and access to inbred, transgenic, and genetically modified strains. But a substantial number of studies have used canine, ovine, porcine, and other large animal models. Studies involving large animals have suffered from a lack of comprehensive analysis owing to the absence of appropriate monoclonal antibodies and reagents used for cell tracking and immunophenotypic characterization. Nevertheless, accumulating data on the safety and efficacy of ASCs suggest readiness for applications in a clinical setting.

Myocardial Infarction: The therapeutic efficacy of ASCs in experimentally-induced myocardial infarction (MI) has been assessed by different groups [58–60]. In these studies, MI was induced by left anterior descending coronary artery ligation. The authors reported improved left ventricular function and improved myocardial function by all measurements including serial echocardiography, histology and immunofluorescence. Left ventricular ejection fraction and fractional shortening were improved and anterior wall thinning was also attenuated after ASC treatment. Post-mortem histological examination showed decreased fibrosis in ASC-treated hearts, in addition to increased peri-infarct

density of both arterioles and nerve sprouts. Moreover, immunofluorescence revealed that grafted ASCs underwent cardiomyocyte differentiation [58]. Human ASCs survived in injured hearts for up to 4 months, as detected by luciferase-based bioluminescence imaging. These positive results have encouraged clinical trials to assess the safety and feasibility of human ASCs for the treatment of MI (NCT00442806).

Bone Defects: The potential of ASCs in treating bone defects in animal models has been explored in several preclinical studies [61–63]. To investigate their in vivo osteogenic capacity to heal critical-size mouse calvarial defects, ASCs were seeded onto scaffolds and implanted into the bone defect. Significant intramembranous bone formation and areas of complete bone regeneration were achieved as shown by X-ray analysis, histology and live micro-molecular imaging. Furthermore, the authors demonstrated that the implanted cells participated to 84–99 % of bone formation by chromosomal detection [61]. Moreover, different results have supported the use of ASC-loaded scaffolds to facilitate spinal fusion [62, 63]. In these studies, there was reduced infiltration of inflammatory cells in the spinal fusion masses with ASC-loaded scaffolds compared to scaffolds alone. Additionally, the ASC-loaded scaffolds showed superior fusion mass mineralization and better remodeling than scaffolds alone.

Diabetes Mellitus (DM): ASCs were shown in many laboratories to differentiate into insulin-producing cells [20, 64, 65]. ASCs ameliorated hyperglycemia in experimentally-induced DM, as after their administrating, fasting blood glucose levels significantly decreased starting from the second week after therapy [66–68]. Moreover, ASCs significantly decreased pancreatic islet damaged induced by stroptozotocin and increased the expression of insulin in pancreatic β cells. Furthermore, their vasculogenesis and angiogenesis properties facilitated engraftment and revascularization of donor pancreatic islets when ASCs were co-transplanted with them and reduced the islet mass required for reversal of diabetes [69]. Additionally, immunomodulatory and anti-inflammatory effects of ASCs could protect donor islets during the early

phase of transplantation and subsequently improve engraftment of donor islets into the recipient organs [70].

Central Nervous System: The differentiation capacity of ASCs into neuron-like cells expressing markers typical for mature neurons has been reported by several laboratories [71–73]. In addition to their neural differentiation capacity, the therapeutic capacity of ASCs in animal models of diseases of the central nervous system has shown promising results [74–77]. Transplantation of ASCs in an animal model of stroke or ischemia reduced both acute cerebral inflammation and chronic brain degeneration, and promoted long-term functional recovery [74, 75]. ASC transplantation has improved motor functions in rat models of spinal cord injury [76]. In experimental autoimmune encephalomyelitis, intravenous administration of ASCs in an animal model of multiple sclerosis, reduced disease severity via modulation of the immune responses, and decreasing the severity of spinal cord inflammation and demyelination, in addition to inducing local neuroregeneration [77].

Cartilage Repair: Cartilage is an avascular tissue, suggesting that an angiogenic signal would not be appropriate when chondrogenesis was the intent. Lee et al. showed that rat ASCs produced large amounts of vascular endothelial growth factor (VEGF), which inhibited proliferation of chondrocytes and increased their apoptosis [78]. However, culturing ASCs with chondrogenic medium significantly reduced VEGF secretion and the detrimental effects of the secreted factors on chondrocyte regeneration. When ASCs were implanted in a rat model of chondral cartilage defects, the degree of healing was not significantly different from that of defects where no ASCs were administered [78]. These data suggest the need for methods to adapt. ASCs to for enhancing cartilage repair by abolishing the damage caused by the secretion of VEGF and other factors that inhibit cartilage regeneration and prevent cartilage repair.

Gastrointestinal Tract (GIT): ASCs were recently shown to suppress effector T-cell responses and have therapeutic effects in some autoimmune diseases [79]. Systemic infusion of ASCs ameliorated the symptoms and histopathologic

severity of colitis, eliminating diarrhea, body weight loss, and inflammation and also increased survival in a murine model of induced colitis [80]. This therapeutic effect was mediated by down-regulating a wide range of proinflammatory cytokines and by increasing IL-10 levels and activating regulatory T lymphocytes. This suggests that ASCs can be an attractive source of cell-based therapy for GIT disorders, and an effective regulator of immune responses in these diseases.

Liver Disorders: The hepatogenic differentiation capacity of ASCs has been investigated in both in vitro [19, 81–86] and in vivo animal models [19, 81, 85]. Infusion of of ASCs in an experimentally induced animal model of liver failure showed enhancement of liver morphology and function. Transplanted mice lived longer, and their liver showed higher proliferation and less apoptosis [84, 87–89].

Vascular Disorders: The angiogenic potential of ASCs have been investigated in an animal model of hind-limb ischemia. Intravenous administration of ASCs in rodents' ischemic hind limbs resulted in better post surgical recovery and prognosis, and histological analysis revealed increased vascular density and reduced muscle atrophy [90]. Interestingly, ASCs were superior to marrow MSCs in their capacity to promote neovascularization in response to vascular ischemia [91]. Administration of ASCs promoted recovery of blood flow in the ischemic limb and boosted neovascularization. In vivo, ASCs showed a significantly higher laser Doppler perfusion index, and better in vitro tube formation compared to marrow MSCs, suggesting that ASCs can achieve highly desirable therapeutic neovascularization in ischemic diseases.

7.5 Clinical Trials Using ASCs

When writing this chapter, the search for clinical trials utilizing either SVF cells or ASCs yielded 141 trials after exclusion of the withdrawn and terminated studies- of which 42 trials have been completed so far (http://clinicaltrials.gov) (Fig. 7.2). Only 3 of the ongoing trials are in phase III. Evidently, there is much discrepancy between preclinical

studies and clinical applications, and therefore applications of ASCs remain for the most part, and up till publication of this book, experimental. Data from these few clinical studies, however, have been encouraging. Safety studies have been most promising, as various forms of treatment, types of cells, and methods of injection have all been well tolerated by patients, with no or mild side effects reported. Slowly emerging data on the efficacy of ASCs have been reported in the following conditions:

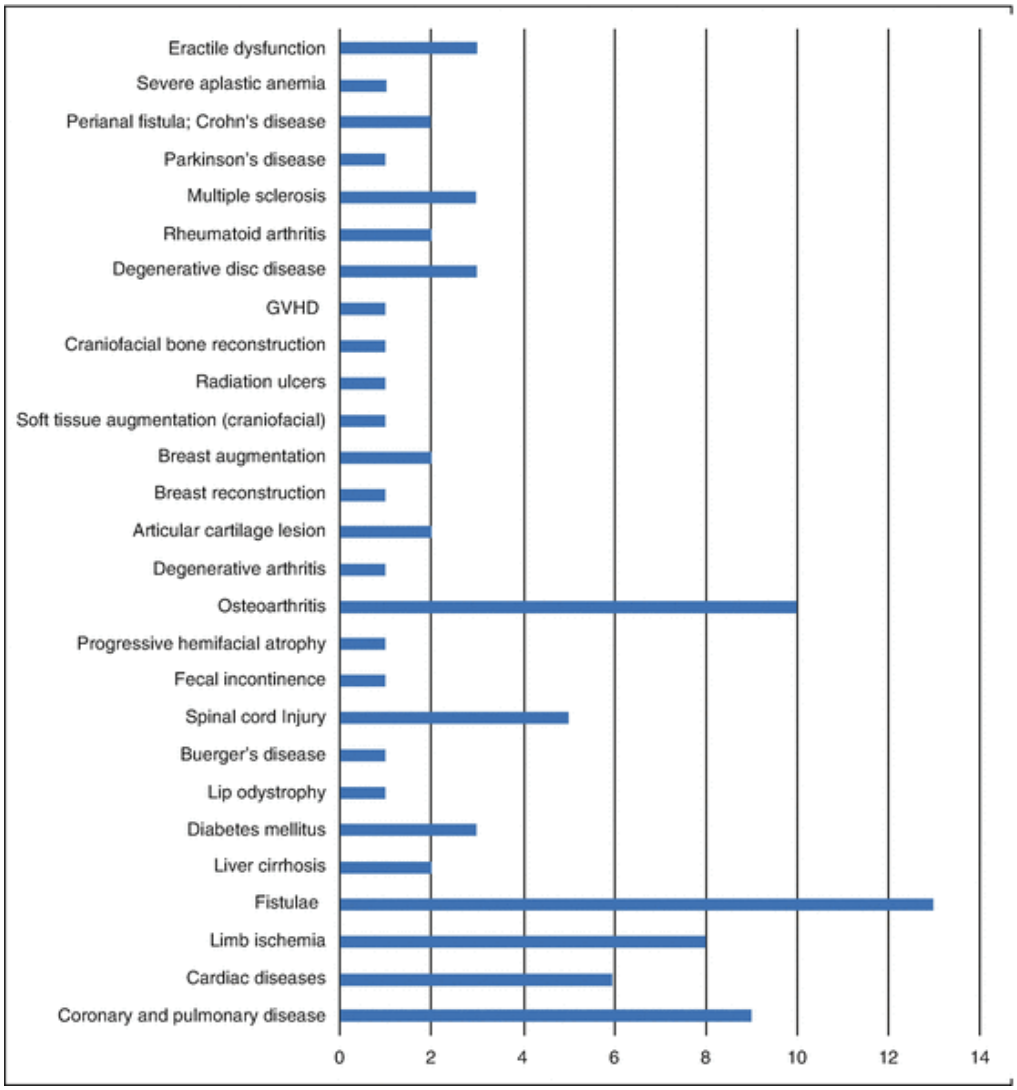

Fig. 7.2 Ongoing clinical trials using adipose stem cells as identified on http://clinicaltrials.gov

Graft-versus-Host Disease (GvHD): Fang and colleagues reported several case studies where ASCs were administered for treating severe and acute GvHD caused by allogeneic hematopoietic stem cell transplantation [92–95]. In these cases, marked improvement in acute GvHD symptoms, such

as diarrhea, skin rash, and liver function, was observed. In another study, six patients with steroid-refractory acute GvHD were infused with ASCs. In five out of six patients, the GvHD was completely resolved.

The same group reported a case study where ASCs were given as a salvage therapy in a case of chronic hepatic GvHD that was resistant to immunosuppressive therapy. In this case, hepatic inflammatory conditions were improved, as evidenced by the decrease in autoreactive T-cell migration to the inflammatory site, the decrease in production of interlukin1, 4 and interferon-γ by mononuclear cells, and the reduction of mutant human leukocyte antigen (HLA)-1 molecules on hepatic cells. Engrafted ASCs seem to have contributed to the regeneration of the liver tissues [95].

Multiple Sclerosis: Autologous SVF cells were used to treat three patients with relapsing-remitting multiple sclerosis [96]. Each patient received two intravenous infusions with autologous adipose derived SVF cells and multiple intrathecal and intravenous infusions of allogeneic $CD34^{+}$ and MSCs over 9–10 days, with no significant side effect reported. In addition to some improvements in functional neurological testing, follow up showed an overall improvement of patients' quality of life. However, because of the small number of patients and the lack of robust analysis and long term follow up, conclusive data about the therapeutic effect of SVF cells on multiple sclerosis necessitate further clinical investigations.

Diabetes Mellitus (DM): In a study published in 2008, Trivedi and colleagues performed the first trial to treat type 1DM using ASCs. Five diabetic patients were included in the study. They received ASCs cotransplanted with unfractionated cultured bone marrow cells. The study followed the patients for a period of 2.9 months, and showed that all treated patients had no reported side effects. Insulin requirements were decreased by 30–50 %, and the level of serum c-peptide levels was increased [97]. Overall improvement in the general health and lifestyle was observed, and none of the patients reported episode of ketoacidosis.

Fistulae: García-Olmo et al. reported using autologous ASCs, collected from lipoaspirate to treat a young female patient suffering from recurrent rectovaginal fistulae due to crohn's disease. The patient's general condition improved, there was good closure of the fistulae up to the 3 months follow up period, and the patient reported absence of vaginal flatulence and rectal incontinence through the vagina [98]. In the phase I clinical trial by the same group, four patients with crohn's disease suffering from 9 perianal fistulae were treated with autologous ASCs. The fistulae were followed weekly for 8 weeks. Six fistulae out of eight showed signs of healing, as determined by complete reepithelization of the external opening, while 2 fistulae did not heal. The ninth patient experienced contamination of the cultured cells, and was excluded from the study. No side effects were reported by the patients during an average of 22 months following surgery. Owing to these promising results, authors recommended proceeding to phase II trials [99].

Multicenter, randomized controlled phase II clinical trials by same group tested the safety of administering autologous ASCs in the treatment of perianal fistulae. Thirty-five complex perianal fistula cases (of cryptoglandular origin, of which 14 cases were associated with crohn's disease) received local injection of 20 million ASCs along with fibrin glue treatment, or using fibrin glue alone. A second dose of ASCs was administered if no healing was observed after 8 weeks. Patients were evaluated at 8 weeks and after 1 year. Coadministration of ASCs with fibrin glue improved the healing of fistulae in both Crohn's and non-Crohn's patients, better than the administration of fibrin glue alone. However, 17.6 % of the treated cases showed recurrence after 1 year. No side effects were reported during the follow-up period, and the authors established the safety of this form of treatment [100].

In a following randomized, single blinded, multicenter phase III clinical trial, 200 adult patients suffering from perianal crypto glandular fistulae randomly received 20 million ASCs with or without fibrin glue. Control patients received fibrin glue alone and were followed up at 24–26 weeks, and after 1 year. If no healing was observed, the

patients received a second dose of treatment. During the follow-up period, 40 % of the patients who received ASCs with or without fibrin glue showed healing after 6 months, while 50 % of the patients healed after 1 year. However, there were no significant differences between the healing in patients treated with stem cells alone compared to fibrin glue alone [101].

In a different study to examine the therapeutic potential of ASCs for healing fistulae associated with Crohn's disease [102], the researchers increased the number of administered ASCs in direct proportion to the size of the fistulae. Fistulae were loaded with ASCs in combination with fibrin glue after intra-lesional administration of ASCs. Complete healing of the fistulae was achieved in 27/33 patients (82 %) by 8 weeks after cell therapy. No side effects related to the injected cells were observed.

Bone Tissue Defects: In a follow up to preclinical studies in which ASCs showed potential to treat bone defects, two clinical case studies were reported where patients received ASCs to repair the defects [103, 104]. In the first case, 7-year-old girl, who had massive calvarial defects due to severe head injury, failed fixation therapy to multifragment calvarial fractures, which left her with an unstable skull. Owing to a deficiency in the autologous cancellous bone from the iliac crest, the calvarial deformity was treated with freshly isolated autologous SVF cells coadministered with fibrin glue. New bone was formed and near complete closure of the calvarial defects, and clavarial continuity were achieved as determined by CT scan.

In the second case, a patient who suffered hemimaxillectomy was treated by reconstruction of the bone defect using microvascular flab consisting of autologous ASCs and beta tricalcium phosphate combined with bone morphogenetic protein-2 (BMP-2) [104]. Autologous ASCs were isolated and cultured using animal-free reagents in combination with beta tricalcium phosphate, and BMP-2 in a clean room in order to composite a flab. Mature bone structure and vasculature were developed in the flab after a period of 8 months, and the flab was transplanted into the defect.

Postoperative healing was achieved, suggesting that the use of autologous ASCs in microvascular reconstruction surgery could be very promising in bone defects.

Osteoarthritis: Recently, Jo et al. carried out a clinical trial to investigate the efficacy and safety of the use of intra-articular injection of autologous ASCs as a therapy for knee osteoarthritis. The trial was divided into 2 phases with a total 18 patients. In phase I, 9 patients were subjected to intra-articular injection of low (1.0×10^7), mid (5.0×10^7), and high doses (1.0×10^8) of ASCs, in 3 groups of patients with 3 patients in each group. In phase II, 9 patients received intra-articular injection of high dose of ASCs. Results were promising in those patients who had received a high doses of ASCs. Western Ontario and McMaster Universities Osteoarthritis index (WOMAC) score (a valid assessment of knee and hip osteoarthritis where an increase in pain and stiffness and a deteriorated function indicate high WOMAC scores) was assessed 6 months post operatively. Reduction in the size of the cartilage defect in medial femoral and tibial condyles was determined by arthroscopy, and histological examination showed that the volume of the cartilage was increased. Interestingly, patients suffered no postoperative side effects. The research group concluded that a high dose of ASCs (1.0×10^8) was safe and effective in the treatment of knee osteoarthritis, shown by enhancement of knee function, decreasing pain, and increasing cartilage regeneration [105].

Neurological Defects: To test the efficacy of stem cell transplant to repair spinal cord injury, Ra et al. infused 8 male patients with a history of spinal cord injury for more than a year with ASCs. A single dose of 4×10^8 ASCs was intravenously administrated in these patients with follow up period of 3 months. No side effects, especially incidences of tumor formation, were observed during the follow-up period, indicating that intravenous administration of ASCs maybe a safe approach to treating spinal cord diseases [106].

Critical Limb Ischemia (* CLI *): ASCs were administered to 15 male CLI patients who suffered ischemic resting pain in one limb with/without nonhealing ulcers, and necrotic foot.

Multiple intramuscular ASCs injections were successfully infused, with no complications during the mean follow-up period of 6 months. Clinical improvement was shown in 66.7 % of patients. Five patients needed minor amputation during the follow-up, and all amputation sites healed efficiently. At 6 months, significant enhancement was noted in functional tests, such as claudication walking distance and pain rating scales. Vascular collateral networks across affected arteries were formed as determined by digital subtraction angiography at baseline and 6 months after ASC implantation [107].

Cosmetic Applications: Koh et al. reported the use of ASCs in the treatment of progressive hemifacial atrophy (Parry-Romberg disease), a disease characterized by atrophy of facial skin, dermis, fat, cartilage and bone. ASC therapy was applied to enhance angiogenesis in order to improve microfat grafting. 10 patients were subjected to injection of microfat grafts in combination with ASCs or microfat graft alone, and followed for an average period of 15 months. Results showed that the survival of the graft that included ASCs injections was better than that without ASCs. The researchers concluded that microfat graft plus ASCs could be used to treat progressive hemifacial atrophy, without the need for the microvascular-free flap transfer that was previously used in the treatment of such cases [108].

Yoshimura and colleagues used a novel procedure called cell-assisted lipotransfer (CAL) to avoid side effects of the lipo-injection for treatment of facial lipoatrophy and for breast augmentation [109, 110]. In CAL, SVF is isolated from half of a fresh adipose sample and then attached to other half of the sample, so that fat in the other sample acts as a scaffold for SVF cells in order to enrich the ASC-poor fat to become ASC-rich fat. Six patients with facial lipoatrophy caused by lupus profundus or Parry-Romberg syndrome were treated; three patients were treated using CAL, while three patients received conventional lipoinjection (non-CAL). Improvement in facial contouring was achieved, and a better outcome was observed in the CAL group [109].

In another study, 40 female patients underwent the CAL procedure for cosmetic breast augmentation. The procedure

was effective without any observed side effect. No postoperative fat atrophy occurred when the patients were followed up for 2 months after the operation. Further clinical studies were required to prove efficacy of this procedure [110].

Cardiomyopathy: To examine the efficacy of autologous ASC infusion in no-option cases of ischemic cardiomyopathy, Perin et al. carried out a randomized, placebo-controlled, double-blinded clinical trial 21 patients were treated with autologous ASCs via trans-endocardial injection, and 6 patients served as control. The patients were followed-up postoperatively for up to 36 months for safety and efficacy of the treatment. In comparison with the control group, ASC-treated cases showed improvement in their total left ventricular mass as shown by cardiac magnetic resonance and wall motion score index. Furthermore, a decrease in inducible ischemia for the 18-month follow up period was shown by single-photon emission computed tomography. It was concluded that trans-endocardial injection of autologous ASCs in no-option ischemic cardiomyopathy patients enhanced myocardial perfusion, ventricular function and exercise tolerance in these cases [111].

7.6 Dedifferentiated Fat Cells

Mature adipocytes undergo dedifferentiation in ceiling culture, giving rise to spindle shaped cells called dedifferentiated fat cells, or DFAT cells. By dedifferentiation, adipocytes lose lipid metabolism function while simultaneously acquire a multipotent capacity [112, 113]. However, the mechanism of DFAT cell dedifferentiation is still unclear. DFAT cells are a homogeneous cell population compared to ASCs driven from SVF, which are heterogeneous cell populations. Because mature adipocytes are available in great abundance in adipose tissue, DFAT cells are considered a promising source of multipotent cells over ASCs [112, 114, 115].

DFAT cells were first isolated from the fat of the bone marrow of metacarpal bones in neonatal calves, and later from subcutaneous adipose tissue [116, 117]. After adipose tissue digestion with the traditional incubation with collagenase, the

floating layer atop the digested fat is collected, filtered and washed then cultured in a flask filled with DMEM with 20 % fetal bovine serum for 7 days. Cells will adhere to the roof of the flask, start to lose their rounded, unilocular appearance, and gradually become spindle-shaped with the break of their lipid droplet into multiple droplets, giving the cells a multilocular appearance. This change is usually followed by complete loss of the lipid droplet after 2–3 weeks in culture [112, 114, 115].

7.6.1 Multipotency of DFAT cells

During the process of generating DFAT cells from mature adipocytes, there is a downregulation of function regulating genes, or genes that are involved in lipid metabolism, such as ADIPOQ, LIPE, PDK4, LPL, FASN, PPARG and FABP4, and simultaneous upregulation in cell proliferation genes (such as SERPINE1, TIMP1, PLAU, SFRP2, AEBP1, PRRX2, PEG10, IGFBP5 and ID2). Genes involved in cell migration, tissue development and altered cell shape are also upregulated [113]. Interestingly, it has been demonstrated that DFAT cells express pluripotency markers similar to ESCs such as OCT4, SOX2, c-MYC and Nanog, in addition to having high alkaline phosphatase and telomerase activity. The latter was shown to be higher in DFAT cells compared to ASCs. Characterized as pluripotent and generated by dedifferentiation, DFAT cells are considered induced pluripotent stem cells [118]. While, DFAT were shown to be similar, they are not identical to MSCs. For example, DFAT cells exhibit fibroblast- like morphology, express CD90, CD105, CD73, CD44 and CD29 surface markers, and are negative for CD34, CD14, CD117, CD133, CD271, CD45 and HLA-DR surface markers. They differentiate into bone, fat and cartilage cells, and have distinct immunomodulatory effects and especially immunosuppressive functions [119]. DFAT cells, however, express the CD31 marker, which is not expressed by MSCs [118]

Mesodermal Lineage Differentiation: DFAT cells were shown to differentiate into osteocytes, adipocytes and chondrocytes in vitro [120]. Rabbit DFAT cells differentiated into osteoblasts in titanium fiber meshscaffold [121].

Adipogenic markers (such as PPARγ, C/EBPα, C/EBPβ C/EBPδ and SREBP- 1c) were still expressed, indicating that fat differentiating ability is retained [112, 120, 122]. Similarly, DFAT cells seem to retain their propensity for adipogenic differentiation when administered in vivo. Subcutaneous injection of DFAT cells over the sternum in mice led to fat pad formation [122].

Myogenesis: When stimulated with myogenic inducing factors, DFAT cells expressed MyoD and myogenin, formed skeletal myotube in culture in addition to multinucleated cells expressing myosin heavy chain [115]. DFAT cells differentiated as well into smooth muscle cells, both in vivo and in vitro. Human DFAT cells expressed smooth muscle actin and contributed to bladder regeneration upon in vivo injection [123]. DFAT cells promoted regeneration of the urethera and uretheral sphincter, and improving sphincter function by differentiation into smooth muscle [124].

Cardiogenesis: DFAT cells have been shown to express cardiac markers when co-cultured with cardiomyocytes or in methylcellulose media. In vivo, injection of DFAT cells into rat ischemic heart lead to cardiac regeneration and enhancement of cardiac vascularity [114]. Eight weeks after DFAT cell injection in acute myocardial infarction rat model, the expression of cardiac sarcomeric actin increased, indicating the cells' cardiomyogenesis potential. Furthermore, capillary density was enhanced in the infarction area. It may be concluded that DFAT cells enhance cardiac tissue regeneration by cardiomyogenesis and improvement of cardiac perfusion [114].

Angiogenesis: DFAT cells showed the capacity to differentiate into endothelial cells, as shown by the formation of vessel-like structured in matrigel. These observations led to the proposal that both endothelial cells and adipocytes may have a common origin. In vivo, DFAT cells have enhanced vascularization of ischemic tissues. Injected DFAT cells significantly increased capillary density. in ischemic muscle tissue in mouse models of ischemia through neovessel formation [18, 114]

Other Lineages Differentiation: **Administration of** DFAT cells improved motor functions in rats that underwent spinal cord injury. Cultured DFAT cells expressed neural markers such as nestin, beta-III tubulin, and GFAP and have been shown to express neurotrophic factors like BDNF and GDNF, both in vitro and after transplantation [125]. Interestingly, markers of lymphoid and myeloid lineages has also been expressed in DFAT cells [113], further confirming their multipotency.

7.7 Conclusions and Future Perspectives

ASCs have demonstrated many biological characteristics that qualify them as a viable and effective source of cell based therapies. The can be obtained in large numbers from several accessible adipose tissues. They secrete myriad cytokines and growth factors, which sustain and promote tissue regeneration, such as hepatocyte growth factor and VEGF [126, 127], perhaps to a higher extent than their bone marrow counterparts [128]. Their immunomodulatory properties may support an anti-inflammatory milieu and immunosuppressive functions, all potentially of great benefit in supporting engraftment and sustainability of the graft.

Nevertheless, so far, comparatively few clinical trials have been carried out to evaluate the therapeutic effects of ASCs compared with the substantial number of published pre-clinical experimental studies [58]. Due to the potentially broad application in the clinical setting, various methods to evaluate the safety, reproducibility and quality of in vitro expanded ASCs are urgently needed. Similar to other types of stem cells, and because of the urgent need for regenerative therapy, ASCs have been rushed into clinical applications despite many questions on their safety, reproducibility, and standardization techniques. Culture expanded cells still require xeno-contaminants, such as bovine serum. Most of the highly attractive immunomodulatory functions of ASCs were performed on cells grown in bovine serum-supplemented culture media. Sporadic reports on the tumor-enhancing properties of ASCs, although not consistent, call for caution in

various clinical applications. Ensuring the genetic stability of the used cells, before and after short-term culture, is necessary for proper monitoring of their tumorogenic potential.

Many of the issues associated with the clinical applications of ASCs stem from assuming similarities to marrow MSCs. However, accumulating data suggest more differences than similarities between the two types of cells. For instance, while MSCs obtained from the bone marrow have shown consistent characteristics regardless of the site or type of their bone source, ASCs lack such consistency. There is substantial evidence that the differentiation capability of ASCs may differ according to the anatomic site of the fat and the donor's age and gender [129]. Furthermore, the key transcription factors and molecular events that initially ascribe ASCs to a particular lineage are still unknown. The heterogeneity of ASCs is another factor that limits clonal analysis and accurate biological characterization. Many stem cell criteria have been attributed to cells within the adipose tissue, and were simply categorized as adipose stem cells. Examples include pericytes, endothelial progenitor cells and DFAT cells. Clearly, better basic biological characterization is required to understand the origin, lineage, phenotype, and functional characters of each type of these cells. However, and despite of these limitations, application of ASCs in the clinical setting is considered a realistic option based on their impressive effects in experimental transplantation, and promising benefits in clinical medicine [130].

DFAT cells are another type of adipose tissue-derived cells that share many benefits with ASCs. Both types of cells are easily available as a byproduct of the increasingly popular liposuction, and the abundance of fat tissue. DFAT cells are more homogeneous and several-fold more abundant in cell cultures compared to ASCs. Robust proliferative and differentiation characteristics of DFAT cells are attractive features for their development and applications in tissue engineering and cell therapy. Given the recent characterization of DFAT cells, long term analysis in culture, genetic stability and understanding the mechanism underlying the generation of these cells remain to be understood. Comparisons that favor

DFAT cells over induced pluripotent stem cells for higher differentiation efficacy are probably premature [131].

The abundance of adipose tissue has thus created new interest in newer types of stem cells that could be purified with relative ease, and collected in large numbers. ASCs provide a unique source for investigating novel medications for a large array of acquired and inherited disorders, and to recognize new molecular targets for drug discovery. Generating large numbers of cells according to Good Manufacturing Practices (GMP) is relatively simple compared to other types of stem cells, including induced pluripotent stem cells. All these factors underscore the high potential of ASCs in regenerative medicine; nevertheless, many important questions on their biology and long-term safety remain to be answered.

Moving ASCs from bench to clinic has been rushed due to their attractive properties. However, extensive and systematic evaluation of the safety, reproducibility and clinical quality of in vitro expanded cells is lacking.

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8. Advances in Umbilical Cord Blood Therapy: Hematopoietic Stem Cell Transplantation and Beyond

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8.1 Introduction

Stem cells represent a wide range of cells from different sources with varying self-renewal capabilities, proliferation capacity, and differentiation potential. The term *stem cell* was initially used to define a cell found in the bone marrow capable of reproducing the full complement of blood cells [1]. With the identification of embryonic stem (ES) cells from mouse and human preimplantation embryos, the definition of stem cell was further delineated to include embryonic and adult, or tissue, stem cells. ES cells are defined as self-renewing cells that are pluripotent, capable of producing all the cells of the developing fetus. Adult stem cells are defined as stem cells with more limited differentiation potential, such as hematopoietic stem cells (HSCs), and incapable of producing all the cells of the developing fetus. Adult stem cells have expanded to include muscle satellite cells, retinal stem cells, neural stem cells, and mesenchymal stem cells (MSCs). Unlike muscle, retinal, and neural stem cells, which are found in specific tissues, MSCs have been isolated from a variety of tissues, including adipose tissue, umbilical cord tissue (CT), umbilical cord blood (CB), bone marrow (BM), and peripheral blood (PB). Although adult stem cells do not have the proliferation and differentiation robustness of ES cells, they have a proven track record in regenerative medicine. Bone marrow transplantation (BMT) has been a standard of care for four decades and represents the best example of cell-based therapy.

Umbilical CB is widely accepted as a rich source of hematopoietic stem/progenitor cells for BMT. The first successful human CB transplantation was performed over 25 years ago after Boyse observed that blood from a syngeneic neonatal mouse could rescue a lethally irradiated mouse [2, 3]. Since then, over 35,000 CB transplants have been performed to date [4]. CB has become an attractive source of HSCs owing to its many benefits over BM and PB. First, CB collection is a noninvasive procedure causing no harm or pain to the donors. Second, the human leukocyte antigen (HLA) matching requirements of CB are less stringent than those of BM. CB is tolerated in patients with one–two HLA disparities out of six HLA loci that are matched for CB, as compared to an 8/8 HLA match required for BM or PB. The less stringent

matching criteria for CB render it a viable option for patients unable to find a perfect BM match, notably for many minority groups and persons of mixed ethnicity who are typically underrepresented in the large BM registries. Importantly, transplantation with umbilical CB is associated with a lower incidence of graft-versus-host disease (GvHD) without compromising graft-versus-malignancy (GvM) effects and lower relapse rates compared to BMT. Lastly, since CB is cryopreserved, it is readily available for transplantation, as opposed to BM, which may take months from identification of a suitable donor to the day of transplantation. Disadvantages of CB are the limited number of cells per collection available for transplantation, restricting their use predominantly to pediatric patients. Additional consequences of a low cell dose include delayed engraftment, increased infection rates, and increased graft rejection.

The worldwide use of CB as a graft source for BMT has been predicated upon the establishment of public CB banks. More recently, the application of CB in the field of regenerative medicine has gained momentum. These studies, at least in the USA, have been dependent predominantly upon private CB banks. This chapter will compare the different CB banking models, review the use of CB for BMT, assess the application of CB in regenerative medicine, and outline the future application of CB in the field of induced pluripotent stem cells (iPSCs).

8.2 Banks Around the World: Characteristics of Public, Private, and Hybrid Banks

Worldwide, stored CB units in public and private (family) banks are estimated at more than four million units [4]. Public CB banks process and store donated CB units, which are made available to patients in need worldwide through multiple CB registries, such as the National Marrow Donor Program (NMDP), NetCord, and EUROCORD. CB registries operate in a manner similar to that of BM registries. Pertinent information of the CB unit, including HLA type and total

nucleated cell number, is listed on the CB registries and is searchable by transplant centers worldwide. Fifty-eight countries house 156 public CB banks [5], and the networking of these public banks through the registries has created a virtual bank of available CB units for use around the world [6]. Approximately 730,000 CB units have been donated and stored in public CB banks [4]. There is no cost to the family associated with donating CB to a public bank. Public CB banks recover their costs when they release a CB unit to a transplant center. For public banks, the larger the inventory, the greater the chance a match will be found for patients from their inventory, but the costs of running a large program will increase. Optimal inventory size, which balances cost and patient needs, has been calculated to assist in forming budgets for operating a public bank. Keeping an inventory of 50,000 units with diverse HLA types to ensure their eventual use translates into a release fee of approximately $15,000/unit to cover the costs of operations, although the actual average cost of a released unit in the USA is approximately $40,000 [7]. This cost takes into consideration the processing and storage costs associated with the CB units that are not used and therefore do not generate a source of income for the public bank. As the uses for CB increase, more units will be released for patient treatment, and the cost of obtaining a publicly banked CB unit should decrease.

Private (family) CB banks process and store CB for a specific individual or family. CB units in a private bank are not listed on registries and are not available for public use. Ninety-four countries have private CB banks with an estimated four million CB units banked solely for private or family use. Private banks charge fees for CB collection, processing, and storage. Typically, private banks have a range of payment options, with most charging a one-time collection and processing fee of US $1200–$1650 in North America and a yearly maintenance fee of US $125–$150, bringing the total cost of storing a unit for 18 years to US $3450–$4350. There are generally no costs related to the release of a CB unit to the family except for courier costs to transport the unit to the treating hospital.

Both public and private CB banks have associated benefits and limitations. For example, public banks increase the likelihood of finding a match for patients in need without financial cost to the donor; however, the donated unit may not be available to the donating family if the need arise. Additionally, the operating costs in the public banks are covered by transplant centers, philanthropic groups, and government funding, which may be unstable over time. Private CB banks, on the other hand, store CB specifically for given families, and it will be available only to the client if the need arises. To address the limitations of both banking models, different hybrid banking models are currently being explored [8]. These models include CB banks that provide both public donation and private storage options and innovations that make privately banked CB units available to the public. Among these innovations is a Spanish hybrid bank that privately banks CB units but also lists them on a BM registry. If a patient who could benefit from a banked CB unit is identified, then the parents must donate the CB unit and are reimbursed the storage fees. Similarly, several banks in Germany offer parents the option to privately bank CB units, which can be listed on registries for public use. However, unlike the Spanish model, parents are under no obligation to release the CB unit if it is a match to a patient. Finally, another hybrid model involves dividing the CB unit into two portions; 80% is made available to the public at large while 20% is maintained strictly for the private use of the family banking the CB unit. It is assumed that the remaining 20% can be expanded into a useful sample. Success in CB expansion is covered in a later section.

CB banks are typically regulated by federal agencies. Additionally, quality-minded CB banks would seek accreditation from one of two voluntary, international organizations, which are committed to promoting quality practices, laboratory processes, and banking to achieve high-quality CB units for administration to patients. These organizations include NetCord-FACT and AABB. Many private (family) banks have now obtained the same accreditation as public banks [5].

8.2.1 Probability of Finding a CB Match

With the publication of large patient studies, it has become clear that CB allows for less stringent HLA matching as compared to BM or mobilized PB. A report by Eapen et al. on 503 children with acute leukemia demonstrated that the 5-year leukemia-free survival rate was highest in the group that received 6/6 HLA-matched CB units (60% survival). Interestingly, survival rates were equivalent for the more stringently 8/8 matched BM (38% survival) compared to the single mismatched (5/6) CB units (45% survival with high dose of cells). Recipients of CB that had two HLA mismatches (4/6) had a survival rate of 33% [9]. The strict 8/8 matching used in the Eapen study, which is required for a BM transplant, limits the availability of potential donors, whereas the allowance for HLA disparity with CB essentially increases the donor pool. In a report on 553 patients searching for unrelated donor BM or CB, the criterion for a suitable donor aimed for a 10/10 match for BM with a minimum of 8/10 HLA match. The criterion for a suitable CB unit, on the other hand, was still kept at 6/6 with a minimum of 4/6 HLA match and a cell dose of at least 1.5×10^7 cells/kg. This study concluded that CB is best for providing an appropriate donor source regardless of race or ethnicity [10].

The optimal graft source for a patient in need of a BM transplant is a perfectly matched sibling; however, only approx. 30% of patients have such a match. The remaining patients must turn to BM registries, marrow drives, or CB banks to find a suitable match. At this point in their treatment cycle, the need for a BM transplant means that all other treatment options have been exhausted. Therefore, finding an appropriate donor is critical. One of the advantages of CB banks is they represent the range of ethnicities of our societies better than BM registries, which tend have a high occurrence of white European heritage represented. The better range of ethnicities in the CB banks might be due to the fact that many nonwhite majority countries have developed CB banks compared to investing in BM registries. Additionally, in many cases, the efforts of the public banks to recruit CB donors that best represent the ethnic/racial diversity within their

immediate community result in a diverse range of CB units that can be used worldwide. For example, the New York Blood Center has exported 5300 CB units to 40 countries [11].

Using a criterion of 6/6 HLA match for CB and 8/8 HLA match for BM, Gragert et al. estimated the probability that different ethnic and racial groups would find an optimal match within the 2012 NMDP database, which consisted of approx. 10^7 BM registrants and 186,166 typed UCB units, for adult (≥20 years) or pediatric (<20 years) patients. Persons of white European descent had the best chance of finding an optimally matched graft, with a 75% likelihood of finding a BM donor, a 17% likelihood of finding a CB unit suitable for an adult patient, and a 38% chance of finding a CB unit for a child. At the other end of the spectrum, African Americans had a 19%, 2%, and 6% likelihood of finding these respective matches. Similar results that emphasized the lack of available BM donors for nonwhite Europeans were observed in a study using a criterion of 10/10 HLA match for a suitable BM donor [10, 12]. Gragert et al. also reported that the likelihood of finding a match improved greatly if the criteria were altered to find acceptable, versus optimal, matches, i.e., minimum 7/8 HLA match for BM and 4/6 HLA match for CB. In this scenario, 97% of the white European group found a BM match, while 96% of adults and 99% of children found CB matches. Among African Americans found 76 % a BM match, while 24% of adults and 95% of children found acceptable CB matches, respectively.

