Gustav Steinhoff *Editor*

Regenerative Medicine - from Protocol to Patient

5. Regenerative Therapies II

Third Edition

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Foreword: Regenerative Medicine: From Protocol to Patient

Third Edition

The vision to unravel and develop biological healing mechanisms based on evolving molecular and cellular technologies has led to a worldwide scientific endeavour to establish *Regenerative Medicine*. This field is involving interdisciplinary basic and (pre)clinical research and development on the repair, replacement, regrowth or regeneration of cells, tissues or organs in congenital or acquired disease. Stem cell science and regenerative biology is prompting the most fascinating and controversial medical development of the twenty-first century. It can be envisaged that this development will establish completely new molecular and cellular techniques for medical diagnosis and therapy. An early rush of scientific development was set up more than one hundred years ago by the physiology of blood regeneration (Hall and Eubanks, 1896) and successful vascular surgical techniques for organ transplantation (Carrel and Guthrie, 1905). However, the clinical realization of allogenic blood transfusion lasted until the discovery of the blood group antigens (Landsteiner and Levine, 1928) and successful routine allogenic organ and bone marrow transplantation even until the end of the last century.

Similar to the field of allogenic cell and organ transplantation it seems that *Regenerative Medicine* again condenses mankind's visions, hopes and fears regarding medicine: hopes of eternal life and effective treatment of incurable disease as well as fears of misuse of technology and uncontrolled modifications of life are polarizing the scientific field. The development and public acceptance of new ethical and regulatory guidelines is a necessary process to support the further clinical development. Nevertheless, the vision of a new medicine using the regenerative power of biology to treat disease and restructure the organism is setting the aim for scientific, technological and medical development. Viewing the great expectations to restructure and regenerate tissue, organs or organisms, the current attempts of scientist and physicians are still in an early phase of development.

The field of *Regenerative Medicine* has developed rapidly over the last 20 years with the advent of molecular and cellular techniques. This collection of volumes on

Regenerative Medicine: From Protocol to Patient aims to explain the scientific knowledge and emerging technology as well as the clinical application in different organ systems and diseases. The international leading experts from four continents describe the latest scientific and clinical knowledge of the field of *Regenerative Medicine*. The process of translating science of *laboratory protocols into therapies* is explained in sections on basic science, technology development, and clinical translation including regulatory, ethical and industrial issues.

This collection is organized into five volumes: *(1) Biology of Tissue Regeneration, (2) Stem Cell Science and Technology, (3) Tissue Engineering, Biomaterials and Nanotechnology, (4) Regenerative Therapies I, and (5) Regenerative Therapies II.*

Biology of Tissue Regeneration (Volume 1) focuses on regenerative biology with chapters on extracellular matrix, asymmetric stem cell division, stem cell niche regulation, (epi)genetics, immune signalling, and regenerative biology in organ systems and model species as axolotl or zebrafish.

Stem Cell Science and Technology (Volume 2) provides an overview as classification of stem cells and describes techniques for their derivation, programming and culture. Basic properties of differentiation states as well as function in human organism are illustrated, and areas of stem cell pathologies in cancer and therapeutic applications for these cells are discussed with emphasis on their possible use in *Regenerative Medicine*.

Tissue Engineering, Biomaterials and Nanotechnology (Volume 3) focuses the development of technologies, which enable an efficient transfer of therapeutic genes and drugs exclusively to target cells and potential bioactive materials for clinical use. Principles of tissue engineering, vector technology, multifunctionalized nanoparticles and nanostructured biomaterials are described with regard to the technological development of new clinical cell technology. Imaging and targeting technologies as well as biological aspects of tissue and organ engineering are depicted.

Regenerative Therapies I (Volume 4) gives a survey on history of Regenerative Medicine and clinical translation including regulation, ethics, and preclinical development. Clinical state-of-the art, disease-specific approaches of new therapies, application technology, clinical achievements, and limitations are described for the central nervous system, head and respiratory system. *Regenerative Therapies II (Volume 5)* contains state-of-the-art knowledge and clinical translation of *Regenerative Medicine* in the cardiovascular, visceral and musculoskeletal systems.

These volumes aim to provide the student, the researcher, the health-care professional, the physician and the patient a complete survey on the current scientific basis, therapeutical protocols, clinical translation and practised therapies in *Regenerative Medicine*. On behalf of the sincere commitment of the international experts, we hope to increase your knowledge understanding, interest and support by reading the book.

After the successful introduction in 2011 with 41 chapters, this work has been actualized and extended for the third edition with into five volumes containing 60 chapters.

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Chapter 1
Blood

Michael Schmitt, Lei Wang, and Mathias Freund

Abstract The transplantation of stem cells from the bone marrow, peripheral blood or cord blood has become a clinical procedure since the 1980s and is now annually performed in 10,000 of patients in the autologous and allogeneic setting world-wide. Refinement of human leukocyte antigen typing as well as recent advances in immunosuppression, anti-infective prophylaxis and therapy as well in supportive care have much improved the outcome of patients with leukemia and lymphoma, aplastic anemia, as well as hereditary diseases of the hematopoietic system. This is still an experimental therapy and patient subgroups that profit most from hematopoietic stem cell transplantation need to be defined. Consideration and classification of co-morbidity indices as well as cytogenetic risk factors are pivotal for making decisions on transplantation modalities. Modern conditioning regimens allow balancing of allo-effects against malignant cells versus normal tissue even in elderly patients. Recent innovations in cellular therapy combine allogeneic stem cell transplantation with genetically engineered or specifically selected T cells and potentially natural killer (NK) cells. Depletion of regulatory T cells and vaccination after allogeneic stem cell transplantation constitute further approaches to improve the long-term outcome of transplanted patients.

Keywords Autologous and allogeneic hematopoeitic stem cell transplantation (HSCT) • Donor lymphocyte infusion (DLI) • Chimeric antigen receptor (CAR) • T cell receptor (TCR) • T cell therapy

1.1 Blood: A Long Way from Replacement to Regeneration

Have you ever asked yourself why the red lights at the crossing are red? It is the color of blood that warns for danger. Blood has ever fascinated man. It is the topic of myths and rites. The shedding of blood initiates deeply rooted fears.

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The first documented attempt to replace this essence of life and to cure deadly disease has been performed in Paris on the 15th of June 1667 by Jean-Baptiste Denis. Blood from a lamb was transfused to a 15 year old boy who survived. More than 300 years later Landsteiner discovered the A, B, O blood group system. He received the Noble Prize for his research in 1930. He laid the fundament for the first successful blood transfusion 1907 in New York by Reuben. 1915 it became possible to conserve blood for transfusion by the addition of citrate. Blood group testing was refined with the discovery of the N, M, and P system and finally in 1939 by the discovery of the Rh system. Blood transfusions increasingly became an essential treatment on the battle fields of the Second World War and later in the Korean War.

As spontaneous recovery from blood loss is evident in healthy subjects and as there are blood diseases in which lifelong blood replacement would be needed the need to advance from replacement to regeneration was obvious. An era of intensive research on bone marrow and its stem cells began in the 1950s.

It was discovered that the infusion of spleen cells promoted blood regeneration and led to survival of supralethal total body irradiation (Barnes and Loutin 1953; Ford et al. 1956; Nowell et al. 1956; Vos et al. 1956). However in 1957 first attempts of clinical bone marrow transplantation in man were unsuccessful in the majority of cases. The reasons were allograft failure and progressive disease (Thomas et al. 1957). Two years later Thomas reported the first successful allogeneic bone marrow transplants from identical twins in two patients with acute lymphoblastic leukemia.

It is remarkable that this program had been started by E.D. Thomas in 1955. It was years before stem cell assays were developed by Donald Metcalf (Bradley and Metcalf 1966) and Leo Sachs (Ginsburg and Sachs 1963). The theoretical and experimental basis of stem cell transplantation had been left behind by clinical application.

Although many human blood stem cell transplantations were carried out between 1958 and 1968, the outcome had not been encouraging. Out of 203 patients transplanted in these times, 125 experienced graft failure, 49 developed lethal graft-versus-host disease (GvHD), and only 11 achieved long-term engraftment. Only three patients were alive when Bortin reported these results in 1970 (Bortin 1970). Many researchers left the field and some voices declared hematopoietic stem cell transplantation as dead.

Those who were not discouraged returned to the laboratory and animal models. After progress in the understanding of the HLA-system and the development of GvHD prophylaxis by immunosuppression transplantation went back to the clinics. The Seattle group realized that patients with far advanced malignant disease and poor general status had a dismal outcome in contrast too patients in the earlier stages (Thomas et al. 1975). It was an enormous adventure at that time to transform this finding into a clinical consequence: to recommend the dangerous procedure to patients in remission, to patients with non malignant or low malignant but long-term dismal disease, and children.

This courage of the early clinical researches and the following developments in high resolution HLA typing, worldwide donor programs, the improvement of the

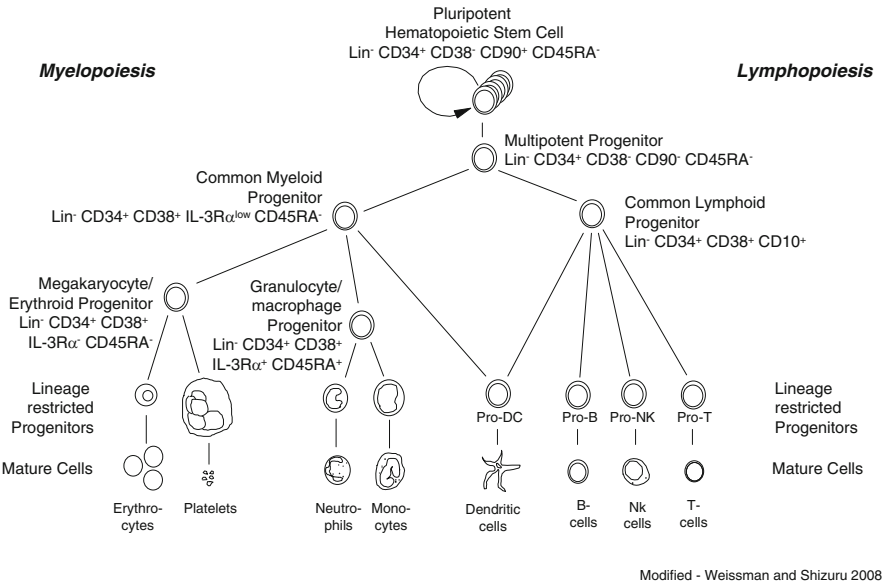

Fig. 1.1 Pedigree of the normal hematopoiesis. Through the influence of cytokines and growth factors like interleukin 3 (*IL-3*), stem cells differentiate into mature cells of the peripheral blood. Partially differentiated progenitor cells are characterized by surface markers, so called "clusters of differentiation" (*CD*) as indicated

conditioning regimen and supportive care have established hematopoietic stem cell transplantation as the first true regenerative therapy. We can learn from that development for other areas of regenerative medicine.

Yet the field of hematopoietic stem cell transplantation is still dynamic. New indications emerge as the procedure is improved. Other indications vanish as their conservative treatment advances. It is the venue of this chapter to give an insight into the actual status of this treatment option.

1.2 Hematopoietic Stem Cells

1.2.1 Basic Properties

Hematopoietic stem cells have the capacity to self-renew and to differentiate in all mature blood lineages (Fig. 1.1). They have been first identified in the mid 1950s by their capability to rescue lethally irradiated mice by reconstituting the entire repertoire of hematopoietic cells. Hematopoietic stem cells are scarce with a frequency of 1:10,000–1,000,000 bone marrow cells. Without stress the majority of stem cells rest in a quiescent state while only a small fraction enters the cell cycle and proliferates to give rise to differentiated progenitors. During infections, acute bleeding, or chemotherapy a large fraction of the stem cells may proliferate.