When private CB banking first appeared, it was viewed as being a source of autologous cells that would only be used by donors in the rare case that their children had developed a disease treatable with their own CB unit. The estimation of the probability for such use was 1/100,000. Since CB is deemed to have the same qualities as BM, the same diseases currently being treated using BM could be treated using CB, with the exception that CB contains fewer cells and therefore would be limited to predominantly pediatric patients. Using these limited criteria for treatment options, autologous CB, whether stored in a public bank or a private bank, would have limited usefulness. Allogeneic use greatly expands the potential end

users of any given banked CB unit. Obviously a CB unit in a public bank, searchable by all treatment hospitals worldwide, would have an increased chance of use compared to a CB unit limited to family members. Studies estimating the use of a family stored unit suggest the frequency of use would be 1/2700—1/4000. This is based on the frequency of specific diseases where CB can be used for treatment [13, 14]. Interestingly, as reported by PGCB, the largest private CB bank, Cord Blood Registry, San Francisco, California, USA, has released 340 units for patient treatment from the 500,000 units stored, including use for regenerative medicine. The actual frequency of use of CB stored at this private CB bank is 1/1470, about twofold more than predicted [5].

A study by Neitfeld et al. aimed to determine the likelihood of a person requiring a BM transplant over his or her lifetime [15]. Under different treatment scenarios such as autologous versus allogeneic CB use, the lifetime probability of a person requiring a BMT was between 1/100 and 1/400, with the probability of use increasing with age. This brings up the importance of developing CB for adults through expansion of the cell population or the use of double-cord transplants; both are discussed in what follows. Studies have also been published that have used estimations and algorithms to determine the optimal size of a CB bank to service the whole country [16]. These studies usually conclude that only a few thousand units will be sufficient to cover a whole population, but the fact that many public banks are not limiting their use only to members of the host country argues that larger banks will be required. Also not addressed is why, with 23 million registrants in the public BM registries, only 30–50% of those seeking a BM unit actually find one. The complexities of any transplant argue that theoretical calculations used to determine an optimal stem cell donor underestimate the multitude of parameters that must be considered.

8.3 Delayed Engraftment for Adult CB Transplants: Problems and Solutions

CB as an HSC source for BM transplantation has the advantages of high tolerance for HLA mismatches owing to naïve T cells, reduced GvHD, presence of GvM effect, and a more complete blood reconstitution compared to BM or PB stem cells. These advantages are balanced against slower immune reconstitution of neutrophils and platelets, which results in the patient's being more susceptible to opportunistic infection, which can account for 23% of mortality [17]. The delay in both neutrophil and platelet engraftment associated with CB transplant can be offset by increased cell dose. For adult patients this becomes an acute problem as the cell dose from a single CB unit is limiting. Banked CB units can range from 400 to 6500 million leukocytes/unit and for optimal doses can reconstitute patients in a range of around 45 kg [18].

The low numbers of hematopoietic stem and progenitors cells available per kilogram of patient body weight, combined with a reduced ability of CB cells to home to the BM, result in delayed engraftment. Studies that looked at immune reconstitution after CB transplants found that there is a delay in the recovery in neutrophils, platelets, and functional T cells [9, 19, 20]. In one example comparing BM to CB as a donor source, the median time to neutrophil recovery was 19 days (9–33 days) for BM and 25 days (9–90 days) for CB. Platelet engraftment was 27 days (12–285 days) for BM and 59 days (12–237 days) for CB [9]. Mature T-cell recovery can take 9–12 months following a CB transplant [21]. Although the advantages to using umbilical CB include less stringent matching criteria that still allow for good engraftment, strong GvM, and reduced GvHD, the delayed reestablishment of neutrophils and platelets results in increased infections. A number of methods are being developed to reduce patients' susceptibility to infection using temporary means, while the donor hematopoietic stem/progenitor cells engraft and start producing neutrophils, platelets, and T cells, while other methods aim to improve the time to engraftment. Since HLA matching and cell dose are indicators of the success of a CB transplant, studies have focused on increasing the number of cells a patient receives by using two CB units for transplant or in vitro culture methods to expand the stem cells in a single CB unit. Similarly, the combination of a CB transplant with a

haploidentical HSC transplant is another method being investigated to increase the cell dose.

8.3.1 Double-Cord Blood Transplant

Although rich in hematopoietic stem/progenitor cells, a limited number of cells can be retrieved from each umbilical cord. Typically, a BM graft provides about 3×10^6 CD34+ cells/kg recipient weight, while CB provides about 2×10^5 CD34+ cells/kg recipient weight. Despite the lower cell dose in CB, cell engraftment and patient survival are similar to a BM graft. Engraftment and survival are only compromised when cell dose is lower than 1.7×10^5 CD34+ cells/kg recipient weight [22]. Owing to its low cell dose, CB transplantation was initially limited to small children. To make CB transplantation available for larger children and adults, double CB transplantations are performed. In double CB transplantation, two partially HLA-matched CB units are transplanted into the patient.

Analysis of 166 allogeneic HSC recipients consisting of 66 8/8 HLA-matched unrelated BM recipients, 45 7/8 HLA-matched unrelated BM recipients, and 55 4/8–7/8 HLA-matched double CB recipients revealed that the 3-year transplant-related mortality was similar for all patient groups, but the 3-year relapse rate was significantly decreased in the double CB group. Furthermore, the 3-year disease-free survival was significantly improved in the double CB group compared to the single mismatched BM recipient group [23].

In a double CB transplant setting, typically one CB unit will dominate and contribute to full-donor chimerism. Neither CD34+ cell dose nor the degree of HLA match is an indicator of the dominating unit as CB units with low CD34+ cell doses (1.5×10^5 cells/kg recipient weight) were able to dominate and contribute to full-donor chimerism. Studies suggest that the number of CD3+ T cells in a CB unit is indicative of which CB unit will dominate in a transplant. The naïve CD8+ T cells contained within the donor CB units are exposed to the alloantigens of the partner unit, which causes their activation. The unit with the relatively higher content of T cells will have

an advantage and eventually reject the second CB donor unit [24, 25].

8.3.2 Stem Cell Expansion

An alternative approach to increasing the cell dose of a CB unit is the in vitro expansion of the HSC population within the CB unit [26–28]. Expansion requires that a suitably matched CB unit be thawed, cultured under appropriate conditions, and transplanted into a patient. Methods incorporating growth factor combinations can result in increases in CD34+ cells in vitro, but no increase in actual long-term repopulating cells [29]. In other studies, modest in vitro expansion of CD34+ cells (10–20-fold) resulted in significant, but modest, increases in long-term repopulating cells [30, 31]. Clinical trials using expanded cells have focused on safety issues. In trials conducted to date, either a portion of the CB was expanded and co-infused with the remaining unmanipulated portion of the CB unit or the expanded CB unit was co-infused with a second unmanipulated CB unit. These trials demonstrated the safety of using in vitro expanded CB cells [29, 32]. Delaney et al. used a modified double CB transplant using two different CB units that could be tracked, and one of the CB units was expanded. They demonstrated a significant reduction in time to engraftment. Patients achieved an absolute neutrophil count (ANC) of ≥500 cells/μL at 16 days compared to 26 days for the control group, which received double-cord transplants with non manipulated cords [33]. Interestingly, the expanded CB unit was dominant during the short-term engraftment phase but did not contribute to long-term engraftment for 80% of the patients. Patients with strong early engraftment of the expanded CB unit did have a faster neutrophil recovery time demonstrating the advantage of using an expanded CB unit. The lack of long-term engraftment of the expanded CB unit in patients mirrors the nonobese diabetic (NOD)/severe combined immunodeficient (SCID) mouse studies demonstrating that large in vitro expansion of CD34+ cells does not seem to include the long-term repopulating stem cells.

Alternatives to cytokine-mediated expansion included the coculture of HSCs with stromal cells as an attempt to recreate the BM niche. Primitive HSCs require interaction with cells of the BM niche, and MSCs isolated from BM grow well and continue to secrete factors that support HSC growth. Although MSCs isolated from CB are different from BM-derived MSCs, CB-HSCs grow well on MSCs from an unmatched, unrelated BM donor. A clinical trial assessing the properties of MSC-expanded CB-HSCs demonstrated that total nucleated and CD34+ cells were increased and the time to neutrophil engraftment shortened, but like other studies using expanded CB, the manipulated graft did not contribute to long-term engraftment. Enhanced short-term engraftment, however, is important for reducing non-treatment-related mortality due to infection [34].

Recently, two studies using small molecules demonstrated expansion of long-term repopulating cells. In the first study, it was hypothesized that OCT4, a well-known pluripotency-associated transcription factor, has a role in maintaining HSCs during expansion [35]. OCT4 levels within a cell are critical for determining whether a stem cell maintains a stem cell identity or whether it differentiates [36]. Increasing the transcription level of OCT4 in HSCs using a small molecule activator, OAC1, along with cytokine supplementation with TPO, SCF, and Flt3L enhanced the expansion of CB CD34+ cells. Although these three cytokines alone are capable of expanding both short-term and long-term repopulating cells [37], augmenting the cultures with OAC1 resulted in the improvement in the number of colony forming units (CFU) and a 3.5-fold expansion of HSCs as assessed by the SCID-repopulating assay [38]. Interestingly, the mechanism of action was via HOXB4 signaling, which had been demonstrated to have a positive effect on HSC expansion [39]. OCT4 can bind to HOXB4 and levels of HOXB4 were increased in OAC1-treated cultures. Furthermore, the addition of blocking siRNA prevented the OAC1-induced expansion of primitive HSCs. This study has not yet been tested in a clinical trial.

In a second study using a similar cytokine-supplemented medium, a screen of 5820 compounds was tested for their

ability to enhance the expansion of human long-term repopulating cells. From this screen, six candidates were identified, with one, a pyrimidoindole derivative, being subjected to further testing. Both long-term repopulating assays and serial transplantation into NOD/SCID gamma mice identified a novel compound (UM721) that resulted in a 13-fold increase in HSCs [40]. Expansion of HSCs has also been successful with a copper chelator [41], Notch [33], and coculture with MSCs [42], and each of these methods is currently being tested in clinical trials [43].

8.3.3 CB/Haploidentical HSC Transplant

An alternative to a double CB transplant or an expanded single CB transplant is a single CB unit combined with a haploidentical BM or PB HSC transplant. As with a double CB or an expanded single CB transplant, the goal of a CB/haploidentical HSC transplant is to increase the cell dose in order to shorten the time to neutrophil and platelet engraftment [44] and, hence, increase patient survival rates in the first 100 days. Since most family members share at least one haplotype, closely related family members (parents, siblings) are potential donors of haploidentical HSCs.

Similar to the double CB unit transplant where one CB unit dominates and is engrafted, evidence from CB/haploidentical transplants has demonstrated the presence of both donor-derived cells over the short term with long-term engraftment coming from the CB unit [45]. Another study compared CB/haploidentical transplants to double CB transplants and found the CB/haploidentical transplants resulted in a shorter time to neutrophil engraftment with 75% of the patients engrafted by 15 days versus more than 21 days for the double CB transplants. Platelet engraftment occurred at 19 days following CB/haploidentical HSC transplant, versus 21–62 days for the double CB transplants, resulting in the patients requiring 50% fewer platelet transfusions [46]. Additionally, recipients of a CB/haploidentical HSC transplant had reduced hospital stays.

Overall, increased HSC dose, even if it comes from an unmatched third-party donor, enhanced short-term engraftment, which has a strong positive effect on patient outcomes. Owing to the low matching success for patients of nonwhite European ancestry and the lack of single CB units with a sufficient cell dose to treat adults, the adoption of double CB transplants or CB/haploidentical transplants expands the potential of CB donations.

8.3.4 Engineered T-Cell Grafts to Enhance Engraftment and Patient Survival

Adoptively transferred T cells found in an HSC graft can undergo a rapid phase of proliferation after transplantation. Antithymocyte globulin (ATG) can be given at the time of HSC transplantation to prevent GvHD. ATG substantially reduces immune competence in patients by reducing the number of circulating T cells. Even in the presence of ATG, some T cells escape and are clonally expanded. CB T cells maintain their naïve T-cell phenotype during expansion because they do not circulate through the patient's thymus. As a result, the T cells have a limited repertoire of T-cell receptors, and any antigen stimulation that occurs results in many cells entering apoptosis. Functional T cells are formed later when blood lymphocyte stem cells (CD34+/CD38+) engraft and undergo de novo thymopoiesis as the cells are educated in the patient's thymus. The rate of functional T-cell recovery is dependent on the quality of the thymus, which is determined through a balance of GvHD and conditioning regimens, such as whole-body radiation, both of which can damage the thymus and delay recovery. Analysis of the viral targets of the early stage T cells versus the later-stage thymic-dependent pathway revealed the latter contained a much larger repertoire of T cells capable of battling infection [21].

After a single CB unit transplant, there is a lag phase between engraftment and the late recovery of functional T cells. One method to bridge this gap and provide the patient protection from infection during this lag phase is the use of in vivo expanded CB-derived T cells. Berglund et al. removed

5% of each CB unit that was being transplanted and used this CB for T-cell expansion for later infusion. T-cell expansion was completed using a combination of CD3 stimulation and cytokine supplementation. The infusion of activated T cells was performed a minimum of 3 weeks post-transplantation of the remaining unmanipulated CB unit. The sample size was small, but the goal to determine the safety of in vitro expanded T cells was successful as none of the patients displayed adverse reactions to the engineered product. This study demonstrated the feasibility of using a portion of the original CB unit as a T-cell source for expansion when allogeneic CB is used for transplantation [47].

8.3.5 Improving Homing of Cord Blood Stem and Progenitor Cells to Bone Marrow

Strategies to improve CD34+ cell homing to the marrow are also being developed. This is important for both unmanipulated and expanded CB units since engraftment is dependent on cells' ability to home to the BM. Successful homing requires that the blood cells be captured by the BM endothelium. This capture is mediated by P- and E-selectins on the surface of the endothelium that interact with P-selectin glycoprotein-1 (PSGL-1) found on the surface of CD34+ cells. To be functional, PSGL-1 is posttranslationally modified by sialylation and fucosylation of O-linked sugars. Studies that neutralized P-selectin or PSGL-1 with antibodies showed reduced homing, and 30% of CB CD34+/CD38^{low} stem cells do not bind P-selectin due to the lack of α1-3 fucosylation of PSGL-1 [48, 49]. Hypothesizing that the ex vivo fucosylation of CB CD34+ cells would enhance engraftment, Xia et al. used GDP-fucose and 1–3 fucosyltransferase to add α1-3-linked fucose. The treated CB cells were used to engraft irradiated NOD SCID mice. The fucosylation of CD34+ cells resulted in improved engraftment [49]. Using a strategy similar to that of Xia et al., a clinical study was developed in which patients received two CB units: one unmanipulated and the other treated with GDP-fucose and 1–3 fucosyltransferase. The study consisted of 22 patients and was compared to 31 historic controls. Median neutrophil engraftment was 17 days

(12–34 days) compared to 26 days (11–48 days) for the untreated controls. Platelet engraftment was also improved, with the treated group demonstrating a median engraftment of 35 days (18–100 days) compared to 45 days (27–120 days) for the controls. These results were statistically significant [50].

8.4 Regenerative Medicine

Currently, clinical trials are under way to explore the application of CB to the treatment of a number of neurological disorders, including cerebral palsy (CP), encephalopathy, stroke, and hearing loss. The rationale for the use of CB in neurological disorders stems initially from the successful application of CB for the treatment of various lysosomal storage diseases or inborn errors of metabolism. These diseases represent approx. 40 rare inherited disorders typically caused by a deficiency in a lysosomal enzyme. The result of the enzyme deficiency is the accumulation of undigested, or partially digested, macromolecules within the lysosomes and subsequent cellular death and organ dysfunction. The manifestations of the disease are variable and depend on the enzyme defect. For example, Hurler's syndrome is due to a lack of the lysosomal enzyme alpha-L-iduronidase, which aids in the degradation of mucopolysaccharides. Children with Hurler's syndrome have both physical and mental impairments and usually die between the ages of 5 and 10 years.

Allogeneic BM transplantations had previously been shown to prevent the progression of certain lysosomal storage diseases. Similarly, the effect of CB transplantation has been investigated in this patient population. Transplantation of unrelated CB into patients with Hurler's syndrome results in sustained engraftment and improved outcomes including reduced organ dysfunction and improved neurodevelopment, growth, development, and overall survival [51, 52]. CB transplantation offers several advantages over BM or mobilized PB: transplantation in children suffering from lysosomal storage diseases, including quicker time to transplant since allogeneic CB is readily available from public CB banks, high rates of engraftment achieving full-donor

chimerism, and normalization of enzyme levels, which is required for enhanced long-term survival. Today, CB is considered the optimal choice for transplantation in children with lysosomal storage diseases [53].

CB is thought to mediate its effects on patients with lysosomal storage diseases via so-called cross correction, whereby CB cells engraft into various nonhematopoietic tissues throughout the body and produce and secrete the normal enzyme, which is subsequently taken up and used by the enzyme-deficient cells. Indeed, engraftment of CB in the brain has been demonstrated through autopsy studies of humans who died months after a CB transplant. These studies identified predominantly donor-derived nonneuronal microglial cells within the brain and, to a lesser extent, donor-derived neurons, astrocytes, and oligodendrocytes [54].

Though CB was initially characterized as a rich source of HSCs, over time it has become apparent that CB also contains nonhematopoietic stem and progenitor cells, which can give rise to a number of different cell types. Unrestricted somatic stem cells from CB have been isolated from CB, expanded, and differentiated into a variety of cell types, including neural, liver, and pancreatic cells, along with chondrocytes, osteoclasts, and cardiac myocytes [55]. CB is also a source of MSCs and endothelial progenitor cells (EPCs); however, the isolation of these cells from frozen CB units has been a challenge. Less than 10% of frozen CB units yield MSC compared to approx. 50% of fresh CB units that were processed within 15 h of collection [56–59]. Vanneaux et al. [60] reported that only 59% of frozen CB units produced EPCs compared to 94% of fresh CB units. In general the yield of angiogenic cells from both fresh CB or PB is low [61]. We previously reported on a population of CD45+ CD34+ cells derived from CB that can be isolated from frozen, banked CB units and manipulated in a simple culture system resulting in up to a 30-fold expansion of cells capable of differentiating into MSC, endothelial cells, and muscle cells [62–64]. These cells together form a multipotential stem cell (MPSC) population with strong therapeutic indications. MPSCs are unique in that they are CD34+ CD45+ but can give rise to

EPCs, circulating angiogenic cells (CACs), and the typical MSCs. MPSCs can be derived from frozen CB with 100% efficiency, and the culture method using FGF4, SCF, and Flt3-ligand greatly increased the yield of cells per banked CB unit. Importantly, following the production of MPSCs, the cells can be refrozen without any loss of potency or viability [63].

The application of CB for the treatment of neurological disorders has been tested in an array of animal models including hypoxic-ischemic encephalopathy (HIE), CP, stroke, traumatic brain injury, and spinal cord injury. In HIE models, the administration of CB has been shown to decrease spastic paresis after perinatal brain damage in neonatal rats [65] and improve motor and cognitive performance [66]. Infusion of CB in a rabbit model of CP demonstrated improved motor performance in a dose-dependent manner [67]. CB reduced the neurological deficit and infarct area in a rat model of stroke. In a model of traumatic brain injury, CB, in conjunction with granulocyte colony-stimulating factor, decreased neuroinflammation, increased neurogenesis, reduced hippocampal cell loss, and stimulated long-lasting recovery of motor functions [68]. Finally, in spinal cord injury models, CB administration results in increased hind limb motor activity [69] and improved neurological function [70].

8.5 Cord Blood for the Treatment of Cerebral Palsy

CP is a chronic condition due to an injury to the brain that affects the communication between the brain and muscles, resulting in uncoordinated movements and postures. The injury often happens before birth, sometimes during delivery, or soon after birth. CP is the most common cause of physical impairment in children. Premature babies (born before 37 weeks) and babies with low birth weight (<2.5 kg) are at greater risk of developing CP. The estimated prevalence of CP is 2.0–2.5 per 1000 live births in Western populations [71].

Currently, there is no cure for CP. Patients are managed through therapy to prevent or minimize physical deformities and discomfort, improve and enhance mobility and motion,

and enhance their overall health to allow them to be as independent as possible at school and in the community into adulthood. Clinical trials are ongoing and investigating the effect of both autologous and allogeneic CB infusion on this patient population.

Two clinical trials studying the effect of allogeneic CB in children with CP have been completed in Korea, and results have been published [72, 73]. The first trial was a double-blind, randomized, placebo-controlled trial designed to determine the efficacy of CB treatment, in conjunction with erythropoietin (Epo), in children with CP [73]. Epo was included in this study because of its neuroprotective and neural repair properties. One hundred and five CP patients between the ages of 10 months and 10 years were enrolled in the study and assigned to one of three treatment groups: (1) allogeneic CB with Epo, (2) Epo with a placebo for CB, and (3) placebo for both CB and Epo. Unrelated allogeneic CB was obtained from the CHA Medical Center Cord Blood Bank. The CB units provided a minimum therapeutic dose of 3×10^7 total nucleated cells/kg and matched the patient for at least four of six HLA types A, B, and DRB1. Patients in the CB/Epo group were also treated with the immunosuppressant cyclosporine for 3 weeks in an attempt to delay/prevent the rejection of the CB cells.

All participants underwent an initial evaluation to establish a baseline for various functional CP measurements. Four main assessments were conducted: gross motor performance measure (GMPM), gross motor functional measure (GMFM), Mental and Motor scales of the Bayley Scales of Infant Development-II (BSID-II), and the functional independence measure for children (WeeFIM). The GMPM and GMFM were used to measure gross motor ability, whereas the BSID-II was used to measure neurodevelopmental progress. There were no significant differences in baseline measurements between the three groups. Functional assessments were repeated 1, 3, and 6 months after the initial treatment.

Results from this study revealed significant improvements at 6 months in all three treatment groups for most of the functional assessments, as compared to the baseline

measurements. At 6 months, the CB/Epo group had significantly greater improvements than the Epo or placebo group in the GMPM, BSID-II, and WeeFIM measurements. Epo alone, as compared to the placebo group, had a minimal effect. Further analysis of the CB/Epo recipients indicated that patients that receive a one-mismatched unit had significantly better outcomes than those that received a two-mismatched unit. A greater total nucleated cell (TNC) and CD34+ dose also led to better outcomes on the GMFM and ESID-II scores, respectively.

Imaging studies were conducted to assess structural and metabolic changes within the brain of the patients. Fractional anisotropy (FA) was used to assess the effect of treatment on white matter integration in response to therapy. Significant FA increments were measured in the CB/Epo group but not the Epo or placebo groups. The changes in the GMPM score measured at 6 months correlated significantly with the FA changes. Positron emission tomography–computed tomography (PET/CT), used to measure metabolic activity, revealed differential activation and deactivation patterns for the three treatment groups.

From this study the investigators concluded that CB/Epo treatment improved motor and cognitive dysfunction in children with CP and that this improvement was accompanied by structural and metabolic changes within the brain. One limitation with this study, however, was the lack of a CB-only treatment group. This was addressed in a follow-up study designed to look at the effect of CB alone on patients with CP [72]. Again, this was a randomized, placebo-controlled, double-blind trial. Thirty-six CP patients, ages 6 months to 20 years, were enrolled and treated with either allogeneic CB or placebo. As before, CB recipients were also treated with cyclosporine. CB units provided a minimum therapeutic dose of 2×10^7 TNC/kg and were at least a 4/6 HLA match. Patient assessments included GMGM, GMPM, and BSID-II scores along with a measurement of muscle strength. Cytokine and receptor measurements in blood samples were also included in patient assessments to determine the effect of CB on the inflammatory response.

Results of this study indicated that the administration of CB in CP patients resulted in greater improvements in muscle strength as compared to the placebo group after 1 and 3 months. CB recipients also showed significant improvements in the GMPM score as compared to the placebo group at 6 months. Similar to the first study, higher CB cell doses and better HLA matches resulted in greater improvements in the patients.

In terms of cytokines and receptors, significant increases in plasma pentraxin 3 (PTX3) were observed in response to CB. CB treatment also resulted in a significantly elevated level of Toll-like receptor 4 (TLR4) in blood cells compared to the placebo group. PTX3 and TLR4 are both part of the innate immune response, and both have been suggested to have neuroprotective roles; thus, the authors have proposed that CB may be eliciting its effect on CP patients through the innate immune response.

While these two studies suggest allogeneic CB may be useful for the treatment of patients with CP, the results are confounded by the presence of cyclosporine. Cyclosporine, an immunosuppressant, was used to delay/prevent the rejection of CB in those patients treated with allogeneic CB. Along with its immunosuppressant activities, however, cyclosporine has also been shown to possess neuroprotective properties [74]. Future studies with autologous CB will have to delineate the role, if any, that cyclosporine plays in this patient population.

Three clinical trials investigating the effect of autologous CB for the treatment of CP are ongoing in the USA. A Phase I double-blind study being conducted in Texas will enroll 30 patients aged 2–10 years with CP. Fifteen patients will be treated with either autologous CB or BM then monitored for changes in gross motor skills and psychological assessments for up to 2 years. Magnetic resonance imaging (MRI) will also be conducted to assess changes in white matter tracts [75]. A Phase I/II single-blind study being conducted in Georgia will enroll 40 patients aged 1–12 years with CP [76]. These patients will be treated with autologous CB then monitored for changes in gross motor skills over a 3- to 4-month period.

The third and most advanced clinical trial, a Phase II double-blind study being conducted at Duke University in Durham, North Carolina, has enrolled 120 CP patients aged 1–6 years [77]. Patients were infused with autologous CB and are being monitored for neurodevelopmental improvements along with morphological changes to the brain by MRI. Imaging studies have demonstrated that the brain damage associated with CP consists of diffuse damage or focal lesions in the white matter. Preliminary results from the MRI studies of a subgroup of patients in this study indicated a strong correlation between increases in white matter connectivity and functional improvement over the first 2-year period of this study. At the time of publication, the investigators were still blinded, and it was unknown whether the children with the improved GMFM scores and the increased white matter connectivity were the ones that received autologous CB [78]. The results of this study are anticipated in the near future.

8.6 Treatment of Hypoxic-Ischemic Encephalopathy with CB Cells

CB has also been tested in a clinical trial for infants with HIE. HIE occurs in children who are deprived of oxygen during delivery and can result in epilepsy, developmental delays, motor impairments, neurodevelopmental delays, and cognitive impairments. Infants born with moderate to severe HIE have better outcomes when treated by moderate hypothermia; however, more than 30% of patients die or survive with impairment. In a Phase I trial, infants diagnosed with HIE were subjected to moderate hypothermia and treated with or without autologous CB. For this trial, the CB was volume and red blood cell reduced and administered fresh with a target of two doses within the first 48 postnatal hours. Twenty-three patients were treated with CB compared to 83 patients treated by hypothermia alone. Both patient populations were monitored for adverse outcomes, including mortality, seizures, and pulmonary hypertension. One-year neurodevelopmental outcomes were assessed using the Bayley III scales, which

assess cognitive, language, and motor developmental domains [79].

Administration of fresh CB to this patient population was deemed safe as there were no differences in deaths, seizures, or pulmonary hypertension between the two patient groups. After 1 year, however, 76% of the infants treated with CB had Bayley III scores ≥85% in all three test domains compared to 41% of the infants that were treated with hypothermia alone. Though these results are intriguing, the number of patients included in this study was small, and results will need to be confirmed in a larger study.

Other ongoing clinical trials investigating the effect of CB on neurological disorders include five trials for stroke, two trials for autism, one for acquired hearing loss, and one for the neurological defects associated with hypoplastic left heart syndrome [80]. Four of the stroke trials are being conducted on adults using allogeneic CB, while the fifth study involves children using autologous CB. The trials for autism, acquired hearing loss, and hypoplastic left heart syndrome all involve the use of autologous CB.

8.7 The Future of Regenerative Medicine with Umbilical Cord Blood and Tissue: Using CB to Produce Induced Pluripotent Stem Cell

Applications of CB have primarily been limited to hematological diseases, but over the years, CB has been applied to neurological injuries, including spinal cord injury, stroke, ischemic brain injury, and CP. With the groundbreaking discovery of iPSCs [81, 82] and with CB being an accessible source of young somatic cells for reprogramming, the use of CB will expand beyond hematological conditions. It is important to keep in mind that current organ transplants for the heart, lung, liver, and kidney use allograft donors. But these transplants require extensive HLA matching to be successful, resulting in a large number of patients who are unable to find a suitable donor and long waiting lists for donor organs. Once a

suitable donor is identified and the transplant is completed, the use of immunosuppression drugs is required to prevent graft rejection. Ideally, being able to generate whole organs from one's own stem cells represents the future of regenerative medicine. This is becoming more practical with the production and increased understanding of iPSCs [81, 83, 84].

Takahashi and Yamanaka first demonstrated in 2006 that a differentiated cell could be reprogrammed back to an ES cell-like state by the forced expression of Oct4, Klf4, Sox2, and c-Myc [81]. They termed these reprogrammed cells iPSCs. Similar to ES cells, iPSCs have the ability to differentiate into all three germ layers and the capacity to proliferate indefinitely. The first reports described the reprogramming of fibroblasts to iPSCs, but in recent years, groups have generated iPSCs from a variety of somatic cell types, including pancreatic beta cells, cardiomyocytes, neural cells, renal epithelial cells, BM cells, PB cells, and CB cells. Among all cell sources, CB cells offer several advantages over other cell types. First, CB is easy to collect and the collection process does not harm the donor. Second, CB cells are young cells that have fewer acquired somatic mutations than adult cells, and they proliferate well in culture. Third, CB is rich in HSCs, and Eminli et al. showed that stem/progenitor cells are easier to reprogram than terminally differentiated blood cells [85]. Moreover, there are currently over four million banked CB units, all of which are potential sources of iPSC lines, and this number continues to grow. Units held in public banks are HLA typed and all units have been characterized for viability and cell number [86]. Importantly, these cells have already been collected and stored, making them easily accessible without having to go back to the original donor to obtain a tissue or cell sample. In the future, it will be important to incorporate into patient consent forms permission to generate iPSC lines that could be available for regenerative medicine.

8.8 Methods of Generating Induced Pluripotent Stem Cells

Since the discovery of iPSCs in 2006 [81], numerous methods have been developed to generate iPSCs. These reprogramming strategies can be broadly grouped as DNA-integrating or nonintegrating methods, each having its own advantages and disadvantages in terms of ease of use, cost, reprogramming efficiency, and clinical application. Here we will describe some of the commonly used genome-integrating and nonintegrating methods of iPSC reprogramming.

8.8.1 Genome-Integrating Methods

Genome-integrating methods are used to stably integrate transgenes into the host genome. Common genome-integrating methods of reprogramming include retrovirus, lentivirus, and *piggyBac* transposon systems. Stably integrated transgenes are not diluted with cell division and therefore allow for higher reprogramming efficiencies compared to nonintegrating methods. While retroviral and lentiviral vectors are permanently integrated into the host genome, *piggyBac* transposons are excisable and allow for the generation of transgene-free and footprint-free iPSC lines.

8.8.2 Retrovirus and Lentivirus

Early reports of iPSC generation mainly used retroviruses and lentiviruses owing to their high infection and reprogramming efficiencies [81, 82, 87, 88]. For both methods, transgenes are randomly and permanently inserted into multiple sites on the host genome. Transgene expression is often constitutive, although drug-inducible systems are available, and transgene silencing is required and a hallmark of fully reprogrammed iPSCs. Retroviruses and lentiviruses are similar in many aspects but they differ in the cells they can infect; retroviruses can infect dividing cells only, while lentiviruses can infect both dividing and nondividing cells.

8.8.3 *piggyBac* Transposons

Since having transgenes, especially oncogenic genes, permanently integrated into the host genome is not ideal for

clinical use, an excisable method of iPSC reprogramming using the *piggyBac* transposon system was developed [89, 90]. In this system, cells are transfected with a transposon encoding the reprogramming factors and a nonintegrating plasmid encoding the *piggyBac* transposase. The transposase recognizes terminal repeats on the transposon and "cuts and pastes" the transgene into the host genome at TTAA sites. The *piggyBac* system can be modified to be a tetracycline (Tet)-On or Tet-Off system, depending on the need. After stable iPSC lines are established, transgenes can be removed without a footprint by reintroducing the transposase. Multiple iPSC lines must be screened to ensure complete removal of transgenes.

8.8.3.1 Nonintegrating Methods

DNA-integrating methods offer high reprogramming efficiencies, but they are not clinically preferred methods of generating iPSCs owing to several safety concerns. For instance, there could be multiple transgene integration sites, and these sites are random (or semirandom in the case of *piggyBac* transposons) and could cause insertional mutagenesis. In the case of retrovirus and lentivirus, transgene integration is permanent, and although transgenes are silenced for successful reprogramming to occur, there is the concern that these transgenes will be reactivated during iPSC differentiation. Transgenes can be removed in the transposon system by the re-expression of the transposase, but numerous iPSC lines must be screened to ensure that all transgenes are excised.

Nonintegrative reprogramming strategies have been developed to generate transgene- and vector-free iPSCs for clinical use. In nonintegrating methods, transgenes are diluted and removed passively through cell division and degradation by the cell. However, since reprogramming factors are lost with each cell division, reprogramming efficiency is often compromised with these methods.

8.8.4 Sendai Virus

A nonintegrating viral alternative to retrovirus and lentivirus is Sendai virus [91–93]. Sendai virus is a single-stranded RNA (ssRNA) virus that replicates in the cytoplasm of the infected cells. Since the virus does not produce DNA and only replicates in the cytoplasm, no genomic material is integrated into the infected cells. Virus vectors are diluted naturally with cell division, or cells with viral vectors can be selected against using an anti Sendai Virus-hemagglutinin-neuraminidase antibody. However, since viral vectors replicate constitutively, it is difficult to establish transgene-free lines. Efforts are being made to make Sendai virus a safer alternative by accelerating the removal of viral vectors. Ban et al. generated a temperature-sensitive strain of Sendai virus by introducing a point mutation in the viral polymerase-related gene [94]. In these temperature-sensitive Sendai viruses, viral replication arrests at a nonpermissive temperature, so the dilution of viral vectors with cell division is quicker.

8.8.5 oriP/EBNA Episomal Vectors

oriP/EBNA1 episomal vector is another example of a nonintegrating method of reprogramming somatic cells to iPSCs [95–97]. The oriP/EBNA1 vector is derived from the Epstein-Barr virus and can be introduced into cells without viral packaging. The vector undergoes extrachromosomal replication once per cell cycle. With drug selection, these vectors can be maintained in about 1% of the transfected cells, and when drug selection is removed, vectors are lost at approx. 5% per cell division. With this method, multiple iPSC lines must be screened to ensure cell lines are free of vectors.

8.8.6 mRNA and Protein

Messenger RNA (mRNA) and protein methods of reprogramming are nonintegrating and DNA-free methods of reprogramming. Modified mRNA encoding the reprogramming factors or proteins is transfected into cells to initiate the reprogramming process. Warren et al. showed that mRNA modified to improve translation, enhance RNA half-life, and escape a cell's antiviral response to ssRNA can

reprogram human fibroblasts to iPSCs [98]. Proteins must be engineered to contain a transduction domain, such as poly-arginine, to allow the protein to cross the cell membrane [99, 100]. Multiple, and often daily, cell transfections are required to successfully reprogram cells owing to the rapid turnover of mRNA and proteins. Although these methods can generate transgene-free and vector-free iPSC lines, the methods are labor intensive, and the reprogramming efficiency is often low compared to other reprogramming methods.

8.8.7 Methods Used to Generate Cord Blood iPSCs

Studies have shown that CB cells can be reprogrammed into iPSCs using both genome integrating and nonintegrating methods, namely, retrovirus, lentivirus, Sendai virus, and episomal vectors. The ability to generate transgene-free CB-iPSCs is important for clinical applications.

8.9 Current and Prospective Applications of Induced iPSCs

Since its discovery about 10 years ago, groups have explored the use of iPSCs in personalized and regenerative medicine, specifically in disease modeling, drug discovery and screening, and cell replacement therapy and tissue regeneration.

8.9.1 Disease Modeling

One application of iPSCs is in disease modeling. In disease modeling, cells from a diseased patient are reprogrammed into iPSCs and differentiated into the affected cell type(s) to better understand disease development and progression in vitro. Since the establishment of human iPSCs in 2007 [82], iPSCs have been generated from numerous diseases, including muscular dystrophy, Down syndrome, Parkinson's disease, type 1 diabetes, Huntington disease, and various cardiovascular diseases [101, 102]. By differentiating the

disease-iPSCs into the target cell types, groups have successfully recapitulated disease phenotype in vitro. For example, iPSCs have been differentiated into cardiomyocytes to recapitulate the abnormal channel function phenotype of long QT syndrome [103–107] and the contractile and electrical abnormality of hypertrophic cardiomyopathy [108, 109].

Conventionally, immortalized cell lines and animal models of human diseases are used to study the development and pathophysiology of diseases. However, disease modeling with iPSCs offers several advantages over conventional methods. To start with, mouse models do not always accurately mimic the human condition or mouse models do not exist for the disease. Fanconi anemia is an inherited autosomal recessive disease caused by defects in the Fanconi anemia pathway, which results in genomic instability and the hallmark spontaneous BM failure. Current mouse models of Fanconi anemia display DNA repair deficiencies similar to the human condition, but they do not exhibit the hallmark spontaneous BM failure [110]. Moreover, the differences in gene order between rodents and humans make it difficult to recapitulate the human disease phenotype. For instance, mouse models carrying the trisomic mutation for Down syndrome do not display the same neurodegeneration and cranial anomalies as the human condition [111, 112]. Lastly, mouse models are often not available for complex, fatal diseases such as hypoplastic left heart syndrome.

There are, of course, limitations to using iPSCs to model diseases. Although iPSCs have proven successful in modeling monogenic diseases, it is still technically difficult to model diseases that are complex or involving multiple genetic components, such as type 1 diabetes, and diseases with a long latency period such as Alzheimer's and Parkinson's diseases. However, groups are investigating methods of "aging" cells in vitro by inducing environmental stress, such as oxidative stress in cell culture. In addition, there are concerns that diseases with a strong epigenetic component may not be faithfully modeled with iPSCs since epigenetic marks are largely erased during the reprogramming process. A last consideration for

using iPSCs to model diseases is whether it is realistic and practical to model a disease with only one cell type in vitro.

8.9.2 Drug Discovery and Screening

A standard drug pipeline consists of four stages: drug discovery, preclinical studies, clinical trials, and, if approved, marketing. Preclinical studies involve the testing of the candidate drugs on cell lines or animal models. If positive effects are seen in preclinical studies, the candidate drugs enter clinical trials, which consist of four phases. Phase I/II of clinical trials focuses on assessing the safety and efficacy of the drug on a small number of participants, while Phase III/IV focuses on assessing the effectiveness of the drug in a larger number of participants and long-term side effects of the drug. From drug discovery to getting the drug to market can take years and millions of dollars.

Unfortunately, many drugs do not make it through Phase I/II clinical trials even if preclinical studies showed promising results. The disparity in results may be due to the use of cell lines and preclinical animal models that do not accurately represent the human disease phenotype. Ideally, drug discovery and screening should be performed on human disease-bearing cells to reduce failure in the clinical trial phase.

In addition to providing a platform to study disease development and progression in vitro, cells differentiated from diseased iPSCs can also be used for drug discovery and screening. As mentioned previously, cardiomyocytes differentiated from iPSCs generated from patients with long QT syndrome exhibit properties of diseased cells [103–107]. Using these differentiated diseased cardiomyocytes, groups have screened and identified drugs that improved disease phenotype and eliminated drugs that exacerbated the condition. For instance, Moretti et al. showed that the addition of isoproterenol exacerbated the disease phenotype, while treatment with propranolol helped reduced catecholamine-induced tachyarrhythmia [103]. These results were replicated by another study by Matsa et al., who confirmed the benefits

of using beta blockers propranolol and nadolol to reduce disease phenotype of long QT syndrome [113]. As a second example, Itzhaki et al. demonstrated that nifedipine, a calcium channel blocker, can reverse disease phenotype [104]. Together, these studies demonstrate the validity and value of using patient iPSC-derived cells for drug screening.

As alluded to earlier, there are several advantages to including diseased patient iPSCs in preclinical studies of the conventional drug pipeline. iPSCs can be patient and disease specific, which opens the door to personalized medicine and allows for high-throughput drug screening on a patient's own diseased cells and not surrogate cell lines and animal models. From a safety point of view, using patient iPSCs as an alternative to human participants for early drug evaluations is advantageous as it does not put participants at risk of possible drug toxicity. Lastly, a diverse collection of diseased iPSCs can be generated from patients differing in genetic background, age, gender, ethnicity, and disease severity. All these factors may influence a patient's drug responsiveness. Hence, diseased iPSCs may provide a better representation of the patient's response to drug than conventional inbred mouse models.

When using diseased iPSCs for drug discovery and screening, several considerations must be made. First, as with all cell culture studies, it is unclear whether and how culture manipulations change cell phenotype. Next, it is also unclear whether the response of a purified population of cells in vitro accurately reflects the response of the tissue or organ as a whole in vivo. Additionally, a reliable readout of cell function must be identified and used to accurately assess drug function and efficacy in vitro. Finally, it is important to address how the drug will be metabolized in the body and its effects on other tissues/organs.

8.9.3 Cell Replacement Therapy and Tissue Regeneration

Like ES cells, iPSCs have the capacity to differentiate into cells from all three germ layers. To direct the differentiation of

iPSCs down a specific cell lineage, researchers attempt to replicate the conditions the cells would experience in the developing embryo in the culture dish. iPSCs can be differentiated in cell monolayers or as embryoid bodies, although growth factors and small molecule concentrations and length of treatment may differ between the two methods. Differentiated cells are characterized by marker expression and physiological or electrophysiological properties. Cell function is often assessed by transplantation into animal models of the disease of interest to evaluate cell survival and engraftment and reversal of disease phenotype. Here, I will focus on progress made in generating dopamine neurons and pancreatic islets from human ES cells and iPSCs.

8.9.4 Dopamine Neurons to Treat Parkinson's Disease

Parkinson's disease is a neurodegenerative disorder characterized by the degeneration of dopaminergic neurons in the substantia nigra pars compacta and the formation of Lewy bodies. Patients with Parkinson's disease typically experience tremors, rigidity, postural instability, and bradykinesia. Some of the current treatments include dopamine replacement therapies, dopamine agonists, monoamine oxidase B (MAO-B) inhibitors, and deep brain stimulations. However, these treatments only treat the symptoms of the disease and do not halt or stop disease progression. Moreover, these treatments become less effective over time.

Since most of the symptoms are caused by the degeneration of dopamine-producing neurons, it is logical to think that replacement of these cells would reverse the disease phenotype. Indeed, early studies on Parkinson's disease used fetal dopamine neurons to replace damaged tissues in patients [114–116]. The transplanted fetal dopamine neurons survived, and patients showed some clinical improvements. Since it is unsustainable to transplant fetal tissues, groups are harnessing the differentiation potential of embryonic and iPSCs to generate dopaminergic neurons in vitro.

Several groups have developed protocols to direct the differentiation of human pluripotent stem cells to dopaminergic neurons [117–120]. These groups demonstrated that gene expression, dopamine production, and the electrophysiological properties of the differentiated neurons were similar to those of endogenous neurons. When transplanted into rodent models of Parkinson's disease, the dopaminergic neurons survived and grafted and were functional as rodents showed improvements in behavioral and motor-related symptoms.

8.9.5 Pancreatic Islet Cells to Treat Type 1 Diabetes

Type 1 diabetes is an autoimmune disease in which the patient's own immune system attacks and destroys pancreatic beta cells and results in the lack of insulin production and the inability to control blood glucose levels. Type 1 diabetes accounts for about 10% of all cases of diabetes. Patients with type 1 diabetes often develop complications such as diabetic retinopathy, kidney failure, and cardiovascular disease. Currently, type 1 diabetes is managed by daily insulin injections. Although pancreas and islet transplantations are available and can result in insulin independence or reduced insulin dependence for patients, the lack of donors makes these options unavailable for many patients. Moreover, long-term graft failure is a reoccurring issue faced by patients. Together these issues illustrate the need for a renewable source of pancreatic beta cells to treat type 1 diabetes.

The ability to self-renew and differentiation into cells from all three germ layers, including pancreatic beta cells, make pluripotent stem cells an attractive cell source for pancreatic beta cell production. The first report to demonstrate the directed differentiated of human pluripotent stem cells into pancreatic insulin-secreting and glucose-responsive cells dates back to 2001 [121]. Since then, numerous studies have reported the differentiation of human ES cells [122–129] and iPSCs [125, 126, 130] into insulin-secreting pancreatic beta cells. In addition to insulin secretion, these studies also

showed that the differentiated pancreatic beta cells were glucose responsive, an important feature of functional pancreatic beta cells.

8.9.6 Issues and Concerns with Using iPSCs in Clinical Setting

There are several issues and concerns that need to be addressed when translating iPSC research from benchtop to bedside. First of all, low differentiation efficiency is a reoccurring problem for differentiation protocols; therefore, improving differentiation efficiency to achieve a relatively pure population of target cells is a priority. On a related note, unlike rodent models, humans require many more cells for transplantation, and therefore the scalability of the differentiation approach and cost of scale-up are important technical and financial concerns in translational research. Next, the major safety concern over using iPSCs in clinical settings is the risk of teratoma formation. To minimize the risk of teratoma formation, strict guidelines for proper monitoring of cellular differentiation and stringent criteria for target cell selection must be developed. Additionally, it is unclear whether somatic mutations from the starting cells used for iPSC generation and the mutations acquired during the reprogramming process will affect cell differentiation and function. This highlights the advantage of using young cells, such as CB, for iPSC generation. Lastly, any newly developed therapy should be compared to current treatment options to ensure that the new therapies are superior with respect to patient safety and quality of life.

To summarize, applications of CB have expanded tremendously over the years. One of these applications is to use CB cells as starting cells to generate iPSCs. Researchers have harnessed iPSCs' ability to self-renew and differentiate into cells of all three germ layers and are applying them to disease modeling, drug discovery and screening, and cell therapy. Results from iPSC preclinical studies and safety results from the first iPSC clinical trial in Japan that started 1 year ago show promising results. With these promising results,

there is great optimism in using iPSCs in the fields of personalized and regenerative medicine.

CB cells has many properties that make them ideal starting material for iPSC generation. About 80–100 mL of CB containing approximately one billion cells can easily and safely be collected from each discarded umbilical cord [18]. CB is also rich in young cells that have fewer somatic mutations, proliferate better in culture, and reprogram into iPSCs more efficiently than adult cells. Lastly, access to HLA-typed and banked CB units worldwide is attractive and desirable for time-sensitive treatments such as cell therapy.

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Part III
Applications of Stem Cells in Tissue Engineering

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9. Dental Pulp Stem Cells in Tissue Engineering and Regenerative Medicine: Opportunities for Translational Research

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9.1 Dental Pulp Stem Cells: Discovery and Terminology

The field of stem cell biology is growing and developing with each passing day. Advancements in this field have led to new discoveries and a better understanding of developmental as well as pathological processes. Consequently, novel therapeutic strategies are being introduced to modern medicine based on these improvements, and that is just scratching the surface.

Stem cells are characterized by their clonogenic proliferation ability and a remarkable capacity for self-renewal and multilineage differentiation [1]. In particular, mesenchymal stem cells (MSCs) can be found in virtually all adult tissues [2]. MSCs from the bone marrow were first identified by their ability to adhere to tissue culture plastic. Since they constitute a heterogeneous population in culture, an attempt was made to define the characteristics of these fibroblast-like cells by introducing minimal criteria. These were discussed by Dominici and colleagues [3] based on the proposal of the International Society for Cellular Therapy (ISCT) in 2006, thus defining MSCs although insisting on the term mesenchymal *stromal* rather than *stem* cells. The criteria are as follows. First, MSCs must be plastic adherent when cultured in standard conditions. Second, MSCs must express CD105, CD73, and CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79α, or CD19 and HLA-DR surface molecules. Additionally, they must retain the ability to differentiate into osteoblasts, adipocytes, and chondroblasts in vitro [3].

In this context, numerous cells with MSC-like properties have been successfully isolated from a variety of human dental tissues [2]. These include alveolar bone-derived mesenchymal stem cells (ABMSCs), dental follicle progenitor cells (DFPCs), dental pulp stem cells (DPSCs) (Fig. 9.1), gingiva-derived MSCs (GMSCs), periodontal ligament stem cells (PDLSCs), stem cells from the apical papilla (SCAP), stem cells from exfoliated deciduous teeth (SHED), and tooth germ

progenitor cells (TGPCs) [2, 4]. Of these, DPSCs have attracted extensive attention. Despite the distinct terminological differences between SHED and DPSCs, cells with MSC characteristics can be obtained from the pulps of a variety of teeth, namely, primary teeth, exfoliated primary teeth, immature permanent teeth, mature permanent teeth, and third molars [5] and, more recently, from the pulps of supernumerary teeth, an otherwise discarded tissue [6].

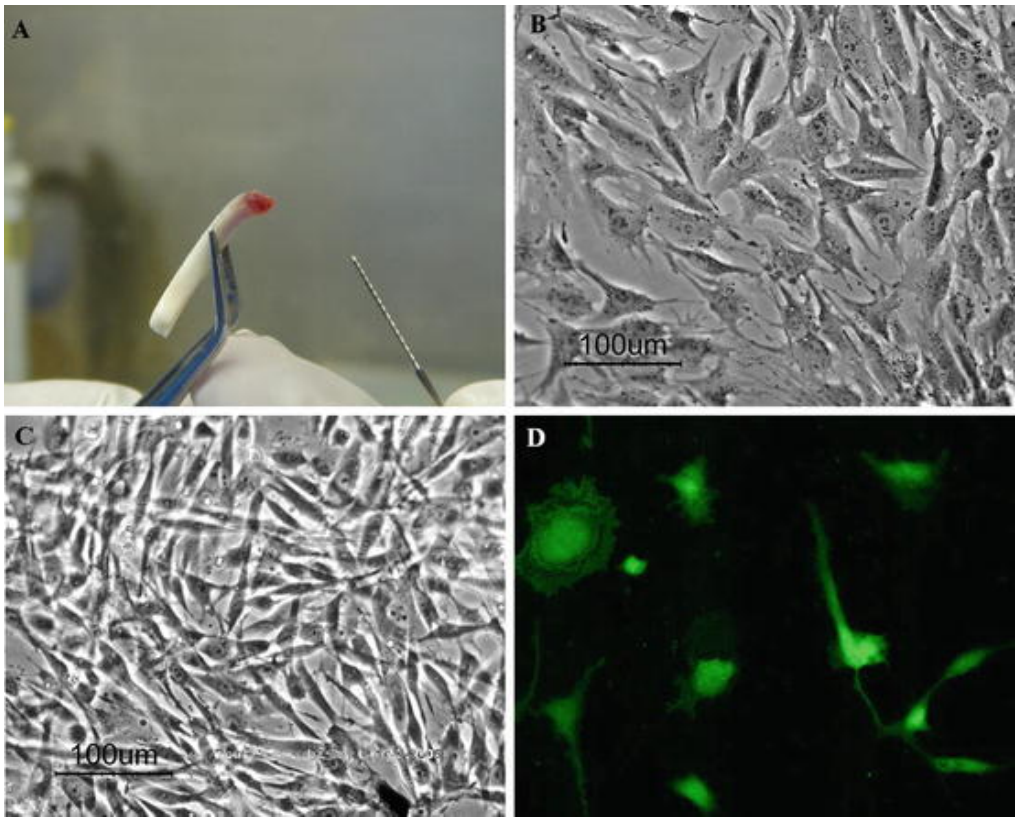

Fig. 9.1 Isolation and characterization of rabbit dental pulp stem cells showing the (**a**) removal of pulp tissue from the apical end of a rabbit incisor for culture, (**b**) rabbit DPSCs 6 days after primary culture (20×), (**c**) passage 2 rabbit DPSCs after 6 days showing a confluent monolayer of cells (20×), and (**d**) confocal laser microscope image of rabbit DPSCs positively stained with a CD271 mouse anti-rabbit monoclonal antibody (original magnification ×40)

One continuous challenge in regenerative dentistry is to identify the ideal cell source for regenerating the dentin/pulp complex [7]. Certainly, the isolation and culture of cells from the dental pulp have long been attempted by many investigators [8]. These early isolated cells frequently demonstrated alkaline phosphatase reactivity, partial cytodifferentiation, and the ability to form mineralized nodules when cultured in the presence of different substrates.

Adult dental pulp is a loose vascular connective tissue surrounded by dentin and consists of a heterogeneous population of cells: the potential preodontoblasts, fibroblasts, stromal cells, endothelial and perivascular cells, neural cells, and others. These cells maintain the homeostasis of dental mineralized tissues. Most adult pulp cells are postmitotic, but some cells may still divide and give rise to new pulp cells, able to differentiate even into odontoblasts and to form new dentin. All these cells and blood vessels are embedded in a specific rich extracellular matrix, which creates a microenvironment permitting repair processes [9–11].

In the year 2000, the term *dental pulp stem cells* was introduced by Stan Gronthos and his colleagues. By demonstrating that a clonogenic population of cells existed in the pulp, they determined that a population of MSCs was indeed present in pulp cell cultures. DPSCs display multilineage differentiation potential giving rise to at least three distinct cell types: osteo-/odontogenic, adipogenic, and neurogenic cells [12]. DPSCs can also undergo differentiation along chondrogenic and myogenic pathways [12]. Additionally, when these cells were combined with a hydroxyapatite-tricalcium phosphate (HA/TCP) carrier and implanted in an ectopic mouse model, dentin-/pulp-like structures could be regenerated after 6 weeks. The transplants were also found to express human-specific transcripts for dentin matrix components, including bone sialoprotein, osteocalcin, and dentin sialophosphoprotein (DSPP) [9].

DPSCs have also been isolated and characterized from several species including rats [13], rabbits [14], and dogs [15, 16]. In an autologous transplantation model, canine DPSC sheet fragments were able to regenerate a pulp-like tissue and newly mineralized dentin [16] when implanted in pulpectomized teeth. Furthermore, numerous cells that stained positive for bromodeoxyuridine (BrdU) could be detected in the pulp-like tissues, indicating that the transplanted cells could proliferate and contribute to the regenerated tissue. Goat DPSCs also reveal similar characteristics to human DPSCs and yet display a higher proliferative capacity (unpublished data).

Interestingly, DPSCs, like other dental tissue-derived stem cells, may be more committed or have limited potency compared to bone marrow mesenchymal stem cells (BMSCs). This is because dental tissues do not undergo continuous remodeling as does bone tissue [12]. Dental tissue is termed *ectomesenchyme*, and DPSCs are neural crest-derived cells that express nestin, which is a neural cell marker [17]. This further distinguishes DPSCs from BMSCs as a unique cell population.

However, postnatal dental pulp contains a diversity of cell types. It has been estimated that less than 1% of these cells have true stem cell-like properties [18, 19]. This has been demonstrated by their ability to efflux the DNA-binding dye Hoechst 33342 [17]. Similar to hematopoietic stem cells, this allows the cells to be later sorted and to purify the stem cell fraction, also called the *side population*. This side population of DPSCs isolated from rats displayed reduction in number with increasing age, a definite implication of pulp healing. The expression of alkaline phosphatase (ALP) was also shown to be less in side-population cells as compared to the main population, indicating an earlier progenitor state of these cells [19].

DPSCs have been frequently compared to another population of dental tissue-derived MSCs: SHED [12]. Despite having lower proliferation rates than SHED, DPSCs, unlike SHED, can successfully give rise to a complete dentin-/pulp-like complex in vivo, while SHED can regenerate dentin-/pulp-like structures only [20]. DPSCs are also a very heterogeneous population, and although they do not show the remarkable osteoinductive capacity of SHED, a subpopulation of DPSCs has been distinguished with distinct osteogenic potential in vivo [21]. DNA microarray analysis has shown that osteoblasts derived from human pulpar stem cells (ODHPSCs) revealed a list of up- and downregulated genes when compared to normal osteoblasts in spite of having a normal phenotype. A detailed overview of DPSC side and subpopulations will be discussed in the following sections.

The objective of precisely identifying and sorting the genuine stem cell population in the dental pulp is of vast

clinical importance for both stem cell-based and cell-free regenerative approaches. In particular, in the realm of an increased focus on cell migration and stem cell recruitment as a prerequisite for tissue repair and regeneration, it will become crucial to understand the dynamics of these sophisticated cells.

9.2 Characteristics of Dental Pulp Stem Cells

As previously discussed, human DPSCs fulfill criteria for MSCs [12]. They stain negatively for CD14, CD34, CD45, MyoD (muscle), neurofilament (nerve), collagen type II (cartilage), and peroxisomal proliferator-activated receptor gamma 2 (fat) and stain positively for CD29 and CD44 [22, 23], stem cell factor (SCF), and CD117 (c-kit) [24], markers associated with the endothelium as the CD106, CD146, smooth muscle (α-smooth muscle actin), bone (alkaline phosphatase, type I collagen, osteonectin, osteopontin, and osteocalcin), and fibroblasts (type III collagen and fibroblast growth factor 2) [12].

DPSCs are also positive for CD73, CD90, and STRO-1 [22, 25]. Other markers, such as the embryonic neural crest cell marker, low-affinity nerve growth factor receptor (LANGFR), or p75 neurotrophin receptor (CD271), have also been shown to be expressed by DPSCs [14, 26, 27]. This explains why these cells may have a major role in neuronal regeneration [28].

The expression of embryonic stem cell markers has also been found in DPSCs [5, 29]. DPSCs have been shown to express Oct-4, Sox2, and c-Myc [30], key reprogramming pluripotency markers. These transcription factors are known to play a regulatory role in the stem cell self-renewal process. Indeed, early human dental pulp cell cultures highly express these markers, but this expression is markedly diminished in later passages. This could be related to the explant culture conditions used, which may reactivate reprogramming factors to maintain pluripotency [30].

It is thus noteworthy to highlight that, although both explant culture and enzymatic digestion techniques employed for the isolation of DPSCs yield cell populations with very similar properties, cells obtained by enzymatic digestion appear to have higher mineralization potential in vitro [1]. This may explain why pluripotency may be better maintained in explant culture conditions [30].

DPSCs also express Nanog, another important embryonic pluripotency marker [31]. The concurrent expression of Nanog and Oct-4 seems to be directly related to the cells' proliferation rate. When Oct-4 and Nanog were depleted, proliferation and osteogenic differentiation of the cells were greatly reduced. In contrast, overexpression of these two factors led to the enhancement of these properties. This might indicate why DPSCs have shown to be a more efficient target population for induced pluripotency studies than other somatic cells [31].

Since the availability of consistent experimental models is crucial particularly in the realm of regenerative endodontic research, characterization of DPSCs from canine premolars has produced important observations. This characterization, although confirming the validity of the dog as a research model for stem cell-based dental tissue-engineering approaches, revealed distinct differences. Human DPSCs appeared to have higher proliferation rates than canine DPSCs [25]. While the expression of STRO-1 was lower in canine DPSCs than human DPSCs, the expression of CD146 was significantly lower in the latter as compared to canine DPSCs.

Furthermore, although displaying multilineage potential, canine DPSCs showed lower adipogenic and neurogenic potentials than their human counterparts, although they were higher than human BMSCs [25]. Interestingly, CD73 and CD90, two of the most important markers for MSCs, were expressed in very low levels by canine DPSCs. Such differences, among others, were explained by the difference in the developmental stage of the teeth from which the DPSCs were isolated: from canine premolars versus human third molars. The latter may represent a population of lower maturity. Differences in marker expression were attributed to

the lack of specific antibodies against canine antigens, and although canine DPSCs displayed marked odontogenic potential, these differences must be borne in mind when applying the results of animal studies to clinical research.

In general, the gene expression [32] and phenotypic profiles of human DPSCs are comparable to those of BMSCs, with the exception of CD106, which is expressed at very low levels in DPSCs (2.2%) as compared to BMSCs (66.3%). On the other hand, DPSCs do not basally express bone sialoprotein or the odontoblast-specific marker DSPP [12], although some researchers have found that the latter is expressed in nonodontogenically induced dental pulp cell cultures but at low levels [22].

Studies of DPSCs have also demonstrated that these cells have distinct immunosuppressive and immunomodulatory properties both in vitro and in vivo [2, 33, 34]. Indeed, DPSCs are capable of activating T-cell apoptosis in vitro [33]. In a coculture model, the proliferation of allogeneic PBMCs in the presence of DPSCs was significantly inhibited, although the PBMCs had been stimulated with a T-cell mitogen [34]. This inhibitory effect appeared to be stronger than that elicited by BMSCs when used in the same experimental model. Furthermore, DPSCs also suppressed B-cell proliferation. These effects seem to be related to cytokine production regulation by DPSCs where IL-10 was upregulated, while IL-2, IL-17, and IFNγ were significantly inhibited in the presence of DPSCs. The authors went on to further elucidate possible mechanisms behind T-cell proliferation inhibition, showing that a key factor in the immunosuppressive ability of DPSCs could be the increased expression of TGFβ1 since the addition of anti-TGFβ1 restored the ability of PBMCs to proliferate in the presence of DPSCs [34].

The immunomodulation properties of DPSCs also clearly involve their expression of Fas ligand. Fas ligand (FasL or CD95L) is a type II transmembrane protein that belongs to the tumor necrosis factor (TNF) family. Binding with Fas receptor, FasL induces a caspase-mediated apoptotic process in many cell types [33]. When the expression of FasL was knocked down in DPSCs by siRNA, it reduced their ability to induce T-

cell apoptosis in vitro. While systemically infused DPSCs showed the ability to ameliorate inflammation-induced colitis in a murine model, this ability was completely lost when FasL was knocked down in the injected cells, highlighting its role in DPSC immunomodulatory mechanisms [2, 33].

Culture supplements can also influence the properties of isolated DPSCs. In accordance with Good Manufacturing Practices (GMPs), there has been an increased concern for employing xeno-free products when designing cell-based human therapies. For instance, human platelet lysate (hPL) has been recommended as a substitute for fetal bovine serum (FBS) for BMSCs [35] and DPSCs [23, 36]. We recently showed that platelet-derived products could condition the wound-healing microenvironment [37–39]. Being rich in a large variety of factors and cytokines that interact during the different phases of wound healing, platelet-rich derivatives can represent a cost-effective, human source of nutrients for culturing cells for therapeutic use.

Although the results of these studies have shown hPL to be an effective substitute for animal-derived serum, their large-scale applicability has been met with challenges. The variability in preparation protocols and the lack of an adequate understanding of how platelet-derived products affect cell proliferation and differentiation are only some of these challenges. Nevertheless, DPSCs can be favorably proliferated and differentiated in hPL, showing in vitro and in vivo performances that appeared to be superior than for DPSCs cultured with 10% FBS [23]. Indeed, DPSCs cultured with 5% hPL proliferated significantly higher than cells in FBS and upon osteo-/odontogenic differentiation; those in hPL showed higher mineralization capacity as well. Interestingly, this seems to be a dose-dependent effect since 10% hPL inhibited cell proliferation of DPSCs. This has also been shown with other cell types [37]. Additionally, higher concentrations also appear to affect other cell functions, such as cell migration and angiogenic ability (unpublished data). These effects may be related to the unique involvement of platelets in both the pro- and anti-inflammatory processes of wound healing. However, the use of human platelet-rich derived products warrants

further study as not only do they appear to be a promising tool for cell-based human therapies, but they are also finding a wide platform for application in translational sciences and regenerative endodontic procedures in clinical settings [40–42].

9.3 Origins and Niches of Dental Pulp Stem Cells

One of the most important aspects of stem cell biology is understanding the in situ "niche" or microenvironment where stem cells lie in the body. The niche is responsible for maintaining and controlling a quiescent stem cell population that is capable of responding according to host requirements.

Within the dental pulp, a perivascular niche for dental pulp MSCs has long been suggested [43–45]. Indeed, most isolated DPSCs appear to have a phenotype consistent with pericytes. Additionally, their preselection using the STRO-1 antibody inferred a possible perivascular niche for these cells [43]. Both CD146 and STRO-1, which can be precisely used for the enrichment of DPSCs, appear to show colocalized expressions on the outer wall of blood vessels in human pulp tissue, further implying that the majority of DPSCs come from the microvasculature. This is intriguing since both DPSCs and pericytes are thought to arise from migratory neural crest cells during embryogenesis [43]. In the rat dental pulp, cells co-expressing CD146 and microtubule-associated protein 1B (MAP1B) were found to be statistically higher in the coronal pulp than in the radicular pulp or periodontal ligament. This was also shown by the messenger RNA (mRNA) expression of CD146, CD105, and CD166, which displayed a similar distribution, indicating that the coronal pulp appeared to harbor more stem cells than the other two regions [46].

The neurovascular bundle has also been identified as a niche using lineage tracing in a mouse incisor model where the Gli1 expression patterns were analyzed in Gli1-LacZ mice. Gli1, which has been hypothesized as a marker for incisor MSCs in vivo, was found to be expressed in the mesenchyme surrounding the neurovascular bundle, centered on arteries and

accompanying nerves, but not veins or capillaries [44]. These data elucidate the important role of nerves of the neurovascular bundle in the MSC niche, particularly via the expression of Sonic Hedgehog (shh) protein. Therefore, MSCs in the mouse incisor originated from periarterial cells in vivo. These periarterial cells, which did not express typical MSC markers in vitro, supported incisor homeostasis and gave rise to the entire MSC population. On the other hand, pericytes made a minor contribution to homeostasis, and their role appeared to be mainly related to injury repair. Indeed, incisor MSCs displayed typical MSC markers in vitro, and yet they were all derived from Gli1+ cells and not from NG2+ cells (pericytes) [44]. Additionally, both glial cells and the endothelium might also play important roles in the niche.

In a recent letter published in *Nature*, the role of glial cells in the MSC niche in the mouse incisor was emphasized [45]. The letter actually described how a significant population of MSCs during development, self-renewal, and repair of a tooth was derived from peripheral nerve-associated glia. Glial cells generated multipotent MSCs that produced pulp cells and odontoblasts. It appears that Schwann cell precursors and Schwann cells contribute to the development, growth, and regeneration of teeth. The results of this paper also suggested that Schwann cells and Schwann cell precursors are dormant neural crest-like cells that can be recruited from nerves and contribute to peripheral tissues and that these cells may be the in vivo origin of neural crest-derived multipotent stem cells identified in cultures of dissociated embryonic and adult tissues [45].

Some studies have also attempted to identify the origin of mesenchymal cells in the pulp using a double transgenic mouse model to determine the contributions of neural crest-derived or mesoderm-derived cells to teeth [47]. To differentiate between the two origins, mice encoding P0-Cre, Wnt1-Cre, Mesp1-Cre, and Rosa26EYFP allowed the tracing of neural crest- or mesoderm-derived cells as YFP-expressing cells. Wnt1 and P0 are expressed in early migratory neural crest, and Mesp1, a transcription factor, is first observed at E6.5 (early gastrulation stages), especially in nascent

mesoderm-derived cells. The authors found that approximately 90% of dental mesenchymal cells from E13.5 or 2-day-old mice were Wnt1/YFP+, whereas only approx. 7% were Mesp1/YFP+, indicating that almost the entire dental mesenchymal cells were neural crest derived [47]. Furthermore, they showed that dental colony-forming unit fibroblasts (CFU-Fs) capable of multilineage differentiation and self-renewal consisted entirely of Wnt1/YFP+ (P0/YFP+) neural crest-derived cells.

9.4 Subpopulations and Side Populations of Dental Pulp Stem Cells: Significance for Therapeutic Applications?

As previously mentioned, DPSCs represent a versatile and heterogeneous population of cells that include sub- and side populations of cells with special characteristics [18, 48–50].

While DPSCs isolated from aged dental pulps appear to have reduced proliferation and differentiation capacities, especially after repeated passaging, a subset of these cells can retain these abilities regardless of donor age. Indeed, isolation of DPSCs on the basis of their migratory ability in response to granulocyte colony-stimulating factor (G-CSF) mobilization has identified a characteristic subpopulation of DPSCs [48]. This subpopulation has been termed mobilized dental pulp stem cells (MDPSCs) and exhibits increased expression of angiogenic/neurotrophic factors with a higher trophic effect on migration, immunomodulation, anti-apoptosis, and angiogenic, neurogenic, and regenerative potentials than total DPSCs. These properties are equally maintained in young and aged MDPSCs [48]. In contrast to DPSCs, MDPSCs show very limited age-related decline in their properties, indicating that this population could be an important target for future regenerative therapies, particularly in light of a continuously aging population. Nevertheless, MDPSCs from aged pulps did display slightly increased expression of senescence-related markers, yet this did not affect their regenerative potential.

Particular side populations have also been isolated and characterized from general populations of DPSCs. As previously mentioned, the active efflux of Hoechst 33342 is an efficient method to identify side populations of stem cells with distinct characteristics. Indeed, side-population cells have been found among porcine DPSCs continuously expressing Bmi1, indicating a longer life span and higher proliferation rates when compared to primary pulp cells [50]. The ability of side-population cells to efflux the dye is mediated by the multidrug resistance transporter of ATP-binding cassette G2 (ABCG2)/breast cancer resistance protein 1 (Bcrp1). These cells appeared to be more primitive than the unfractionated porcine DPSC population, and while the latter failed to undergo chondrogenic differentiation, side-population cells were very chondrogenic.

In rat dental pulp, approximately 0.40% of pulp cells in young rats and 0.11% in aged rats comprised side-population cells [18]. Side-population cells expressed higher levels of nestin than the main-population cells, and while both side- and main-population cells maintained their differentiation capacities, the former seemed to be more primitive cells or *true* stem cells. Another striking observation is that side-population cells have also been isolated from human adult dental pulp in response to ischemic culture [49, 51]. In these studies, ischemic culture conditions were elicited by serum starvation of the cells and culturing them in hypoxic conditions (2% O_2) for 48 h following an initial 24 h in normoxic conditions. Side-population cells isolated following ischemic culture were much greater in number (five times) than those isolated from normally cultured cells. Expression of the multidrug resistance transporter of ATP-binding cassette G2 (ABCG2) also increased in side-population cells with ischemic culture, highlighting a possible role of ABCG2 in stem cell recruitment [49]. Since ABCG2 acts as a pump for cytotoxins and since increased expression of this protein is found around inflamed dental pulp tissue, this could indicate a role of these so-called side-population cells in response to injury by migrating to the site of damage upon their activation by repair signals.

Among porcine primary dental pulp, side-population cells with enhanced angiogenic properties have been isolated. When porcine dental pulp cells with stem/progenitor cell properties (initially characterized as $CD34^+$, $VEGFR2/Flk1^+$) were further subfractionated into $CD31^-;CD146^-$ and $CD31^+;CD146^-$ cells, distinct properties were found [52]. The $CD31^-;CD146^-$ cells expressed much higher levels of stem cell markers compared to the $CD31^+;CD146^-$ population. These former cells also displayed enhanced neurogenic potential. In addition, they had strong vasculogenic potential both via direct differentiation and through paracrine-mediated mechanisms since the conditioned medium of $CD31^-;CD146^-$ cells enhanced survival and proliferation of human endothelial cells [52]. These observations underpin the importance of adequately defining a true stem cell population with therapeutic potential.

9.5 Dental Pulp Stem Cells in Health and Disease

Being encased in a hard structure, dentin, the dental pulp's ability to expand and respond to injurious stimuli is limited. The dental pulp tissue is hence commonly exposed to inflammatory stimuli, and while chronic pulpal inflammation may be detrimental to the tooth's vitality, inflammation does play a role in regulating angiogenic responses and wound-healing processes such as reparative dentinogenesis.

Pulpal healing has been linked to the ability of pulp cells to secrete growth factors including angiogenic and neurotrophic factors [53]. Interestingly, short-term exposure to tumor necrosis factor-α (TNF-α), a proinflammatory cytokine, was shown to induce apoptosis in DPSCs by triggering the nuclear factor kappa B (NF-kB) signaling pathway associated with an upregulation of vascular endothelial growth factor (VEGF) expression. On the other hand, prolonged exposure to TNF-α resulted in increased proliferation of DPSCs with enhanced angiogenic signaling. At the same time, the telomere length and mineralization potential of DPSCs were significantly reduced after prolonged exposure to TNF-α [54]. These results

indicate that chronic inflammation results in telomere shortening with enhanced proliferation of DPSCs while negatively impairing their differentiation potential.

Infection appears to be the most determinant factor in whether or not pulpal healing will take place. When a “sterile” inflammatory response such as that induced by the use of calcium hydroxide or more recently mineral trioxide aggregate occurs, the dentin-pulp complex usually heals by inducing dentinal bridge formation. This acute local inflammatory response has been shown to be triggered by the local rise in pH seen in response to these materials [55]. This response results in local tissue necrosis, thereby causing necrotic cells to release low levels of inflammatory cytokines, which remove the dead ells and may subsequently trigger a reparative dentinogenic response. Molecular signals for repair are also released from their sequestered locations within the dentin extracellular matrix (ECM) [53]. These are imperative not only for reparative dentinogenesis but for inducing repair processes of angiogenesis and neurogenesis as well. These signals include factors such as VEGF, platelet-derived growth factors, transforming growth factor-β1 (TGF-β1), fibroblast growth factor 2, and bone morphogenetic protein-2 and bone morphogenetic protein-4. Complement activation also appears to be directly involved in initiating the inflammatory response as well as being involved in regeneration. For example, C5a has been shown to be involved in the recruitment of pulp progenitor cells, which express the receptor C5a.

Several of the current regenerative approaches have targeted the mechanisms of releasing latent growth factors in an attempt to trigger endogenous regeneration of the dentin-pulp complex [55]. The use of low-power laser was recently shown to enhance tertiary dentin formation by the activation of latent endogenous TGF-β1, thus directing resident dental stem cell differentiation [56]. Such a discovery sheds light on the crucial role of TGF-β1 in low-power laser-induced reparative dentinogenesis and enhances the concept of activating stem cells for endogenous tissue repair, taking us closer to clinical application of regenerative therapies.

Besides acidic signals coming from carious lesions, agents such as ethylenediaminetetraacetic acid (EDTA) can also release fossilized or latent growth factors upon removal of the smear layer and surface demineralization of dentin. Contrarily, agents such as 2-hydroxyethyl methacrylate (HEMA), which is an important component of dental composite restorative materials, besides being toxic to cells, have been demonstrated to hinder the migration of DPSCs [57]. These observations may explain why pulpal healing responses beneath dental adhesives in vivo are quite poor and dentinal bridges are seldom seen beneath these materials when they are used for direct pulp capping.

As a result of this renewed interest in the inflammation/regeneration interplay, research is now focusing on scaffolds that can control inflammation, thereby indirectly influencing pulpal regeneration. Macrophages appear to be major contributors in inflammation/regeneration, particularly the reversible switch between M1 (proinflammatory) and M2 (proresolving) macrophages [58]. Supporting this critical transition from an M1 phenotype to an M2 phenotype may well be the target of bioactive scaffold design for pulpal regeneration in the future.

Owing to the immunomodulatory features of stem cells, DPSCs themselves may also play a direct role in modulating the inflammatory response of pulp after injury. One alluring revelation is that DPSCs can be isolated not only from healthy pulps but from inflamed ones as well [59]. Although these cells exhibited lower proliferation rates than their counterparts from normal tissues, they had similar immunophenotypes. It appears that the inflammatory milieu has a negative impact on the proliferation and differentiation capacities of DPSCs isolated from inflamed pulps. Nevertheless, these cells retained their ability to regenerate dentin/pulp complexes when transplanted in an in vivo mouse model. The fact that viable DPSCs can be isolated from inflamed pulp tissue offers a new perspective on the previously limited source of DPSCs.

Similarly, DPSCs isolated from normal pulps appear distinctly different from those isolated from deep carious pulps in their proteomic profiles [60]. DPSCs isolated from dental

pulps under deep carious lesions actually displayed higher proliferation rates than normal DPSCs. Most differentially expressed proteins between these two populations were found to be related to cell proliferation and differentiation, cell motility and cytoskeleton, and antioxidative function. This higher proliferation rate could be partly related to the upregulated expression of T-complex protein 1 subunit beta (CCT2) in DPSCs from deep carious pulps. Increased expression of chloride intracellular channel protein 4 (CLIC4) could also account for this observation and may additionally play a role in the enhanced osteogenic differentiation of these cells when compared to their normal counterparts [60]. DPSCs from deep carious pulps were also found to express higher levels of antioxidative proteins, which may protect these cells from oxidative stress. Other proteins were found to be downregulated in deep carious DPSCs, such as stathmin, a protein with an essential role in regulating cytoskeleton dynamics.

The dentin-pulp complex is definitely a unique tissue whose intricacies we are just beginning to unravel. However, recent discoveries of the various properties of DPSCs have undoubtedly added to the puzzle of understanding the nature of this tissue.

9.6 Neurogenic and Angiogenic Potentials: Therapeutic Prospects?

We have already established that dental pulp stem cells have distinct angiogenic and neurogenic potentials owing to their specific origin and special characteristics. These properties have led researchers to explore unorthodox applications for these stem cells in various regenerative applications.

Human DPSCs express a variety of angiogenesis-related molecules including both pro- and antiangiogenic factors [61, 62]. Vascular endothelial growth factor VEGF is highly expressed by DPSCs, as are interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1), and these were all found in both conditioned medium and in cell lysates. The latter two appeared to enhance endothelial cell migration, an important

phenomenon in any revascularization strategy. Plasminogen activator inhibitor-1 (PAI-1) and endostatin were found both at the mRNA and protein levels. Using the chick chorioallantoic membrane assay, DPSCs could also significantly induce blood vessel formation [61]. Likewise, $CD31^{-}$;$CD146^{-}$ side-population porcine pulp cells could significantly induce neovascularization in a model of mouse hind limb ischemia [52]. Similarly, human DPSCs enhanced angiogenesis in a mouse skin wound healing model via paracrine-mediated mechanisms as they did not themselves engraft in the healing tissue at the injury site [63].

It appears that both Akt and ERK pathways are involved in DPSC-mediated endothelial cell migration, with VEGF playing a crucial role [61, 63]. This has also been delineated by the fact that the angiogenic capacity of murine DPSCs appears to be VEGF receptor-2 (VEGFR2) dependent. In coculture conditions with endothelial cells, DPSCs appear to assume a pericyte location, highlighting a pericyte-like function in addition to what was previously believed that DPSCs lie in pericyte-like locations [64]. This represents true data of the function of DPSCs as pericytic cells. Using an angiogenic inhibitor as soluble Flt (sFlt), the amount of blood vessels induced by murine DPSCs significantly decreased, indicating that sFlt inhibited VEGFR2 and downstream ERK signaling in DPSCs. Knockdown of VEGFR2 resulted in downregulation of VEGFA, VEGF receptors, and ephrinB2 and decreased the angiogenic induction of DPSCs in vivo.