The regenerative capacity of the stem cells has its evidence in the fact that despite the short lived nature of blood cells a continuous supply of these cells is given even in very long living persons without clinical signs of insufficiency. The self renewal potential of the hematopoietic stem cells is associated with the activity of telomerase. The telomeres at the end of the chromosomes shorten during cell division. This process is reduced by telomerase, a reverse transcriptase which synthesizes new telomeric DNA (Morrison et al. 1996). Telomere shortening is associated with cell cycle arrest, replicative senescence and chromosomal instability. It might be an inhibitory mechanism against the evolution of malignant cell clones.

Despite the activity of telomerase in hematopoietic stem cells, their replication capacity is limited. Serial stem cell transplantations in mice can be done with minimal stem cell numbers for five to seven times until hematopoietic insufficiency occurs (Harrison and Astle 1982). On the other hand it should be noted that transplantation is a severe stress for stem cells. The regenerative potential of stem cells under normal conditions is enormous. It has been concluded from these mouse experiments that hematopoietic stem cells should be able to function normally through at least 15–50 life spans. Therefore hematopoietic insufficiency should not be expected in even very old subjects.

1.2.2 Characterization of Hematopoietic Stem Cells

The study of hematopoietic stem cells is difficult because of their low frequency in the bone marrow. Specific markers or tests for a definitive identification of stem cells are lacking. So in most instances methods have to be combined for the characterisation of stem cells.

1.2.2.1 Surface Markers

In mice hematopoietic stem cells are fairly well characterized by surface markers. A single murine bone marrow cell which is CD34−/lo, CD117+ Sca1+ (stem cell antigen) and negative for lineage-specific antigens is capable of self renewal and multilineage differentiation when transplanted into a recipient mouse (Ema et al. 2000). In humans the phenotypic properties of hematopoietic stem cells are far less well defined.

1.2.2.2 Stem Cells and the Concept of “Niches”

There exists a more than 30 year old concept that the number and behavior of hematopoietic stem cells (HSCs) is regulated by physically discrete locations within the bone marrow for which the French term “niches” was coined. Despite the fact that the precise identities of the niche cells are not yet well defined and controversial, there is an increasing body of evidence that HSCs are retained within the niches by specific adhesion molecules and chemokine gradients (Papayannopoulou and

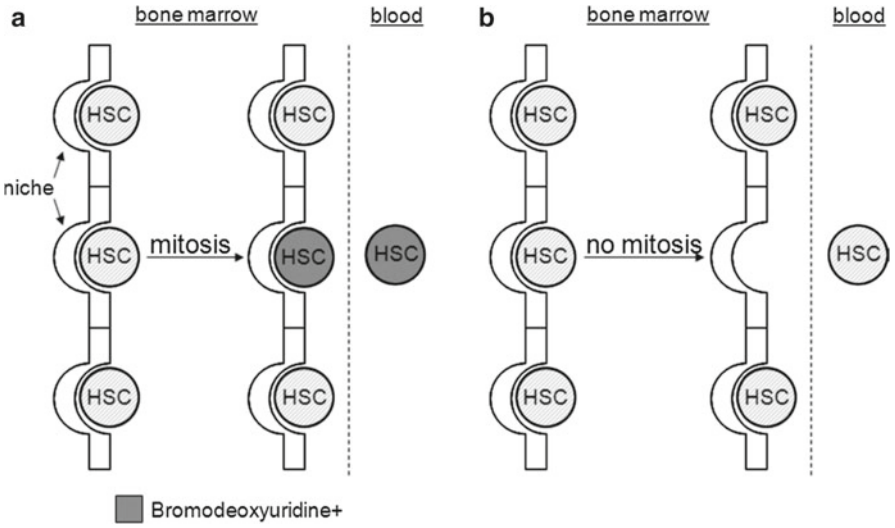

Fig. 1.2 HSC egress is either division dependent or independent (from Bhattacharya et al. 2009). HSCs can either undergo an extrinsically asymmetric division, in which one daughter cell is positioned away from a supportive niche and can thus intravasate to the blood (**a**) or can exit the supportive niche in the absence of cellular division (**b**). In the former model, all HSCs in the blood would be expected to have incorporated BrdU (*gray shaded cells*) after an appropriate feeding period, while the latter model would predict similar low BrdU incorporation rates between bone marrow and blood HSCs

Scadden 2008). By these interactions, HSCs can be assured that they receive appropriate supportive signals that allow them to retain their stem cell identity. In contrast to this concept, there are data suggesting that recipient bone marrow can be readily displaced by transplanted marrow in an efficient and linear dose-dependent manner, even in the absence of conditioning (Colvin et al. 2004). These authors have described a model where HSCs do not reside locked into fixed positions in the bone marrow, but instead they would receive their regulatory signals through limiting quantities of freely diffusible factors.

Work by Irvin Weissman and co-workers (Bhattacharya et al. 2009); (Czechowicz et al. 2007) clearly demonstrated that a certain degree of HSC replacement occurs even in the absence of conditioning. Recent studies could demonstrate that egress of HSCs can be stimulated pharmacologically through administration of plerixafor (AMD3100), an inhibitor of CXCR4. This resulted in the clearance of niches from HSCs. As HSCs and progenitors have been demonstrated to circulate under physiological conditions, a steady-state HSC egress from niches might also allow the engraftment of donor HSCs. One to five percent of HSCs in the murine model enter into the circulating pool every day.

Weissman's group fed their mice with bromodeoxyuridine and found out that HSCs in the circulating pool incorporate the dye at the same rate as bone marrow HSCs (Fig. 1.2). This suggests that HSCs egress from the bone marrow to the blood without cell division and can leave behind them vacant HSC niches (Bhattacharya et al. 2009).

Fig. 1.3 Stem cell donor during leukapheresis. A leukapheresis device (Spectra Optia™) is put into a circuit between the left and the right cubital veins. It separates white blood cells containing stem cells by a density gradient method

1.2.3 Stem Cell Sources

Initially, bone marrow was obtained from healthy HLA-matched sibling donors of the patients. Donors were subjected to intubation and anesthesia. In a prostate position bone marrow was aspirated from the upper posterior iliac crest and transferred into a transfusion bag. After stem cell counting and microbiological and virological evaluation of the bone marrow blood preparation the stem cell containing bone marrow is transfused into the patients by the way of a central venous catheter.

Bone marrow aspiration under sterile conditions in the operation theater is costly and cumbersome. Moreover, stem cells mobilized from the bone marrow niches into the peripheral blood by subcutaneous administration of granulocyte-colony stimulation factor (G-CSF) or plerixafor to the donor will result in a 1 week earlier engraftment when compared with bone marrow. Therefore most of the stem cell preparations given nowadays to adult patients are peripheral blood stem cells collected by leukapheresis (Fig. 1.3). Through magnetic cell separation using anti-CD34 monoclonal antibodies labeled with magnetic beads (Fig. 1.4) a highly purified fraction of CD34+ stem cells can be prepared. Only for younger patients with e.g. aplastic anemia (see below), bone marrow stem cells are preferred due to the better reconstitution of the bone marrow with all its components (Schrezenmeier et al. 2007).

A novel source for hematopoietic stem cell is cord blood. Its application has been initiated by Eliane Gluckman at the Hôpital Saint Louis, Sorbonne VII Paris

Fig. 1.4 Purification of stem cells through a magnetic cell separation device (CliniMACS® by Miltenyi, Bergisch Gladbach/Teterow, Germany). Cells (in the *red* bag above the device) are labeled by monoclonal antibodies (*moAbs*) against the stem cell marker CD34. These moAbs are coupled with magnetic beads. The beads stick to a magnet during the first run of a buffer (the bag with a clear solution on the right side) though the column. When the column is detached from the magnet, a second eluate containing the marker-positive (stem) cells can be obtained

(Czechowicz et al. 2007) and has been practiced in more than 4,000 patients in Japan (Kodera 2008). Hitherto, the transplantation of cord blood derived stem cells is restricted by the number of stem cells from this source when put into relation with the average body weight of a European patient undergoing HSCT. Application of several cord blood preparations as "dual" or even "triple" cord blood transplantation gets into practice (Arachchillage et al. 2010). While the extended time of engraftment make the patient even more prone to opportunistic infections, cord blood stem cells do obviously not have to be HLA-identical Mismatches cause less GvHD in the cord blood setting than in the setting of PBSCT or bone marrow transplantation (Barker et al. 2010).

1.2.4 Stem Cell Doses for Transplantation

Different sources of stem cells like bone marrow, peripheral blood and cord blood can yield different amounts of stem cells with various pros and cons (Table 1.1):

For autologous transplantation one would like to administer 2×10^6/kg body weight (BW) of the recipient. In the case of allogeneic HSCT the desirable CD34+

Table 1.1 Stem cell sources and stem cell doses

Source	Pro's/con's	CD34+ stem cell count
Bone marrow	Aspiration requires general anesthesia	Median of 2.8×10^6/kg body weight (BW)
Peripheral blood	Easy collection, but G-CSF side effects	Median of 7.0×10^6/kg BW
Cord blood	Easy, immediately available, partial HLA mismatches acceptable	Median of 0.2×10^6/kg BW

stem cell count would be at least $>2.5 \times 10^6$/kg BW, better $>5.0 \times 10^6$/kg BW for peripheral blood stem cells, and at least $>1.0 \times 10^6$/kg BW, better $>2.5 \times 10^6$/kg BW for bone marrow blood stem cells.

1.3 Principles of Hematopoietic Transplantation for Regeneration in Blood Diseases

Hematopoietic stem cell transplantation can be performed in two principally different situations: (1) Stem cells can be harvested in a patient with malignant disease and can be used to induce and/or accelerate hematopoietic regeneration after myelosuppressive or myeloablative treatment procedures. (2) Stem cells from a healthy volunteer donor can be transplanted for hematopoietic recovery of patients with non-malignant and malignant blood disorders.

1.3.1 Autologous Transplantation

The rationale in the autologous setting is to deliver as intensive cytotoxic treatment to the patient as possible. The basis of this concept is the finding that in a certain dose range of irradiation or cytotoxic treatment, the effect on the tumor increases in a steep linear relationship (Fig. 1.5). However this dose range is not equal in all tumors and with increasing doses there is increasing damage to hematopoiesis and organs. As organ damage may occur later in some agents an autologous transplantation of hematopoietic stem cells might open a therapeutic window for dose intensification.

This concept (Fig. 1.6) has been proven most convincingly in lymphomas, Hodgkin's disease and multiple myeloma. Attempts to apply high dose chemotherapy in other diseases as sarcomas or some other solid tumors have not been as successful, probably due to the fact that chemotherapies active in these diseases are very toxic to the organs so that the window opened by autologous hematopoietic transplantation is small or non-existing. Although autologous transplantation is given even in disseminated hematologic disease as the acute leukemias, this treatment principle is not convincing in these entities as potentially tumor stem cells are also re-infused with the graft. Purification of grafts by chemical of immunologic

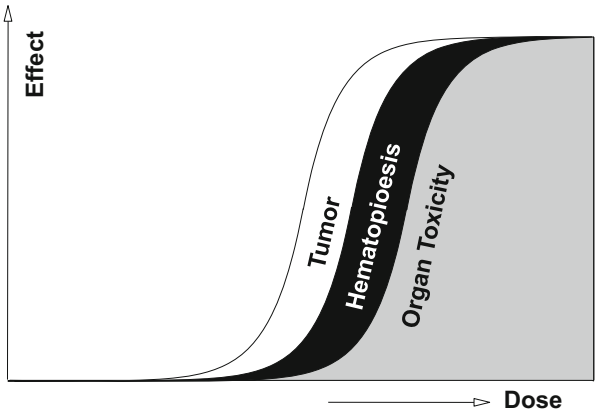

Fig. 1.5 Dose of cytotoxic treatment and toxic effects on tumor, hematopoiesis and organs. The higher the dose, the myelotoxicity or even organ toxicity

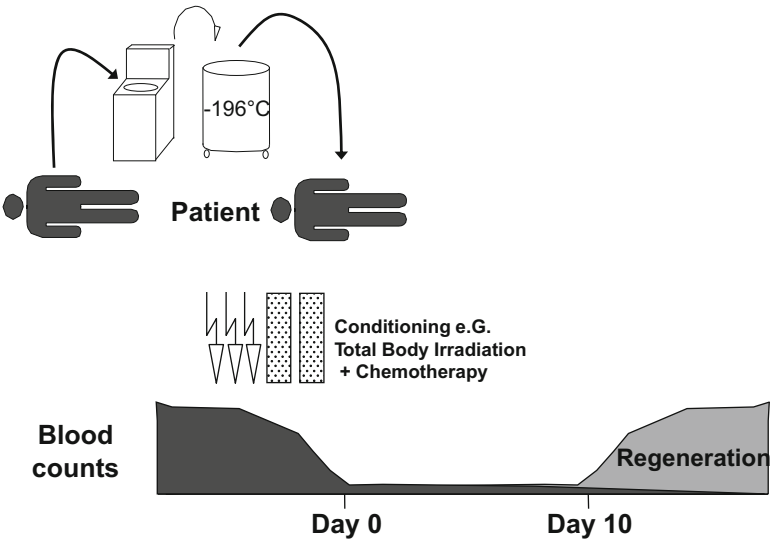

Fig. 1.6 Schema of autologous transplantation. Stem cells are collected from a hematological patient by leukapheresis after mobilization with e.g. cyclophosphamide and granulocyte-colony stimulating factor (*G-CSF*). Stem cells are stored in liquid nitrogen. The patient undergoes a conditioning regimen with e.g. total body irradiation (*TBI*) and cyclophosphamide. Thereafter the autologous stem cells are given back to the patient. The blood counts drop under a conditioning regimen of TBI and chemotherapy. Regeneration of hematopoiesis starts by day 10 after transplantation

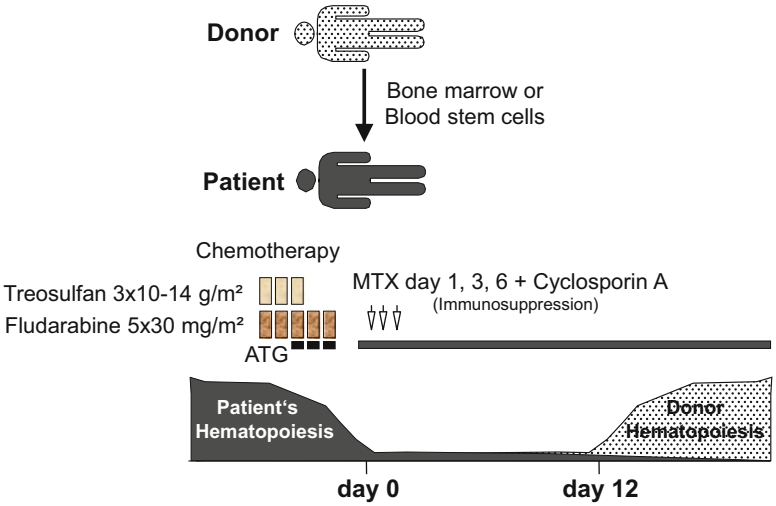

Fig. 1.7 Schema of allogeneic transplantation with the treosulfan-fludarabine conditioning regimen. After a conditioning with a treosulfane and fludarabine containing chemotherapy regimen the patient obtains stem cells from the bone marrow or peripheral blood of a healthy donor. To avoid graft rejection immunosuppression with e.g. methotrexate (*MTX*) or cyclosporine A is administered. As early as by day 12 reconstitution of the hematopoietic system is effective

methods have not yielded better results and are hampered by side effect e.g. immunosuppression by T-cell depletion in CD34+ selected grafts.

1.3.2 Allogeneic Transplantation

The basic concept of allogeneic transplantation is to replace malignant or deficient hematopoiesis by tansplantation from another individual (Fig. 1.7). Consequently allogeneic transplantation has been first applied in irradiation injuries and aplastic anemia.

As immunocompetent cells are normally transferred with the graft and as minor immunologic disparities might lead to a graft-versus-host reaction concomitant immunosuppressive prophylaxis has to be given in the allogeneic setting. However in contrast to organ transplantation the immunosuppression can be omitted in most patients after immunologic reconstitution.

Allogeneic transplantation is widely applied in children and adult patients with genetic diseases, immunodeficiency syndromes, aplastic anemia, leukemias and lymphomas.

1.4 Diagnostics and Indications of "*blood*" Regenerative Therapies

Histocompatibility is a basic prerequisite for allogeneic transplantation. Future concepts of allogeneic transplantation might overcome this principle to some extent. Another very important limitation for the application of allogeneic transplantation is the presence of comorbidities of the patients which become more prevalent with increasing age. A third overwhelming important factor for the outcome after autologous and allogeneic transplantation is the disease status and risk group. The latter is discussed later.

1.4.1 HLA Compatibility

The human leukocyte antigen (HLA) system is the human equivalent of the major histocompatibility complex. T cell recognition of peptide epitopes derived from functional or structural proteins is essential for the engraftment or rejection of the transplant and directly linked to GVHD and GVL reactions. Two classes of HLA molecules can be distinguished: HLA-A,-B and -Cw are class I molecules with three alpha subunits and a beta-2 microglobulin molecule, while class II molecules such as HLA-DR,-DQ and –DP are composed of two alpha and two beta subunits (see Fig. 1.8) (Klein and Sato 2000a, b). In their groove, HLA-ABC molecules present 9–11 amino acid residues long peptides to the T cell receptor of CD8+ T lymphocytes while class II HLA-D molecules present 15–20mer peptides to CD4+ T lymphocytes (Rammensee et al. 1993).

Minor histocompatibility antigens (miHAgs) such as HA-1 (den Haan et al. 1998) can both elict GVHD (Mutis et al. 1999) but can also contribute to the recognition of leukemic blast i.e. to the GVL (Goulmy et al. 1996) effect. Moreover natural killer cells, their surface molecules and killer cell inhibitory (KIR) molecules contribute GVHD and GVL, and are of pivotal importance for allogeneic stem cell transplantations in the haploidentical setting (Moretta et al. 2009).

1.5 Standardized Treatment, Technologies

1.5.1 Autologous Hematopoietic Stem Cell Transplantation (HSCT)

1.5.1.1 Conditioning Regimens

Stem cell transplantation requires always the preparation of the bone marrow compartment in particular but also the whole patient to receive the graft. The term "conditioning" has been coined for this central process of the stem cell transplantation

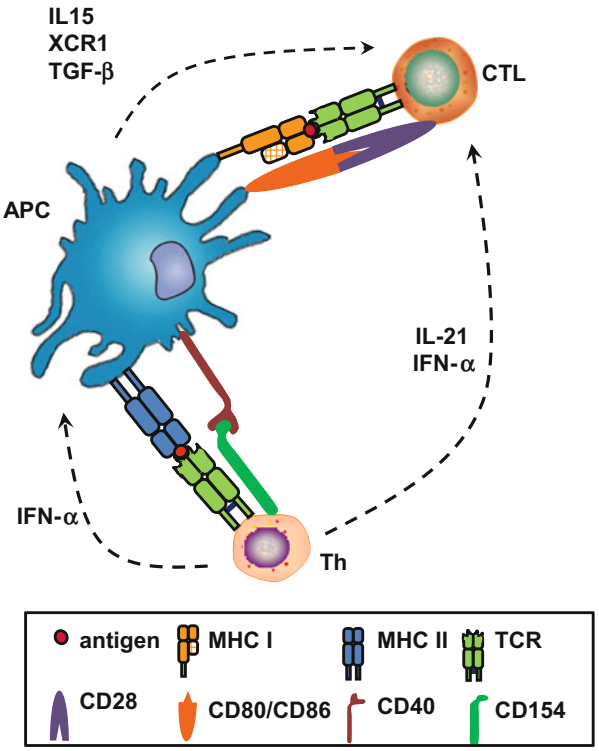

Fig. 1.8 Recognition of antigen epitopes by CD4+ and CD8+ T lymphocytes. Peptides derived from antigens are presented on HLA-molecules class I and II. A second signal is effective by binding of costimulatory molecules with their ligands. Interaction of T helper cells and cytotoxic T cells occurs through interleukines. (*CTL* CD8+ cytotoxic T lymphocytes, *Th* CD4+ T helper cells, *APC* antigen presenting cell, *TCR* T cell receptor, *IL* Interleukin, *DC* clusters of differentiation, *CD28, CD40, CD80, CD86, CD154* Co-Stimulatory molecules, *HLA* human leukocytes antigen, *TGF β* Transforming growth factor beta, *XCR* chemokine (C motif) receptor 1, *IFN-α* Interferon-alpha, *IL-15/-21* Interleukin-15/ -21)

which constitutes an integral part of the HSCT. However, there is an ongoing debate on how to bring the patient in the best condition possible to accept the graft.

The "creation of space" has been postulated as a major goal of conditioning. The original concept was that immature progenitor cells occupy circumscriptive bone marrow niches to gain the necessary support from feeder cells of the stroma for their proliferation and maturation. To this end the patient's stem cells have to be eradicated to provide the donor stem cells with access to these important niches so that engraftment might occur. The concept of reduced intensity conditioning (see below) with a fading in-fading out phenomena of donor stem cells versus host stem cells has at least to some extent relativated this concept. One might not necessarily have to eradicate the complete bone marrow to give a graft a realistic chance to engraft successfully. However, this depends on the underlying disease and the dynamic of the malignant clone.

In general the antitumor activity of the conditioning regimen is also needed to further reduce the tumor burden before transplantation. This is particularly true in autologous transplantation where no graft-versus-tumor effect is present.

For autologous HSCT standard conditioning regimens are in common practice according to the disease entity. For patients with multiple myeloma, 200 mg/m^2 melphalan has shown the best survival. For patients older than 60 years this dose might be reduced to 140 mg/m^2. Lymphoma patients (Hodgkin's disease (HD) and Non-Hodgkin-Lymphoma (NHL)) obtain most commonly a combination of carmustine (bis-chloronitrosourea; BCNU), etoposide, cytarabine and melphalan (Mills et al. 1995). Purging of transplants for lymphoma patients constitutes an interesting approach; however it can be associated to hypoglobulinemia or secondary malignancies such as MDS or AML (Gyan et al. 2009). For patients with acute myeloid leukemia (AML), autologous HSCT has become rather rare, even more in Europe than in the US. The reasons are multiplex. In the last decade there has been no major progress in that field. Some centers report higher survival of patients receiving a purged transplant. But this has never been proven in a randomized trial. Most importantly, only half of the patients allocated to autologous HSCT for AML reach the transplantation because of relapse of the disease or a poor graft. For acute lymphoblastic leukemia the picture is even clearer. Several studies showed no difference for the comparison of chemotherapy versus autologous HSCT or even a significantly inferior outcome for auto-HSCT (Goldstone et al. 2008).