Culture conditions can additionally modify/enhance the angiogenic potential of DPSCs [65]. By coculturing human DPSCs with endothelial progenitor cells in the presence of platelet-rich plasma (PRP), the expression of angiogenesis-related markers such as VEGF, PDGF, Flk-1, and SDF-1 was enhanced. This, coupled with the effect of PRP in vivo on increasing endothelial progenitor cell tube vessel formation, elucidates some of the important mechanisms by which the regenerative potential of these cells can be enhanced for future applications.

Although the angiogenic potential of DPSCs was the focus of previous studies, their neurogenic potential is also quickly

gaining appeal. Transplantation of human DPSCs 24 h after focal cerebral ischemia in a rodent model caused significant improvement in forelimb sensorimotor function at 4 weeks posttreatment [66]. Indeed, other studies showed that human DPSCs could differentiate into Schwann-like cells and their conditioned medium contained numerous neurotrophic factors that play a role in neural regeneration [67]. Again, these functions in vivo appeared to be mediated via paracrine mechanisms rather than through merely neural replacement by the DPSCs.

After complete spinal cord resection in rats, the injection of human DPSCs led to a marked recovery of hind limb locomotor functions when compared to the effects of bone marrow-derived cells [68]. By inhibiting cell apoptosis in response to injury and by inhibiting the action of many axon growth inhibitors as well as differentiating into mature oligodendrocytes, human DPSCs can act both directly and indirectly via trophic mechanisms to treat spinal cord injury. Human DPSCs can also confer neuroprotective and neuritogenic properties to axotomized retinal ganglion cells, opening up new arenas for their use in retinal nerve repair [28].

9.7 Dental Pulp Stem Cells: From Tissue Engineering to Regenerative Endodontics

The definite primary goal of using DPSCs has targeted the regeneration or engineering of the dentin-pulp complex. There have been numerous strategies involved, including the more traditional cell-seeding approach onto a variety of scaffolds to the more recent 3D-based scaffold-free culture techniques. Throughout the previous sections, we discussed the versatility of DPSCs as a MSC population with several examples of their ability to regenerate dentin-/pulp-like tissue in vitro and in vivo [69]. While several animal studies have been conducted, efficient experimental models such as the tooth slice/scaffold model may offer elegant approaches for translational research in the field of dental pulp engineering [70].

It is now imperative to identify a new field called *regenerative endodontics*. Regenerative endodontics is perhaps the epitome of the clinical translation of regenerative dental applications. While the terminology was introduced in 2007 [71], the field has greatly evolved since that time. It is defined as the use of biologically based procedures designed to replace damaged tooth structures such as dentin, root structures, and cells of the pulp-dentin complex. Interestingly, the concept behind this approach was actually introduced in the early 1970s by Nygaard-Ostby and Hjortdal [72]. They found that when root canals of vital teeth were cleaned and bleeding was induced, followed by partial filling of the root canal space, new fibrous connective tissues were regenerated in the apical portion, and healing was found. At that time, they did not find healing to have occurred in any of the necrotic teeth included, although the sample size was quite small.

Up until this time, the potential of the field has mainly relied on so-called revascularization procedures, whereby apical bleeding is induced in immature necrotic (dead) teeth and the developing blood clot acts both as a scaffold and vehicle for delivering bioactive molecules. The process of initiating bleeding itself appears to trigger the recruitment of endogenous stem cells via homing mechanisms. So the concept relies on the classic definition of tissue engineering and regenerative medicine whereby cells, scaffolds, and morphogenic signals, as well as microenvironmental cues, are involved in the regeneration process. However, there are many challenges to this approach, such as that of regenerating the dentin-pulp complex in mature teeth or when bleeding cannot be induced. In such cases, the use of more complex approaches becomes fundamental [73].

Several dental pulp engineering strategies have focused on the delivery of appropriate morphogenetic signals and designing suitable scaffolds (Fig. 9.2). Enhancing cell attachment and the use of angiogenic factors (such as VEGF) in injectable scaffolds (hydrogels) such as self-assembling peptides are important strategies [74]. It is also crucial to design strategies that can control or direct the fate of either transplanted or recruited DPSCs once they arrive at the site of

injury. From a clinical perspective, eliminating infection, modulating the inflammatory response, and stimulating angiogenesis are key factors to guarantee a favorable biological response. It is also important to identify key morphogenic signals (their concentration and spatiotemporal patterns of release are also important) involved in the process of dentin/pulp repair and regeneration. As previously mentioned, agents such as EDTA can release fossilized growth factors from the dentin matrix, and these may themselves act to recruit resident stem cells [57].

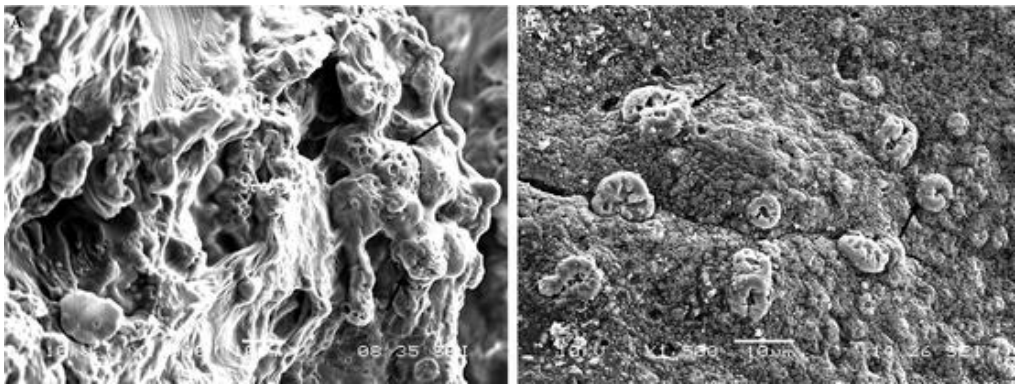

Fig. 9.2 Scanning electron microscope images of DPSCs seeded onto different scaffolds showing typical formation of cellular aggregates and nodule-like structures 12–14 days after culture without differentiation inducers (*arrows*). (**a**) DPSCs cultured onto porous collagen scaffolds for 14 days (×1000); (**b**) DPSCs cultured onto porous 50/50 poly(lactic-co-glycolic acid) sheet scaffolds for 12 days (×1500)

The complete regeneration of pulp tissue following pulpectomy has been shown in an autologous canine model [75]. This approach relied on the transplantation of CD105+ in addition to SDF-1 delivered via an implanted collagen scaffold. The verification of the regenerated pulp tissue was done via examination of both gene and protein expressions, and it revealed the similarity of the regenerated pulp tissue to the native pulp. It is noteworthy that these authors intentionally widened the apical diameter to 0.7 mm to allow a source of vascularization. The width of the apical diameter has been suggested to be a determining factor for the success of endodontic revascularization procedures [76].

In an attempt to simulate a more clinical approach, DPSCs combined with PRP were injected into the prepared and enlarged root canals of mature teeth in dogs [77]. The

regenerated tissues appeared to be cemental, periodontal-like, and bone-like tissues rather than dentin-/pulp-like tissues, and it appeared that this treatment did not produce any results different from using the blood clot alone or DPSCs alone.

One intuitive approach was to combine the effects of calcium hydroxide, a classic material routinely used for vital pulp therapy procedures with DPSCs [78]. DPSCs pretreated with a low concentration of calcium hydroxide had higher mineralization ability compared to untreated cells. Extensive dentin-like tissue was also regenerated in vivo upon the autologous transplantation of calcium hydroxide-treated DPSCs. Additional data demonstrated the effects of calcium hydroxide on enhancing the migration, proliferation, and differentiation of DPSCs, elucidating previously suggested scenarios of the role of calcium hydroxide in dentin regeneration following pulp capping procedures. Other approaches have explored the use of a tissue-engineered transplant composed of cell sheet fragments of DPSCs and platelet-rich fibrin (PRF) granules as a more clinically directed approach [16]. The implantation of the DPSC/PRF constructs allowed the regeneration of homogeneous and compact pulp-like tissues. A clear abundance of blood capillaries and deposition of a neo-dentin matrix were found 8 weeks after transplantation of these constructs.

While DPSCs can be appealing for cell-based therapies, many issues remain to be explored, such as the ability of these cells to regenerate a complete dentin structure. Although several scaffolds have been used, the unique architecture and composition of native dentin have directed some researchers to devise scaffolds based on a natural dentin matrix [79]. Dentin matrix scaffolds were prepared from human third molar dentin tissues following exposure to citric acid and treatment with EDTA. These were then seeded with DPSCs and ectopically transplanted in the dorsum of nude mice. The results showed that dentin-like tissue regenerated in the transplants and these tissues were positive for two dentin-specific markers: dentin matrix protein 1 (DMP-1) and DSPP [79]. Furthermore, cells in the regenerated tissue stained positive against an antibody

for human mitochondria, indicating the direct participation of the transplanted DPSCs in the regenerated tissue.

The shifting attention toward 3D naturally derived extracellular matrix scaffolds has also introduced the concept of a scaffold-free approach, namely, the use of pre-vascularized microtissue spheroids [80, 81]. This approach hypothesizes that by allowing the cells to self-assemble into a 3D tissue, the physiological interactions between the cells better simulate the natural situation and eliminate the influence of a secondary material. Using 3D agarose petri dishes (micromolds), DPSCs could assemble into 3D spheroids 3 days following culture. These spheroids were then implanted into pretreated root slices and transplanted ectopically in the mouse. This system resulted in the regeneration of pulp-like tissue similar to native tissue. Furthermore, when these spheroids were cocultured with human umbilical vein endothelial cells (HUVECs), the regenerated pulp-like tissue was attenuated and displayed a significantly higher number of blood vessels than in the transplants with DPSCs alone.

9.8 Dental Pulp Stem Cells and Bone Tissue Engineering

DPSCs can also represent a suitable model for the study of bone differentiation due to their osteogenic ability compared with other cell types harvested from the adult human body. This feature, together with their easy availability, high accessibility in the oral cavity, and their resistance to cryopreservation, makes DPSCs very interesting for use in bone tissue engineering procedures in combination with scaffolds [82]. It has been demonstrated that DPSCs, when undergoing differentiation into preosteoblasts, deposit an extracellular matrix that becomes calcified woven bone without the need of osteoinductive templates, after in vivo transplantation is remodeled into a lamellar bone. Additionally, these cells appear to be good candidates for bone tissue reconstruction protocols and bone regeneration models because of their good cellular morphology and high bone morphogenetic protein (BMP)-2 and VEGF secretion [83–86].

Numerous studies have evaluated the multipotency of DPSCs. These studies have demonstrated that DPSCs are able to differentiate into osteoblasts when cultured in osteogenic medium supplemented with dexamethasone, β-glycerophosphate, and ascorbic acid [2, 87]. Apparently, all the characteristics of DPSCs have suggested that these cells are more suitable than BMSCs for mineralized tissue regeneration, particularly for orofacial bone regeneration [11, 43, 88, 89].

Consecutively, DPSCs have been used to enhance osseointegration of titanium dental implants [90]. Titanium (Ti) implants are popularly used in dentistry owing to their good biocompatibilities, resistance to corrosion, and mechanical properties. The fundamental aspect in the performance of an implant is the mechanical and biological behavior of its interface with the surrounding microenvironment of the periodontium. A stable biological and noninert interface between the biomaterial surface and the surrounding tissue is a vital prerequisite both for immediate implant loading and for the long-term success of such implants. This interface is achieved by a biological process (partially regulated by mechanical and material properties) known as osseointegration of the implant, which is an intimate connection of the implant within the bone by means of an appropriate and sufficient growth of new bone on the surface of the implant [91].

There is potential to enhance osseointegration of prosthetic implants by modifying the biologic modulus at the implant interface with osteoblast-like progenitor cells that are capable of self-renewal and can be experimentally directed into an osteoblast lineage in vitro. Human cells have been used to seed porous metal implants. Embryonic stem cells, fetal osteoblasts, and mesenchymal precursors such as adipose tissue-, bone marrow-, or dental pulp-derived cells have high differentiation potential in in vitro experiments, having not only demonstrated good adhesion but also shown osteogenic differentiation, proliferation, and mineralized matrix formation [92, 93].

DPSCs were challenged with two titanium surfaces, either in plain cultures or in a roller apparatus within a culture

chamber, for hours up to a month. During the cultures, cells on the titanium surfaces were examined for histology, protein secretion, and gene expression. Results showed that complete osseointegration using human DPSCs was obtained: these cells were able to quickly differentiate into osteoblasts and endotheliocytes and produce bone tissue along the implant surfaces [93]. In a study done on dogs comparing BMSCs to DPSCs, complete osseointegration of dental implants and tissue-engineered bone was higher using DPSCs in comparison to BMSCs and periosteal cells after 8 weeks with the highest osteogenic level of bone-implant contact. The authors concluded that DPSCs may be a good source of tissue-engineered bone around dental implants [90, 94]. Since it has been proven that even teeth with damaged dental pulps are able to maintain their stem cells with high proliferative and differentiation capacity, the validity of the dental pulp as a source of stem cells may replace the bone marrow from iliac crest [95]. This evidence lays the groundwork for the possibility of local injection of DPSCs during implant placement to enhance bone regeneration and implant stabilization [96, 97].

Although the field of dental implantology has revolutionized dental therapy for the past 40 years, it has not yet met all expectations. During function, implants are subjected to various constraints, such as twisting, bending, and stretching; lateralization may become unbearable for the bone, causing shear stresses and cellular damage by friction and resulting in marginal bone loss over time [98, 99]. For these reasons, polyether ether ketone (PEEK) was proposed as a viable alternative material for dental implants because their elastic modulus is close to the bone and computer-assisted modeling presents promising clinical results [100]. To increase the cell adhesion and osteogenic potential of PEEK surface functionalization in vivo, the physicochemical surface characteristics were modified by creating a synthetic matrix from polyelectrolyte multilayers and protein deposition, then spraying the surface with DPSCs, which resulted in superior osseointegration of PEEK implants [101].

These observations have paved the way to clinical application. Although the majority of studies evaluating in vivo applications of DPSCs have been performed on animal models, there have been a few clinical trials. In one study, 17 patients participated in a clinical study aiming to evaluate the effect of DPSCs seeded onto a collagen sponge on bone formation subsequent to DPSC transplantation in the extraction sockets of third molars. After 3 months, significant bone regeneration was found in the side that received the DPSCs compared to the biomaterial alone. Using in-line holotomography, an advanced phase-imaging method using synchrotron radiation, this regenerated bone appeared to be uniformly vascularized and composed of compact lamellar bone. In this context, compact bone regenerated after DPSC engraftment in the mandible could be considered of fundamental importance to limit pathologic fracture and to offer better quality of life in oral cancer patients [102, 103]. Human clinical trials using allogeneic DPSCs with PRP mixed with tricalcium phosphate for the treatment of mandibular osteoradionecrosis revealed appreciable bone formation from the second month onward [104].

Another important issue for the clinical translation of DPSCs for bone tissue engineering is donor age. It was shown that DPSCs can be isolated from donors of all ages, including patients up to 67 years old. DPSCs from aged donors show a better proliferative ability at lower in vitro passages (maximum p2). When DPSCs are cultured on nanostructured hydroxyapatite (HA) scaffolds, they maintain their biological properties. Although in monolayer conditions DPSCs derived from the senior patient group showed a low proliferative ability, when cultured on HA nanostructured granules and used in vivo to repair critical size defects, they showed the same ability as the younger group in terms of the time to repair the defects and the quality of the extracellular matrix [105, 106].

9.9 Future Areas of Research

Based on all the characteristics of DPSCs, they will surely become targets for the discovery of further cutting-edge

applications in tissue engineering and regenerative medicine (Fig. 9.3). Their use has already been explored as therapeutic tools to enhance wound healing and treat neurodegenerative diseases. One important and perhaps still underrated tool is the secretome of these cells, which may actually represent a changing front in how regenerative therapies are designed for clinical application, especially since the conditioned medium of DPSCs has been shown to be a rich source of bioactive molecules.

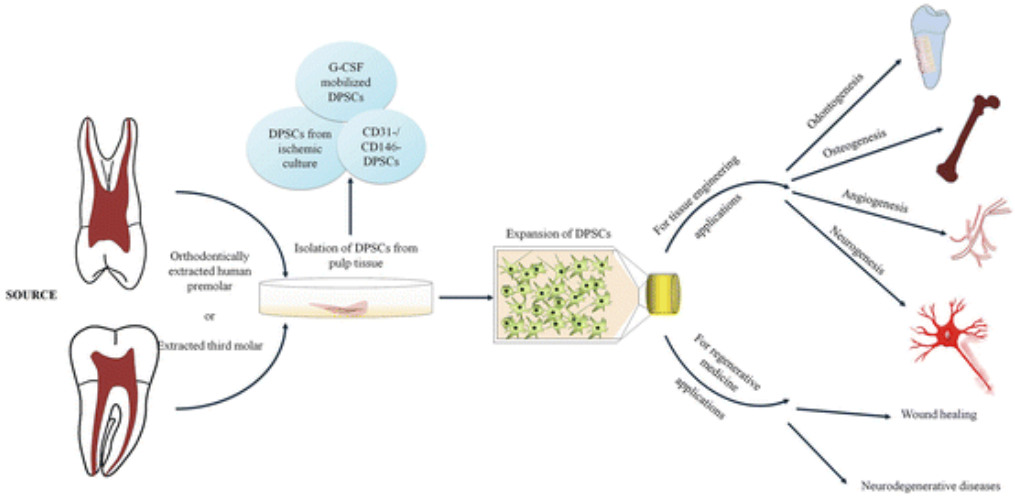

Fig. 9.3 Applications of dental pulp stem cells (DPSCs) in tissue engineering and regenerative medicine

A unique structure that is currently under investigation in MSCs is the exosome. Exosomes from various cells appear to be involved in important physiological and pathological processes such as immune responses, angiogenesis, inflammation, tumor metastasis, spreading of pathogens or oncogenes, and processes [107, 108]. Proteomic analysis of exosomes from a variety of stem cells has revealed that they could indeed be considered vehicles for drug delivery, thereby further narrowing the gap between bench top and bedside [109, 110]. Exosomes isolated from SHED have been demonstrated to suppress carrageenan-induced acute inflammation in mice [111] and appear to have antiapoptotic functions on human dopaminergic neurons [112]. However, no similar studies investigating the effects of exosomes from adult DPSCs were found at the time this chapter was written; thus, it may represent a rich new area of research.

Another area that warrants further research is utilizing tools to trigger endogenous regeneration, i.e., rely on endogenous stem cells rather than exogenously delivered cells to induce dentin/pulp regeneration. For example, a recently introduced nanoparticulate bioceramic putty (iRoot BP Plus) could activate dental pulp cell migration, a mechanism that apparently involves fibroblast growth factor receptor signaling as well as mitogen-activated protein kinase and Akt pathways [113]. This result was also observed in vivo in a rat pulp repair model. The hypothesis that the release of chemotactic signals can induce migration of endogenous dental pulp cells has also been demonstrated by the fact that dental pulp cells could effectively migrate into a 3D collagen scaffold in response to stromal-derived factor-1 (SDF-1) and basic fibroblast growth factor (bFGF) [114].

The seminal work by Jeremy J. Mao's group in New York in 2010 [115] showed that by implanting endodontically treated real-size native human teeth in a nude mouse ectopic model, the delivery of basic fibroblast growth factor or vascular endothelial growth factor (bFGF or VEGF) yielded recellularized and revascularized connective tissue that integrated into the native dentinal wall in root canals. When delivery of these factors (bFGF, VEGF, or platelet-derived growth factor) was combined with a basal set of nerve growth factor and bone morphogenetic protein-7, cellularized and vascularized tissues were generated. This study represented the first formal introduction of pulp regeneration via chemotaxis-induced homing.

Similarly, the use of stem cell factor (SCF), a powerful chemokine that binds to the c-kit receptor CD117, could enhance the proliferation and migration of DPSCs [116]. Upon in vivo implantation in a collagen scaffold in an ectopic mouse model, neovascularization and cell recruitment were significantly increased, indicating a role of SCF for inducing cell homing, angiogenesis, and tissue remodeling.

Efficient drug delivery vehicles will still need to be designed to promote the sustained and controlled delivery of such chemotactic factors that can stimulate the migration and, henceforth, differentiation of dental pulp stem cells in situ.

These strategies would also likely benefit cases in which the remaining pulp tissue is inflamed, in light of the recent discovery of potent DPSCs from inflamed pulps.

An impressive new clinical trial is under way based on the ability to mobilize DPSCs in response to G-CSF. Mobilized DPSCs in addition to G-CSF can completely regenerate the pulp tissue in an in vivo pulpectomized model [117]. Although good manufacturing procedures still need to be optimized, the clinical implication of this approach is clear, especially since this subset of cells appears to retain its characteristics irrespective of the age of the donor. If one is to realize the true clinical applicability of these cells in the near future, particularly in the field of regenerative endodontics, it is imperative to focus on strategies that are feasible, practical, economic, and efficient (Fig. 9.4).

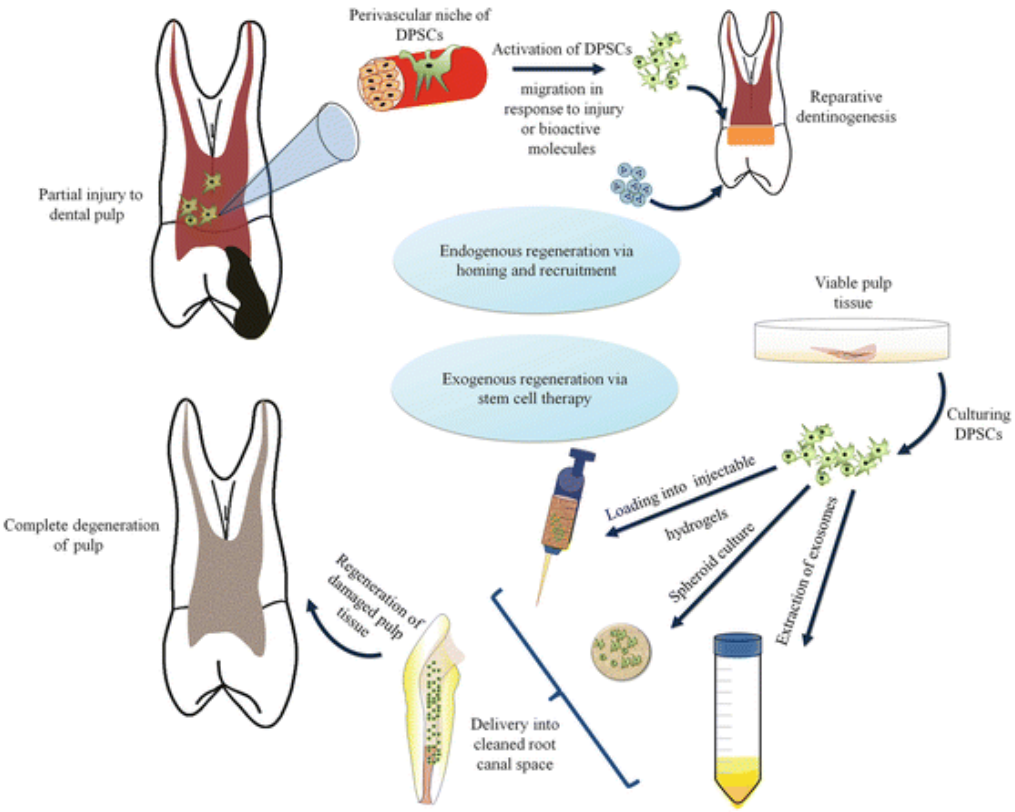

Fig. 9.4 Strategies for regeneration of dentin-pulp complex using DPSCs via endogenous or exogenous regenerative strategies

9.10 Concluding Remarks

As the field of tissue engineering and regenerative medicine continues to evolve, so does the clinical need for applicable regenerative strategies that can be implemented in the clinic that are also economic and easy to apply. The discovery of DPSCs and their potential for regenerating the dentin-pulp complex and their application in regenerative endodontics are only a few of these strategies. As the aging population is continuously growing, there is increasing demand for a better quality of life, especially while living with diseases such as diabetes, Parkinson's, Alzheimer's, and other neurodegenerative diseases. Hence, the role of stem cells will undoubtedly increase in the coming decades, and with more understanding of the biology and pathology of these cells, more efficient ways to utilize them for therapy will become available. Stem cells derived from the dental pulp have shown promise in several of these applications and will probably become the focus of more studies and clinical trials in the very near future.

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10. Stem Cell Therapy and Tissue Engineering in Urogenital Diseases

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Abbreviations

AMDC Autologous muscle-derived stem cell

ASC Adipose tissue-derived stem cell

BMP Bone morphogenetic protein

BMSC Bone marrow-derived stem cell

ECM Extracellular matrix

ED Erectile dysfunction

EFG Epidermal growth factor

EPCs Endothelial progenitor cells

ESCs Embryonic stem cells

HGF Hepatocyte growth factor

HO-1 Heme-oxygenase-1

HSC Hematopoietic stem cell

IGF Insulin-like growth factor

iPSCs Induced pluripotent stem cells

MSCs Mesenchymal stem cells

PD Peyronie's disease

PDE-5 Phosphodiesterase-5

PLA Polylactic acid

SMC Smooth muscle cell

STING Subureteric transurethral injection

SUI Stress urinary incontinence

TA Tunica albuginea

UCB Umbilical cord blood

UC Urothelial cell

UVJ Ureterovesical junction

VD Voiding dysfunction

VEGF Vascular endothelial growth factor

VUR Vesicoureteral reflux

10.1 Introduction to Stem Cells

The field of stem cell research offers promising alternatives in the treatment of several diseases. This chapter will attempt to summarize recent breakthroughs in stem/progenitor cell maintenance and differentiation related to the regeneration of the genitourinary apparatus. Before discussing this matter further, a brief introduction to stem cell biology will be presented. In 1961, Till and McCullough reported the four basic characteristics of a stem cell: self-renewal and regenerative capacity, multipotency, and the ability to differentiate into any other cell lineage [1].

10.1.1 Embryonic Versus Adult Stem Cells and Their Properties

Adult and embryonic stem cells (ESCs) are the two major types of stem cells, and they possess distinct properties [2, 3]. In adults, the bone marrow is the primary source of stem cells. Adult stem cells are classified as (1) hematopoietic stem cells (HSCs), which differentiate into either red blood cells or lymphoid/myeloid lineage-specific white blood cells [4], and (2) mesenchymal stem cells (MSCs) [5], which differentiate into adipocytes, chondrocytes, and osteoblasts [6]. Adult MSCs are also found in all postnatal organs and tissues, and they play important functions in tissue injury repair and general homeostasis [7]. These cells are some of the best promising tools for regenerative medicine because of their sustained proliferative capacity and their multipotent differentiation potential [6, 8].

10.1.1.1 Embryonic Stem Cells and Induced Pluripotent Stem Cells

ESCs, induced pluripotent stem cells (iPSCs), and postnatal adult stem cells are interesting sources of stem cells for regenerative medicine. ESCs are isolated from the early morula stage embryo or from the inner cell mass of blastocysts, are able to self-renew and expand, and have the potential to differentiate into any type of somatic tissues [9]. In fact, ESCs are potentially able to self-renew indefinitely

because their telomeres can remain intact. On the contrary, adult stem cells, which are derived from different tissues, lose telomeric DNA during aging. Indeed, their telomere shortening has been reported both in vitro and in vivo during cell division [2, 3].

In principle, forced expression of Oct-4, Sox2, c-Myc, and KLF4 transcription factors can transform any somatic cells into ESC-like cells, which have been termed *induced pluripotent stem cells*, iPSCs. iPSCs appear to possess the same potential and properties when compared to ESCs, while their generation is not dependent on an embryo [10]. Even with improved iPSC generation [11–13], reactivation of the c-Myc retroviral transgene has been shown to increase tumorigenicity of chimeric mice obtained via blastocyst injection of selected iPSCs [14]. In later studies, it was reported that murine iPSCs could be generated without the c-Myc transgene. Chimeric mice derived from c-Myc-deficient iPSCs did not develop tumors over the course of those studies. Furthermore, the absence of the c-Myc transgene resulted in a better isolation of iPSCs without drug selection [15, 16]. Both ESCs and iPSCs have numerous medical applications due to their pluripotency, but the relative difficulties with their genetic manipulation and the ethical considerations surrounding their use represent formidable limitations. In contrast, postnatal adult stem cells are often immunocompatible, and no ethical concerns related to their use restrict their therapeutic potential.

10.1.1.2 Mesenchymal Stem Cell Biology

MSCs, which were first isolated from the rat bone marrow by Friedenstein and Petrakova, demonstrated osteogenic differentiation potential [17]. MSCs are multipotent stem cells with high proliferative capacity and the ability to differentiate into various cell types. For instance, they have the potential to differentiate into adipocytes [18], cartilage [19], bone [20], and nervous tissues [21–23]. Experimental procedures have shown successful applications of MSCs in a collagen matrix to repair Achilles tendon [24] and to regenerate muscles [25]. The identification of a specific markers that distinguish MSCs

is not yet available, but studies have reported that these cells can express VCAM-1, Thy1, Sca-1, CD29, CD44, CD73, CD105, CD166, and MHC class I, but lack CD45, CD11b, and c-Kit expression (reviewed in [26]). MSCs have been used to regulate immunological responses and treat degenerative diseases, but concerns have been raised regarding their ability to cure diseases in clinical trials [27].

For instance, MSCs can improve tissue repair because of their self-renewal capacity and because they can induce multilineage differentiation within a target site (reviewed in [28]). MSCs exert immunomodulatory effects on tissues and can direct residing stem cells or progenitor cells to the area of injury [29]. Many treatments, especially for the management of urogenital diseases, have used stem cell therapy. However, a few animal experimentations and clinical trials have shown promising results. For example, stem cells were used in regulating the mechanisms involved in voiding deficiencies, leading to the cure of stress urinary incontinence [30–33]. Other therapeutic effects of delivering MSCs to an injured site have been reported in animal models of Cisplatin-induced acute kidney injury [34], ischemia [35], Adriamycin-induced nephrotic syndrome [36], and mesangioproliferative anti-Thy1.1 glomerulonephritis [37]. A rat kidney transplantation model has even been developed to study chronic allograft nephropathy [38]. Most of these studies showed that MSCs were able to provide a therapeutic environment via the secretion of paracrine factors such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin-like growth factor-I (IGF-I) [39–42], and erythropoietin [43]. These stem cells may also exert a therapeutic effect because of their involvement in the secretion of bioactive factors with antiapoptotic, anti-scarring, or neovascularization effects. Heme oxygenase-1 (HO-1) [44], the SDF-1-CXCR4/CXCR7 axis [45], and CD44/hyaluronic acid interactions [46] were reported to contribute to the important role of MSC-mediated protection in acute tubular injury or renal ischemia-reperfusion injuries. Nonetheless, many challenges remain to translate the promising animal model results into clinical trials. For example, aberrant differentiation of intraglomerular MSCs into adipocytes and

glomerular sclerosis was observed in an experimental setting that aimed at preventing renal failure [47].

10.1.1.3 Extra-Embryonic-Derived Stem Cells

The placenta is composed of three layers: the decidua, from maternal origin, and the chorion and amnion, both from fetal origin [48]. Placental mesenchymal cells are derived from fetal origin [49], and the amniotic fluid of both murine and human pregnant females has been identified as an alternative source of MSCs [50, 51]. Amniotic fluid fills the amniotic cavity and allows the exchange of substances between fetus and mother, protects the developing fetus, and allows it to move and grow within the cavity [52]. In 2003, cells expressing Oct-4, a transcription factor expressed by ESCs and partially responsible for the pluripotency of these cells, were isolated from the amniotic fluid [53]. Extra-embryonic-derived stem cells from the placenta are classified as amniotic mesenchymal cells, amniotic epithelial cells, chorionic mesenchymal cells, and chorionic trophoblast cells. In 2007, stem cells isolated from the amniotic fluid were defined as multipotent stem cells that do not form teratomas when implanted in immunodeficient mice [54]. The lack of tumorigenic potential of these MSCs could be considered an advantage over human ESCs for medical applications. The ability of amniotic fluid-derived MSCs to differentiate into adipocytes has not been detected, suggesting that the adipogenic potential is present in adult bone marrow cells but not in cells isolated during the embryonic period [54, 55]. Furthermore, stem cells isolated from the amniotic fluid were less differentiated when compared to their bone marrow counterparts. It has also been reported that these cells have shorter proliferative cycles [54, 55].

MSCs have been purified from the umbilical cord, umbilical cord blood (UCB), and peripheral blood, although the properties of the cells isolated from these various sources present contradictory results. For example, some studies could not detect the presence of MSCs in the UCB [54, 56], while

others could [57]. Umbilical cord MSCs showed a cardiomyocyte-like phenotype [58], while UCB-MSCs seem to contain two populations of cells, osteoclasts and mesenchymal-like cells, whether the cord blood was harvested from preterm or term deliveries [59]. UCB-MSCs cultured in vitro give rise to cells that display mesenchymal progenitor features. On the one hand, UCB-derived mononuclear cells can give rise to adherent cells with an osteoclast phenotype. These cells are multinucleated, express the common leukocyte antigen CD45 and the osteoclast-related antigen CD51/CD61 (vitronectin receptor), and display a strong tartrate-resistant acid phosphatase activity (as previously reported in [60, 61]) [59]. On the other hand, cells with the mesenchymal phenotype have a fibroblast-like morphology and express several mesenchymal progenitor-related antigens (SH2, SH3, SH4, ASMA, MAB 1470, CD13, CD29, and CD49e) [6, 59, 62, 63]. A study by Erices and colleagues suggests that, compared with term cord blood, preterm cord blood is richer in mesenchymal progenitors resembling hematopoietic progenitors [59]. Additionally, UCB-MSCs seem to possess immunomodulatory capacities. Indeed, they may be able to suppress lymphocyte proliferation and decrease proinflammatory cytokine production (interferon gamma and tumor necrosis factor) [64].

10.1.1.4 Endothelial Progenitor Cells and Adipose-Derived Mesenchymal Stem Cells

Fat is an abundant and accessible source of stem cells. Adipose tissue-derived stem cells (ASCs) are extracted from adipose tissue and characterized as plastic-adherent and multipotent cells [65]. ASCs are considered both MSCs, thus progenitors of cell types derived from the mesoderm, and stromal cells. As ASC cultures are passaged, the cell populations become homogeneous and exhibit fibroblastoid morphology. ASCs can be distinguished by the expression of CD73, a marker of mesenchymal cells, in addition to the bone marrow progenitor marker stromal-derived factor-1 [66]. It has also been reported that ASCs are $CD31^{+}$, $CD34^{+/-}$, $CD45^{-}$,

$CD90^+$, $CD105^-$, and $CD146^+$. Studies have shown that $CD34^+$ ASCs have increased cell division potential, while $CD34^-$ ASCs are more multipotent [67, 68]. Compared to ASCs, endothelial progenitor cells (EPCs) are $CD31^-$, $CD34^+$, $CD45^-$, $CD90^+$, $CD105^-$, and $CD146^+$ [69]. EPC have the ability to differentiate into endothelial cells, which constitute the lining of blood vessels [70, 71]. When uncontrolled, EPCs participate in pathological angiogenesis, which contributes to tumor growth. However, their presence in blood has also been associated with better outcomes for cardiovascular disease and wound healing in nontumorigenic settings [72–74].

10.2 Kidney Regeneration: Stem Cell Approaches

Early renal lineage specification, nephric duct elongation, and movement of the ureter towards the bladder are key events that ensure normal renal and urinary tract function [75]. The kidney is a highly specialized filtrating organ that eliminates waste products from the circulation and preserves electrolyte levels and pH balance of the body fluids and thus plays a key role in proper maintenance of bone mineralization and blood pressure. Most of these roles are carried out by the nephron, the functional unit of the kidney [76, 77].

Acute renal failure causes injury to renal tubular epithelial cells mainly. Over time, injured tubules regenerate via cell proliferation from surviving dedifferentiated cells, from renal stem cells that reside inside the kidney and migrate to the site of regeneration, or from bone marrow-derived stem cells (BMSCs) that are attracted to the injured epithelium and undergo cellular differentiation [78]. Kidney stem cells are found in the renal tubules and the papilla. Glomerular parietal epithelial cells have self-renewing potential and can generate podocytes and proximal tubular cells [79]. These progenitors can grow and differentiate in response to renal injury, but they often fail to correct injuries resulting from chronic renal diseases in which, as a consequence, patients require long-term dialysis or renal transplantation (reviewed in [80]).

10.2.1 Renotropic Factors and Kidney Repair Mechanisms

Renotropic factors that induce remodeling and maturation of the epithelium after renal injury or contribute to kidney development have been identified over the years. Hepatocyte growth factor (HGF), epidermal growth factor (EGF), IGF-I, heparin-binding EGF-like growth factor, platelet-derived growth factor, bone morphogenetic protein-7 (BMP-7), and uterine sensitization-associated gene-1, a BMP antagonist, are among the renotropic factors that can enhance kidney recovery [81–87]. In contrast, activin A, a member of the transforming growth factor-beta superfamily, has been identified as an inhibitor of renal organogenesis [88]. Most of the renotropic factors can promote tubular cell proliferation, but the exact mechanisms involved in cell maturation are not completely understood. It is also unclear whether these renotropic factors can promote renal regeneration via the activation of some renal stem/progenitor cells.

10.2.2 Cellular Therapy Strategies for Renal Regeneration

Stem cells can be used for kidney repair. This form of therapy is suitable for acute renal injury and acute renal failure. Stem cell therapies often require stem/progenitor cells to differentiate in a dynamic environment. Biochemical and physiological events need to be coordinated in order for the stem cells to properly engraft and function in vivo.

10.2.2.1 In Situ Tissue Regeneration

In situ tissue regeneration depends on mobilization of the host endogenous stem cells or tissue-specific progenitors to the site of injury. A role for BMSCs in promoting both endothelial and epithelial renal cell proliferation in response to injury has been reported [89]. Therefore, new therapeutic approaches have focused on the enhancement of these progenitor cells'

recruitment and delivery to the kidney in order to treat organ diseases and injury.

Stem cell factor and granulocyte colony-stimulating factor are two cytokines reported to induce HSC homing to an injured kidney, leading to an increase in the kidney's ability to recover from acute injury [90, 91]. Contradictory findings regarding the use of these cytokines suggest that this protocol of HSC mobilization is associated with granulocytosis, in which a high concentration of activated granulocytes worsens acute ischemic injury [92].

10.2.2.2 Exogenous Stem Cell Therapy

Studies have shown that transplantation of HSCs into injured kidneys may play a role in vasculogenesis instead of actual tubulogenesis [93]. The recruitment of BMSCs may not be enough to induce kidney repair, which is mainly stimulated by the proliferation of intrarenal and intrinsic epithelial cells [94–96]. Nevertheless, HSCs may contribute to the repair mechanism by secreting regenerative factors, rather than replacing damaged cells [96].