1.5.1.2 Mobilization

A standard protocol for the mobilization of autologous stem cells requires cyclophosphamide 1.5 g/m^2 on day 1 followed by 10 μg/kg BW/day G-CSF on days 2–12. Stem cells can be collected on days 10–12 when the WBC count reaches 8 G/L post nadir. In the case of poor mobilizers plerixafor (AMD3100) might be given in concert with G-CSF: on day 4 give additionally plerixafor at a dose of 160–240 μg/kg BW i.v. or i.m. 6–12 h before the intended harvest.

1.5.2 Allogeneic Hematopoietic Stem Cell Transplantation (HSCT)

1.5.2.1 Conditioning Regimens

The favorable results of allogeneic stem cell transplantation depend not only on chemotherapy and irradiation, but also on the allo-effect which is introduced by the graft from a family donor or unrelated donor. Therefore conditioning regimes for allogeneic HSCT must include immunosuppression to prevent a host-versus-graft reaction. Transplanted donor cells might be immediately attacked by immune cells of the host. Natural killer (NK) cells, T lymphocytes as well as dendritic cells (DCs)

are involved in the complex interplay. There is a particular need for immunosuppression in the case of increasing human leukocyte antigen (HLA) disparities. The risk for graft rejection is also increased through pre-sensitization against minor histocompatibility antigens (miHAgs), e.g. through multiple blood product transfusions into the host preceding the allogeneic HSCT.

From the historical perspective, HSCT has been understood as a potential cure for patients irradiated through atomic bomb explosions or nuclear accidents. Therefore, total body irradiation (TBI; 12 Gray [Gy]) was tested first as conditioning method. TBI was efficient in eliminating the hematopoietic system, but TBI alone could not eradicate the leukemic clone. Only by adding cyclophosphamide (Cy) to TBI in patients at early stage disease, the first successful allogeneic HSCTs could be performed in the 1970s. In further studies TBI was replaced by the "radiomimetic" busulfan (Bu), i.e. Bu/Cy. Other alkylating drugs like melphalan (Mel) or carmustine (BCNU), as well as "leukemia-specific" drugs like cytarabine (ARA-C), etoposide (ETO) and 6-thioguanine (6-TG) followed in a conditioning regimen termed BACT. As for TBI/Cy and Bu/Cy there are several differences in toxicities (more venous occlusive disease [VOD], more permanent alopecia with Bu/Cy), but both regimens are comparable in terms of long-term survival of the patients with the exception of ALL, where TBI/Cy is more effective.

With regard to the reduction of transplantation-related mortality (TRM) and the quality of life (QoL) the concept of "reduced intensity conditioning (RIC)" was born. In preclinical experiments the requirements for a stable engraftment were evaluated, and subsequently low and even lowest dose TBI (2–4 Gy) were used as well as conditioning regimens with fludarabine (Flu), Bu, Mel and Cy. RIC concepts became particularly interesting in the context of donor lymphocyte infusions (DLIs) which where inaugurated at the begin of the 1990s as a tool to bring patients with myeloid disease back into remission.

A revolutionary development in recent conditioning regimens is the postponement of cyclophosphamide after transplantation of the graft. Allo-HSCT has two major limitations. The first relates to the procedure's toxicity, including conditioning regimen toxicity, graft-versus-host disease (GVHD), and infection. The second limitation is the lack of histocompatible donors. A human leukocyte antigen (HLA)-matched sibling or unrelated donor cannot be identified expeditiously for up to 40 % of patients. In general HSCT from partially HLA-mismatched donors, or HLA-haploidentical relatives has been complicated by unacceptably high incidence of graft rejection, severe GVHD, and non-relapse mortality. Recently, Luznik et al. have developed a method to selectively deplete allo-reactive cells in vivo by administering high doses of cyclophosphamide in a narrow window after transplantation (Fig. 1.9). Using high-dose, post-transplantation cyclophosphamide, crossing the HLA barrier in allo-HSCT is now feasible and donors can be found for nearly all patients (Luznik et al. 2012; Kanakry et al. 2014).

In haploidentical hematopoietic stem cell transplants, post-transplant cyclophosphamide together with standard prophylaxis reduces the incidence of GVHD to acceptable rates without the need for T cell depletion. In matched related and unrelated donor settings, cyclophosphamide alone or in combination with solely

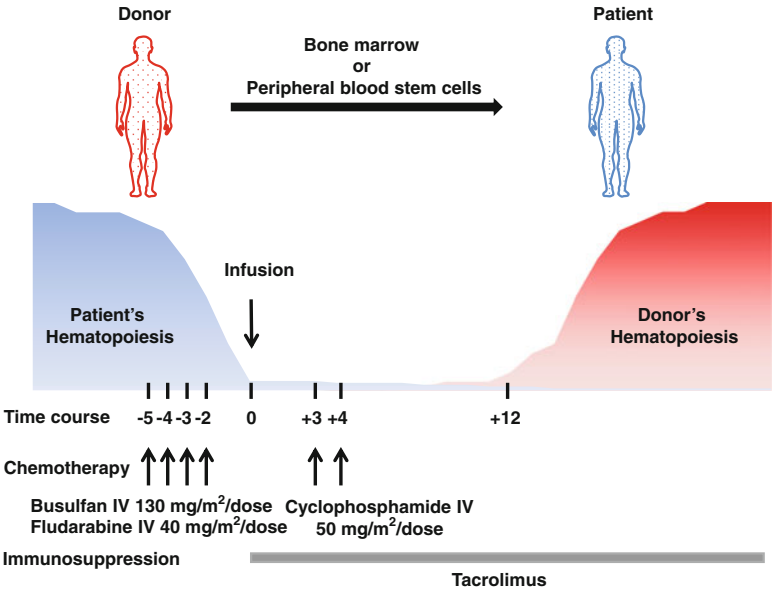

Fig. 1.9 Transplantation platform. The treatment schema is shown. Busulfan dosing was adjusted based on measured pharmacokinetics. Only Tacrolimus was administered as graft-versus-host disease prophylaxis after post-transplantation cyclophosphamide on days +3 and +4

Tacrolimus has produced encouraging results. Interestingly, only a low incidence of chronic GVHD has been observed (Al-Homsi et al. 2015).

1.5.2.2 Graft-Versus-Host Disease (GvHD)

Graft-versus-host disease (GvHD) constitutes one of the most serious complications after allogeneic stem cell transplantation. The underlying pathomechanism is the recognition of host-specific proteins by T cells of the donor which were transferred together with the donor stem cells or maturated thereof. Moreover dendritic cells of the host are involved in this complex interaction of the immune system depicted schematically in Fig. 1.10.

Clinical manifestations of GvHD can be detected on skin, liver, and GI tract. A macula-papular rash can develop on the upper part of chest and back as well as a palmo-plantar exanthema. It might involve the entire integument and might lead to desquamation or even the development of bullae. Cholestatic hepatopathy with classical jaundice, increased serum levels of bilirubin as well as of cholestatic enzyme (whereas transaminases rather show no specific changes) are signs of GvDH of the liver. This needs to be differentiated from the veno-oclusive disease (VOD) which occurs rather early after allogeneic HSCT even before immunoreconstitution and subsequently the basis for a GvHD occurs. VOD is characterized by liver pain, ascites, impaired flow of intrahepatic veins and elevated serum levels of bilirubin and cytokeratin 17.

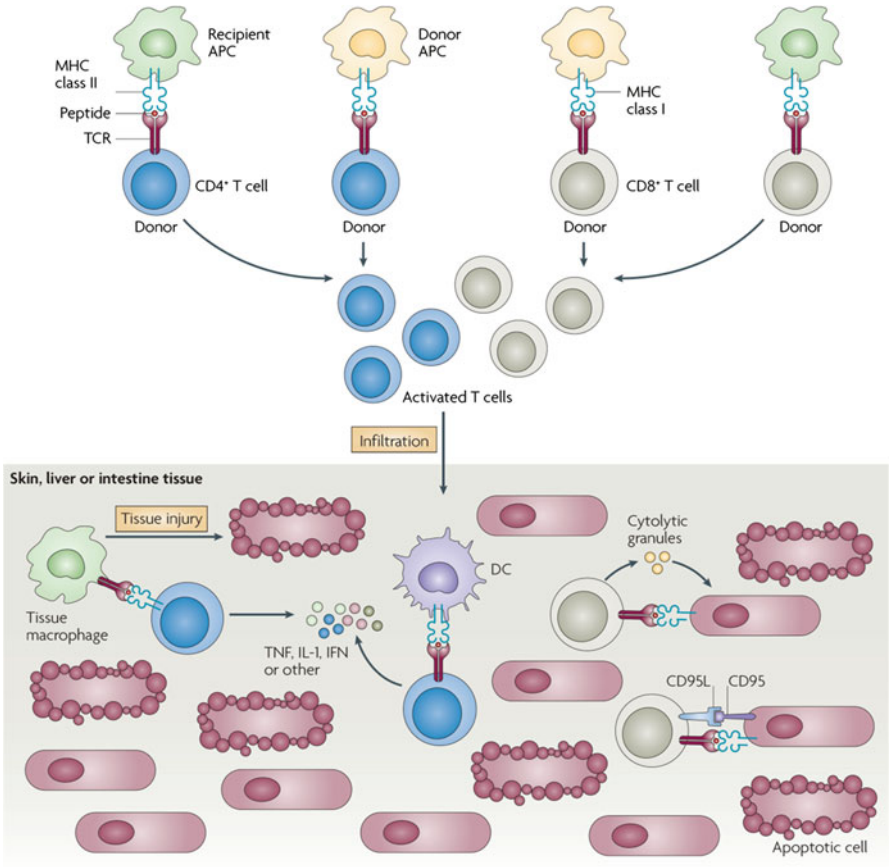

Fig. 1.10 GvHD – mechanism of injury by tissue-infiltrating alloreactive T cells. Activated alloreactive CD8+ T cells require direct cognate interactions with MHC-class-I-expressing parenchymal targets (non-haematopoietic tissues such as skin, liver or intestine) to induce injury through the expression of CD95 ligand (*CD95L*) and the production of cytolytic granules. By contrast, infiltrating CD4+ T cells can mediate graft-versus-host disease (*GVHD*) without directly contacting MHC-class-II-expressing non-haematopoietic tissues. CD4+ T cells could be activated locally by tissue macrophages and dendritic cells (*DCs*) to release inflammatory mediators, such as tumour-necrosis factor (*TNF*), interleukin-1 (*IL-1*) and interferon (*IFN*), or alternatively may activate recipient antigen-bearing macrophages which induce tissue injury. *APC* antigen-presenting cell, *TCR* T-cell receptor (Shlomchik 2007)

GvHD of the GI tract manifests as nausea, vomitus, loss of appetite, diarrhea and consequently weight.

The classical grading system inaugurated by Glucksberg et al. in 1974 stages four grades for each organ system according to the percent of integument with maculo-papular rash, the serum level of bilirubin and the quantity of diarrhea (Glucksberg et al. 1974).

Besides the acute type of GvHD described above which is defined as a GvHD starting within the first 100 days from the day of allogeneic HSCT, there exists also

a chronic form of the disease. Chronic GvHD is characterized by the clinical feature of sclerodermia, Sjögren's syndrome, primary biliary cirrhosis, wasting syndrome, bronchiolitis obliterans (BO), as well as chronic cellular immunodeficiency.

Of note, the desirable Graft-versus-leukemia (GVL) effect seems to be interwoven with the noxious GvHD which can be explained by the fact that a general T cell activation can always results in an activation of an anti-leukemic T cell clone. Therefore, patients with a grade I to II GvHD show a better overall survival as the anti-leukemia/-lymphoma effect overweighs the immune aggression of the graft towards the recipient's organs.

1.5.2.3 Immunosuppressive Agents Against GvHD

In an attempt to alleviate the immune attack of the graft, an appropriate immunosuppressive prophylaxis as well as in the case of occurrence of GvHD an intensified immunosuppressive therapy is mandatory.

Standard drugs for GvHD prophylaxis include anti-thymoglobuline (ATG), campath (a monoclonal antibody against CD52, a pan-lymphocyte marker), methotrexate (MTX), cyclosporine A (CSA/CyA), mycophenolate mofetil (MMF) or its pro-drug MPA. Therapy of GVHD consist primarily of (methyl-) prednisolone, tacrolimus, sirolimus, everolimus, basiliximab/anti-interleukin-2, rituximab, blockers of tumor necrosis factor alpha (anti-TNFa), pentostatin. Extracorporeal photopheresis (ECP) can often cause tremendous improvement of GvHD without putting the patient at an increased risk for infectious disease. Mesenchymal stem cells (MCSs) are under current investigation for the treatment of GvHD and hold promise.

1.6 Clinical Experiences with Stem Cell Transplantation as a Paradigm of Regenerative Medicine

1.6.1 Genetic Diseases

1.6.1.1 Hemoglobinopathies (Thalassemia and Sickle Cell Disease [SCD])

Stem cell transplantation constitutes the only curative therapy for patients with thalassemia or sickle cell disease, although significant progress has been made in the field of supportive care such as oral therapy with iron chelators. Particularly in adulthood, severe affection of parenchymal organs by hemoglobinopathies is obvious and lifethreatening. According to the recommendations of the EBMT (2008) (http://www.ebmt.org/EBMT_Handbook.html), definite indications for HSCT constitute transfusion-dependent alpha- or beta-thalassemia major, transfusion-dependent HbE/beat-thalassemia when the patient is not older than 16 years and has a HLA-identical family donor. In special circumstances thalassemia in adult patients between 17 and 35 years might be considered.

Definite indications for HSCT in patients with sickle cell disease (SCD) depend on the existence of one or more of the following clinical complications: (1) SCD-related neurological deficit, stroke or subarachnoid hemorrhage. (2) Recurrent sickle chest syndrome or failure of response to therapy with hydroxyurea (HU) or contraindication of HU. (3) Recurrent and severe debilitating pain due to vaso-occlusice crises despite therapy with HU or contraindication of the drug (Amrolia et al. 2003a, b).

Conditioning regimens for hemoglobinopathies include Bu/Cy or Treosulfan/Cy in concert with campath/alemtuzumab or ATG. The transplantation-related mortality is very low with 2–5 % and the risk of graft failure less than 5 %. Three independent factors for the outcome of HSCT for thalassemia in children have been established by the Pesaro classification: hepatomegaly, portal fibrosis as diagnosed by liver biopsy and lack of compliance with iron chelation. By positive selection of CD34+ stem cells with a minimal contamination of T cells HSCT was also possible in the haploidentical setting from mother to child in the absence of a HLA-matched donor (Sodani et al. 2010).

For SCD there is no good score, however SCD patients who suffer from impaired quality of life (QoL) as stated above profit most from HSCT. The results of HCST for SCD are enchanting with an overall survival is very favorable with about 90 %, a disease free survival about 85 %, and both TRM and graft rejection <10 % (Bhatia and Walters 2008).

1.6.1.2 Severe Combined Immunodeficiency (SCID)

SCID usually manifests as opportunistic infections in early childhood such as cytomegalovirus or fungal infections, or as absence of thymic shadow on chest X-ray films lymphocytopenia. Allogeneic HSCT constitutes the sole cure for these young children and procures a survival rate of more than 90 %. In the absence of an appropriate donor, MUD or haploidentical family donors are also acceptable. The usual conditioning regimen consists of Bu/Cy with the addition of ATG in the case of a MUD. As GvHD caused by the patients inability to reject the allogeneic cells, usually the graft is CD34-positive selected. However, the patients require a protective environment and anti-infective prophylaxis as well as intravenous substitution with immunoglobulins over at least 3 months. Due to the absence of B-cell engraftment in about 40 % of the patients, these SCID patients require life-long immunoglobulin replacement therapy. In the meantime tremendous progress has been made and the results for haploidentical donors are as good as for HLA-genoidentical donor (Antoine et al. 2003).

Gene therapy for SCID-X1 patients has been undertaken with success (Hacein-Bey-Abina et al. 2003) as well as for SCID-ADA (Aiuti et al. 2009). Nevertheless many points regarding infectious complications and secondary neoplasia through vector integration are still poorly understood and need elucidation (Neven et al. 2009).

1.6.2 Aplastic Anemia

Aplastic anemia (AA) is a life-threatening disorder of the hematopoietic system characterized of bi- or pancytopenia. Severe AA (SAA) is defined by insufficient marrow production in at least two hematopoetic cell lines, and very severe AA (VSAA) if the neutrophil count is less than 0.2 G/L. Differential diagnosis of AA comprises paroxysmal nocturnal hemoglobinopathy (PNH), congenital dysplastic anemia (CDA), hypoplastic myelodysplastic syndrome (MDS). The ultimate goal of therapy for AA is freedom from transfusions. This can be achieve either by immunosuppressive therapy including CSA, ATG and prednisolone, or HSCT. The 5-year survival data are with 70–80 % similar for both treatment options (Bacigalupo and Passweg 2009).

BMT is superior over peripheral blood HSCT in AA (Schrezenmeier et al. 2007). A survival benefit of BMT over mere immunosuppressive therapy (IS) has been established for patients younger than 40 years with VSAA and in patients in whom ISA failed.

The standard regimen for conditioning comprises Cy 200 mg/kg BW with or without ATG. With ATG less chronic GvHD and a better survival has been observed than with Cy alone (Storb et al. 1994). For patients older than 30 years, also non-myeloablative conditioning regimens with fludarabine have been successfully established (Piccin et al. 2010). There is rather limited information on the use of haploidentical donors and cord blood in patients with AA to make a definite statement at that time. The right immunosuppressive treatment after HSCT for AA is an ongoing debate. ATG, CSA and steroids are the standards. Some favor the addition of G-CSF to CSA and ATG. Recently, the supplier withdrew the equine ATG from the market and replaced it by rabbit ATG which is produced in the same manner, i.e. by stimulation with human thymocytes.

1.6.3 Myeloproliferative Neoplasias (MPN) of the Hematopoiesis

Chronic myeloid leukemia (CML) as one type of MPN is classically characterized by the translocation t(9;22) resulting in the Philadelphia chromosome has been considered as the paradigm of diseases which can be cured by HSCT. Through the advent of the tyrosinase kinase inhibitors (TKIs) such as imatinib mesylate (IM), dastinib and nilotinib, this feature has completely changed (Deininger 2008). IM has replaced interferon-alpha as first line therapy for CML. From the IRIS study the following results were reported: The cumulative best complete cytogenetic response (CCyR) rate was 82 %; 63 % of all patients randomized to receive imatinib and still on study treatment showed CCyR at last assessment. The estimated event-free survival at 6 years was 83 %, and the estimated rate of freedom from progression to AP and BC was 93 %. The estimated overall survival was 88 % – or 95 % when only

CML-related deaths were considered. This 6-year update of IRIS underscores the efficacy and safety of imatinib as first-line therapy for patients with CML.

Therefore any decision on HSCT for CML has to take into account the following aspects: age of the patient, disease phase, duration of disease, nature of stem cell donor and the genders of recipient and donor, as well as the response of the patient to TKIs. As more and more TKIs like e.g. DCC-2036 targeting the difficult-to-treat mutation T315I (Chan et al. 2011) are available on the market one might suggest patients to use one TKI after the others as patients with HIV infection take different combinations of highly active anti-retroviral therapy (HAART) after the other, thus living with their disease for decades without dying from the disease. These considerations have been implemented in an EBMT risk factor score.

The role of allogeneic stem cell transplantation in chronic myeloid leukemia is being reevaluated. Whereas drug treatment has been shown to be superior in first line treatment, data on allo SCT as second line therapy after imatinib failure are scarce. Three year survival after transplantation of 56 patients in chronic phase was 91 % (median follow-up 30 months). Transplantation related mortality was 8 %. In a matched pair comparison of transplanted and non-transplanted patients, survival was not different. Three year survival after transplantation of 28 patients in advanced phase was 59 %. Eighty-eight percent of transplanted patients achieved complete molecular remissions. We conclude that allo SCT could become the preferred second line option after imatinib failure for suitable patients with a donor (Saussele et al. 2010).

Allogeneic HSCT is certainly not an option for CML patients in full manifest blast crisis; here newer TKIs are anyway more successful. After achieving a second chronic phase allogeneic HSCT is feasible.

Standard conditioning for HSCT in CML patients are Bu/Cy or TBI/Cy. An interesting aspect is that non-myeloablative HSCT followed by donor lymphocyte infusions (DLIs) for female patients with CML in fertile age open them a therapeutic option to bear a child after HSCT (Ringhoffer et al. 2007). This might require asservation of oocytes prior to HSCT. In contrast TKIs constitute an absolute contraindication to pregnancy because of their high teratogenic potential.

1.6.4 Acute Myeloid Leukemia (AML)

Acute myeloid leukemia (AML) constitutes a clonal disorder of the myeloid lineage which is associated with unlimited proliferation and loss of differentiation. AML is the most common adult leukemia with an incidence of about 3 per 100,000 inhabitants. Several characteristic chromosomal aberrations such as translocations t(8;21), t(15;17), t(9;11) and others can be detected in half of the patients. The majority of the remaining AML patients can be characterized by different mutations of genes like the *FMS-like tyrosine kinase type 3* gene (*FLT3*), the *mixed lineage leukemia* (*MLL*) gene, *the nucleophosmin 1* (*NPM1*) gene, the gene encoding *runt-related transcription factor 1* (*RUNX1*), the *tet oncogene family member 2* (*TET2*), and others (Dohner and Gaidzik 2011).

Several systems have been established to stratify AML patients according to these genetic abnormalities for their relapse risk. This risk stratification has led to treatment schedules with a different intensity of polychemotherapy to counterbalance the effects and side effects of cytostatic drugs used in these treatment algorithms (Dohner et al. 2010). To achieve a high rate of complete remissions (CR) in patients younger than 60 years old, the application of four chemotherapy cycles with cytarabine is considered to be essential (Dohner et al. 2010). Elderly patients with AML might be better treated by daunorubicin (Lowenberg et al. 2009). However, there is a big difference between CR which can be achieved in 60–80 % of adults younger than 60 years on one hand, and the 25–30 % 5-year survival rate of all AML patients. This clearly indicates that despite hematological CR, a sufficient number of leukemic cells can hibernate in their bone marrow niches to survive conventional chemotherapy. Here HSCT which combines chemotherapy, irradiation as well as the allo-effect can eradicate such hibernating leukemic (stem) cells (Koreth et al. 2009).

However, there is a transplantation-associated mortality (TAM) of 20–25 % which is made up by opportunistic infections, by GvHD as well as by relapse of the disease. Standard conditioning consists of busulfan and cyclophosphamide or TBI, but phase III trials including fludarabine and treosulfane are also underway at our institution and others (Casper et al. 2010; Beelen et al. 2008).

In AML patients at relapse or with refractory disease, a conditioning regimen inaugurated by the group around Hans-Jochem Kolb has proven great efficacy with even up to 30 % long-term survivors in this deleterious situation: after a block of fludarabine, amsacrine and cytarabine, the patient in aplasia receives a "non-myeloablative" conditioning with only 2 × 2 Gy TBI and standard dose of cyclophosphamide. The acronym FLAMSA has been coined for this particular conditioning approach (Schmid et al. 2006). Donor lymphocyte infusion might even improve the outcome of this approach if given at day +100 or later in the absence of GVHD (Schmid et al. 2007).