The ability of MSCs to treat renal dysfunctions has been demonstrated in many models of injury and is primarily attributed to the cells' capacity to secrete renotropic factors such as VEGF, HGF, and IGF-I [40–42]. Other paracrine factors, such as erythropoietin [43] or HO-1 [44], contribute to the expansion of some renal cell populations and lead to better recovery from injury when MSCs are implanted in the kidney. Although renal implantation of MSCs has been shown to prevent experimental kidney failure in some models, it has not always been successful in the regeneration of intraglomerular tissues. In fact, it has been reported that the technique leads to aberrant differentiation of MSCs into adipocytes in the kidney in addition to glomerulosclerosis [47].

ASC therapy has shown promising results for kidney protection against various injuries, such as ischemia-reperfusion injury, mainly by suppressing oxidative stress and inflammatory responses [97] and by improving revascularization in atherosclerotic renal artery stenosis

models [98, 99]. MSCs cultured in vitro with growth factors derived from a neuronal cell line [100] or human embryonic MSCs treated with VEGF before implantation [101] have contributed to better renal repair mechanisms. In other models, murine ESCs were reported to differentiate into renal epithelia in response to nephrogenic factors [102] or to differentiate in vitro into renal tubular cells [103]. Furthermore, murine ESC-derived embryoid bodies hold the potential to induce progenitors that can integrate into renal tubules in vivo [104], which opens a multitude of treatment options when progenitor cells are cultured in defined factors.

EP cells also show therapeutic potential as they have been shown to successfully home to the kidney and to participate in the restoration of renal function in the case of renovascular disease [105, 106]. Research using human-derived amniotic fluid stem cells is ongoing and shows encouraging results for the treatment of kidney injuries, such as delaying tubular necrosis and progression of renal fibrosis [107–109]. Similarly, human iPSCs have been successfully used to generate nephrogenic intermediate mesoderm under various conditions [110, 111]. The differentiation of human iPSCs into kidney structures [112] will lead to the development of novel therapies for many kidney dysfunctions and ultimately to the reconstruction of renal components or even artificial kidneys.

10.2.3 Tissue Engineering for the Treatment of Kidney Disease

The worldwide prevalence of chronic kidney disease is rapidly increasing, but therapeutic options for end-stage disease, namely, dialysis and transplantation, remain limited. Although renal transplantation is the optimal therapy, it is limited by organ donor shortage, rejection problems, and long-term immunosuppressive drug use. To address these issues, the concept of whole-kidney tissue engineering has emerged. The kidney is composed of cellular components, extracellular matrix (ECM), and blood vessels. Although many protocols using electrospinning and 3D printing for the fabrication of renal ECM have been proposed, it is extremely difficult to

regenerate an organ with such complexity and specificity as the kidney. A promising alternative approach is through the decellularization of the whole xenogenic or allogeneic kidney. With this technique, which has been used in rodent, porcine, and human kidneys [113–116], cell lysing solutions are perfused through the renal vessels to remove antigenic parenchymal tissues from the renal ECM.

The resulting ECM is supposed to regulate kidney regeneration and differentiation when seeded with stem cells. After verification of the decellularization process, the recellularization process that follows aims to endothelialize the renal vasculature and replenish the renal epithelial cells. Many entry points have been explored for stem cell delivery, including the renal artery, the renal vein, and the ureter, as well as under the renal capsule. Different cell types have been used, including neonatal kidney stem cells, adult renal stem cells, human umbilical vein stem cells, ESCs, and iPSCs [117]. Recent reports have shown the feasibility of differentiating iPSCs into specific renal progenitor cells (ureteric bud or metanephric mesenchyme) with the potential to eventually generate mature renal cells in large quantities [118].

10.3 Ureter Replacement Using Tissue Engineering Advances

Ureteral tissue engineering has been undermined mainly because of the false impression of a small number of cases requiring complex surgical ureteral reconstruction. However, with advances in endourology, the incidence of ureteral damage has been increasingly reported, and ureteral defects vary in size. While bridging short defects is usually surgically achievable, the reconstruction of long defects require extensive surgical repair that is not always possible and may cause complications, including metabolic disorders and tissue harvest problems. Hence, tissue engineering proposes novel therapeutic alternatives utilizing urothelial-lined grafts. Unseeded synthetic or naturally derived biomaterials have been used for ureteral replacement in a few animal studies and resulted in ureterohydronephrosis owing to the lack of normal

tissue formation [119]. Cell-seeded matrices seem to be the ideal solution for tubular ureteral regeneration. Although many scaffolds have been tried, none has been successful to date. The ideal scaffold should withstand mechanical loads when attempting to repair the unsupported ureter [120]. The seeded cells should include both urothelial cells (UCs), to act as a blood-urine barrier, and smooth muscle cells (SMCs), to induce ureteral contractions for urine transport. Autologous urinary tract cells are preferred [121], but if they are unavailable, stem cells constitute a viable alternative. ASCs and BMSCs have been explored in a few studies for ureteral regeneration [122–124]. Tubular grafts made of bladder matrix seeded with SMCs on one side and BMSCs on the other side were implanted to replace 4-cm ureteral defects in rabbits after being preimplanted for a 2-week conditioning period in the rabbits' omenta. This resulted in successful repair with no stricture or hydronephrosis [123]. ASCs were differentiated into UCs through indirect coculture protocol and seeded on tubular polylactic acid (PLA)/collagen scaffolds [122]. Strong evidence of differentiation into the urothelial lineage was detected with cytokeratin-18 and uroplakin-2. When implanted subcutaneously in athymic mice, the differentiated cells in the graft survived, stratified, and exhibited urothelial markers. In another study, ASCs were seeded on decellularized rabbit aorta and cell-seeded scaffolds were used to replace a ureteral defect. Sixteen weeks after animal implantation, the graft consisted of a well-organized muscle layer and stratified urothelium similar to the native tissue, and there was no evidence of strictures or hydronephrosis [120].

10.4 Bladder Regeneration: Function-Based Therapeutic Strategies

Due to its function as a dynamic reservoir of urine, the bladder has distinctive structural and regulatory mechanisms that need to be carefully considered when regenerative therapies are considered. The bladder epithelium, or urothelium, has cellular, intercellular, and architectural features that make it expansible, durable, and resilient to lifelong persistent

irritation by urine [125]. Additionally, the detrusor muscle that surrounds the mucosa has a peculiar pattern of fiber arrangement to enable instant and complete emptying of the bladder once it is contracted [126]. The neurological control of the bladder and its urethral sphincter is regulated by autonomic, sensory, and motor neural interactions [127]. Disturbance in one or more of these elements can result in dysfunction in the vital process of urination, which could significantly impair a patient's quality of life and, in severe cases, cause disability or even death.

Urine stem cells have been isolated and differentiated into specialized cells to offer a readily accessible cell source for various applications [128]. Although a general depot of adult stem cells exists in the fat and bone marrow, lineage-specific stem cells are believed to dominate in specific organs, such as the skin and the cornea [102, 129]. Urothelial adult stem cells are thought to be slow cycling in vivo (3–6 months), clonogenic, highly proliferative, and located in protected sites. These cells are commonly identified by their localization in the basal layer of the urothelium and by being label-retaining cells with high expression of β-4 integrin [130, 131]. The identification and isolation of these cells are important for the tissue engineering of urothelium-lined organs, including the bladder, the urethra, and the ureters. Bladder smooth muscle progenitor cells can be harvested from bladder biopsy and expanded in vitro to be seeded on scaffolds.

10.4.1 Bladder Replacement Methods

After surgical removal of the urinary bladder, or cystectomy, bladder reconstruction is a critical step in the patient's life. Significant morbidity and mortality often occur due to the incorporation of intestinal segments into the urinary tract [132, 133]. Therefore, exploring new therapies is essential.

Tissue engineering using cell-seeded scaffolds has been considered for urinary bladder reconstruction [134]. This method involves the seeding of a scaffold with autologous bladder SMCs and UCs. The use of autologous cells may not always be possible, as in cases of cancer [135] or benign end-

stage bladder diseases [136]. In this situation, stem cells can be isolated from other tissues, including the adipose tissue, bone marrow, and amniotic fluid. They can be seeded on scaffolds and transplanted in either differentiated or undifferentiated form. However, current data show that in vivo differentiation occurs only in a small percentage of the delivered cells [137]. Stem cells have shown a good potential for urothelial differentiation. This has been achieved by using conditioned medium [138, 139] with or without growth factors, such as all-trans-retinoic acid [140]. Both direct culture (seeding stem cells with UCs) and indirect coculture (using a Transwell migration assay (ThermoFisher Scientific, Life Technologies Inc., Burlington, ON, Canada)) have been attempted with variable outcomes, although cell-to-cell contact in direct culture appears to be an enhancing factor for stem cell differentiation [141, 142]. Stem cell differentiation into SMCs is more feasible and can be achieved by the use of growth factors [143] or coculture with SMCs [137].

iPSCs from adult tissues, such as skin fibroblasts, urinary tract stromal cells, and urine-derived cells, have also been used and were subsequently differentiated into UCs and SMCs [144–146]. However, urinary tract-derived iPSCs are believed to have superior differentiation properties than cells from other sources, which should emphasize the epigenetic differences between individual iPSC lines and stress on the importance of organ-specific iPSCs for tissue-specific studies [144–146].

10.4.2 Stem Cell Therapy for the Treatment of Voiding Dysfunction

Voiding dysfunction (VD), or disorders of urine storage or emptying, can affect patients' quality of life and interfere with social activities. Current therapies for VD are insufficient and often fail to correct the underlying pathophysiology of the disease. Stem cell therapies have also been studied in this field and were shown to cause positive clinical response, either due to differentiation or, more likely, to indirect paracrine effect associated with the release of growth factors and cytokines. The latter mechanism could lead to modulation of local and

systemic inflammatory responses and mobilization, stimulation, and differentiation of native stem cells in addition to the enhancement of vascularization of regenerating tissues and reduction of fibrosis [147]. A variety of stem cell types have been explored for the treatment of VD, including the bone marrow, skeletal muscle, and ASCs [148, 149]. ASCs are the most popular cells owing to their easy harvest, high yield of stem cells, and better smooth muscle differentiation potential compared to other types [150]. They have been shown to improve VD in animal models of bladder overactivity and hypoactivity associated with different etiologies, such as diabetes mellitus, radiation induced skeletal muscle-derived multipotent stem cells transplantation for bladder dysfunction reconstitution, or in the case of hyperlipidemia [147–150].

In one study, a 79-year-old patient with a hypoactive bladder managed with clean intermittent catheterization underwent autologous muscle-derived stem cell (AMDC) transplantation. The AMDCs were isolated from the thigh muscles, expanded in vitro, and injected in the bladder wall. During the 1-year follow-up period, the subject did not report gross hematuria, urgency, frequency, or infection. Functionally, there was a reduction in maximum cystometric capacity from 844 to 663 mL. The patient was able to void small amounts but continued to require self-catheterization 1 year after AMDC injection. The authors concluded that intradetrusor injection of AMDCs is a safe, minimally invasive, and a promising treatment option for bladder hypoactivity [151].

10.4.3 Stem/Progenitor Cell Treatment for Vesicoureteral Reflux

Primary vesicoureteral reflux (VUR) is a congenital anomaly of the ureterovesical junction (UVJ) that is due to poor valvular mechanism of the distal ureter and allows backflow of urine from the bladder to the kidney. Stem cells and muscle progenitor cell therapies are viable alternatives for the recovery of this muscle defect at the ureteral orifice. One

clinical study utilizing autologous chondrocytes was conducted in 29 children with VUR. Overall, VUR was corrected in 24 of the 29 patients (83%) [152]. Despite the high efficacy and safety of the procedure, this therapeutic option is associated with high costs that limited its more widespread use in clinical applications. In a porcine model, autologous fibroblasts were injected at the UVJ using the modified STING technique [153]. After the sacrifice of the animals, the labeled implanted autologous fibroblasts were detected at the UVJ, suggesting that in vitro-expanded fibroblasts could survive in vivo and represented a potential corrective therapy for VUR. Urine-derived stem cells appear to be a good option for VUR treatment due to their easy harvest, enhancement of vascularity, and readiness for muscle differentiation [154].

10.4.4 Urinary Incontinence Treatment with Stem Cells

Stress urinary incontinence (SUI) is a widespread disorder, particularly in women [155], due to inherent predisposing anatomical and physiological factors specific to the female urethra. In addition, SUI is commonly initiated or aggravated by female-specific physiological stages, such as pregnancy, vaginal birth, and menopause, or pathological conditions like uterine fibroids and tumors [156]. The current treatment of SUI relies on pelvic floor exercises in mild cases and surgical interventions in severe cases. The surgical methods aim to provide support to the urethra using various artificial tapes or autologous tissue slings. Each one of these methods has its adverse effects and postoperative complications [157, 158]. More recently, the injection of bulking agents around or through the urethra to treat SUI has gained some popularity. Many agents have been used, with varying success and complication rates. Collagen-, fat-, Teflon-, silicon-, and carbon-coated beads are common examples of the various agents that are used [159]. A recent systematic review in the Cochrane database showed that current data are insufficient to prove a benefit of these therapies. Indeed, saline injections were found to have an effectiveness similar to that of bulking

agents, and serious side effects have been reported with some of these agents [160]. Consequently, stem cell therapies have emerged as the next generation of therapies in SUI and have recently attracted attention. Animal studies have shown potential benefit in treating SUI. Sectioning of the pudendal nerves or vaginal distension is usually used to create animal models mimicking SUI. Muscle-derived stem cells have been the most widely used source of stem cells and are believed to provide stem cells that are able to differentiate into committed striated muscle cells more than other stem cell sources. However, the use of many other sources of stem cells has been attempted with comparable success. To evaluate the success of stem cell therapy in SUI animal models, multiple outcome measures have been used, including leak point pressure, intravesical pressure, maximum bladder volume, urethral functional length, maximum urethral closure pressure, and morphological examination of the sphincter muscle and matrix [161].

ASCs have been injected in rat models of SUI using intravenous or transurethral routes and shown significant improvement in terms of increased elastin content and voiding function measured by cystometry [162]. Adding nerve growth factor and PLA to ASCs for injection in the rat urethral sphincter has improved stem cell proliferation in vivo in a dose-dependent pattern. Such factors appear to improve stem cell survival and functional performance of the urethra compared to using ASCs alone [163]. Furthermore, human amniotic fluid stem cells have a favorable safety pattern and, owing to their low immunogenicity and tumorigenicity, seem to be of potential benefit for the restoration of normal urethral function in animal models of SUI [164]. A triple stem cell therapy approach has used human amniotic stem cells processed in the stage of early differentiation into three lineages in vitro (myogenic, neurogenic, and endothelial). This triple approach was able to improve SUI signs in the animal model compared to using only one or two types of differentiated cells [165]. A gene therapy strategy of inducing urine-derived stem cells to overexpress VEGF showed improvement of the sphincter composition, especially the nerve fibers, muscle cells, and vascularization [166].

The reconstruction of stem cell-based tissue-engineered slings to support the urethra has also been investigated. A silk scaffold covered with bone marrow-derived MSC sheets was implanted as a sling to support a rat's urethra and showed better matrix deposition compared to a silk sling alone [167]. Likewise, a combination of ASCs and silk fibroin microspheres was able to maintain improvement in SUI longer than silk fibroin microspheres alone [168].

10.5 Tissue Engineering of the Urethra: An Advancing Solution for Long Segment Urethral Defects

Multiple urethral pathologies necessitate extensive reconstruction, which can be limited by a lack of donor tissues. Regenerative medicine using scaffolds or cell-seeded grafts has been applied in preclinical studies and clinical trials [134]. Those techniques rely on the use of acellular matrices or synthetic scaffolds, alone or seeded with urinary tract progenitor/stem cells. Many preclinical and clinical trials have been successful using synthetic or acellular matrices coupled with progenitor cells from the bladder or buccal mucosa [169–172]. Stem cells can be used as a source of cells and scaffolds for the construction of tissue-engineered urethral grafts. Considering the efficacious production of biomaterials from human ASCs using the self-assembly technique, and because of their favorable mechanical characteristics for bladder replacement [173], an ASC-based scaffold is another appealing alternative for urethral replacement. Many stem cell types have been used for urethral tissue engineering, including ESCs [174], BMSCs [175], ASCs [121, 176], and urine-derived stem cells [176]. ASCs and urine-derived stem cells are the most convenient and useful cell sources owing to their simple harvest, high stem cell yield, and easy differentiation, especially into SMCs.

ASCs have been used to replace the urothelium [121] and smooth muscle [176]. In the former study, ASCs were differentiated into UCs and seeded on bladder acellular matrices for implantation in rabbits. The urethral continuity

and caliber were preserved, and the labeled differentiated UCs survived and formed a multilayer structure. In the second study, ASCs were chemically induced with 5-azacytidine to differentiate into SMCs under the effect of mechanical extension stimulation. The autologous induced cells, together with oral mucosal epithelial cells, were seeded on a polyglycolic acid mesh to replace urethral defects in dogs. Those urethras architecturally resembled nearby native urethras. Therefore, with their multiple advantages, ASCs can significantly contribute to tissue engineering for urethral replacement.

10.6 The Penis: Highly Developed Strategies for Functional Restoration Using Stem Cells

10.6.1 Tunica Albuginea

The tunica albuginea (TA) envelops the penile erectile bodies and is composed of organized fibrillar collagen interlaced with elastin fibers, which allow tunica expansion. It protects erectile tissue, promotes penile rigidity and length, and participates in the veno-occlusive mechanism. Penile reconstruction might be required for various conditions, including trauma, neoplasm, congenital anomalies, and Peyronie's disease (PD). Multiple surgical techniques have been developed to simplify the functional and esthetic restoration of the penis [177]. PD has two phases. The acute phase is characterized by pain and induration, while the chronic phase involves formation of a fibrous plaque causing penile curvature. PD is a progressive disorder, with up to 48% of men having disease progression if left untreated [178]. Evidence suggests that there is no benefit from medical therapy and that surgery is the mainstay of treatment once penile curvature occurs. However, surgeries for PD have generally been challenging as a result of the limited availability of suitable local tissues [179, 180].

Stem cells have been used in the treatment of PD. Recently, Castiglione et al. injected ASCs intratunically in a

rat model of PD during the acute phase of the disease [181]. They prevented fibrosis and elastosis and maintained erectile function. This treatment was not successful in terms of curing well-formed plaques and their resulting deformities. As a result of the unfavorable outcomes associated with off-the-shelf grafts, there has been increased interest in cell-seeded grafts as ideal TA grafts. Stem cells have been used to improve the characteristics of these off-the-shelf grafts and reduce the accompanying inflammation. In one study, allogeneic ASCs were seeded on the small intestinal submucosa and implanted in rats [182]. These cell-seeded grafts resulted in cavernosal tissue preservation and maintenance of erectile response. Cell-seeded grafts led to better outcomes with regard to the architecture of the tunica and to erectile function than scaffolds alone.

10.6.2 Erectile Dysfunction and Stem Cell Replacement

Erectile dysfunction (ED) has been reported to have a prevalence of no less than 52% [183] and to cause major morbidity and distress for men and their partners [183]. The main causes of ED include aging, diabetes mellitus, and cavernous nerve injury during radical prostatectomy [184]. The imperfect efficacy and risk of complications associated with existing therapies for ED have urged the scientific community to look for new treatment modalities, including stem cell replacement. All available treatment options for ED tend to alleviate symptoms rather than correct the underlying pathology. Stem cell therapy aims to replenish damaged endothelial cells and SMCs and to prevent further apoptosis and fibrosis. Among the different types of stem cells tested for ED treatment, ASCs have been the most frequently investigated, mostly because of their easy harvest, abundance, and established efficiency in other medical venues and because of the availability of separation devices. Both stromal vascular fraction of the adipose tissue and ASCs have been successfully used in ED research [185]. An in vitro model of the cavernous tissue showed that ASCs contributed to the repair of endothelial damage and decreased apoptosis resulting from

diabetes mellitus. ASCs also demonstrated the ability to undergo differentiation into endothelial cells and SMCs [186]. When used in the treatment of ED due to type 1 or 2 diabetes in rats, ASCs showed an increase in intracavernous pressure and improvement of ED, together with improvement of blood glucose levels [187, 188]. Autologous ASCs were able to treat both acute and chronic (4 weeks) cavernosal nerve injury-induced ED [185]. When used in combination with PDE-5 inhibitors or growth factors, ASCs had a synergic effect on therapeutic efficacy [189, 190]. In a model of resected cavernous nerves, ASCs were seeded on an autologous vein graft or on the adipose tissue biomatrix and had beneficial effects on penile histology and functional outcomes [191, 192].

Intracavernous injection of ASCs is the preferred stem cell delivery method for ED, especially in the case of cavernous nerve injury. It has been associated with the rapid disappearance of injected stem cells from the penis, thereby minimizing therapeutic efficiency in chronic disease models, such as those of diabetes [193]. Other routes of delivery include periprostatic injections [194], subtunical implantation [195], and nerve or tunical grafts, coupled with biomaterials [181, 189]. Although intravenous administration of ASCs has shown efficacy in ED after radiation [196], it can be associated with severe adverse effects. The main mechanism of ASC-mediated repair in treating ED is largely dependent on paracrine actions, with scant evidence of cell engraftment [193].

10.7 Testicular Stem Cell Therapy for the Treatment of Infertility and Hormonal Deficiency

10.7.1 Infertility

Different modalities of antineoplastic treatment, including radiotherapy, new targeted therapies, and cytotoxic drugs, may result in permanent damage to both testicular germ cells and somatic cells (Sertoli and Leydig cells). Attempting to

preserve fertility in those patients, especially children, is of fundamental importance. In a 2014 study where the testicular tissue of cryopreserved newborn mice was cultured on agarose gel after thawing, it displayed spermatogenesis up to sperm formation. Microinsemination was achieved with round spermatids and sperm, leading to eight offspring that grew well and mated successfully. In vitro spermatogenesis of cryopreserved tissues appears to be a potential approach to fertility preservation in male cancer patients in the future [197].

10.7.2 Hormone Replacement Therapy

Long-term exogenous testosterone therapy is associated with serious side effects, including excessive erythropoiesis, bone density changes, and even infertility [198]. To overcome these problems, the transfer of Leydig cells from normal men's testes to hypogonal men has been proposed in order to provide adequate endogenous testosterone. In a rat model, isolated Leydig cells from the testes of normal rats were injected into castrated rats in an encapsulated form to isolate them from the hosts' immune system. The castrated rats kept serum testosterone levels up to 40% of normal levels for a maximum period of 43 days without any human chorionic gonadotropin stimulation [199].

10.8 Conclusions and Future Considerations

Many challenges face the extensive use of stem cells for patient treatment in clinical practice. Strenuous methods to characterize transplanted cells and the lower therapeutic efficacy of stem cells in chronic diseases in comparison to their efficacy in acute injuries are among those difficulties. Specific technical problems relating to the transplantation of human cells in animals and new delivery procedures present further challenges in the design and execution of preclinical animal studies for stem cell therapies. Moreover, stem cell integration within tissues must be improved. The best condition in which stem cells can be delivered still needs to be

defined, especially the use of differentiated versus undifferentiated stem cells. It would be exciting to explore whether predifferentiation of stem cells before transplantation would improve their effects without affecting their secretome power. Lastly, there is no final agreement concerning the optimal number of cells per treatment, the number of cell injections, or even the best mode of delivery. Hence, following animal models for long periods, consistent protocols, and further clinical trials are required to confirm stem cell therapeutic efficacy and safety before widespread use.

In the search for novel therapeutic alternatives in the treatment of urogenital diseases, different types of stem cells have been the focus of numerous in vitro and in vivo studies. Stem cells can be used alone for cellular therapy or coupled with scaffolds for tissue engineering of genitourinary organs. Although limited clinical trials have been performed to date, those available show encouraging results. More in vitro experiments, in vivo implantations, and well-designed clinical trials are required to ensure their efficacy before common use.

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11. Stem Cell Therapy for Autoimmune Disease

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11.1 Introduction

The immune system is the body's form of defense and helps to protect it against infectious organisms such as bacteria and viruses. The immune system typically attacks and kills foreign infectious agents and does not recognize and kill the body's normal cells. However, the immune cells sometimes recognize and attack normal self-cells; this is called autoimmune disease (AD). AD reflects a dysfunctional condition of the immune system, in that it cannot identify self-antigens as self.

When ADs occur, some cells of the body are killed, causing functional disability and morbidity in tissues or organs. ADs are the third most common category of disease in the USA, after cancer and heart disease, affecting approximately 5–8% of the population [1], with a higher

prevalence in women [2, 3]. Some tissues and organs commonly affected by ADs include the endocrine system, connective tissue, gastrointestinal tract, heart, skin, and kidneys. To date, more than 80 ADs have been identified, which include 15 common diseases that are divided into two groups. The first group is organ-specific ADs, in which immune cells attack specific cells in one organ. Examples of these include type 1 diabetes, Addison's disease, Hashimoto's thyroiditis, Graves' disease, Sjögren's syndrome, vitiligo, pernicious anemia, glomerulonephritis, myasthenia gravis, Goodpasture's syndrome, autoimmune hemolytic anemia, idiopathic thrombocytopenic purpura, and pulmonary fibrosis. The second group is systemic ADs, in which immune cells attack numerous different cells in some organs, such as systemic lupus erythematosus (SLE), rheumatoid arthritis, systemic sclerosis (SS), ankylosing spondylitis, and polymyositis.

ADs are currently treated by conventional approaches based on immunosuppressive drugs, such as corticosteroids, cyclophosphamide, azathioprine, and methotrexate. However, these drugs are not effective in some patients and have high rates of side effects and toxicity [4, 5]. For a long time, some mechanisms and features of ADs were clearer, and some novel therapies were developed that brought tremendous benefits and few side effects. This chapter summarizes and analyzes certain mechanisms of stem cell transplantation to treat ADs and discusses the roles and potential of stem cell therapy for treating ADs.

11.2 Hematopoietic Stem Cell Transplantation

11.2.1 Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) have a long history from discovery to application. The first suggestion of the existence of HSCs was presented in 1956, when Ford et al. determined that transplantation of the spleen and bone marrow could save patients subjected to previously lethal doses of radiation in

World War II [6]. However, the existence of HSCs was subsequently confirmed in these tissues by Becker et al. [7] in mouse and rat models [7]. HSCs in humans were characterized and cultured in the 1980s [8–10].

HSCs have now been identified as adult stem cells that can self-renew for a long time and differentiate into all types of blood cell. They can be isolated from various tissues, including adult bone marrow, some fetal tissues (liver, spleen, thymus), the umbilical cord, and peripheral blood. HSCs from these sources have been shown to express common markers, such as CD34, CD38, CD90, CD133, CD105, CD45, and c-kit, and are negative for lineage markers (lin^-), such as lymphocytes and monocytes. However, human and mouse HSCs exhibit numerous differences in this regard. Markers of mouse HSCs include $CD34^{low/-}$, $SCA\text{-}1^+$, $Thy1.1^{+/low}$, $CD38^+$, $c\text{-}kit^+$, and lin^-, but those of human HSCs include $CD34^+$, $CD59^+$, $Thy1/CD90^+$, $CD38^{lo/-}$, $c\text{-}kit/CD117^+$, and lin^-. Nonetheless, HSCs form heterogeneous populations that contain some cells that do not express these markers. For example, in humans, HSCs have been reported to have the marker profile of $CD34^-CD38^-$ [11, 12] or to lack the expression of c-kit [13].

Clinically, most laboratories have used the second two-platform methods: the ISHAGE protocol, published by the International Society of Hematotherapy and Graft Engineering, to determine and count HSCs using flow cytometry. Using this method, an HSC is recognized from the profile $CD34^+CD45^{dim}$ [14, 15]. HSCs were first applied clinically in 1968, when they were used to treat sex-linked lymphopenic immunological deficiency in a 5-month-old boy in the form of the bone marrow cells of his sister, aged 8 years [16]. The second case involved the treatment of a 2-year-old child with Wiskott–Aldrich syndrome, who received an allograft from a sister, herself having X-trisomy (47,XXX) [17]. HSC transplantation is currently used to treat several diseases, not only of hematologic origin, but also immunological disorders, solid tumors, and inborn errors of metabolism.

11.2.2 Hematopoietic Stem Cells for ADs

Preclinically, autologous, syngeneic, and allogeneic HSC transplantations have been performed in animal models of autoimmune diseases, including experimental autoimmune encephalomyelitis [18, 19], experimental autoimmune myasthenia gravis [20], adjuvant-induced arthritis [21], collagen-induced arthritis [22], type 1 diabetes (NOD mice) [23], and SLE-like ADs (MLR/lpr mice and NZB/W F1 mice) [24, 25]. The results from these studies showed that HSC transplantation can cause diseases to go into remission or can induce immune tolerance.

HSCs were used for the first time for autoimmune diseases in 1997, specifically for severe and therapy-refractory autoimmune diseases [26]. The European Bone Marrow Transplantation (EBMT) database PROMISE is the largest database of transplanted patients with autoimmune diseases, currently including data on more than 1000 such patients from 172 institutions in 27 countries. Pilot studies were conducted on SS [26, 27], rheumatoid arthritis [28], SLE [18, 29, 30], multiple sclerosis (MS) [29], and hematological ADs, such as idiopathic thrombocytopenia [31], autoimmune hemolytic anemia, and Evans syndrome, which showed that HSC transplantation had improved in patients with such conditions.

For example, the study of Gratwohl et al. [32] showed that autologous HSC transplantation helped the 5-year progression-free survival rate to reach more than 50% [32]. Some clinical benefits were also recorded as structural changes, such as fibrosis and microvessel rarefaction, both typical features of SS after HSC transplantation [33, 34]. Although HSC transplantation has been used to treat various ADs, MS is its main indication. To date, more than 12 trials have been conducted with more than 400 patients treated in this way [35]. A review of these trials showed that the rate of disease stabilization reached 70% and patients showed improvement for at least 3 years after transplantation. The second most common disease treated by HSC transplantation is SLE. In these patients, HSC transplantation was also associated with good results; namely, the 5-year disease-free rate was

approximately 50%. Transplant-related mortality was also reported to vary from 4 to 12% depending on the institution [36, 37]. In other ADs, HSC transplantation also achieved high rates of remission, such as 11 of 12 patients with severe, therapy-refractory Crohn's disease achieving remission [38] and 50% of patients with the same condition remaining insulin-independent at a median of 30 months after HSCT, among those with type I diabetes mellitus [39].

11.2.3 Mechanism of HSC Transplantation for ADs

Although reports have shown that HSC transplantation can cure ADs, the exact mechanism behind this therapy remains unclear. To date, in almost all studies, it has been considered that HSC transplantation removes the autoreactive effector and inflammatory cells that cause ADs. It was hoped that the newly transplanted HSCs would reset the immune system (Fig. 11.1). However, the HSC transplantation procedure actually destroyed almost all immune cells of the "diseased" immune system. After HSC transplantation, it was hoped that the de novo generation of naïve T lymphocytes would occur and a normal immune system could replace the old one. However, Tehlirian et al. [40] showed that the reinfusion of HSCs could cause aplasia. They demonstrated that high-dose cyclophosphamide therapy without the reinfusion of stem cells could still be an effective treatment for SS [40]. In fact, lymphotoxic chemotherapy such as cyclophosphamide reduced the effects of autoantibodies as well as the toxicity of autoreactive T cells.

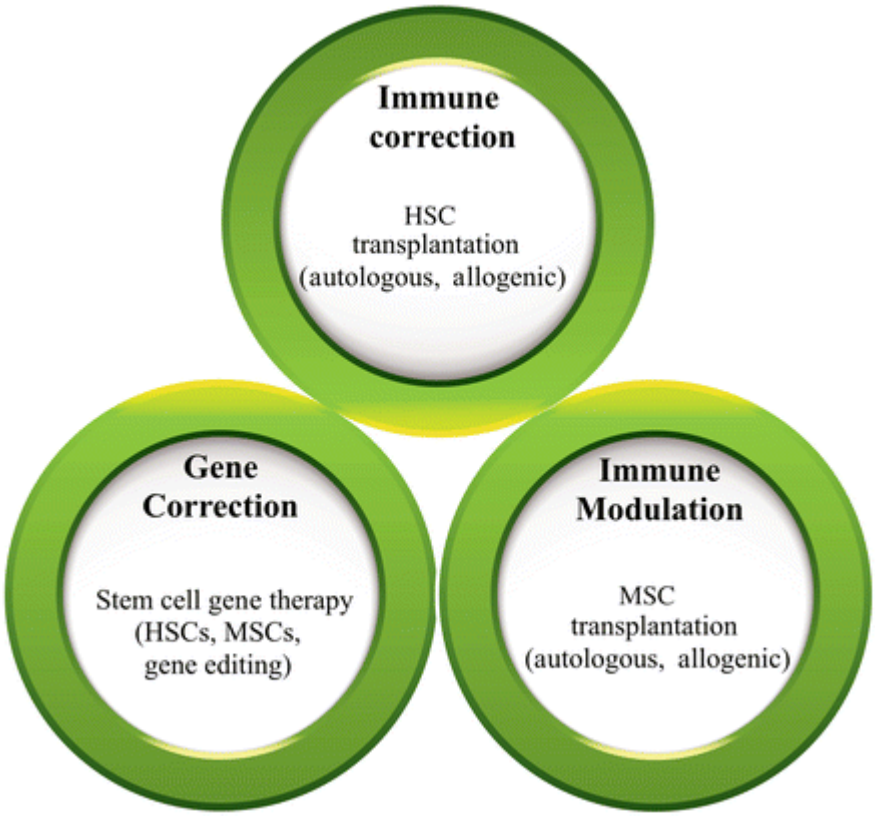

Fig. 11.1 Three approaches to treating ADs based on stem cells: immune correction, immune modulation, and gene correction. The immune system can be corrected by HSC transplantation, while immune modulation is usually performed by MSCs. The latest approach, related to gene correction, is also recommended in some recent studies

However, some of the following lines of evidence suggest that HSC transplantation also plays roles in reestablishing immunological tolerance: (1) autologous HSC transplantation leads to an increased number of regulatory, FoxP3-positive T cells [41]; (2) the reactivation of thymic function after autologous HSC transplantation may lead to a tolerant, "juvenile" immune system [42, 43]; and (3) antithymocyte globulin directly targets long-living, autoantibody-producing plasma cells by complement-mediated lysis and apoptosis [44].

According to this principle, ADs could be effectively cured by HSC transplantation; however, relapses also sometimes occurred after treatment. This is related to (1) the persistence of autoreactive cells such as long-lived plasma cells [45], (2) de novo emergence of AD in a highly predisposed host, and (3) polymorphisms of genes involved in the innate immune system [46].

11.3 Mesenchymal Stem Cell Transplantation

11.3.1 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are adult stem cells that can be isolated from various sources. They were first discovered in the 1960s and called MSCs by Friedenstein et al. [47]. MSCs are multipotent stem cells that can differentiate into various types of mesoderm cell, such as osteoblasts, adipocytes, and chondroblasts. In recent studies, MSCs were demonstrated to be able to differentiate into other germ layer cells, such as neurons, beta cells, and hepatocytes.

In contrast to HSCs, MSCs can be isolated from various sources, such as adipose tissue [48, 49], peripheral blood [50–52], umbilical cord blood [53–55], banked umbilical cord blood [56, 57], umbilical cord [58, 59], umbilical cord membrane [60], umbilical cord vein [61], Wharton's jelly [62], placenta [63], decidua basalis [64], and ligamentum flavum [65]. They could also be isolated from amniotic fluid [66, 67], amniotic membrane [68, 69], dental pulp [70, 71], chorionic villi of human placenta [72], fetal membranes [73], menstrual blood [74, 75], breast milk [76, 77], and urine [78, 79]. Although MSCs from these different sources are not identical, they exhibit some common characteristics, which are considered to be minimal criteria for defining MSCs [80], as follows:

1. MSCs must adhere to plastic under standard tissue culture conditions.

2. MSCs must express some specific markers, such as CD73, CD90, and CD150, and lack the expression of CD14, CD34, CD45 or CD11b, CD79 alpha or CD19, and human leukocyte antigen - antigen D related (HLA-DR).

3. MSCs must successfully differentiate into osteoblasts,

adipocytes, and chondroblasts in vitro.

Given their high differentiation potential, MSCs have been preclinically and clinically applied for the treatment of degenerative diseases, such as for repairing injured cartilage and promoting the healing of bone. Moreover, MSCs have a special feature related to immune modulation or immunosuppression, which led to their transplantation being considered as a novel therapy for ADs.

11.3.2 MSC Transplantation for ADs

MSCs have been successfully applied for both preclinical and clinical treatments of various ADs, such as SLE [81–83], CD [84, 85], multiple system atrophy (MSA) [86, 87], MS [88, 89], and amyotrophic lateral sclerosis (ALS) [90–92].

11.3.2.1 Crohn's Disease

CD, also known as Crohn's syndrome or regional enteritis, is a type of inflammatory bowel disease that can affect the gastrointestinal tract [93]. CD usually has some characteristic signs and symptoms, such as abdominal pain, diarrhea, fever, and weight loss [93]. Other complications outside the gastrointestinal tract have also been detected, including anemia, skin rash, arthritis, eye inflammation, and fatigue.

The first report on the application of MSCs in the treatment of CD was published in 2005. The results showed that the local injection of MSCs from adipose tissue led to the healing of fistulas without side effects [94, 95]. These results were confirmed by a recent study by the same group in 2015 [96]. To date, more than ten clinical trials using MSCs on more than 500 CD patients have been conducted. Almost all trials used adipose stem cells, but a few used bone marrow-derived MSCs (BM MSCs) (Table 11.1). Both autologous and allogeneic MSCs could improve the disease. Regarding the route of administration, it is noteworthy that only one study used the intravenous injection of allogeneic MSCs from bone marrow [98]; in almost all trials, the cells were delivered intralesionally.

Table 11.1 Clinical trials using stem cells for the treatment of Crohn's perianal fistula

Study design	Source of cells	Results	Year	Reference
Phase I clinical study (n = 4)	ASC (autologous)	Complete closure: 50% of patients 75% fistulas	2005	[94]
Open-label, multicenter, phase II study (n = 14)	ASC + fibrin glue (autologous)	Fistula healing: 71% vs. 14%	2009	[95]
Prospective study (n = 10)	MSC (autologous)	Reduction in CDAI, PDAI, and pain/discharge PDAI scores	2011	[97]
Open-label phase II study (n = 10)	MSC (allogeneic) IV	Reduction in CDAI and fistula drainage	2011	[98]
Retrospective follow-up of Garcia-Olmo phase II study (n = 5)	ASC + fibrin glue (autologous)	58% sustained fistula closure at end of follow-up by mean 3 years	2012	[99]
Open-label, multicenter, dose escalation phase I study (n = 10)	ASC (autologous)	Healing in 50% receiving $\geq 2 \times 10^7$ cells/mL	2013	[100]
Open-label, multicenter, phase II study (n = 42)	ASC (autologous)	Fistula closure in 82% PP, 67% ITT analysis	2013	[101]
Open-label pilot study (n = 24)	ASC (allogeneic)	Complete closure: 56.3%	2013	[102]
5-year follow-up of 2011 study (n = 10)	MSC (autologous)	37% fistula relapse-free 4 years later	2015	[84]
1-year follow-up of 2013 study	ASC (autologous)	Complete closure maintained in 75% at 2 years ITT analysis	2015	[103]
Retrospective, open label (n = 3 with CD)	ASC (allogeneic and autologous)	Healing in 2/3 CD fistula patients	2015	[96]
Double-blind, placebo-controlled study, phase II	MSC (allogeneic)	Healing up to 85%	2015	[104]

Source: Clinicaltrials.gov. *ASC* adipose-derived stem cell, *CD* Crohn's disease, *CDAI* Crohn's disease activity index, *ITT* intention to treat, *IV* intravenous, *MSC* mesenchymal stem cell/mesenchymal stromal cell, *PDAI* pouchitis disease activity index, *PP* per protocol, *SC* stem cell

Regarding the dose to be administered, the suitable dose of MSCs has yet to be fully determined. Some trials used varying doses ranging from 3.5×10^6 to 30×10^6 cells [94]; in another study, 15.8×10^7 cells were used [101]. Interestingly, in a recent study, Molendijk et al. showed that a dose of 9×10^7 MSCs had the best response rate, which was greater than those of 10^7 and 3×10^7 cells [104].