The data of the Acute Leukemia Working Party (ALWP) of the Euopean Bone Marrow Transplantation (EBMT) Society demonstrate in a large cohort of 2,100 patients that autologous HSCT in patients with AML in first remission can result in an outcome at 5 years with an overall survival of 51 %, a relapse rate of 53 % and a TAM of only 9 % (Breems and Lowenberg 2007). The relapse rate is too high in this autologous setting. Moreover, it is not possible to collect enough stem cells in more than half of the AML patients and early relapse of the disease might even prevent autologous transplantation.

Therefore, the autologous approach has been given up in many centers and has been clearly surpassed by allogeneic HSCT. Patients at low cytogenetic risk and with a certain mutation status (NPM1pos. while FLT3-ITDneg.) might be sufficiently treated by chemotherapy. The majority of AML patients with intermediate cytogenetic risk allogeneic HSCT should be performed if a HLA-matched sibling is available for transplantation. Patient with high cytogenetic risk and a low or moderate co-morbidity score will profit from early transplantation in first CR. Patients at relapse of the disease will also profit from allogeneic HSCT. A special type of

AML, the acute promyelocytic leukemia (APL; former AML FAB M3) characterized by the translocation t(15;17) is never recommended for HSCT.

1.6.5 Acute Lymphoblastic Leukemia (ALL)

Treatment results in adult acute lymphoblastic leukemia (ALL) have improved considerably in the past decade, with an increase of complete remission rates to 85–90 % and overall survival rates to 40–50 %. Superior chemotherapy and supportive care, the integration of hematopoietic stem cell transplantation (HSCT) into frontline therapy, and optimized risk stratification were important developments. Even more impressive is the success of targeted therapies in subgroups of ALL. In the formerly most unfavorable subgroup, Philadelphia chromosome (Ph)/BCR-ABL-positive ALL, survival now ranges from 40 % to 50 % after incorporating imatinib in combination chemotherapy. In mature B-ALL, survival rates increased above 80 % with the combination of short intensive chemotherapy and rituximab. The prerequisite for comprehensive therapy is standardized and rapid diagnosis and classification as the basis for treatment stratification (Gokbuget and Hoelzer 2009).

Allogeneic hematopoietic stem cell transplantation as part of post-remission therapy improves survival for adult patients with high-risk acute lymphoblastic leukemia. A meta-analysis of seven studies including almost 1,300 patients demonstrated that patients in the donor groups had significantly better survival than patients in the no-donor groups (hazard ratio [HR], 1.29; 95 % confidence interval [95 % CI], 1.02–1.63 [P=0.037]). In high-risk patients with Philadelphia-positive ALL, the superiority of the survival advantage was even greater (HR, 1.42; 95 % CI, 1.06–1.90 [P=0.019]). A meta-regression analysis revealed that compliance with allogeneic HSCT showed a significant and positive correlation with survival (coefficient, 0.022; P<0.01). This suggests that allogeneic HSCT improves the outcome of adult patients with high-risk ALL. Allogeneic HSCT should be considered for such patients if a suitable donor is available (Yanada et al. 2006).

1.6.6 Chronic Lymphocytic Leukemia (CLL)

Chronic lymphocytic leukemia (CLL) constitutes the most common leukemia in western industry countries. In recent years both autologous and allogeneic HSCT have been exploited to treat CLL patients. Peter Dreger and others defined criteria for poor-risk CLL patient according to the EBMT CLL transplant consensus (Dreger et al. 2007): these are defined as non-response or early relapse (within 12 months) after purine analogue-containing therapy, relapse with 24 months after purine analogue combination therapy or treatment of similar efficacy (i.e. autologous HSCT) or p53 deletion/mutation (del17p13) requiring treatment. There is now only limited hope that autologous HSCT could cure CLL. Rather there is cumulating evidence

that the graft-versus-leukemia (GVL) effect is crucial for the therapy of CLL by allogeneic HSCT.

Several leukemia-associated antigens (LAAs) have been identified in CLL patients (Giannopoulos et al. 2009). Allogeneic HSCT from matched related or unrelated donors can overcome the treatment resistance of poor-risk CLL as defined above. Even with reduced-intensity conditioning, allogeneic HSCT in CLL is associated with significant mortality and morbidity due to (chronic) GVHD, which has to be weighed against the risk of the disease when defining the indication for transplantation. Therefore, it can be regarded as a reasonable treatment option only for eligible patients who fulfill accepted criteria for poor-risk disease. If allogeneic HSCT is weighted, it should be performed before CLL has advanced to a status of complete refractoriness to assure an optimum chance for a successful outcome. Prospective trials are underway to prove whether allogeneic HSCT might indeed change the natural course of poor-risk CLL (Dreger 2009), particularly del17 patients and patients after the use of the anti-CD52 monoclonal antibody alemtuzumab (Brown 2011).

By the advent of novel therapeutical agents such as the Bruton kinase inhibitor ibrutinib and the monoclonal antibody of atumumab, the indication of allo-HSCT for CLL needs to be redefined (Dreger et al. 2014).

1.7 Conclusions and Future Perspectives on "*blood*" Regenerative Therapies

Autologous and allogeneic hematopoietic stem cell transplantation is well established as a treatment modality to escalate antineoplastic therapy in some diseases and to replace deficient or neoplastic hematopoiesis in others. Alas, the results of autologous transplantation are hampered by a high incidence of relapses and the limiting barrier for the expansion of allogeneic transplantation is the increasing incidence of GvHD in older patients and with increasing HLA disparities.

In autologous transplantation the improvement of remission quality and the prolongation of remission duration by introduction of new anti-neoplastic agents into remission and maintenance is intensively studied. We will not extend on this perspective as it is not in the center of interest in regenerative medicine.

In allogeneic transplantation GvHD on one side and immune competence after transplant on the other side is correlated with the presence and absence of different immune cells in the graft. These cells become increasingly well defined and methods are developed to isolate and manipulate them. Therefore much hope rests on engineered grafts for the improvement of allogeneic transplantation. The result of this development might be the elimination of virus infections after allogeneic transplantation and the enhancement of graft-versus-tumor effect.

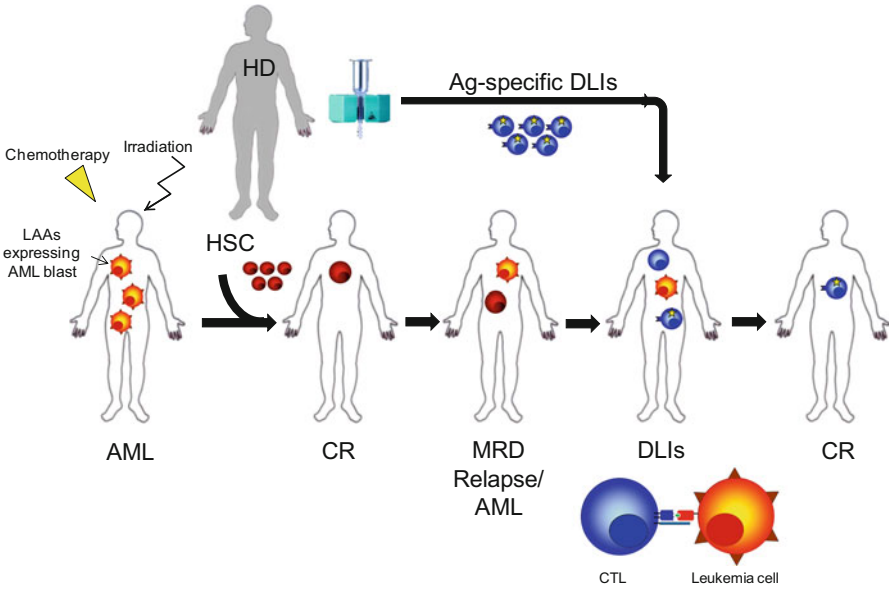

Fig. 1.11 GVL effect after HSCT and DLIs/Ag-specific CTLs. After chemotherapy and irradiation, HSC are infused for the re-population of the bone marrow. However, the graft exerts not only a GVL effect, but potentially also causes GvHD. In case of relapse, either DLIs or antigen-specific CTLs are infused with a good outcome against leukemia cells due to the GVL reaction. *LAA* leukemia associated antigen, *HD* healthy donor, *HSC* hematopoietic stem cell, *DLIs* donor lymphocyte infusions, *CR* complete remission, *MRD* minimal residual disease, *AML* acute myeloid leukemia (Taken from our recent publication (Casalegno-Garduno et al. 2010))

1.7.1 Selective Donor Lymphocyte Infusions (sDLIs)

Multimers such as tetramers, pentamers, or streptamers, mimick the immunological synapses that naturally occur between T cells and antigen presenting cells (APC), through the T cell receptor (TCR) and major histocompatibility complex (MHC). Multimers are used to select either virus- or antigen-specific T cells (Casalegno-Garduno et al. 2010). However, only streptamers are currently available at GMP level.

Streptamer selection might facilitate the adoptive transfer of T cells, thus dissecting both GvHD from GVL effects. Streptamers can select either antigen- or virus-specific CTLs from seropositive donors (Fig. 1.11). This multimer is reversible and the CTLs remain functionally active.

We have own experience with the transfer of CMV-specific T cells into patients after allogeneic stem cell transplantation who suffered from recurrent CMV antigenemia. In two patients, the course of CMV antigenemia was definitively controlled after a single DLI with CMVpp65 specific CD8+ T cells (Schmitt et al. 2010).

1.7.2 Translation Towards Leukemia Antigen-Specific DLIs

In an ongoing study (Wang et al. 2010), we observed a significant difference in the frequency of WT1-specific CD8+ T cells in healthy donors and AML patients. It was possible to purify the WT1-specific T cells labeled with specific streptamers coupled with magnetic beads by MACS™ columns, and we achieved a purity of up to 90 %. In our eyes this approach holds promise for clinical application in patients after allogeneic stem cell transplantation and detectable (minimal) residual disease to achieve a GVL effect without GvHD. This approach is currently extended to other LAAs.

1.7.3 Ways to Improve the Homeostatic Expansion of Antigen-Specific CD8+ T Cells

With the paradigm of CMV-specific T cells we demonstrated that adoptively transferred antigen-specific cells can expand by homeostatic expansion in the periphery bypassing thymic priming (Surh and Sprent 2005). A similar homeostatic expansion of leukemia antigen-specific T cells is highly desirable. This might be facilitated by the depletion of CD4 + CD25hi regulatory T cells (Tregs), the shift in the balance of CD8 vs. CD4 + CD25hi Tregs. To this end several methods are under current investigation (Mielke et al. 2011). Administration of T cells by cytostatic drugs like cyclophosphamide and fludarabin as well as total-body irradiation might cause at least to some extend lymphodepletion. Most importantly, vaccination against leukemia-antigens like WT1, PR1 or RHAMM (Schmitt et al. 2008) has been performed after chemotherapy and autologous stem cell transplantation. Now the challenge is to administer vaccines after allogeneic stem cell transplantation to booster the anti-leukemic GvL effect and to maintain this reaction for an extended time period. The format of post-transplantation vaccines (Rezvani 2011) might be a peptide (Rapoport et al. 2011) or a truncated protein. Dendritic cells transfected with RNA encoding the leukemia antigen (Van Tedeloo et al. 2010) constitute a further option as well as K562 cells transfected with the gene encoding granulocyte macrophage colony stimulating factor (GM-CSF), a vaccine designated GVAX.