11.3.2.2 Multiple Sclerosis

MS is the most common autoimmune inflammatory demyelinating disease of the central nervous system (CNS), which eventually leads to demyelination and axonal loss. Its main cause is an immune response to myelin proteins. There are three forms of disease evolution. About 80% of patients have the relapsing-remitting form, and two-thirds develop a secondary progressive form 10–15 years after disease onset; the remaining 20% of patients develop a progressive form right at onset, namely, primary progressive multiple sclerosis.

The first report on the use of autologous MSCs delivered intrathecally for treating MS showed that their transplantation was not associated with a significant clinical improvement and caused adverse events in ten patients [105]. In more recent studies, Yamout et al. [106] and Karussis et al. [107] showed that MSC transplantation helped to increase the proportion of CD4+CD25+ regulatory T cells, decreased lymphocyte proliferation, and decreased activated markers of dendritic cells (DCs) [106, 107].

Certain clinical benefits were identified in a recent study in which autologous MSCs were infused into patients with MS. Specifically, their visual function [108] improved, and they exhibited transient disease stabilization [109]. In almost all clinical trials to date, it was autologous MSCs that were

transplanted. In addition, MSCs were isolated from bone marrow and were administered to patients in one of two ways: (1) intravenous injection and (2) the introduction of cells directly into the cerebrospinal fluid via an intrathecal injection. Although no clinical trials have yet compared the efficacy and safety of MSC transplantation between these two approaches, some independent trials showed that intrathecal injection may be less safe than its intravenous counterpart. In fact, certain side effects were detected in MS patients who had undergone the transplantation of BM MSCs, particularly in one patient who received a very high dose of cells [106]. Regarding the efficacy of these approaches, results have shown that introducing cells directly into the cerebrospinal fluid via an intrathecal injection may be more effective for improving symptoms than an intravenous injection.

Two mechanisms have been hypothesized to explain the roles of MSCs in MS. The first is related to the immunomodulation performed by MSCs. Intravenously injected MSCs would promote the release of cytokines, trophic factors, and microvesicles. These factors can suppress chronic autoimmunity and CNS injury [110, 111]. The second mechanism is related to the differentiation of MSCs into neurons. Studies have shown that MSCs can penetrate the blood–brain barrier and enter the CNS [112, 113]. The delivery of cells directly into the cerebrospinal fluid results in being as close as possible to lesions without the risk of spinal cord damage, facilitating their differentiation into neurons.

Recently, MSCs were differentiated in vitro into neural progenitors (MSC-NPs). In animal models, the transplantation of MSC-NPs was shown to improve neurological functions [110]. Following this preclinical trial, a clinical trial was approved in the USA for the transplantation of MSC-NPs to treat MS patients. From the initial results, no adverse effects were noted in the first ten patients treated [114].

11.3.2.3 Systemic Lupus Erythematosus

SLE is a systemic autoimmune disease which can affect the skin, joints, kidneys, brain, and other organs. Other common

symptoms include chest pain, fatigue, fever, general discomfort, malaise, hair loss, mouth sores, sensitivity to sunlight, and skin rash.

The transplantation of MSCs for SLE patients produced remarkable results. Almost all clinical trials showed that MSC transplantation was effective for conditions across the SLE spectrum. Sun et al. [115] successfully treated some SLE patients who were unresponsive to monthly intravenous cyclophosphamide and oral prednisone (≥20 mg/day) [115]. All patients significantly improved at 1, 6, and 12 months' follow-up, as shown by clinical symptoms, urinary protein, and immune cell functions. In particular, the level of T regulatory cells increased at 3 months' follow-up, and no complications were detected after 12–18 months' follow-up [115].

In a larger trial, Liang et al. [116] showed that the transplantation of allogeneic BM MSCs from non-HLA-matched healthy family members effectively improved clinical and serological features. Surprisingly, proteinuria decreased significantly 24 h after transplantation. Anti-double-stranded DNA antibodies also decreased significantly at 1 month and 3 months post-transplantation [116].

In a recent phase II study, Wang et al. [117] used allogeneic umbilical cord-derived MSCs (UC-MSCs) to treat SLE. With 4 years of follow-up (mean 27 months), patients exhibited better clinical results, with an overall survival rate of 94%; in addition, approximately 50% of patients achieved and remained in clinical remission at 4 years, although disease relapse occurred in 23% of the patients [117]. Similar results were also recorded in clinical trials using allogeneic BM MSCs. No difference in the clinical efficacy was found between allogeneic BM MSCs or UC-MSCs. MSC infusion was also shown to promote remission in multiorgan dysfunctions including lupus nephritis [81], diffuse alveolar hemorrhage [118], and refractory cytopenia [119].

In the last year, the first multicenter clinical trial involving the transplantation of allogeneic UC-MSCs to treat SLE was reported. The results showed that 32.5% of patients achieved a

major clinical response and another 27.5% of patients achieved a partial clinical response during a 12-month follow-up [82]. According to this study, several patients experienced disease relapse after 6 months, indicating the need to repeat MSC transplantation after 6 months.

11.3.2.4 Systemic Sclerosis

SS is a systemic connective tissue disease. The characteristics of SS include essential vasomotor disturbances, fibrosis, and subsequent atrophy of the skin, subcutaneous tissue, muscles, and internal organs.

In contrast to other ADs, few clinical studies have used MSC transplantation to treat SS. In a recent study by Akiyama et al. [120], allogeneic MSCs were transplanted into five patients with SS. The results showed that this transplantation triggered the induction of T-cell apoptosis, lymphopenia, and Treg induction, leading to skin ulcer healing in one case and significant improvements in the skin score, Health Assessment Questionnaire results, and autoantibody titer in the entire group [120].

11.3.3 How Can MSCs Cure ADs?

11.3.3.1 MSCs Regulate Immune System Activities

In contrast to other stem cells, MSCs have a remarkable capacity to regulate immune responses, both in vitro and in vivo. MSCs were demonstrated to affect the immune system cells, namely, T lymphocytes [121–123], B lymphocytes [124–126], natural killer (NK) cells [127, 128], and DCs [129, 130]. MSCs were found to suppress T-cell proliferation induced by cellular or nonspecific mitogenic stimuli [122], alter the cytokine secretion profile of naïve and effector T cells [121], and promote the expansion and function of Treg cells [123]. In the case of B lymphocytes, MSCs can also inhibit their proliferation [125], affect their chemotactic properties [126], and suppress their terminal differentiation [124]. For NK cells,

MSCs were approved to alter their phenotype and suppress proliferation, cytokine secretion, and cytotoxicity against targets expressing HLA class I [127, 128]. Finally, in DCs, MSCs can influence the differentiation, maturation, and function of monocyte-derived DCs [130]; suppress DC migration, maturation, and antigen presentation [129]; and induce mature DCs into a novel Jagged-2-dependent regulatory DC population [131] (Fig. 11.2).

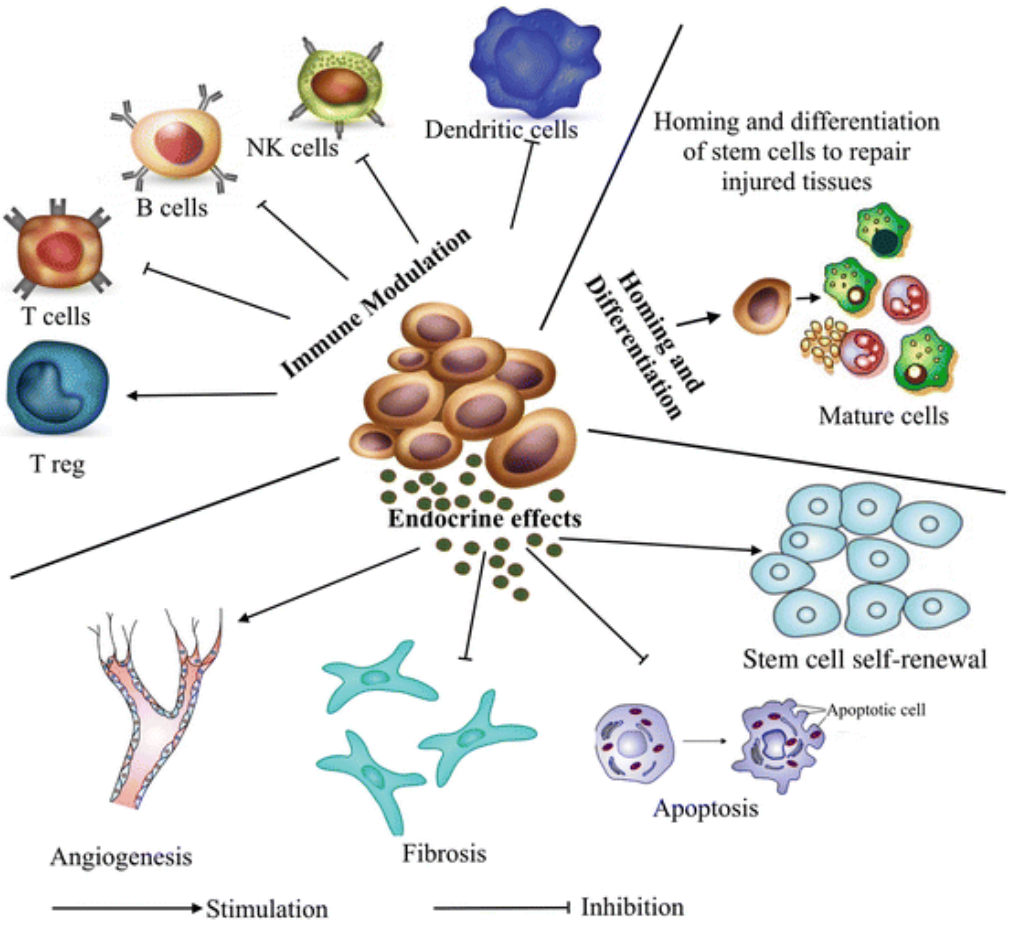

Fig. 11.2 Some mechanisms of MSCs for autoimmune diseases. To date, at least three ways for MSCs to affect ADs have been identified. (1) MSCs can modulate the host's immune system; (2) MSCs can home and differentiate into specific cells that replace the injured cells in some tissues; (3) MSCs release cytokines and growth factors that can inhibit fibrosis as well as apoptosis, trigger the self-renewal process of stem cells, and stimulate angiogenesis

These effects of MSCs are related to certain bioactive molecules (Table 11.2). Almost all of these molecules are anti-inflammatory agents, such as IL-10, prostaglandin E2, and interleukin-1 receptor antagonist. Important molecules related to antiproliferative activity include transforming growth factor

β-1 (TGFβ1), hepatocyte growth factor (HGF), and human leukocyte antigen G isoform (HLA-G5).

Table 11.2 Important bioactive molecules secreted by MSCs and their functions

Bioactive molecules	Functions
Prostaglandin-E2 (PGE2)	Antiproliferative mediators [132]
	Anti-inflammatory [133]
Interleukin-10 (IL-10)	Anti-inflammatory [134]
Transforming growth factor β-1 (TGFβ1), hepatocyte growth factor (HGF)	Suppress T-lymphocyte proliferation [122]
Interleukin-1 receptor antagonist	Anti-inflammatory [135]
Human leukocyte antigen G isoform (HLA-G5)	Antiproliferative for naïve T cells [136]
LL-3	Antimicrobial peptide and reduce inflammation [137]
Angiopoietin-1	Restore epithelial protein permeability [138]
MMP3, MMP9	Mediating neovascularization [139]
Keratinocyte growth factor	ALVEOLAR epithelial fluid transport [140]
Endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), placental growth factor (PlGF), and monocyte chemoattractant protein-1 (MCP-1)	Enhance proliferation of endothelial cells and smooth muscle cells [141, 142]

In some AD animal models, MSC transplantation regulated the immune system functions (Table 11.3).

Table 11.3 Immunomodulatory effect of mesenchymal stromal cells in animal disease models

Disease model	Anti-inflammatory MSC effects	References
Type 1 diabetes	↑ Regulatory T cells	[143–146]
	$T_H1 \rightarrow T_H2$	[144, 145]

Disease model	Anti-inflammatory MSC effects	References
	↓ Inflammatory T cells	[143, 145]
	↑ Tissue repair	[144, 147, 148]
Pancreatic islet transplantation	↑ Islet survival	[149–151]
	↑ Regulatory T cells	[149, 151]
	↓ T_H1 cytokines	[151]
	↓ T-cell responsiveness	[150]
Experimental autoimmune arthritis	↓ Inflammatory cytokines	[152–155]
	↑ Regulatory T cells	[132, 152, 154, 156]
	↓ T-cell responsiveness	[125]
	↑ IL-10	[154–156]
	↓ T_H1/T_H17 cells	[154, 156]
	↑ T_H2 cells	[132, 154]
Graft vs. host disease	↓ Autoantibodies	[157]
	↓ Inflammatory cytokines	[158–161]
	↑ Regulatory T cells	[162]
	↓ T-cell proliferation	[161]
	↓ T_H1 cells	[163]
Experimental autoimmune encephalomyelitis	↓ T-cell responsiveness	[164]
	↓ Autoantibodies	[165]
	↓ CNS infiltration	[165–167]
	↓ Inflammatory cytokines	[166–168]
	$T_H1 \rightarrow T_H2$	[169]
	↓ T_H17 cells	[168]

Disease model	Anti-inflammatory MSC effects	References
Inflammatory bowel disease	↓ Inflammatory T cells	[120, 170, 171]
	↓ Inflammatory cytokines	[170–173]
	↓ T-cell responsiveness	[170]
	↓ Growth factor expression	[172]
	↑ FasL-mediated T-cell apoptosis	[120]
	↑ Regulatory T cells	[170, 171, 173, 174]
	↓ Intestinal $CD4^+$ T-cell infiltration	[170, 171, 173]
	↑ Anti-inflammatory cytokines	[120, 170, 171, 173, 174]
Systemic lupus erythematosus	↓ Anti-dsDNA antibodies	[115, 175–177]
	↓ T-cell frequency	[177]
	↑ Regulatory T cells	[115]
	↑ Anti-inflammatory cytokines	[175]
	↓ Inflammatory cytokines	[175]
	↓ Plasma cells	[115]
	↓ T_H17 cells	[115]
Experimental autoimmune uveitis	↓ Inflammatory cytokines	[178, 179]
	↑ IL-10-producing B cells	[178]
	↓ T_H17 cells	[178, 180]
	↓ T_H1 cells	[178]
	↓ Inflammatory T cells	
	↑ Regulatory T cells	[181]

Disease model	Anti-inflammatory MSC effects	References
	↑ IL-10	[178]

11.3.3.2 MSCs Contribute to the Repair of Injured Tissue

The immune responses involved in ADs can result in tissue injury, resulting in reduction of physiological functions, and death of local cells. MSCs could differentiate into tissue-specific cells to replace injured cells.

MSCs could also produce factors that trigger tissue healing. For example, they were demonstrated preclinically and clinically to be a source of cytokines and growth factors, which affect injured cells in the vicinity [182]. The MSC secretome has been shown to be responsive to stress, including physiological changes (hypoxia or anoxia), small-molecule stimulation, and cytokine treatment [183].

11.4 A Comparison of Allogeneic MSC Transplantation and Autologous HSC Transplantation

It appears that ADs can be treated by both HSC and MSC transplantation. However, to date, no comparative studies have been undertaken to determine which therapy is more suitable. Based on various recently published independent clinical trials, initial observations showed that MSC transplantation has some advantages over HSC transplantation. For example, in the treatment of SLE using both HSC and MSC transplantation, allogeneic MSC transplantation showed superior clinical efficacy comparable to that of HSC transplantation (Table 11.4). Almost all outcome data showed that allogeneic MSC transplantation is better than autologous HSC transplantation, in particular that the cost for MSC transplantation is lower than that of autologous HSC transplantation.

Table 11.4 Comparison of allogeneic MSC and autologous HSC transplantation

	Allogeneic MSC transplantation (references)	**Autologous HSC transplantation**
Overall survival rate (%)	92.5–94 [82, 117]	81 ± 8 [184]
Rate of clinical remission (%)	50–60 [82, 117]	20–66 [185]
Rate of relapse (%)	17.5–23 [82, 117]	32 [185], 56 ± 11 [184]
Rate of TRM (%)	0 [82, 117]	2–12 [36, 185]
CYC conditioning regimen	Not necessary [117]	Necessary
Cost	20,000	60,000 [186]

CYC cyclophosphamide, *TRM* transplant-related mortality

11.5 Conclusion

ADs have poor progression, which is related to injury to cells as a result of abnormal immune system activities. For a long time, ADs were treated using immunosuppressive drugs with low efficacy. After more than 10 years of stem cell transplantation for ADs, patients can now anticipate the development of novel methods using stem cells to treat their diseases. Theoretically, HSC transplantation should reset a patient's immune system; however, this technique is expensive and difficult and has a high rate of relapse. MSC transplantation however is a promising alternative therapy. In contrast to HSCs, MSCs have both high differentiation potential and high immunomodulating functions. Preclinically and clinically, MSCs can correct the patient's immune response, which significantly reduces autoimmune reactions. In fact, in an increasing number of updated and reported clinical trials, MSCs have been used to effectively treat different types of ADs. Despite that some of the mechanisms of therapy remain unclear, along with the limitation of a small number of patients, these initial results are encouraging and show that MSC transplantation is especially promising in treating AD.

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12. Treatment of Hepatic Malignances and Disorders: The Role of Liver Bioengineering

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12.1 Introduction

The liver is the largest gland in the body, and it performs several hundred vital functions, predominantly related to metabolism and homeostasis. It is important in amino acids, proteins and enzyme synthesis, energy storage (glycogen and fat storage), production of cholesterol and bile, and

detoxification and elimination of drugs and xenobiotics. Hepatocytes, the major cell type in the liver, carry out most of the aforementioned functions. Some of the other cell populations in the liver are constituted by cholangiocytes, Kupffer cells, sinusoidal endothelial cells, stellate cells, stromal cells, and hematopoietic cells, with each one of them having a specific function. Moreover, being the central organ for a diversity of vital functions, the liver serves as a prime example of regeneration because it holds a major regenerative capacity following loss of up to three quarters of its mass due to partial hepatectomy or toxic injury. This adaptive response and robustness of liver regeneration is crucial to ensure the body's metabolic functions, and thus, an unhealthy liver may have a profound negative impact on metabolism and homeostasis. Hence, novel therapies that ensure some degree of recovery from liver disease, are greatly sought worldwide. This chapter describes some of the most common liver diseases and several novel regenerative therapies that are currently in development or already translated into the clinical setting.

12.2 Liver Diseases

A variety of factors, such as toxins, infectious agents, immune-mediated insults, tumors, and hereditary defects, can cause various liver diseases. Depending on the severity and duration of the insult, the result may be either acute liver failure (ALF) or chronic liver disease (CLD). According to the American Liver Foundation, more than 30 million people in the USA have a liver disease. CLD and cirrhosis alone were responsible for approximately 10 deaths per 100,000 people in all age groups in 2010, with the death rate being almost 30 per 100,000 people in the age group of 50+ years and the total deaths numbered at 31,903, making them the most common cause of death among all liver diseases [1]. CLD and cirrhosis are indeed the 12th leading cause of death in all ages, but among people 45 years and older, CLD and cirrhosis are the fifth leading causes of death in the USA [2]. In the past decade, the death rate due to CLD and cirrhosis has often seen yearly increases, and according to government census reports,

the number of Americans aged 65 and older is projected to be 88.5 million by 2050, more than double the projected number of 40.2 million in 2010 [3]. As a result, it is reasonable to expect an increase over the coming years in the number of patients with CLD and cirrhosis. Primary liver cancer is the second leading cause of death among other liver diseases and led to the death of approximately 24,550 people in 2015 according to the American Cancer Society. More than 90% of the people diagnosed with liver cancer are older than 45 years of age and thus present a bigger threat in the near future with the increasing percentage of the elderly population worldwide [4]. At the same time, a variety of early-stage liver diseases caused by factors ranging from drugs, toxins, and alcohol to lifestyle and various preexisting diseases, lead to CLD and, ultimately, end-stage liver failure. This progression pattern makes it difficult to predict mortality rates for various early-stage liver diseases.

Many factors are involved in causing different types of liver diseases. The failure of numerous hepatic metabolic and synthetic functions is usually the pathophysiological consequence of liver disease. Etiologic risk factors involved in some of the major liver diseases will be discussed in this section.

12.2.1 Hepatocellular Carcinoma

One of the most deadly malignant tumors is hepatocellular carcinoma (HCC), with 5-year survival rates ranging from 3 to 28% based on the stage of progression [5]. It is the fifth most common cancer worldwide and fourth most common cause of cancer-related deaths [6]. HCC is usually accompanied by cirrhosis and hepatitis B or C viral (HBV or HCV) infection, which compromise the survival rates of diagnosed patients [7]. Alcoholic liver disease, metabolic liver disease, and damages caused by toxins and drugs are other risk factors involved in the progression of HCC [8]. Owing to the coexistence of various risk factors in patients diagnosed with HCC, several etiological factors are involved in the development of HCC. The disease progression of HCC is directly affected by these factors, making it a complex disorder with poor prognosis [9].

Liver cirrhosis triggered by HBV and HCV infection is thought to be the primary risk factor in the development of HCC [7]. Activation of stellate cells, a hallmark of liver cirrhosis, resulting in increased production of cytokines and growth factors that affect hepatocyte proliferation, may also cause tumor formation [10].

12.2.2 Hepatic Fibrosis and Cirrhosis

Several etiological factors contribute to the development of liver cirrhosis. Following liver injury caused by a variety of factors discussed earlier, usually quiescent stellate cells become activated, resulting in the synthesis and secretion of extracellular matrix (ECM) molecules and cytokines as a step in the repair process. This "scarring" process promotes hepatic fibrogenesis, which is enhanced by several liver cell populations in a diseased liver, leading to increased ECM synthesis by stellate cells [8]. Liver fibrosis is a result of the anomalous continuation of fibrogenesis, and it progresses at variable rates depending on the cause of the liver disease and environmental and host factors [11]. Cirrhosis, an advanced stage of liver fibrosis, compromises exchanges between hepatic sinusoids and adjacent hepatic parenchyma owing to the formation of scar tissue in the space of Disse. Impaired liver function, portal hypertension, and development of HCC are the major pathophysiological consequences of liver cirrhosis [11]. Alcohol abuse, HBV and HCV infection, and fatty liver disease are the main causes of the development of cirrhosis, despite the fact that most other liver diseases, if untreated, can cause liver damage to an extent that leads to cirrhosis.

12.2.3 Alcoholic Liver Diseases

Alcohol consumption accounted for 3.8% of all deaths worldwide in 2004 [12]. Moreover, alcohol-related liver deaths accounts for up to 48% of the cirrhosis-related deaths in the USA [13]. Overconsumption of alcohol over a long period of time generally causes ALD, but other factors, such as diet, hepatitis infection, and coexisting nonalcoholic fatty liver

disease (NAFLD), play an important role in its progression [14]. Not all heavy alcohol drinkers develop ALD, as the extent of liver injury caused by alcohol consumption is dependent on multiple factors, such as dose, time and type of alcohol consumption, drinking pattern, age, ethnicity, and other risk factors such as iron overload, obesity, and genetic factors [14, 15]. ALD can eventually lead to the development of the patients advanced liver disease with concomitant cirrhosis in up to 50% of cases [16]. The toxic effects of ethanol on the liver include disruption of the lipid portion of cell, altering the capacity of liver cells to cope with environmental toxins and acetaldehyde, an oxidative product of ethanol-mediated toxicity [8]. ALD presents as a broad spectrum of liver diseases, ranging from alcoholic fatty liver (steatosis) disease to more severe forms of liver injury, including alcoholic hepatitis (AH), cirrhosis, and HCC [13].

12.2.4 Nonalcoholic Fatty Liver Disease

NAFLD is characterized histologically by fatty infiltration in the hepatocytes accompanied by inflammation or hepatocyte necrosis resembling AH and can progress into cirrhosis [8]. It represents the spectrum of liver disorders associated with hepatic steatosis, not being caused by alcohol consumption. The prevalence of NAFLD is estimated between 16 and 23% of the adult population, making it the most common liver disease in the USA [17], and is increasing its incidence in Western countries. Obesity, type 2 diabetes mellitus, and hyperlipidemia are the major risk factors leading to the progression of NAFLD [18]. The severe form of fatty liver disease, NASH, generally progresses into cirrhosis and liver cancer. Several mechanisms have been proposed to understand the pathogenesis of NAFLD explaining the basis of fat accumulation, liver injury, and fibrosis occurring during its progression [19–21]. Although there is increased understanding of pathogenesis of fat accumulation, clear elucidation of the mediators and mechanism of hepatocyte injury, mediators of stellate cell activation, and fibrosis is lacking [22].

12.2.5 Acute Liver Failure

ALF, also called fulminant hepatic failure (FLH), is a syndrome resulting from the rapid loss of hepatocyte function usually associated with coagulopathy and encephalopathy in patients with no preexisting liver diseases [23]. Viral infection, idiosyncratic drug-induced reactions, toxins, metabolic abnormalities, and vascular disasters are the principal factors leading to ALF [8]. Viral hepatitis (A, B, and E) is the major culprit for ALF in developing nations, while acetaminophen toxicity is the most common cause of ALF in the USA and UK [24]. ALF is an unusual syndrome with approximately 2000 cases per year in the USA with high mortality rates ranging between 40 and 90% [25]. ALF affects the physiologic status of almost every organ system in the body as a consequence of the involvement of liver in functions affecting almost all organs. During the progression of ALF, the most important liver functions are altered, leading to hyperbilirubinemia, hyperammonemia, altered drug metabolism, and the metabolic dysfunction of carbohydrates, lipid, and proteins.

12.2.6 Viral Hepatitis

Viral hepatitis is an inflammatory disease of the liver caused by various viruses, namely, hepatitis A, B, C, D, and E. Hepatitis A, B, and C are the more common types of viral hepatitis found in the USA [26]. These viruses can spread through food, water, infected blood, and bodily fluids. Viral hepatitis is usually acute with a short-term infectious stage, but HAV (hepatitis A virus), HBV, and HCV can also cause chronic hepatitis, which accounts for approx. 82% of the total number of liver cancer cases in the world. About 60% of these cases are associated with cirrhosis, cancer, liver failure, and HBV, while 22% cases are associated with HCV [27]. Hepatitis C is the major cause of liver transplantation in Europe and the USA and is responsible for an increase in the number of deaths due to HCC [28].

12.2.7 Inborn Errors of Metabolism

Defects in an enzyme or transport protein by genetic mutations can produce alterations in a metabolic pathway and lead to inborn errors of metabolism [8, 29]. Some of these metabolic diseases can cause major structural damage to the liver and ultimately lead to liver failure or cirrhosis. These errors of metabolism could also damage other organ systems. Approximately 15–25% of liver transplantation in children [30] are a result of a group of inborn errors of metabolism. A shortage of livers for transplantation, combined with the possibility of long-term graft rejection, has led to hepatocyte transplantation as an alternative approach to treating these disorders, although complete correction of the errors of metabolism has not yet been achieved [29, 30].

12.3 Therapies for Liver Diseases

Various liver diseases require variable methods of management and care, as the symptoms vary greatly based on the type and duration of the disease. For example, a patient with severe ALF may only survive with immediate liver transplantation, but a patient with a progressive chronic liver disease may be treated with pharmacological interventions before the disease progresses to the end stage, where liver transplantation may be the only alternative for survival. To date, liver transplantation remains the only option for all chronic-/end-stage liver diseases. However, other treatment strategies have been implemented in the therapeutic management of acute and chronic liver diseases:

1. An antidote can be used to reduce hepatic injury and reverse ALF in certain cases. *N*-acetyl-cysteine (NAC) is a well-known antidote that provides cysteine, which helps replenish hepatic glutathione stores and protects the liver from free-radical injury arising from acetaminophen, carbon tetrachloride, or trichloroethylene [31].
2. In hepatic encephalopathy (HE), the first step in management is to reduce the production and absorption of ammonia, either by reducing protein intake or administering nonabsorbable disaccharides or antibiotics

administering nonabsorbable disaccharides or antibiotics [8]. The last option, if these therapies do not produce satisfactory results, is liver transplantation.

3. Therapeutic agents such as immunomodulatory compounds, inhibitors of stellate cell activation, antioxidants, and modulators of collagen synthesis and degradation are some of the treatments used for progressive liver fibrosis, when it is reversible. Treatment of liver cirrhosis varies significantly based on the nature of the disease. For example, alcoholic cirrhosis is treated by abstaining from alcohol, while cirrhosis caused by viral hepatitis is treated by interferon immune therapy and ribavirin or any of the novel drugs available on the market for hepatitis C [32].

4. Treatment of NAFLD is generally focused on correcting underlying risk factors such as weight control and diabetes control [17].

5. The etiological factors in HCC have a big impact in determining treatment strategies. If an HCC patient has a compromised liver function due to cirrhosis, surgical resection of the tumor may not be ideal because of the risk for postoperative decompensation [7]. Most of the patients that eventually progress to end-stage liver disease (cirrhosis) are then recommended for orthotopic liver transplantation.

12.4 Liver Transplantation

Liver transplantation is the substitution of a diseased liver with a healthy donor organ; thus, it serves as a viable treatment option for patients with acute liver failure, end-stage liver disease, hepatic malignancies, and metabolic diseases [8]. Except in the case of specific disorders, transplantation is

usually the treatment of choice for liver disease complications, rather than the underlying illness. The survival rate of liver transplantation patients has increased owing to improvements in immunosuppression drug regimens, transplantation techniques, and preoperative care. The most recent 1-year survival rate is 88.2% [33]. However, there are still great risks in liver transplantation, including the inherent risk of surgery, disease recurrence, and long-term immunosuppression. These risks must be weighed against the benefits of surgery, which vary from patient to patient but include improvements in the quality of life, survival, and prevention of long-term complications [8].

Patients with advanced chronic liver failure, acute liver failure, decompensated cirrhosis, and hepatocellular carcinoma are potential candidates for liver transplantations. The primary liver diseases in these cases include chronic hepatitis C and B, alcoholic liver disease, cryptogenic and primary biliary cirrhosis, sclerosing cholangitis, and hepatitis. More uncommon indications for liver transplantation include hepatic tumors, metabolic and genetic disorders, and some vascular disorders [8]. Differences in the specific disease have implications for the transplant evaluation process and recovery post-transplantation. Cardiopulmonary diseases, advanced pulmonary disease, malignancy outside of the liver, active alcohol and drug use, and anatomic abnormalities precluding liver transplantation are reasons for the removal of patients from the transplant lists [34]. Prior to 2002, organ allocation was based on a first-come first-served basis. The major issue with this method was that patients who were at the greatest risk of dying were not given the highest priority. However, the organ allocation system is now driven by disease severity, mostly based on the model for end-stage liver disease (MELD) score. The MELD score attempts to determine disease severity and potential for survival post-transplantation and is currently used by the United Network for Organ Sharing in the USA. MELD scores are calculated using laboratory values of serum bilirubin, serum creatinine, and international normalized ratio for prothrombin time, and a high MELD score is associated with decreased survival rates [33]. In general, patients

displaying signs of end-stage liver disease or a MELD score of ten and above should be referred for transplant evaluation.

The most recent data published by the Organ Procurement and Transplantation Network in 2011 indicated that approximately 15,000 people were on the waiting list to receive a donor liver. Only 5805 transplants were performed that year, with 2456 patients dying while on the waiting list and another 482 patients removed because they were too sick for transplantation [33]. These numbers highlight the serious challenge of organ shortage in liver transplantation. Of the 5805 patients who received liver transplants in 2011, 5617 were from deceased donors, with the remaining small percentage from living donors. While living donor liver transplantation (LDLT) provides a means to expand organ availability, stringent donor and recipient selection criteria and concerns over donor complications have kept the number of LDLTs relatively low [35].

Other approaches to expanding the donor organ supply have included donation after cardiac death [36, 37], the use of hepatitis C- and hepatitis B-positive donors [38–40], and split-liver transplantations [41, 42]. Split-liver transplantation involves splitting adult donor livers into left lateral and extended right grafts for transplantation into both pediatric and adult patients [41]. The results of this technique have been comparable with whole-graft transplantation, and increasing the use of this technique could amplify the total number of transplant recipients in the USA by 1000 annually [42]. Nevertheless, almost two-thirds of people waiting for a donor liver never receive one, even with these recent attempts to alleviate donor organ shortage.

12.5 Cellular Therapies

There are many potential clinical applications for liver cell transplantation, such as the treatment of metabolic disorders and acute liver failure and the management of chronic liver failure. The intact host liver architecture harbors cells with the potential to proliferate and repair damaged tissue. The first successful hepatocyte transplantation was performed in 1992

in a woman with familial hypercholesterolemia. After ex vivo transduction with a retrovirus encoding for the human low-density lipoprotein (LDL) receptor, the patient's own hepatocytes were infused into the liver through the inferior mesenteric vein. Improved levels of LDL and high-density lipoprotein were detected throughout the next 18 months, and transgene expression was also detected in a liver biopsy [43]. Despite these promising results, other patients were treated following this first successful transplant without showing clear benefits [44]. Since then, hepatocyte transplantation has been used in the treatment of metabolic diseases, with varying degrees of success, including alpha-1-antitrypsin deficiency [45, 46], Crigler-Najjar syndrome type 1 [45, 47–49], factor VII deficiency [50], glycogen storage disease [45, 51], infantile Refsum's disease [52], progressive familial intrahepatic cholestasis [48], ornithine transcarbamylase deficiency [53–55], and citrullinemia [45]. It has also been used as a support treatment of acute [46, 53, 56] and chronic liver diseases [56–58] by bridging severely ill patients to orthotopic liver transplantation. To minimize the potential risks of the transplant, only a small number of hepatocytes were engrafted in the recipient liver in many of these trials. Some studies showed that hepatocyte engraftments of 1–5% of the total liver mass led to better clinical outcomes, even if they were short lived [45].

Because of the shortage of donor livers available for cell isolation and the inability to expand primary hepatocytes in vitro, there is a limitation on the numbers of obtainable primary human hepatocytes. Furthermore, the organs available for cell isolation are usually rejected after transplantation, leading to variable cellular viability and variable survival following cryopreservation. This limitation makes it necessary to use alternative cell sources in research on novel liver cellular therapies, such as the use of immortalized hepatocytes [59], human fetal liver cells [60], which have the particular advantage of being able to differentiate into adult hepatocytes and cholangiocytes, the biliary epithelial cells in the liver [61]. Other highly promising sources for cell therapy include induced pluripotent stem cell (iPSC)- or human embryonic stem cell (hESC)-derived hepatocytes [62, 63] and adult stem

or progenitor cells [64–66]. Both hESCs and iPSCs were shown to differentiate into hepatocytes using growth factor signals that mimic embryonic development [67, 68]. These cells were able to demonstrate hepatic functions after transplantation into rodent models [62, 63].

Despite the promising data these therapies have shown in early-stage clinical applications, many barriers must still be overcome to make it a viable and reproducible treatment (Table 12.1). It is necessary to suppress the proliferation of host hepatocytes in the treatment of metabolic disorders and to allow donor cells to repopulate the liver in a suitable way. To enhance the proliferation of transplanted cells, irradiation of the host liver [72–74] or suppression of host hepatocyte proliferation through drug treatments [75] has been researched in preclinical studies. Technological improvements are also used to measure the amount of donor versus host cells following transplantation to evaluate the treatment success. In rats, for example, luciferase imaging is used to see engrafted cells; nevertheless, this treatment option is not useful in humans because of the thickness of the abdominal wall. One strategy to resolve this issue is to use creatinine kinase gene as a marker for donor hepatocytes: once expressed, it produces phosphocreatine in the liver and allows for P-31 magnetic resonance imaging of the engrafted donor cells [76]. Advances in immunosuppression protocols or the development of cell transplantation methods lacking immunosuppression would accelerate the field of liver cellular therapy.

Table 12.1 List of cell therapy procedures performed in clinical trials

Disease/syndrome	Cell type	Benefits/disadvantages	Reference
Familial hypercholesterolemia	Hepatocytes	– Patients improved their health status	[43, 44]
Crigler-Najjar syndrome type I	Hepatocytes	– Not all patients had a clear benefit from the procedure	[47, 49]
Severe ornithine transcarbamylase deficiency	Hepatocytes	– These trials presented low efficacy and lack of long-term therapeutic effect	[54]
Glycogen storage disease type 1a	Hepatocytes	– Reduced availability of human hepatocytes	[51]

Disease/syndrome	Cell type	Benefits/disadvantages	Reference
Peroxisomal biogenesis disease	Hepatocytes		[52]
Acute liver disease	Hepatocytes		[46, 53, 56]
Chronic liver disease	Hepatocytes		[53, 58, 69]
Chronic liver disease	Bone marrow mesenchymal stem cell	– High expansion capabilities and differentiation into hepatocytes	[48, 70, 71]
		– Great promise for regenerative medicine applications	
Chronic liver disease	Hematopoietic stem cell	– More extensive safety evaluation is needed	[54, 65]
Chronic liver disease	Fetal liver progenitor/stem cells	– Clinical amelioration was limited in a period of time	[60]

12.6 Gene Therapy Treatments for Liver Disease

Gene therapy holds great promise for the treatment of inborn errors of metabolism and other liver diseases. The principle of gene therapy is the introduction of genetic material into cells to produce a curative biological effect [77]. This genetic material can come from native or chimeric genes (which are artificial genomic constructs that code for therapeutic molecules). Both genes are used to direct the synthesis of therapeutic proteins inside transduced cells, and sub genomic DNA and RNA molecules (which include ribozymes, antisense molecules, and RNA decoys [78]) are used to modify the expression of endogenous genes. Despite the fact that gene therapy is today a powerful tool to regulate biological function in diseased tissues, much remains to be investigated to improve its efficacy and minimize toxic side effects. The field is actually moving, and the combination of what is already known about vectors used in gene therapy with

stem cell technology seems to be an emerging novel technology in the treatment of liver disease.

12.6.1 Vectors for Gene Therapy

Genetic material is transferred to target cells using vectors. An ideal vector for gene therapy should have low antigenic potential, high capacity, high transduction efficiency, and controlled and targeted transgene expression [79]. Normally, viral vectors have better transduction efficiency and longer duration of transgene expression than nonviral vectors. These nonviral vectors (like liposomes, DNA-protein complexes, and naked DNA) have some advantages over viral vectors: they are easier to handle, they have high capacity for DNA sequences, they have low toxicity, they can be specifically targeted to a tissue, and they are not immunogenic, which allows for repeated vector administration. However, nonviral vectors have low transduction efficiency [79]. Another technique is the use of the liposome gene transfer system, which consists of DNA surrounded by a liposomal coat that allows the genetic material to be absorbed and taken up by the cells via endocytosis. DNA-protein complexes can be used as a gene transfer system that uses membrane molecules as receptors for mediated endocytosis. The specific expression by hepatocytes of specific membrane receptors, such as the asialoglycoprotein or the transferrin receptor, allows for the targeting of these receptors for the endocytosis of DNA-protein complexes. Naked DNA or plasmid DNA can be directly injected into tissues and is often taken up by numerous cell types, including liver cells, muscle cells, and skin cells [79].