1.7.4 Genetic Engineering of T Cells

Recently, the treatment paradigm for cancer, including hematological malignancies, is gradually shifting from relatively nonspecific therapies towards targeted personalized therapies (Vanneman and Dranoff 2012). As one example, immunotherapy has opened new avenues for the treatment of hematological malignancies.

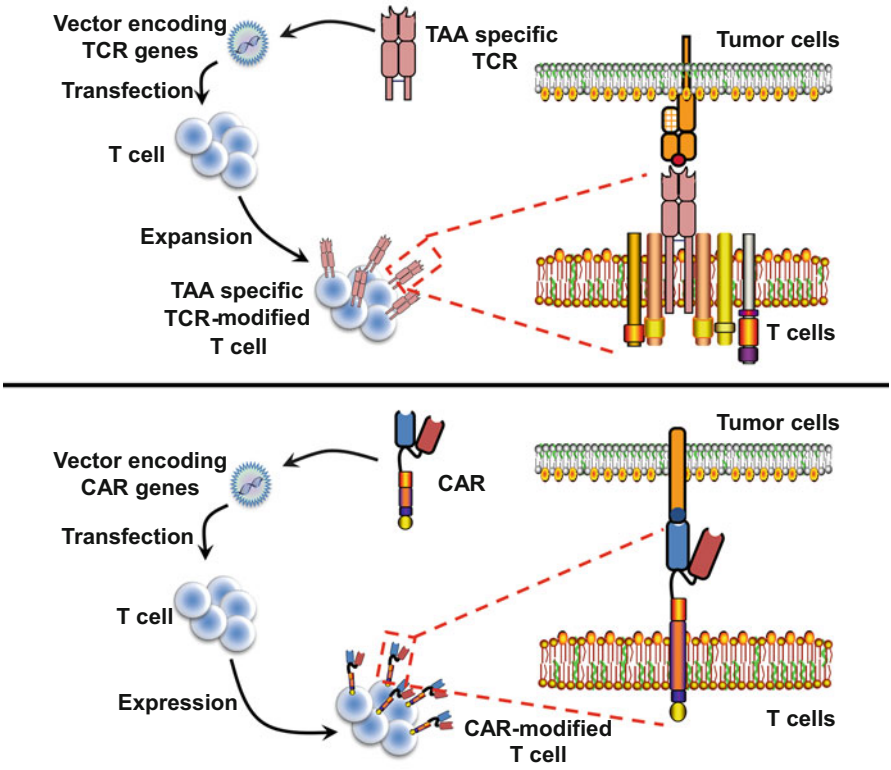

Fig. 1.12 Genetic engineering of T cells. (**a**) T cell receptor therapy (*upper panel*). The genetic information for a T cell receptor (*TCR*) recognizing a particular tumor associated antigen (*TAA*) is transferred into T cells from the patient (or stem cell donor) by using retro-/lenti-viral vectors or RNA. T cells gain specificity for the respective TAA and can be expanded to attack tumor cells bearing the TAA peptide on their HLA class I molecules on the surface of the tumor cells. This peptide can be derived from surface, but also from intracellular, even intranuclear antigens. (**b**) Chimeric antigen receptor (*CAR*) T cell therapy (*CART*) (*lower panel*). The basic principle of the therapy is the same as above. However a single chain fragment from a monoclonal antibody recognizing a specific antigen epitope from the TAA has been genetically fused with the intermembraneous and intracellular part of a TCR. Thus HLA-specificity could be circumvented. But only surface- or membrane-bound molecule can be targeted by CART

Immunotherapy, as "the breakthrough of the year for 2013", harnesses the immune system to eliminate tumour cells, constituting a totally different way of treating hematological malignancies (Couzin-Frankel 2013). It includes diverse strategies that range from antibody- and tumour antigen (TA)-peptide-based approach to genetically engineered and chimeric antigen receptors (CARs)-transfected T cell therapy (Fig. 1.12).

Although derived from self tissue, tumour cells can express certain molecules, termed tumour antigens (TAs), which can be used to discriminate tumour cells from most normal tissues. T cells, via their T cell receptor (TCR), can sometimes respond

to TAs but, because tumours are derived from self tissue, a process that is termed immune tolerance usually operates that renders T cells unable to respond to tumour cell antigens. During the process of immune tolerance induction, which operates throughout the lifetime of an individual, tumour-specific T cells are either deleted or rendered non-responsive to self molecules, including TAs. Therefore, except in relatively rare cases, tumour specific T cells cannot be isolated from most individuals with cancer. However, T cells that are reactive against tumours can be generated using genetic engineering, which involves inserting into the T cells genes encoding cell surface receptors that are able to detect TAs. These receptors can be TCRs, derived from responding patients or mice, or CARs, which are synthesized using molecular biology techniques (Kershaw et al. 2013).

1.7.4.1 Genetically Engineered T Cell Therapy

Overcoming immunosuppression in patients and reconstituting cellular immunity after HSCT, as well as enhancing the GvL effect while decreasing GvHD is a highly desirable goal. Transduction of T cells with a specific TCR can be transferred into the transplanted patient. It might offer an effective antitumor treatment strategy. Recently, TCR-engineered precursor cells as a controllable immunotherapeutic modality with significant anti-leukemia activity were generated. The co-transfer of engineered precursor T cells gives rise to mature T cells that display specific antigen recognition upon induction in leukemia-bearing mice. After antigen exposure, effector memory and central memory populations are generated. It showed that early induction of the TCR is a prerequisite for the development of a mature T-cell population with defined TCR specificity by favoring the differentiation into CD8+ T cells and allowing a leukemia-reactive T-cell subset to escape negative selection (Hoseini et al. 2015). Furthermore, in a study of generating NY-ESO-1-specific redirected T cells with multiphenotypes, antigen-specific effector cytokine release and target cell lysis have been detected in vitro. In vivo, the functional activity of these redirected T cells was also conformed, which could protect mice against NY-ESO-1 positive myeloma cells. Results from a phase II clinical trial to evaluate the safety and activity of autologous T cells genetically engineered to express an HLA-A2 restricted, affinity enhanced NY-ESO-1/LAGE-1 specific-TCR have shown prolonged T cell persistence, homing to the bone marrow, and antitumor activity. Encouraging clinical results were obtained as stringent complete response/complete response (sCR/CR) in 2/15 (13 %), near complete response/CR (nCR/CR) in 10/15 (67 %) and partial response/CR in 3/15 (20 %) after day 100 post-transplant infusion (Schuberth et al. 2013; Stadtmauer et al. 2013; Kalos et al. 2012).

1.7.4.2 CAR-Transfected T Cell Therapy

CARs, chimeric immunoglobulin-TCR molecules were first synthesized by Gross in 1989 (Gross et al. 1989). Typically, CARs consist of an extracellular target-binding module, comprising variable domains of both heavy (VH) and light (VL)

chains of a tumor-specific monoclonal antibody, a transmembrane domain, the intracellular signaling domain, with one or more costimulatory signaling molecules. CARs endow T cells with MHC-unrestricted ability to avoid tumor escape through down regulation the expression of MHC molecule. Alternatively, because of the target-binding moiety derived from antibodies with affinities several orders of magnitude higher than TCR, CARTs can bypass the fundamental issue of central tolerance. In preclinical study, gene-modified T cells directed against the carbohydrate antigen Lewis Y (LeY) was generated, with antigen-restricted INF-g secretion and cell lysis in vitro. Its antitumor efficacy in vivo was demonstrated by delaying growth of myeloma xenografts in NOD/SCID mice, and superior OS (Peinert et al. 2010). Moreover, other CARTs against CD38, B-cell maturation antigen, CD44v6 or CS1 also have shown an anti-myeloma effect, respectively (Carpenter et al. 2013; Casucci et al. 2013; Mihara et al. 2012; Chu et al. 2014). Considered a potent approach in cancer immunotherapy, CARTs have debuted in the clinical arena. A number of clinical trials of CARTs directed to a variety of antigens are under way. Several centers have focused some effort to studies of CD19-directed CARTs and to other B cell markers such as immunoglobulin light chains and CD20, in part because hematopoietic cells have been extensively characterized and the expression of their surface molecules is often lineage-dependent. Recently, a phase 1 dose-escalation trial reported that CD19-CARTs therapy is feasible, safe, and mediates potent anti-leukemic activity in children and young adults with chemotherapy-resistant B-precursor acute lymphoblastic leukaemia. All toxicities were reversible and prolonged B-cell aplasia did not occur (Lee et al. 2015).

Suggested reading for more information on all aspects of hematopoietic stem cell transplantation:

1. The EBMT Handbook – Haematopoietic Stem Cell Transplantation, 6th Edition. J. Apperley, E. Carreras, E. Gluckman, T. Masszi, Forum Service Editore, Genoa, Italy, 2012. http://ebmtonline.forumservice.net/
2. The BMT Data Book Including Cellular Therapy. 3rd Edition. Reinhold Munker, Gerhard C. Hildebrandt, Hillard M. Lazarus, Kerry Atkinson. Cambridge University Press, Cambridge, UK, 2009. ISBN 9781107617551 paperback.
3. Hematology 2014. The American Society of Hematology Education Program Book. http://asheducationbook.hematologylibrary.org/
4. Clinical Bone Marrow and Blood Stem Cell Transplantation. 3rd Edition. Kerry Atkinson, Richard Champlin, Jerome Ritz, Willem E. Fibbe, Per Ljungman, Malcom K. Brenner. Cambridge University Press, Cambridge, UK, 2004. ISBN 0-521-82912-7
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Chapter 2
Vascular Regeneration Therapy: Endothelial Progenitor Cells for Ischemic Diseases

Masaaki Ii, Atsuhiko Kawamoto, Haruchika Masuda, and Takayuki Asahara

Abstract Since the discovery of circulating endothelial progenitor cells (EPC) in adult human peripheral blood, EPCs are believed to home to sites of neovascularization, where they contribute to vascular regeneration by forming a structural component of capillaries and by secreting angiogenic factors, thereby enhancing vascular and blood flow recovery in ischemic tissue. This therapeutic strategy has been effective in animal models of ischemia, and we and other clinical trials have demonstrated that it was safe and feasible for treatment of critical ischemic limb and cardiovascular diseases. However, the decline of EPCs in the peripheral blood and evidence that several disease states reduced EPC number and/or function have prompted the development of several strategies to overcome these limitations, including the administration of genetically modified EPCs that overexpress angiogenic growth factors. To optimize therapeutic outcomes, investigators must keep refining methods of EPC purification, expansion, and administration, and to develop techniques that overcome the intrinsic decline and phenotypic deficiencies of EPCs. In this chapter, we have illustrated EPC biology and the therapeutic potential of EPCs for vascular regeneration demonstrating our data of clinical study.