Because of their natural infectivity, viral vectors are the most efficient vehicles for gene transfer. These vectors are produced by deleting some or all viral genes and replacing them with sequences that encode the therapeutic molecule [80]. Examples of viral vectors are adenovirus, retrovirus, adeno-associated virus (AAV), herpes virus, lentivirus, baculovirus, SV40 virus, and vaccinia virus. Adenovirus (which consists of a double-stranded DNA virus) has been found to have a natural tropism to the liver, with a high

efficiency for transducting nondividing cells [81]. They are also very efficient in the infection, either in vivo or in vitro, of normal hepatocytes [82], with the disadvantage that they can produce immune responses. AAVs (which are nonpathogenic human parvoviruses) are generated by the deletion of all viral genes except for the inverted terminal repeat genes. They are successfully used in gene therapy owing to their capacity to transduce both dividing and nondividing cells and because they are able to maintain long-term transgene expression either by integrating into the host genome or by persisting in the cells as episomal forms [83]. Furthermore, AAVs also demonstrate excellent liver tropism after systemic injection. It has been demonstrated that AAV-mediated gene transfer of factor IX in the liver of mice induces consistent curative levels of active factor IX [84]. It has also been shown that adjuvant treatment with genotoxic agents (like gamma irradiation) improves the expression of the AAV transgene in vivo and in vitro.

12.6.2 Gene Therapy for Patients with Inborn Errors of Metabolism

Gene therapy using recombinant AAV (rAAV) is applied in the treatment of various diseases related to metabolic disorders such as alpha-1-antitrypsin (AAT) deficiency, tyrosinemia, and lysosomal storage disorders (LSDs). Some clinical trials have been completed over the last decade for the treatment of patients with AAT deficiency [85]. This genetic disorder consists of the accumulation of mutant AAT in hepatocytes and the decrease of this protein in serum levels. A reestablishment of AAT plasma levels may be seen in patients treated with rAAV. Paulk et al. showed in 2010 that AAV could also be used to correct hereditary tyrosinemia in a mouse model in which an inborn error of metabolism resulted in the inability to break down amino acid tyrosine in an adequate way. This disorder includes symptoms like liver and kidney disturbances and mental retardation [86]. Patients with LSDs are also being treated using gene therapy. The therapeutic targets in the treatment of LSD are the hematopoietic progenitor cells (HPCs), because genetically modified hematopoietic-derived cells can provide a source of

secreted enzyme that would have direct access to the circulation [87]. Hematopoietic-derived cells can repopulate the fixed tissue macrophage system and deliver the enzyme directly to multiple organs [87]. Intravenous delivery of early-generation adenoviral vectors produced high-level expression of lysosomal enzymes, which resulted in therapeutic levels of circulating enzyme and a reduction of storage material in murine disease models of Pompe, Fabry, and Wolman diseases [88, 89].

Recent studies combined stem cells and gene therapy technologies in the treatment of liver diseases. One of these studies was performed using mesenchymal stem cell (MSC)-based gene delivery for the treatment of AAT deficiency [90]. The advantages of using MSCs are that they can be expanded in vitro, they retain their multi lineage differentiation potential, and they can differentiate into hepatocytes both in vivo and in vitro [91]. The viral vector used in this study was rAAV. The vector with the corrected AAT was transduced into MSCs, and when the rAAV-transduced AT-MSCs were transplanted into mice, there was long-term transgene expression in the recipient liver and sustained serum levels of the transgene product human alpha-1 antitrypsin (hAAT). This combinatorial method using stem cells and gene therapy using viral vectors could help to move the field forward.

12.6.3 Gene Therapy for Patients Infected with Hepatitis B and C

Patients who suffer from chronic hepatitis B and hepatitis C are usually treated with interferon α (IFN-α). However, this treatment has only a 40% success rate in patients with chronic hepatitis B and 20–30% success rate in the case of hepatitis C [80]. To date, many clinical trials have used genetic vaccines against HBV and HCV. In one of these, scientists studied patients with chronic hepatitis B treated with lamivudine and then immunized with DNA vaccine encoding all HBV antigens and a genetically engineered mutant human IL-2 (hIL-12 N222L), which induced a sustained virological response (SVR) [92]. After 1 year, they observed that half of

the vaccinated patients had undetectable viremia, while the other half had no changes in serum viral loads. There are also gene therapy approaches designed to directly target viral replication. One of these procedures uses RNA interference (RNAi), which consists of the silence of genes in a sequence-dependent manner [93]. It has been shown that siRNA can alter the course of HBV and HCV infection by mediating viral RNA degradation and inhibiting viral RNA translation and replication. However, the strategy has some limitations. On the one hand, the high mutation rate in the viral genome means it does not degrade, and in the other, the inhibitory molecule must reach a high enough percentage of the infected hepatocyte to produce a therapeutic effect.

12.6.4 Gene Therapy for Patients with Liver Cirrhosis

Ueki et al. demonstrated in 1999 a remarkable decrease in fibrosis along with an improvement in hepatocellular function after transducing cirrhotic livers with vectors expressing hepatocyte growth factor (HGF) [94]. These same results have also been reported in rat cirrhotic livers after a long-term expression of a vector encoding for IGF-1 [95]. Nonetheless, there is a concern about using IGF-1-based gene therapy for liver cirrhosis because this deficiency is a premalignant condition and IGF-1 is an antiapoptotic growth factor, which may favor tumor development [80, 82].

12.6.5 Gene Therapy for Acute Liver Failure

Of all the critical conditions, ALF is one of the most challenging to treat. A potential approach to preventing ALF is to target key molecules involved in hepatocyte death such as Fas/FasL, TNFα, TRAIL, or TGFβ [80]. A study performed in 2006 showed that treatment with adenovirus-mediated dominant negative form of the Fas-associated death domain (FADDdn), which consists of a downstream signaling molecule for Fas and TNFRs, inhibited TNF-/galactosamine-

mediated hepatocellular apoptosis, leading to a significant decrease in serum transaminase levels [96].

12.6.6 Gene Therapy for Patients with Liver Cancer

HCC is the most common primary liver cancer. Gene therapy strategies for patients who suffer from HCC include gene replacement, antisense strategies, drug sensitization, genetic immunotherapy, and antiangiogenesis. The mechanism of gene replacement for patients with HCC is to replace the nonfunctional tumor suppressor genes. Many approaches have been used to restore p53 function with an adenoviral vector that expresses the wild-type p53 form, called Gendicine. It has been used in combination with chemotherapy, showing optimistic results for the treatment of HCC [82]. However, to completely inhibit tumor growth using gene transfer of p53, there needs to be 100% transduction efficiency, which is not possible with the gene vectors currently available. Nonetheless, it has been described that the introduction of the wild-type p53 form to tumor cells represses the transcription of vascular endothelial growth factor (VEGF), leading to an antiangiogenesis effect within the tumor [97]. Although replacing the p53 mutation with the wild-type p53 appears to be a promising treatment, the responsible genetic defect that cells suffer during their path toward a malignant state is not always originated by a p53 mutation or inactivation.

Targeting responsible oncogenes is another option for HCC. An inhibition of tumor cell growth in vitro, along with decreased tumorigenicity in nude mice after transduction of HCC cells with retroviruses that carry antisense RNA directed against the N-ras oncogene, has been observed [98]. After transfection of HCC cells with plasmids containing antisense RNA targeting fibroblast growth factor (FGF-2), mRNA led to the inhibition of FGF-2 synthesis and to a loss of tumorigenicity in nude mice. The apoptotic capability of transformed hepatocytes can be restored by antisense inhibition of insulin-like growth factor I (IGF-1). However, some limitations on this approach to treating liver cancer

include the inability to determine exact targeted delivery to the tumor and half-life of the antisense therapeutics, which only allow transitory inhibition of gene expression.

Drug sensitization is also an alternative approach to HCC treatment. The objective of this therapy is to release a suicide gene encoding for a foreign enzyme that converts a nontoxic product into a lethal drug metabolite [82]. The best known suicide gene is HSV-tk, which, once expressed in tumor cells, causes the conversion of ganciclovir (a nontoxic prodrug) into a phosphorylated toxic compound that stops DNA synthesis by inhibition of DNA polymerase [79]. Although this therapy has promising effects for the treatment of HCC because of the large number of cancer cells killed, the main limitation is that HSV-tk/ganciclovir has toxic side effects not only on the cancerous cells but also on nontumoral tissue.

12.7 Liver Bioengineering

Despite the fact that actual techniques of living donor and split-liver transplantation have mitigated the donor shortage problem, there is still a huge need to find an alternative to liver transplantation. Owing to the shortage of donor livers for transplantation (common to many other organs), regenerative medicine approaches have emerged with the main objective of stimulating the regeneration of the diseased liver itself or replacing it with a bioengineered liver fabricated ex vivo and transplanted into the patient. This last approach is still years, even decades, away from successful clinical application, but recent advances in the liver regenerative medicine field have yielded promising results. Organ engineering requires the use of a supporting scaffold, which can be made of synthetic materials or by decellularized organs. This scaffold is then seeded with cells and matured in a bioreactor to create functional tissue. It has been successful in rodent livers using decellularized scaffolds seeded with both animal and human cells [34, 99]. The main obstacle in translating this approach is the generation and seeding of enough cells into the scaffold to create a fully functional tissue that can successfully provide different liver functions. It also needs to be transplanted into a

patient without clotting or rejection problems. Once these hurdles are resolved, regenerative medicine could probably solve the the issue of donor organ shortage.

Nowadays, tissue decellularization techniques represent a succesful tool to obtain matrices and scaffolds for the fields of tissue engineering and regenerative medicine. The use of detergents like Triton X-100 or SDS makes whole-organ bioscaffolds more readily available [34, 100]. To generate these whole-organ scaffolds, the detergent solution is perfused through the organ vascular network, keeping it intact and fully decellularizing the organ (Fig. 12.1). This perfusion decellularization method has proven very efficient because it not only preserves the microarchitecture of the organ but also allows the retention of many bioactive signals (e.g., cell-adhesion peptides, ECM proteins) that are difficult to replicate in vitro and that support cell attachment and viability [101]. Another fundamental advantage in the use of organ scaffolds in bioengineering organs for clinical purposes is the preservation of ECM components among species. Thus, decellularization techniques combined with recellularization with human cells may be a viable approach to alleviating the shortage of organs for transplantation [102]. Liver decellularization has been done using a perfusion method and a bioreactor system to deliver human cells inside organ scaffolds [34, 103]. The bioreactor provides a continuous flow of culture media supplied with growth factors and gases, which allow for proper cell growth in the 3-D liver structure [34]. Bioengineered livers showed a typical hepatic architecture with biliary ductular structures (cytokeratin 19 positive) along with clusters of hepatocytes (albumin and cytochrome P450 isoforms 2A, 3A positive in the parenchymal space of the liver). Humanized bioengineered livers also exhibited a vascular network coated with endothelial cells expressing von Willebrand Factor (vWF) and endothelial nitric oxide synthase (eNOS). Furthermore, the confluent endothelium layer prevented platelet adhesion and aggregation, which is important for blood flow without coagulation after transplantation [34]. This technology has been adapted to decellularized porcine liver, maintaining a patent vascular network and an intact ECM; however,

complete recellularization to obtain a functional liver with a clinically relevant size has still not yet been accomplished [104]. Nonetheless, the recellularization of porcine liver scaffolds with human cells could change the liver transplantation medical field and lead to a significant increase in the number of organs available for transplantation.

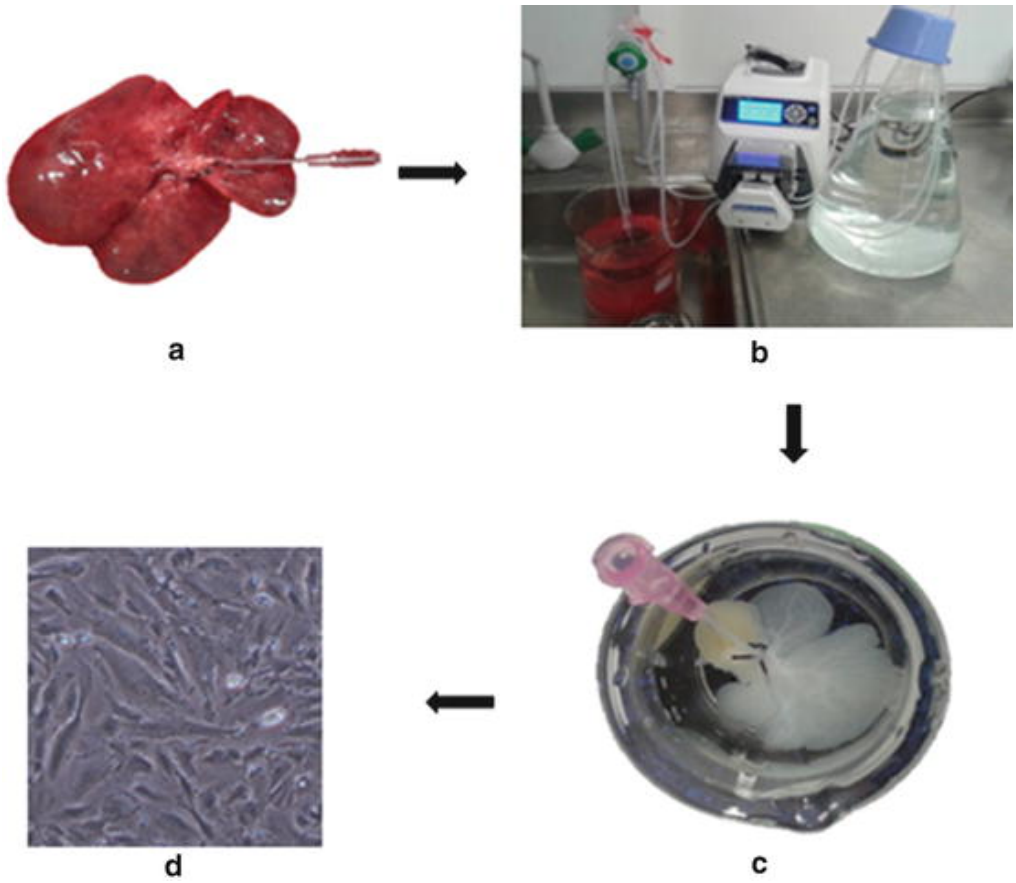

Fig. 12.1 The process begins with liver isolation and cannulation (**a**). Then it is connected to a peristaltic pump (**b**), which allows water and detergent perfusion throughout the liver in order to decellularize it (**c**). Finally, this bioscaffold is seeded with human cells (**d**)

12.8 Liver Assist Devices

The number of patients waiting for a liver transplantation exceeds the number of performed liver surgeries, causing higher mortality rates for end-stage liver disease. Because of this problem other alternatives have been investigated to reduce the number of patients waiting for a suitable donor. Extracorporeal liver support devices emerged as a solution; its function is to detoxify the blood or plasma to compensate for the lack of function of the injured liver. There are two kinds of

liver assist device: artificial liver (AL) devices and bioartificial liver (BAL) devices, which differ in the system used for detoxification.

Initially, these kinds of devices tried to compensate for the loss of liver functions with a detoxification of the blood. However, with a series of improvements, liver assist devices can allow patients to recover from an acute liver injury, preventing in many cases transplantation [105].

12.8.1 Artificial Liver Devices

Through techniques such as hemofiltration, hemodialysis, hemodiafiltration, plasmapheresis, hemadsorption, plasma fractionation, and albumin dialysis, AL devices are capable of removing toxins from blood and plasma [105–107]. In dialysis-based devices, toxins are adsorbed and removed from the blood when the patient's blood crosses a low-to-medium permeability membrane while a suspension of activated charcoal and cation exchange is being pumped to the other sides of the dialyzer [105, 108].

The two principal albumin dialysis systems, the molecular adsorbent recirculating system (MARS) and fractionated plasma separation and absorption system (Prometheus), have been widely assessed in clinical trials. In MARS an albumin-impermeable membrane (50-kDa cutoff) divides the high-flux albumin-coated dialyzer from albumin-filled dialysate. Hence, the toxins transfer to the albumin solution in the dialysate side after having been dissolved and passed through the membrane [109, 110]. On the other hand, in Prometheus devices, the albumin and protein-bound toxins pass across an albumin-permeable membrane (250-kDa cutoff). Finally, the toxin-free albumin returns to the patient thanks to special adsorbents that take away the toxins [107–109].

Various clinical studies in patients with ALF and acute-on-chronic liver failure have reported that liver dialysis, MARS and Prometheus, produces improvements in several functions as well as increased life expectancy [107].

12.8.2 Bioartificial Liver Devices

Because AL devices are not capable of providing essential and metabolic hepatic functions [111], BAL devices were designed to provide such functions thanks to the combination of hollow fibers or porous matrix membrane with primary hepatocytes or hepatoma cell lines [111, 112]. An ideal method would be to use primary human hepatocytes, but, as mentioned earlier, their availability is low and [108, 112] they have a limited proliferative capacity in vitro. Hence, alternative hepatocyte sources and hepatoblastoma cell lines have been used, keeping in mind a possible xenoresponse or retroviral transmission [113, 114].

Furthermore, cell morphology and metabolism should be preserved because isolated cells need to be immobilized in an adequate platform where nutrients and oxygen must be delivered at physiological levels. In addition, to prevent an immune rejection, a porous membrane is needed to divide the cells from the blood or plasma, which should also allow passage of toxins and proteins [114, 115]. In the near future, BAL systems should also be able to provide a personal response according to patient needs [116].

12.8.3 Available BAL Devices

Up to the present moment, the most frequent BAL devices (Table 12.2) utilized in clinical trials are HepatAssist and ELAD [110, 122]. Whereas ELAD uses human immortalized C3A cell line derived from the human hepatoma cell line HepG2, the other systems employ porcine hepatocytes [123]. In most cases where these devices were used, the majority of them recovered entirely; therefore, a liver transplantation was not necessary.

Table 12.2 Summary of developed and published bioartificial devices

Device	Reference
Extracorporeal Liver Assist Device (ELAD): Vital Therapies Inc., San Diego, CA	[117]
HepatAssist: HepaLife, Boston, MA	[118]

Device	Reference
Bioartificial Liver Support System (BLSS): Excorp Medical Inc., MN	[119]
The Academic Medical Center-bioartificial liver (AMC-BAL): Hep-Art Medical Devices B.V., Amsterdam, The Netherlands	[120]
Modular extracorporeal liver support device (MELS): Charité Virchow Clinic, Berlin, Germany	[121]

Currently, several studies of BAL devices are carried out in the USA, Europe, and Asia to evaluate safety and efficacy in the treatment of various end-stage liver diseases. To date, none of the BAL devices has been approved by the FDA for commercial use.

In many cases liver assist devices represent a hopeful solution for patients waiting for a liver transplant; however, it is still necessary to resolve many issues for the purpose of using the devices efficient on all patients. Although AL systems are easy to use and improve patient survival, they are not capable of replacing critical synthetic and metabolic functions of the liver. On the other hand, in various clinical trials, BALs have been shown to have enough potential. Nevertheless, it would be required to determine optimum cell sources, optimize bioreactor technologies, and reduce costs.

12.9 Future Directions

12.9.1 Liver ECM Support

In the field of bioengineering, the ECM is quickly becoming a central biomaterial in drug development and in bioengineered tissues for transplants. The ECM provides a large amount of molecules such as proteins or growth factors, which are crucial for the maintenance of liver cell phenotypes [124]. Owing to the development of liver decellularization, the introduction of ECMs in experimental liver bioengineering approaches has been steadily growing [34, 99, 124–127]. Hence, a possible solution to improving the effectiveness of bioartificial liver systems could be the introduction of a decellularized liver matrix. It has been very difficult for any devices to be accepted for clinical trials because of their inability to significantly

improve survival rates [128–131]. This deficiency may be attributed to the use of synthetic material where the cells are seeded, thus a native liver ECM would be an attractive choice [132, 133] to increase cellular functionality and lead to greater levels of protein secretion and waste metabolism elimination.

12.9.2 Hepatocyte Injection and Encapsulation

Cell encapsulation increases the therapeutic potential of hepatocytes because of the inclusion of decellularized matrix proteins in a semipermeable membrane. The encapsulation system avoids an immune rejection and the destruction of implanted allogeneic or xenogeneic donor cells [134, 135]. In addition, the capsule allows for the passage of several molecules such as therapeutic products or metabolites.

Alginate-encapsulated hepatocytes support phases I and II metabolic pathways when seeded in bioreactors. Their activity has been evaluated by 7-ethoxycoumarin hydroxylation (ECOD) and uridine diphosphate glucuronosyltransferase (UGT) activity [136]. Several in vitro and in vivo studies have been reported on encapsulated hepatocytes and hepatocyte-like cells, that are capable of synthesizing albumin, metabolizing drugs, and generating ammonia [132, 133, 137]. Despite improvements in acute liver failure models [132, 133], the efficiency and functioning of encapsulated cells must be increased.

Using ECM in a capsule's membrane could increase liver cell function by mimicking the natural liver microenvironment. Hence, molecules present in the native liver ECM (such as adhesion molecules, growth factors, and matrix collagens) have been added to the capsule to maintain the function of several hepatic cell types [138, 139]. However, the next step will be to determine the most important components of the liver matrix that maintain cellular viability and cell phenotype.

Despite the fact that encapsulated hepatocytes cannot be injected into the liver because of the risk of clogging the liver

vasculature, they can be implanted in extrahepatic sites from where they can support liver functions like the peritoneal cavity, which provides an ideal site for introducing a huge number of encapsulated hepatocytes that have the capacity to improve patient health [140, 141]. By means of diffusion through the semipermeable peritoneal membrane, the blood is cleared of waste products like ammonia [142]. On the other hand, products made in a healthy liver can exit the liver and enter the circulatory system through drainage of the cavity into lymphatic vessels. Both improvements in several serum biochemical parameters (alanine aminotransferase, aspartate aminotransferase, albumin levels, total bilirubin) and an increase in life expectancy have been demonstrated when encapsulated cells were transplanted in the peritoneal cavity of acute liver failure models [138, 143].

Finally, lymph nodes are a well-vascularized structure, so they have easy access to oxygen and nutrients. For this reason, they have proven to be an ideal place to inject hepatocytes. Recently, it was reported that hepatocytes were capable of engraftment in lymph nodes and could generate small clusters of functioning hepatocytes, sufficient to rescue a lethal mouse model of tyrosinemia [144, 145]. Hence, many studies should focus on investigating whether more injury models might benefit from cells used at this site.

12.9.3 Growth Factor Therapies

HGF, epidermal growth factor (EGF), and IGF-1 are growth factors that can be used to treat several liver deficiencies. HGF is a potent mitogen that is secreted by mesenchymal cells and that, by binding its receptor, c-met, induces cell proliferation [146, 147]. This produces morphologic and mitogenic modifications in tissue organization and liver regeneration [148–150]. Higher amounts of albumin and urea have been obtained in vitro when hydrogels were combined with HGF in liver cultures [139, 151]. Hence, growth factor therapies based on preventing fibrosis of the tissue may prove vital to repairing tissue mass following an acute injury or stress [149, 152].

EGF is another growth factor that activates the cell division of hepatocytes, which operates through the tyrosine kinase EGF receptor (EGFR), and it is crucial for normal liver regeneration [153, 154]. IGF-1 is a hormone that activates the growth of almost all cell types. In combination with EGF, it is able to induce cell replication and decrease the symptoms of a steatotic liver [155, 156].

However, the short half-life of growth factors in circulation reduces the efficacy of these therapies. Thus, new studies that extend their half-life and devise a better therapeutic control are needed to allow for their successful utilization. An alternative for carrying out this goal is to construct microspheres combining growth factors that can be delivered or targeted toward liver proteins in patients [157, 158]. Nevertheless, the protein quantities and the number of microspheres are still limited. Another approach is to activate transplanted cells to secrete the desired proteins. Several cells in the liver, such as sinusoidal endothelial cells, Kupffer cells, and hepatic stellate cells, have the capacity to secrete growth factors and cytokines upon activation [159]. Hence, the production of HGF has been stimulated in stellate cells, with hepatocyte-conditioned medium, IGF-1 [160], or the amino acid leucine [161]. Transgenic lentiviral vectors may be another method for inducing a continuous expression of these growth factors [162, 163]. However, the risks involved in this form of gene therapy await proper evaluation.

12.10 Concluding Remarks

Currently, liver transplantation is the only therapeutic option for curing certain liver diseases, although it is still in its youth. Therefore, other alternatives, such as cellular and genetic therapies or tissue engineering, have been proposed and developed to further develop and improve existing techniques. However, their clinical application is still limited mainly owing to their long and difficult development process. Nonetheless, the future is promising, with novel techniques and therapies underdeveloped, hopefully, soon to be transferred into the clinic.

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13. Advances in Micro- and Nanotechnologies for Stem Cell-Based Translational Applications

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The application of stem cell-based therapeutics in improving human health is the ultimate anticipated outcome of stem cell research. Yet there exists a recognized lag between laboratory discoveries confirming the potential of stem cells and their actual application in routine clinical care. This lag can be

appreciated if we contrast the number of research articles published in the field of stem cells with the number of products used in clinical practice. For example, a search for the keyword “stem cell” found 279,213 publications on PubMed, whereas only five products, all based on hematopoietic stem cells, were approved for therapeutic use by the US Food and Drug Administration [1]. This chapter will provide an overview of emerging technologies applied to help overcome such challenges with a highlight on their potential for clinical applications.

13.1 What Do Stem Cells Need from Technology?

13.1.1 Stem Cell Tissue Processing

One of the anticipated technologies is a comprehensive platform capable of (1) safely obtaining the human donor’s tissue through a minimally invasive biopsy, (2) processing the tissue sample in an automated and efficient way, and (3) providing an adequate number of functional stem cells. This requires the integration of numerous steps of automated tissue dissociation, cell filtration, centrifugation, and cell sorting, as well as a biomimetic stem cell culture system for cellular expansion and differentiation. This futuristic platform can provide high-quality stem cells ready for transplantation or can be tailored to test the effect of regulatory biomolecules or drugs on stem cells of interest (e.g., cancer stem cells).

13.1.2 Stem Cell Identification

There is no single gold standard marker for identification and isolation of all types of stem cells. *Stem cell* can be considered a generic functional term that describes a heterogeneous population rather than a well-defined cell type. These cells differ significantly in their sources, hierarchies, and functions. Therefore, current isolation methods rely on cellular expression (positive selection) or the absence of expression (negative selection) of multiple markers (e.g., surface receptors, stem cell-associated genes, or other intracellular

enzymes and proteins). The application of multiple markers, however, is technically demanding and is not appropriate for routine use in moderately or poorly funded clinical settings. Furthermore, it becomes increasingly challenging to make an objective comparison of stem cell studies when variable combinations of markers have been used.

Fluorescence-activated cell sorting (FACS) and magnetic cell sorting are standard tools for the isolation of stem cells. The basic cost of the equipment involved is composed of the operational cost of multiple antibodies, fluorophores, or labeled magnetic beads. The side population method is another technique that avoids using multiple markers and depends on the ability of stem cells to efficiently efflux the Hoechst 33342 dye [2]. Despite the low cost of the dye, its use requires a UV laser, which is available only in costly multiparameter flow cytometers and microscopes. Likewise, adherence to plastic is used traditionally for isolating mesenchymal stem cells; however, this method produces cells of considerable heterogeneity and variable stemness capacities [3]. Such barriers should be viewed as opportunities where technology can offer novel and economic solutions in stem cell research and applications.

13.1.3 Controlling Stem Cell Fate and the Stem Cell Niche

In order for stem cells to be clinically relevant, they must efficiently expand, differentiate into a specialized cell type, and precisely engraft into the target tissue to repair a defective function. Expansion requires that stem cells proliferate in the undifferentiated state into clinically useful cell numbers without affecting their multipotency. Differentiation of stem cells into a specialized cell type of interest is not a challenge by itself, and most stem cells start to differentiate once exposed to the appropriate growth factors. Difficulties, however, arise in the long-term commitment of stem cells to their the new fate. Frequently, differentiated cells can be short lived when transferred to an in vivo environment, which is a much more complex environment than a culture plate [4].

Likewise, inefficient engraftment could mean that stem cells transferred to a patient to treat a liver disorder, for example, might never reach the liver and be lost in other tissues. Accordingly, differentiation of stem cells in vitro is not equivalent to a successful clinical outcome. Rather, it should be considered the first step in a series of intricate physiological events.

Many challenges are related to the dissimilarity between the over-simplified conditions used for stem cell culture in the laboratory and the complex native microenvironment of stem cells in the human body, generally referred to as the *stem cell niche*. This niche encompasses all biological, mechanical, biophysical, and topographical cues that compose the physiological environment of stem cells. The simulation of only a single factor, such as oxygen concentration in the culture system, could enhance the multipotency of stem cells and recover some of their in vivo properties [5]. A similar impact can be induced by providing the proper surface topography, stiffness, and fluid dynamics of the extracellular fluid or by introducing cells that naturally coexist with stem cells [6, 7]. Therefore, constructing a niche that can precisely mimic these factors is crucial to eliminating the discrepancy between research and real therapeutic outcomes.

In this chapter, lab-on-chip technologies and nanotechnologies are highlighted as being among the most promising tools that can contribute to advancing stem cell research toward applicable therapies. In addition, other emerging technologies (e.g., quantum dots and immunosensors) with applications in the stem cell field are briefly surveyed.

13.2 Microfluidics for Stem Cell Applications

Microfluidics has attracted prominent attention in cell-based applications and is playing an increasingly recognized role in stem cell research. Initially, microchannels with various shapes, sizes, and internal anatomies were developed, followed by the introduction of digital microfluidics that

allows the automated mobilization of discrete fluidic droplets on a microchip surface. Both of these approaches are open for unrestricted designs and can miniaturize almost any biological reaction traditionally conducted at the macro scale.

13.2.1 Primary Tissue Dissociation

Tissue dissociation is the first step in human tissue processing for the purpose of isolating stem cells. Conventional dissociation involves multiple manual steps such as cutting, mincing with or without adding tissue dissociation enzymes, centrifugation, cell sieving and multiple wash steps, and pipetting. Enzymes such as trypsin, collagenase, accutase, and others are used to break down the collagen fibers of the connective tissue and the adhesion molecules that hold cells together. Stem cells constitute approximately 1–2 % of the cell population in a given adult tissue [8]; thus, the rarity of these cells makes initial tissue processing very critical. Since the isolation of stem cells is largely dependent on identifying specific surface receptors, it is assumed that enzymatic dissociation may alter these receptors, so that subsequent isolation methods may miss such altered cells. Accordingly, the tissue dissociation process requires further development to achieve greater efficiency and minimal cell loss. Microfluidic platforms that could offer efficient and safe dissociation of human tissue utilizing shear stress and fluid flow forces have been recently developed [9].

13.2.2 Stem Cell Isolation

The ability to accurately isolate stem cells from a mixed cell population is imperative for clinical applications, and thus purity is an essential parameter in cell separation methods. Even after a stem cell population, there can be different types of stem and progenitor cells that need to be sorted. Variations in size [10, 11] and deformability [12] could be used as indicators for variable differentiation potentials.

13.2.2.1 Miniaturized Flow Cytometry

The working principle is that cells of interest are detected and sorted while flowing, which has been intensively reviewed by Bhagat et al. and Tsutsui et al. [13, 14]. There are two approaches: labeled and label-free detection methods. Briefly, in a labeled approach, an immunoassay or other methods are used to label stem cells [15], which are then flushed to pass through an imaged region, followed by cell sorting and collection into different gates. When this process is miniaturized, the possibilities become unlimited [16, 17]. Fluid flow conditions can be modified based on hydrodynamic fluid flow (Fig. 13.1a) [18], dielectropohoresis (Fig. 13.1b) [19], electrowetting (Fig. 13.1c) [20], or a magnetic field (Fig. 13.1d) [21] to enable sorting of stem cells.

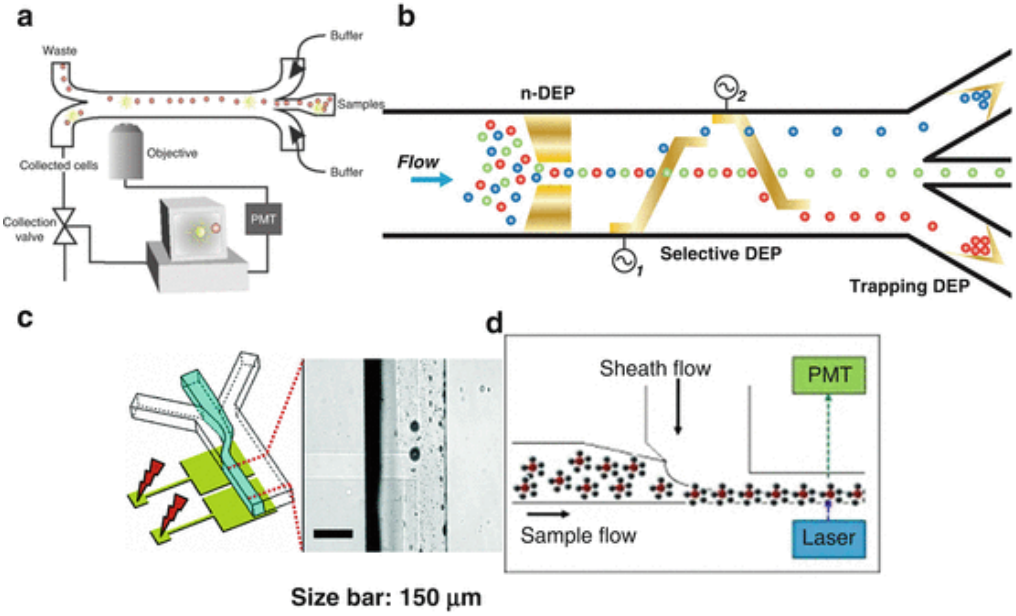

Fig. 13.1 Modified microfluidic flow cytometry has been proposed to sort stem cells based on hydrodynamic fluid flow (a) (reprinted with permission from [18] ©RSC), dielectrophoretic forces (b) (reprinted with permission from [22] ©Springer), electrowetting (c) (reprinted with permission from [20] ©ACS), or magnetic field (d) (reprinted with permission from [21] ©AIP)

Label-free detection relies on the intrinsic properties of cells of interest that can adequately distinguish and isolate them from other cells, without the need for attachment to additional markers. These properties include size, shape, and deformability and adhesion properties, which will be discussed in subsequent sections. There are detailed reviews on label-free cell sorting using microfluidics [22, 23]; we will highlight several techniques briefly in this chapter.

13.2.2.2 Size-Based Sorting of Stem Cells

In general, label-free methods are attractive not just as simple and cheap methods, but more importantly, because the yield of cells is ready to use without any attached biomarkers. It is believed that some types of stem cell could be of smaller size compared to differentiated cells, and in these cases, size-based sorting would be a straightforward label-free method to isolate stem cells. There is also evidence for a direct correlation between the size of a stem cell and its differentiation capability. Very small embryonic-like (VSEL) stem cells were isolated from bone marrow showing a significantly higher pluripotency than their larger counterparts from the same tissue. However, one of the obstacles in applying size only-based stem cell isolation is the lack of solid data about the characterization of this subset of small cells. Microfluidic size-based sorters can be a convenient and effective tool for precisely isolating a pure population of small and very small cells for further understanding of their role and behavior. Figure 13.2 highlights some size-based sorting methods that have been applied to separate stem cells including weir-type filters [24, 25], pillar-type filters [22], cross-flow filters [26, 27], and porous membrane filters [28]. The weir-type filter has been used in several applications where the clogging problem was alleviated to an extent [29]. Among these four types of filter, the cross-flow filter has been demonstrated to be superior to other reported methods since it is less prone to clogging by cell debris and other tissue components.

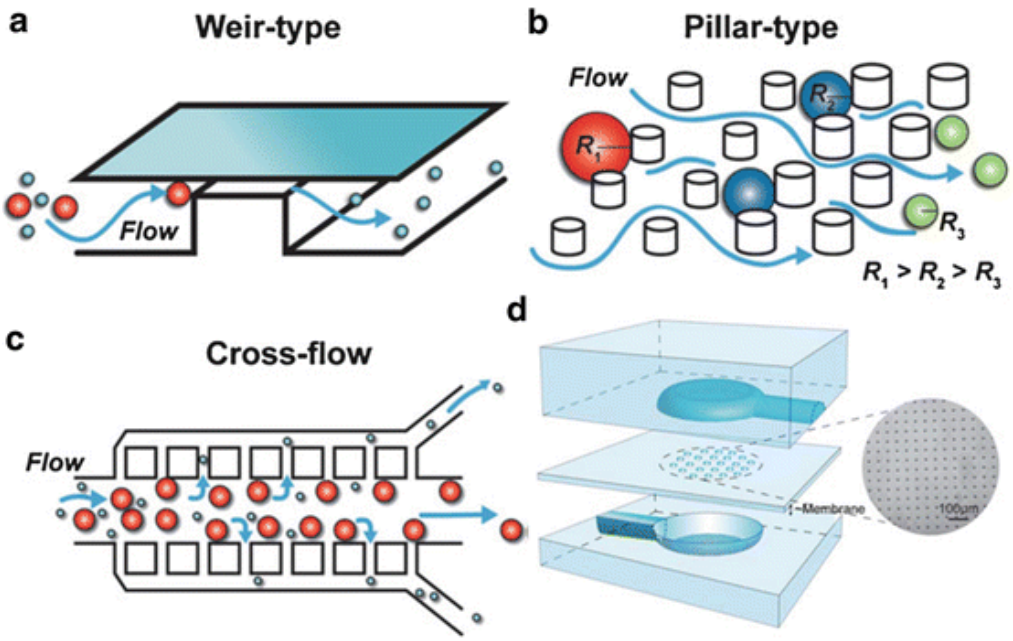

Fig. 13.2 (**a**) Schematic of weir-type filter that allows small particles to pass through, whereas large particles are trapped. (**b**) Schematic of pillar-type filter, allowing particles with diameters smaller than the spacing between particles to pass through; thus it suffers from the issue of potential channel blockage similar to the weir-type filter. (**c**) The cross-flow filter discards large particles during the filtration procedure, allowing much larger sample volume to be processed. Reprinted with permission from [22] ©Springer. (**d**) A porous membrane filter with pore sizes ranging from 2.5 to 16.6 μm, which can be integrated easily with other channel components. Reprinted with permission from [28] ©RSC

13.2.2.3 Adhesion-Based Sorting of Stem Cells

Stem cells contain membrane proteins including adhesion molecules leading to distinct adhesion properties, which have been used for the separation and sorting of stem cells. For instance, Fig. 13.3a shows a microfluidic system that enables microstem cell high-efficiency, adhesion-based recovery. Compared to flow cytometry-based cell separation, this approach uses a simple experimental setup, although the throughput remains lower than in conventional flow cytometry [30].

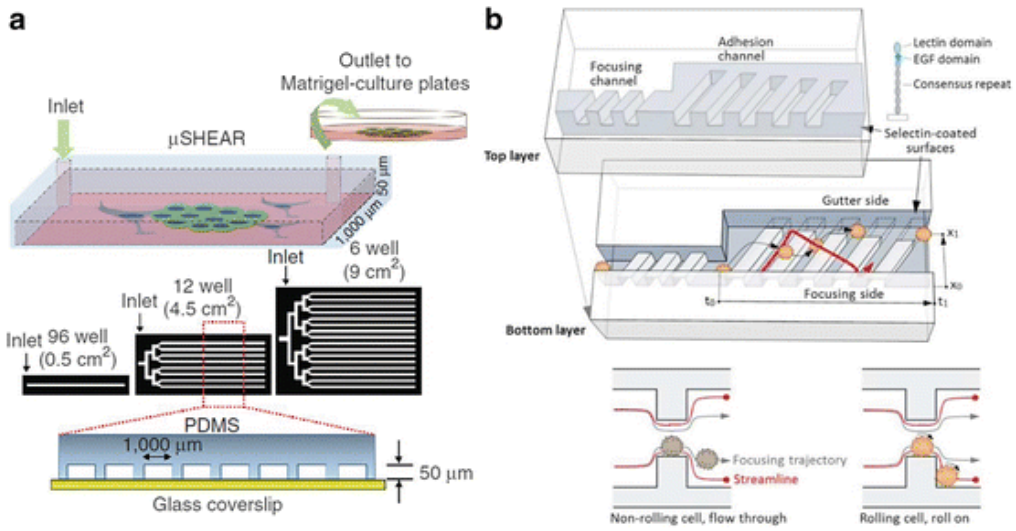

Fig. 13.3 (**a**) Isolation of stem cells based on differences in adhesion strength to a particular surface in a microstem cell high-efficiency, adhesion-based recovery device. Reprinted with permission from [30] ©Nature Publishing Group. (**b**) A cell rolling cytometer where the microfluidic channel walls are coated with molecules that bind with specific cells, regulating their passing velocities and directions. Reprinted with permission from [31] ©RSC

In addition, a cell rolling cytometer was introduced to separate stem cells [31–33]. The principle behind this method is to coat molecules specific to stem cells on microfluidic channels, which can change the flow velocity and direction of the stem cells while having no effect on other types of cells without the specific membrane protein expression (Fig. 13.3b). Thus, this method can provide a label-free method of stem cell separation in a continuous manner, producing a much higher throughput than the aforementioned adhesion-based separation approach [31].

13.2.2.4 Optical Sorting of Stem Cells

A force can be exerted on small particles using lasers, which have been combined with microfluidic flow cytometers for optical sorting of cells of interest [34–36]. As shown in Fig. 13.4a, the optical force is applied on traveling cells to regulate their flow direction, and the cells can be collected for further analysis [37]. However, this method works best when there is a detectable difference in the refractive indices of the trapped objects and the surrounding medium. Dholakia et al. overcame this limitation with beads coated with specific cell antigens that are known to work well with the surrounding medium

[38]. A related but different approach uses a pulsed laser diode to create bubbles that push cells one way or another toward the desired flow channels (Fig. 13.4b) [39].

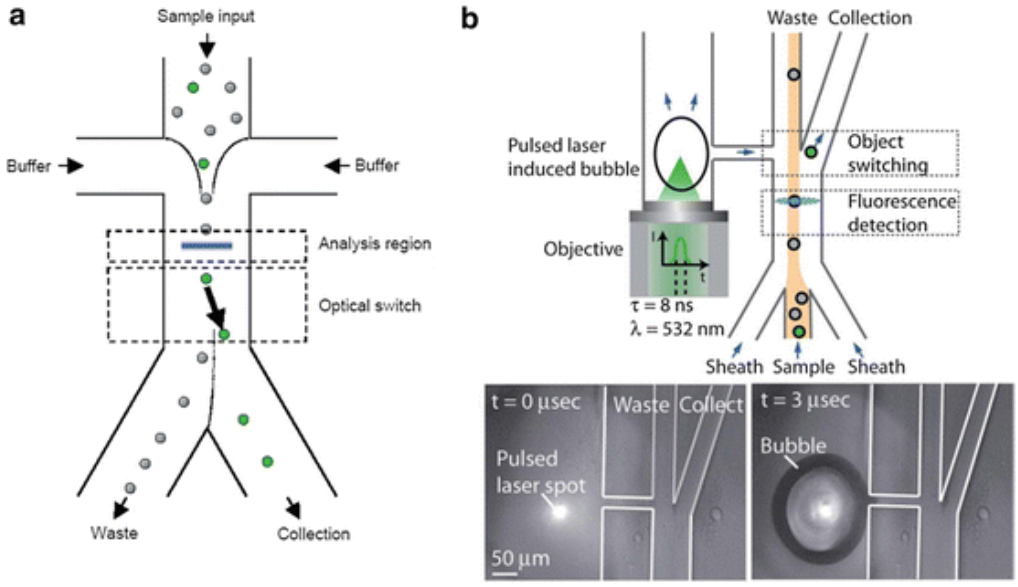

Fig. 13.4 Optofluidic devices for sorting stem cells. (**a**) In an optical flow cytometer, a buffer is used as a sheath flow to align cells to the central region, and the cells are then analyzed by fluorescence detection and directed by laser to specific collection ports. Reprinted with permission from [40] © Nature Publishing Group. (**b**) Detecting a cell of interest triggers a pulsed laser, which induces an air bubble, deflecting the cell to the collection channel. Reprinted with permission from [39] ©RSC

In addition, dielectrophoresis-based sorting is an emerging field [41] whose findings have been successfully applied on many types of cells, including stem cells. Surface acoustics is also an emerging method for cell sorting [42–44], which has not yet been applied in the field of stem cells.

13.2.3 Culture of Stem Cells on Chip

13.2.3.1 Two-Dimensional Culture

Each of the standard methods for the culture of stem cells has its own advantages and limitations. Multiwell plates are easy to use, but they only offer basic biological factors in the form of a static culture medium without providing physical and mechanical regulatory factors necessary for genuine stem cell behavior. Meanwhile, cell culture bioreactors can be used in the perfusion of medium with controlled composition and biophysical cues for the culture of stem cells. However, the

operating volumes of bioreactors are significantly larger than those of well plates, leading to high operating cost when expensive media components are required. Microfluidics can potentially overcome such limitations thanks to its small device size, high throughput, local cellular microenvironment regulation, and automation. Indeed, microfluidics is expected to be the next-generation platform for stem cell culture [45–58].

As pioneers in this field, Figallo et al. proposed a microfluidic perfusion culture platform, made of an array of 12 microbioreactors [47] (Fig. 13.5a). The platform integrates a bioreactor (represented by each individual culture well) and a microfluidic device (represented by the independent flow control for each well). The microbioreactor array has the advantages of both microwell plates (small volume, high throughput, independent culture wells) and perfusion bioreactors (steady-state dynamic flow, enhanced mass transport, application of physical signals). The microfluidic system was used to culture human embryonic stem cells, showing good control over cell seeding densities and a regulated differentiation into smooth muscle cells as evidenced by the expression of actin. In addition, Gomez-Sjoberg et al. built a fully automated cell culture screening system based on a microfluidic chip with various culture media compositions in 96 culture chambers (Fig. 13.5b) [48]. Based on this platform, a comprehensive control over culture conditions (e.g., cell seeding density and rate of media change) was realized, leading to successful stem cell culture for several weeks.

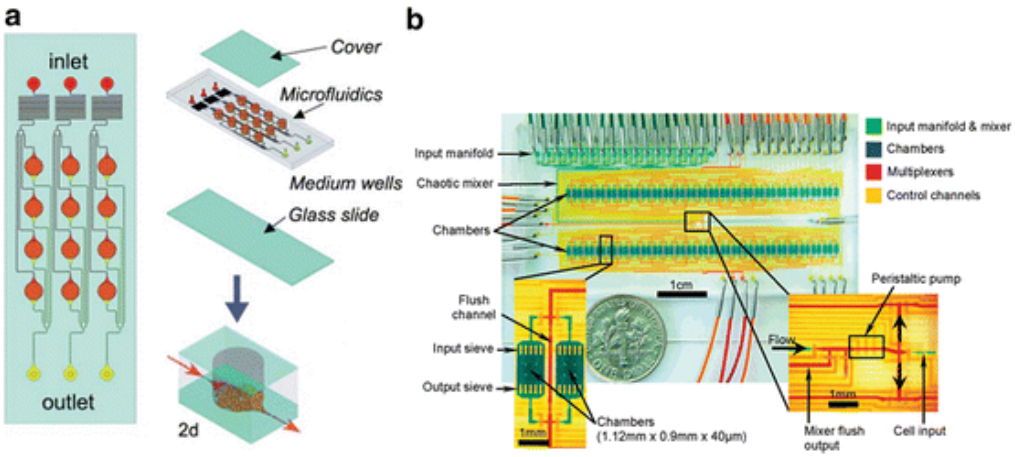

Fig. 13.5 (**a**) A microfluidic device includes independent bioreactors connected by microfluidic channels to enable perfusion culture of stem cells. Reprinted with permission from [47] ©RSC. (**b**) A versatile, fully automated, microfluidic stem cell culture system where each chamber can be individually addressed. Reprinted with permission from [48] ©ACS

13.2.3.2 Three-Dimensional On-Chip Culture of Stem Cells

A major limitation of two-dimensional (2D) microfluidic culture systems is their difference from the in vivo microenvironment, where stem cells actively interact with the extracellular matrix (ECM), neighboring cells, and biochemical and biophysical factors incorporated into a three-dimensional (3D) spatial orientation. To address this need, several recent developments have been made in microfluidic systems to provide three-dimensional on-chip culture of stem cells [59–66].

As shown in Fig. 13.6a, Toh et al. developed a microfluidic system to enable continuous perfusion culture of stem cells in a 3D extracellular matrix and allow an optimal cell-to-matrix interaction [60]. A solution containing extracellular matrix ECM and suspension cells was flushed into the middle channel, which was further converted into the solid state by cross-linking of the ECM. Culture media and growth factors were subsequently supplied in the two outer channels. Using this system, bone marrow mesenchymal stem cells were perfusion cultured for up to 1 week, well preserving their 3D architecture and differentiation capabilities. Likewise, droplet microfluidics was used to form 3D multicellular spheroids for the culture of stem cells [64]. In a double-emulsion setup, cells and extracellular matrix aggregated into spheroids in an initial flow-focusing compartment were further encapsulated into a hydrogel layer to produce spheroid-encapsulated microgels (Fig. 13.6b). In this study, the encapsulation of human mesenchymal stem cell spheroids in alginate was achieved showing evidence of enhanced osteogenic differentiation under the effect of such conditions.

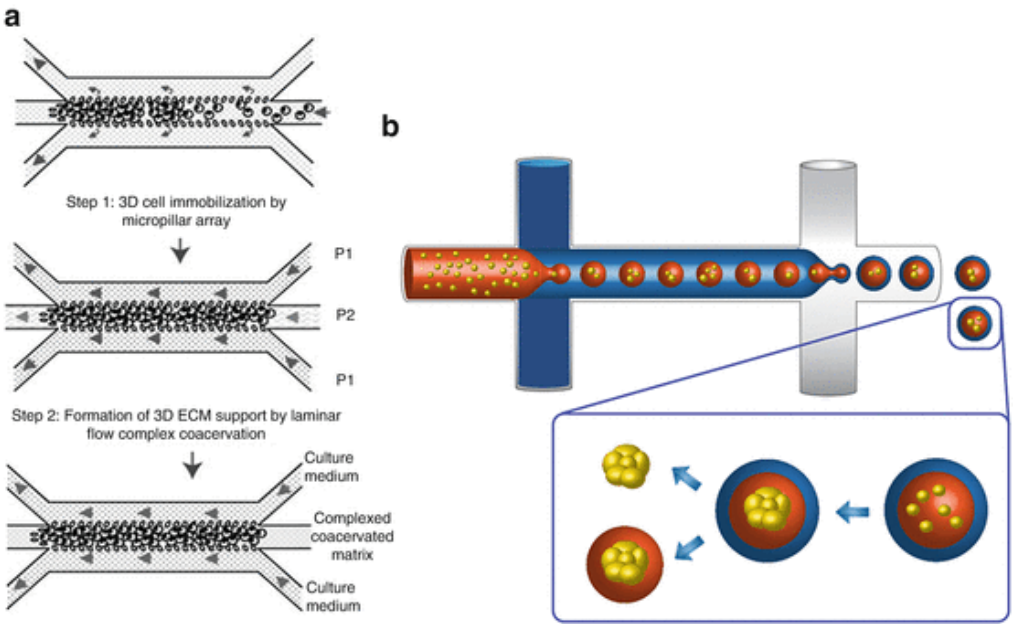

Fig. 13.6 (**a**) Microfluidic device that provides a 3D culture system for stem cells in its middle channels, while the outer channels provide biophysical and biochemical factors necessary for stem cell proliferation. Reprinted with permission from [60] ©RSC. (**b**) A microfluidic Microfluidic device that enables the formation of double-emulsion droplets where stem cells are aggregated into single spheroids. Reprinted with permission from [64] ©RSC

As a powerful tool that gives precise control over fluidic conditions, microfluidics has been used to provide a well-regulated biochemical gradient to induce stem cell differentiation [67–77]. Kawada et al. presented a membrane-based microfluidic device that allowed spatiotemporally variable culture conditions to enhance the differentiation of induced pluripotent stem cells (iPSCs) [67]. As shown in Fig. 13.7a, the membrane-based microfluidic device consists of upper and lower channels separated by a porous membrane. Cells placed on the membrane in the upper channel receive nutritional and growth support media by a dynamic laminar flow in the lower chamber that is delivered through the membrane pores. In that setup, stem cells can sustain the influence of a dynamic concentration gradient without the effect of excessive mechanical shear stresses, similar to what occurs in vivo. In a different model, Liu et al. proposed a microfluidic device that can produce a gradient of extracellular matrix the ECM to regulate the differentiation of stem cells and allow a quantitative and comparative estimation of the conditions that promote an optimal outcome (Fig. 13.7b). As a demonstration, bone marrow-derived mesenchymal stem cells

showed differential adhesion capacities on the patterned surface, and the spreading of stem cells was influenced by the changing gradient of the ECM [68].

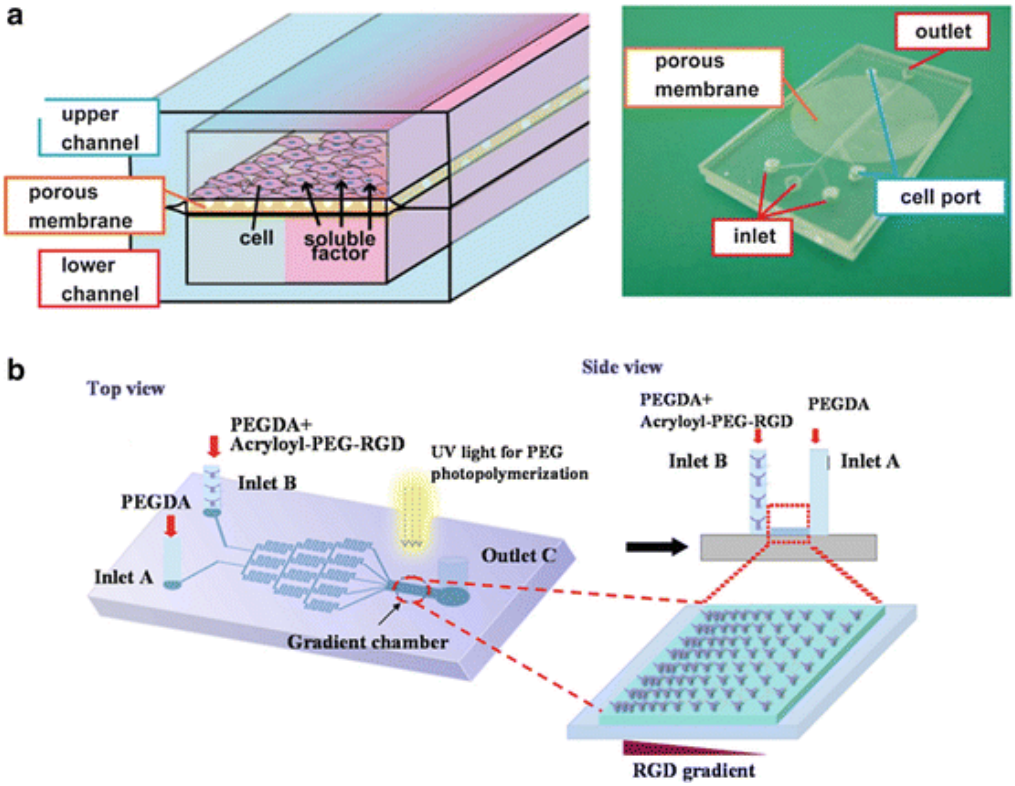

Fig. 13.7 (**a**) Membrane-based microfluidic device capable of forming regulated pattern of soluble factors to stimulate stem cell differentiation. Reprinted with permission from [67] ©RSC. (**b**) The gradient-generating microfluidic device could precisely control the spatial distribution of biomolecules on a biomaterial surface for quantitative mesenchymal stem cell studies. Reprinted with permission from [68] ©AIP

13.2.3.3 On-Chip Formation of Embryoid Bodies

Embryonic stem cells often form embryoid bodies in suspension culture, which may contain a variety of differentiated stem cells. Microfluidic culture systems have been developed to form homogeneous embryoid body units that are similar in size and cellular differentiation status [78–90]. Hwang et al. used microwells of different sizes to accurately regulate the size of embryoid bodies in an attempt to control the fate of these stem cells. It was observed that by confining embryonic stem cell aggregates in smaller microwells, stem cells differentiated toward endothelial

lineages, whereas embryoid bodies in larger microwells showed a trend toward cardiogenic differentiation (Fig. 13.8a) [81]. In addition, Tung et al. presented a method for the fabrication and operation of a 384-well hanging drop culture plate. To form hanging drops, the cell suspension solution was pipetted from the top side through specific access holes; thus, the culture medium was guided to the bottom surface to form cellular spheres (Fig. 13.8b). This model provided a regulated 3D culture microenvironment for the high-throughput formation of embryonic bodies and a novel tool to study human embryonic stem cells [87].

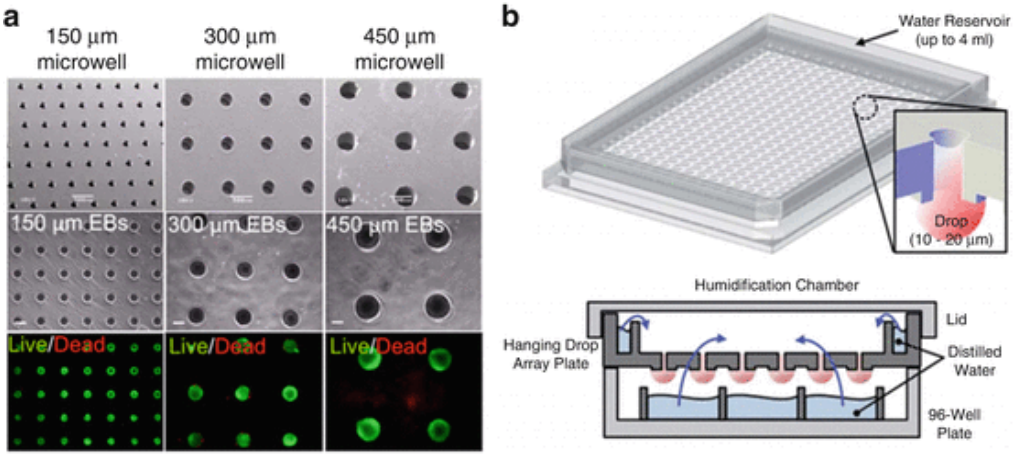

Fig. 13.8 (**a**) Arrays of hydrogel microwells to form embryoid bodies of increasing size. Reprinted with permission from [81] ©PNAS. (**b**) Schematic illustration of 384-well hanging drop spheroid culture array plate, its cross-sectional view, and a final humidification chamber which enabled the culture of three-dimensional spheroids in the hanging drop array plate. Reprinted with permission from [87] ©RSC

13.2.3.4 On-Chip Patterning of Surface Topography for Stem Cell Niche

In the native in vivo conditions of stem cells, cellular interactions with the ECM and topographies at the micro- and nanoscales are believed to be crucial for determining stem cell fate. Several microfluidic approaches have been developed to mimic the natural extracellular environment, which will be briefly described in the following section [91–95]. Derda et al. generated arrays of self-assembled thiols on patterned gold surfaces to allow selective surface patterning of the ECM [92]. Through variations in the type and the coating density of the

ECM, the researchers enhanced the proliferation of embryonic stem cells in the undifferentiated state. Such a model can be valuable for comparing large number of extracellular matrix proteins or other compounds for their effect on various cells simultaneously and under the same conditions. Besides patterning of the ECM, the surface topography at the micro- and nanoscale was also engineered to regulate stem cell behavior. For example, Yim et al. created nanopatterns in the form of parallel grooves on polydimethylsiloxane (PDMS) using a nano-imprinted Si master mold. As a result, mesenchymal stem cells showed obvious cytoskeleton arrangements and reduced expression of focal adhesion molecules compared to those cultured on a nonpatterned surface [93].

13.2.3.5 On-Chip Stem Cell Coculture

Human embryonic stem cells are often cultured on top of a feeder cell layer of inactivated fibroblasts to maintain their pluripotency in the undifferentiated state. Microfluidic approaches have been developed to attain improved functionality and control over coculture conditions [88, 96–104]. For instance, a PDMS microfluidic device with fibronectin-coated microwells was seeded with inactivated murine embryonic fibroblasts to produce a successful coculture with human embryonic stem cells (Fig. 13.9a) [97]. This platform allowed a reproducible size control of cultured cells and demonstrated efficient preservation of the pluripotency of human embryonic stem cells.

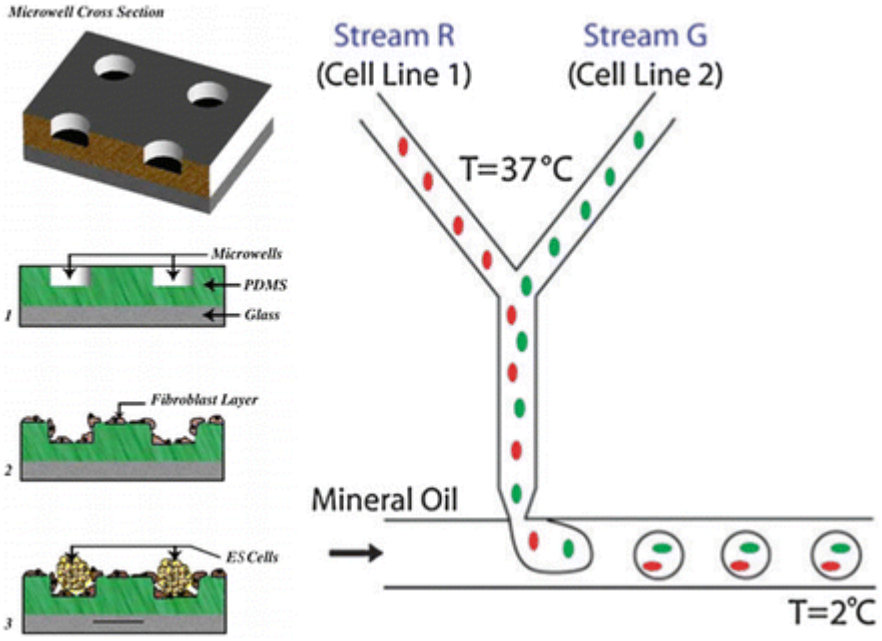

Fig. 13.9 (**a**) Schematic of microwell-based platform showing a coculture of embryonic stem cells with feeder cells. Reprinted with permission from [97] ©ELSEVIER. (**b**) Microfluidic system for coculture of stem cells by encapsulating two types of cells in microdroplets using the conventional T-junction structure. Reprinted with permission from [102] ©RSC

In addition, Tumarkin et al. presented a microfluidic device for high-throughput generation of hydrogel microdroplets of cocultured stem cells [102]. Encapsulation of different cell populations in microdroplets was realized by a T-junction design where two streams of distinct cell suspensions were mixed together and emulsified to form precursor droplets (Fig. 13.9b). The co-encapsulation of two types of hematopoietic progenitor cells was achieved at various cell ratios, depending on the controlled flow rate of each cell stream. The resultant in-droplet paracrine factors in between different cells appeared to modulate the viability of these cells. Moreover, to show the potential of this design for cell screening, the same platform was used to investigate the effect of specific growth factors on defining subpopulations of cells with a distinct response to such growth factors.

13.2.3.6 On-Chip Culture of Stem Cells: Impedance Evaluation

Impedance spectroscopy has long been considered an effective tool for noninvasive monitoring of cellular processes,

including the process of stem cell differentiation [105–109]. As a demonstration, Hildebrandt et al. used micropatterned electrodes to monitor the 2D cell layers of human mesenchymal stem cells. The impedance spectra of osteogenic-treated human mesenchymal stem cells were of significantly higher amplitude compared to undifferentiated control cells [105]. In addition, Bagnaninchi et al. cultured adipose-derived stem cells seeded on multielectrode arrays, and variations in impedance spectroscopy through induction of stem cell toward osteoblasts and adipocytes were observed [106]. Likewise, Stephens et al. applied microelectrode array technology to monitor the characteristis and activity of neural stem cells during differentiation into specialized neurons using a rat hippocampal model [108].

13.2.4 Single-Cell Analysis of Stem Cells

The blossoming of stem cell research has brought with it the need for platforms that enable high-throughput analysis of an individual stem cell. Single-stem cell analysis can offer insight into the molecular mechanisms of stem cell differentiation, including the intracellular and intracrine factors. Lindstrom et al. developed a microfluidic device for single-cell culture that enabled the study of thousands of individual stem/progenitor cell samples simultaneously [110]. As shown in Fig. 13.10, a standard size microfluidic device was designed to accommodate 672 wells of 500 nL capacity each, which provided a low-cost high-throughput platform without losing compatibility with standard detection equipment. The platform was able to maintain the culture of embryonic and neural stem cells and the clonal analysis of single cells using flow cytometry.

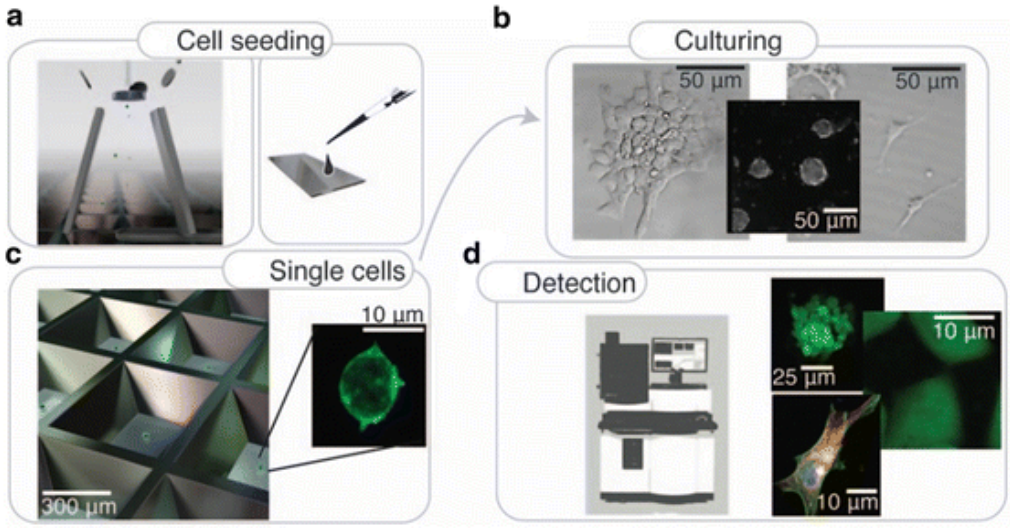

Fig. 13.10 Microfluidic approach showing cell seeding (**a**) and (**b**) culture (**c**) and characterization (**d**) of stem cells at single-cell level. Reprinted with permission from [110] ©PLOS

Genetic modification of stem cells to perform a specific required function is a major step for both research and clinical applications. The current conventional methods for gene transfer into stem cells, such as bulk electroporation, suffer from a limited yield of DNA transfection. To address this obstacle, Valero et al. developed a microfluidic device for electroporation of single stem cells to transfer the MAPK3 gene with high efficiency [111]. As shown in Fig. 13.11a, the microfluidic device contains two channels that were connected by microholes to act as trapping sites for living cells. After individual cells were positioned, electric fields were generated by patterned electrode arrays to facilitate parallel DNA transfection, while each event can be individually regulated.

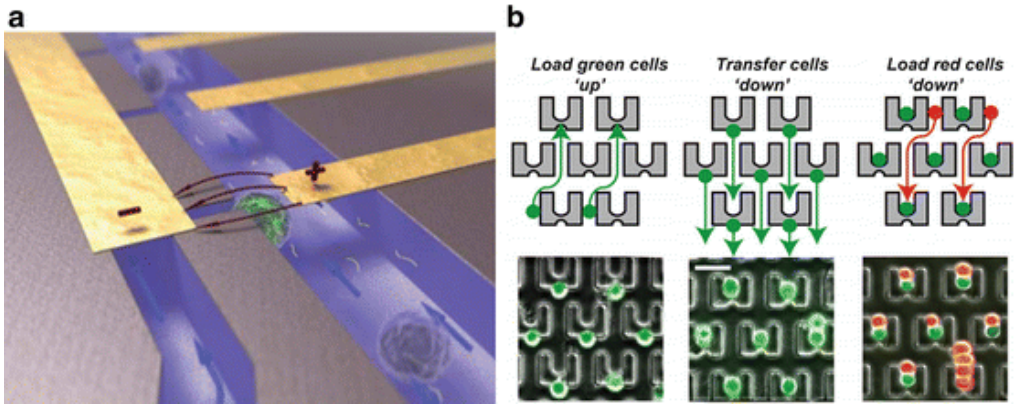

Fig. 13.11 (**a**) Schematic of microfluidic device used in electroporation of single stem cells. Reprinted with permission from [111] ©RSC. (**b**) Three-step procedure of microfluidic device to fuse two types of cells. Reprinted with permission from [112] ©Nature Publishing Group

Besides electroporation, microfluidic devices have been used to pair thousands of stem cells for cell fusion studies [112]. As shown in Fig. 13.11b, a microfluidic device is composed of a large array of cellular trapping sites. By regulating the flow direction, in each trapping site, two cells (one cell for one type) can be paired for subsequent cell fusion using electrical or chemical stimuli. Additionally, reprogramming of the somatic fibroblast cells was possible in hybrids with embryonic stem cells.

13.3 Nanotechnology for Stem Cell Applications

13.3.1 Nanofibers for Stem Cell Niche

The use of nanomaterials to address the mechanical and topographic aspects of the stem cell niche has made substantial progress. The nanotopography of the extracellular surroundings is well sensed by stem cells. It defines important cytoskeleton effects, adhesion properties, and biological responses that together determine its eventual fate [113]. Notable efforts have been made to mimic the extracellular niche using different nanoscale materials to control the surface topography, including the control of the size of scaffold fibers, surface pores, and the attachment to growth factors and surface coatings. The simplest modification in these factors could have a significant effect on stem cell fate. For example, changing the density of nanoposts on the surface provided for cell growth, it was adequate to switch the fate of mesenchymal stem cells between the adipogenic and osteogenic lineages [114]. The stiffness of the extracellular matrix ECM is also a decisive factor that can switch the differentiation of embryonic stem cells into neural or osteogenic progenitors [115]. These findings have important implications when we about clinical scenarios and how surface topography and matrix mechanical cues should be included in our planning and expectations of stem cell behavior once the cells are transferred in vivo. Several models have been developed that could have application in the near future. An interesting illustration of a clinically implantable cellular cardiac patch was shown in the

model by Khan et al. Cardiomyocytes were derived from iPSCs grown on a nanofiber scaffold composed of polylactide-co-glycolide (PLGA). The nanofibers were aligned to mimic the fibrous skeleton of the heart muscle. The differentiated cardiac cells aligned in the correct anatomical distribution over the nanofiber skeleton, whereas they grew completely in a random fashion on the flat surface of the regular culture plate [116].

13.3.2 Nanocarriers for Stem Cell Targeting

Driven by multiple barriers and inadequacies of delivering medications systemically, especially those with serious toxicity such as cancer therapies, nanomedicine has acquired an intense research focus parallel to what occurred in stem cell research. Nanoscale molecules, often inactive, can carry other active molecules into the cell. The ultimate goal of nanoscale-based drug delivery is to sustain the effectiveness of medications without causing systemic adverse effects for patients. This could be realized if these medications are distributed only to the diseased cells [117]. This goal is particularly imperative in the stem cell field, where stem cells can function as vehicles to carry nanomolecules to the target tissue or, conversely, nanocarriers can be used as vehicles to carry biomolecules required for stem cell function. Either way, there have been several promising developments with preliminary success toward achieving this goal. For example, cytokines and growth factors necessary for stem cell proliferation, such as the vascular endothelial growth factor, were transported by nanocarriers made of natural compounds (chitosan and heparin) into mesenchymal stem cells [118]. Conversely, the migration properties of mesenchymal stem cells are considered beneficial from the point of view of drug delivery. They have been used in transferring cytotoxic agents to cancer tissue owing to what is believed to be a tumor-tropic effect; nevertheless, this phenomenon is not yet fully understood. Examples include lipid nanocapsules (LNCs) and poly-lactic acid nanoparticles, which were used to carry coumarin-6 to mesenchymal stem cells with the aim of using

mesenchymal stem cells as vehicles to transfer a cytotoxic agent to glioma, an aggressive brain tumor [119]. Likewise, mesenchymal stem cells were loaded with nanoparticles linked to a porphyrin-based compound to add a photosensitivity property to the compound. Upon laser activation, mesenchymal stem cell death and the consequent death of osteosarcoma cells were initiated [120]. Likewise, human cord blood mesenchymal stem cells were used as vehicles, which were first induced to express interleukin-15 (IL-15) through viral transduction, leading to significant antitumor activity of pancreatic cancer in mice. The method was successful in triggering a long-lasting and transferable immune response, showing a significant potential in cancer prevention studies [121].

Cancer stem cells are believed to be responsible for cancer initiation, aggressiveness, recurrence, and multiple drug resistance. Identification of cancer stem cells remains a challenge, as with other types of stem cells. Targeting cancer stem cells is an emerging direction that can benefit from nanocarrier-based delivery to produce new cancer therapies. Nanocarriers were investigated for their ability to target specific markers on these cells. Both passive nonspecific delivery and active specific delivery were employed with variable success. For example, LNCs were linked to a monoclonal antibody against AC133, an epitope on CD133 that was shown to correlate with stemness capacity [122]. Also, gold nanoparticles were linked to the cytotoxic agent doxorubicin to target breast cancer stem cells. This method increased the efficacy of the medication compared to traditional delivery. In a similar model, researchers were able to successfully target $CD44^{+}$ cancer stem cells using a nanocarrier composed of chitosan molecules coating doxorubicin. The cytotoxicity of the drug increased significantly without causing noticeable general side effects in the animal model [123]. Nevertheless, the field is still in its early stage, thus requires and so extensive evaluation of the effectiveness and safety profile of the currently used nanoparticles is required. Note also that not all nanoparticles result in desirable effects on stem cells. For example, silver

nanoparticles, although known for their biocompatibility and antimicrobial effects, inhibited stem cell differentiation and in high doses caused stem cell toxicity [124].

13.3.3 Quantum Dots

Another challenge for stem cell clinical applications is the lack of a reliable and safe method of tracking transplanted stem cells to ensure their engraftment by the target tissue and, more importantly, the long-term persistence of their regenerative function. Quantum dots (QDs), which are efficient semiconductor fluorescent nanoparticles, represent a promising solution for monitoring clinical outcomes of stem cell therapy. QDs were used to label human mesenchymal stem cells and efficiently allowed their in vivo tracking using near-infrared-based imaging [125]. Mesenchymal stem cells were also tracked by cadmium oxide-selenium QDs, revealing their differential distribution in the pancreas of normal or diabetic mice [126]. However, one concern with QDs is their reported cytotoxicity, especially with the popular cadmium-based QDs [127]. Graphene QDs were also reported to cause adverse effects in human neural stem cells, though they seem to be of a safer profile in terms of their effect on viability, proliferation, and differentiation [128].

13.4 Biosensors and Immunosensors

Immunosensors and immunoprobes are closely related technologies that encompass a wide range of methods. The basic concept includes the use of a modified surface sensitive to an antigen-antibody reaction. This reaction stimulates a transducer to generate a characteristic quantifiable signal using electrochemical, optical, or piezoelectric detection methods. Such a technology tool is in its early development for stem cell applications; however, it could be of particular potential for enabling point-of-care clinical applications. One looked-for application is to enable the detection and probing of cancer stem cells. Since these cells are involved in initiation and recurrence of cancer, their early detection and eradication could indeed revolutionize cancer screening, prevention, and

cure. For instance, an immunoprobe that used an EpCAM-based sensor was able to capture 125 prostate cancer cells accurately, and most importantly cells remained viable for further analysis. Similarly, melanoma cells were immuno-sensed with a high sensitivity, being able to detect as few as 20 cells per/mL. The immunosensor relied on the detection of a melanocortin 1 surface receptor using modified silica nanoparticles. Extending this to stem cells, gold nanoparticle-based immunosensors were also used to quantify Nanong, a popular marker of stem cell pluripotency, with very good sensitivity [129–131].

13.4.1 Stem Cell-Based Biosensors

Stem cells themselves have been considered as cellular sensors that can demonstrate the adverse effect of drugs on physiological functions. Diverse technological tools were coupled with stem cell-based sensors to quantify the extent of that cellular effect. For example, human embryonic stem cells were differentiated into cardiomyocytes that were exposed to cardiac medications. Using a semiconductor sensor excited by light called a light-addressable potentiometric sensor (LAPS), cells could respond with different action potentials that are translated into a measurable photocurrent. This method shows several advantages suitable for miniaturized biomedical applications [132].

13.5 Considerations and Risks

The outburst of micro- and nanotechnology applications in the stem cell field promises substantial advancement and can offer definitive solutions to current barriers and the delay in clinical applicability. Unfortunately, this delay has been impatiently perceived in poorly regulated clinical settings, leading to shortcuts of unreliable stem cell therapies without adequate quality control. Nonetheless, in human-related therapies, overcaution must always be exercised without rushing the application of new materials into living systems. Robust toxicity and adverse outcome studies need to be conducted in advance. Some of the presented technologies in this chapter

were associated with adverse outcomes on cells such as gold, silver, and titanium nanoparticles. Reported alteration of gene expression toward more proliferation and DNA damage could have a significant effect in the case of stem cells. Normal stem cells, with their inherent proliferative capacity, may be readily transformed into cancer stem cells that can initiate and maintain the whole malignant process [133, 134]. In view of these risks, biomaterials incorporated into stem cell therapies would ideally be biocompatible and noninteractive with cellular elements. This can be achieved if more investment is directed toward the fabrication of technological tools such as nanofibers, microfluidic systems, immunosensors, and others using biological materials, especially those derived from suitable human sources.

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Erratum

Erratum: Chapter 6 Clinical Applications of Stem Cells in Women's Reproductive Health

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In the original version of this chapter, the names of second and third authors were not correct. They should read as Sherif Abdelkarim Shazly and Ahmed Abobakr Nassr, respectively.

Index

A

B

C

D

E

F

G

H

I

L

M

N

O

P

Q

R

S

T

U

V

W

X

X-linked adrenoleukodystrophy (ALD)