Keywords Stem/progenitor cell • Cell therapy • Ischemia • Angiogenesis • Regeneration

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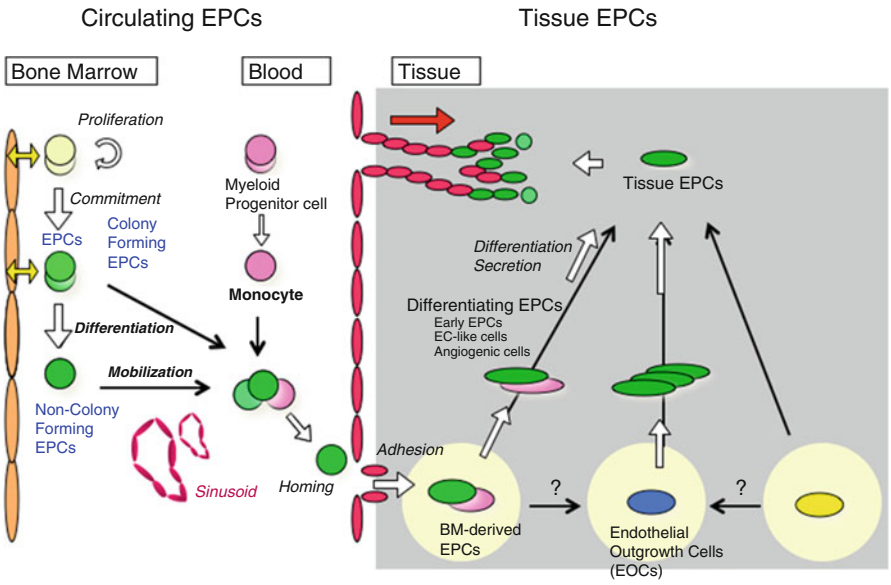

Fig. 2.1 Kinetics of circulating EPCs and tissue EPCs. The relationship among EPCs in the bone marrow (BM), blood, and organ tissues, and their differentiation cascade is represented in the figure. *HPP* high proliferative, *LPP* low proliferative, *ECFC* endothelial colony forming cell

2.1 Introduction

Recently, endothelial progenitor cells (EPCs) have been isolated from adult human peripheral blood (PB) (Asahara et al. 1997). EPCs are shown to be derived from bone marrow (BM), and to accumulate in active angiogenic foci and participate in neovascularization following ischemic insults (Asahara et al. 1999a, b), exhibiting common stem/progenitor cell characteristics. The evidence that BM derived EPCs home to sites of neovascularization differentiating into endothelial cells (ECs) in situ is consistent with "vasculogenesis", a critical paradigm well described in embryonic neovascularization, but recently proposed in adults in which a reservoir of stem/progenitor cells contribute to post-natal vascular formation (Fig. 2.1). The discovery of EPCs has therefore drastically changed our understanding of adult blood vessel formation specifically in ischemic tissue. The following issue highlights the potential utility of EPCs for therapeutic angio/vasculogenesis in ischemic diseases, updating the notion of EPC biology.

2.1.1 Biological Characteristics and Definition of EPCs

Endothelial Progenitor Cells or "EPCs" were originally described as blood bound cells with the ability to differentiate into the endothelial lineage (Lyden et al. 2001). Believed to be a "progenitor cell", EPCs were thought to be able to reside in their

immature state and upon the encounter of appropriate stimuli to migrate, proliferate or differentiate into a more mature lineage, capable of either direct contribution to or at least support of regenerative processes, namely the regeneration of the injured cardio-vascular system.

EPCs are currently believed to be represented by the following hallmarks: (1) the ability for endothelial lineage commitment, and the acquisition of an EC specific or "EC-equivalent" phenotype, (2) initial immatureness, while preserving the competence to differentiate, indicated by a primitive progenitor cell phenotype and the (partial) lack of mature EC markers, and (3) the presence of pro-angiogenic and vasculogenic properties, with a strong biological activity towards neo-vascular formation resulting in functional recovery and regeneration of the injured vascular system. Besides these general hallmarks EPCs can be distinguished and subdivided into various categories:

2.1.1.1 Tissue EPCs vs. Circulating EPCs

Based on their in vivo classification, one can distinguish between "tissue EPCs" and "circulating EPCs". Tissue EPCs are characterized by their adhesive nature and the fact that they can be isolated directly from organ tissues, representing either EPCs in the wake of differentiation originating from the circulation, so called "homed-down" circulatory EPCs, endothelial outgrowth cells (EOCs) of a yet to be defined origin, or cells of the endothelial lineage which are directly derived from organ-based stem and progenitor cells such as cardiac stem cells (Beltrami et al. 2003), neural stem cells (Ii et al. 2009), myogenic stem cells or mesenchymal stem cells (Kovacic and Boehm 2009) (Fig. 2.1). On the other hand, circulating EPCs are cellular components of blood which can be isolated from peripheral blood (PB), umbilical cord blood (UCB), bone marrow (BM), and from organs or organ blood vessels. Circulating EPCs emerge as floating, non-adhesive cells present in and moving throughout the circulatory system. A "suspended, non-attaching blood cell state" is therefore most characteristic for circulating EPCs which can mobilize and be recruited from preservative and educational niches in the BM into the blood stream, and home to sites of ischemic and/or vascular distress, contributing to the regeneration of the target tissue by transforming into adhesive EPCs.

2.1.1.2 Hematopoietic EPCs vs. Non-hematopoietic EPCs

Circulating EPCs can be subdivided into two main categories, hematopoietic lineage EPCs (h-EPCs) and non-hematopoietic lineage EPCs (nh-EPCs) (Fig. 2.1). The h-EPCs originate from BM and represent a pro-vasculogenic subpopulation of hematopoietic stem cells (HSCs). The h-EPCs can enter circulation upon stimulation as cellular components of blood, compromising a possibly heterogeneous cell population, represented by for example colony forming EPCs, non-colony forming

"differentiating" EPCs, myeloid EPCs or angiogenic cells etc.. The nh-EPCs are not HSC-derived cells, which can be isolated from blood or tissue samples via the help of adhesive cell culture techniques and distinguished by their rather obvious EC (-like) phenotype. The origin of nh-EPCs remains to be clarified, but they are generally thought to be derived from non-hematopoietic tissue prone lineage stem cells or organ blood vessels.

The h-EPCs can be further subdivided into three distinct classes. The first class is represented by EPCs which can be classified as direct descendant of HSCs, which can form immature hematopoietic-like EPC colonies and commit into circulating EC-like cells. The second class is represented by myeloid cells derived from myeloid progenitors, already committed to the myeloid lineage, but still capable to differentiate into EC-like cells, mimicking an endothelial cell phenotype. The third type is represented by cells, loosely termed circulating angiogenic cells (CACs), which can give rise to EC-like cells and contribute to neovascularization mainly by the secretion of pro-angiogenic growth factors. The characterization and identification of HSC-derived EPCs are tightly linked to and associated with the methods and markers already applied in the hematopoietic field. EPCs and HSCs can both be isolated using antibodies)against various cell surface markers, including membrane receptors like CD34, CD133, Flk-1/KDR, CXCR4, CD105 (Endoglin) for human samples (Asahara et al. 1997; Quirici et al. 2001; Peichev et al. 2000; Handgretinger et al. 2003; Gehling et al. 2000; Friedrich et al. 2006; Eggermann et al. 2003) and receptors like c-Kit (Patschan et al. 2006), Sca-1 (Hamada et al. 2006; Iwakura et al. 2003) and CD34 (Patschan et al. 2006; Heeschen et al. 2003) in combination with Flk-1 (VEGFR2) in case of mouse samples. Nevertheless, the identification of a unique combination of receptors specific and selective for primary EPCs, enabling an unambiguous distinction between EPCs and HSCs is still missing. The)introduction of a definitive assay system capable of clearly distinguishing between EPCs and HSCs, thus enabling the identification of the long sought precise primary EPC phenotype, is highly anticipated but still missing.

2.1.1.3 Colony Forming EPCs vs. Non-colony Forming EPCs

Colony Forming EPCs

A novel recently developed EPC colony forming assay (EPC-CFA) system, capable to address and overcome most of the above mentioned limitations of the classical assay systems, is challenging several of the predominant classical opinions about EPCs, and enabling an until now missing differential hierarchic view on EPCs. We recently reported one of the first examples of such an assay system, initially designed to work with mouse samples. c-Kit$^+$/Sca-1$^+$/Lineage negative (KSL) cells were used as a putative murine hematopoietic EPC-enriched cell population, allowing the identification of two clearly distinguishable types of colonies (small and large colonies) that in turn correspond to two distinct EPC populations, primitive (small) and definitive (large) EPCs, respectively (Kwon et al. 2008; Tanaka and Sata 2008;

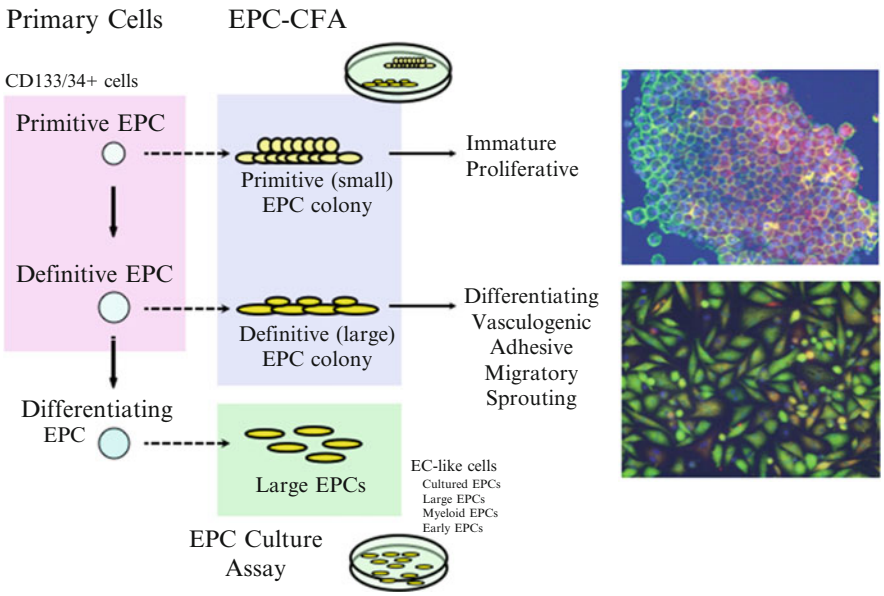

Fig. 2.2 Differentiation cascade of blood EPCs and in vitro EPC assay system. (1) Heterogeneous cell populations including myeloid cell-, lymphoid cell-, and EPC-aggregates are assessed by Hill's colony assay system (2) relatively purified EPC-rich cell populations including primitive (small) EPCs and definitive (large) EPCs are assessed by EPC-CFA, and (3) small/large EPCs, monocytic EPCs, and angiogenic monocyte/macrophages are assessed by culture EPC assay system

Kamei et al. 2010) (Fig. 2.2). The concept of an EPC-CFA was recently introduced and further developed for analysis of human EPC samples (Masuda et al. unpublished data). The EPC-CFA enables hereby not only the EPC-colony formation analysis of single and/or bulk cells from EPC-enriched arbitrary fractions or non-selected cell populations but allows also the cell fate analysis of primary and/or suspension culture cultivated single and/or bulk cells. It can further be easily combined with a classical HPC colony assay system, thus allowing a direct and comprehensive elucidation of the differences and similarities between EPCs and HPCs via the clarification of the cell fate of each cell type. The use of such an EPC-CFA allows not only the elucidation of a possible but so far elusive differentiation hierarchy of EPCs, but can be further used to identify and characterize the parameters associated with proliferation, commitment, and differentiation of EPCs in vitro and in vivo.

Indeed, application of EPC-CFA on human CD34+ or CD133+ stem/progenitor cells enabled the identification of small and large distinct colony types each derived from a single cell, small-EPCs and large-EPCs, respectively (Fig. 2.2). Small-EPCs showed a higher rate of proliferative activity with a higher number of cells being in the S-phase, when compared to large-EPCs. Interestingly, large-EPCs showed a

significantly higher rate of vasculogenic activity with overall increased potential for cell adhesion and tube-like structure formation in vitro as well as a high in vivo de novo blood vessel forming activity following transplantation of these cells into a murine ischemic hindlimb model, as compared to small-EPCs. In contrast to small-EPCs, large-EPCs did not form secondary colonies but gave rise to isolated endothelial cell (EC) like cells when reseeded. Due to the observed in vitro (by FACS analysis) and in vivo characteristics of these colony types, small-EPCs were further characterized and believed to represent "primitive EPCs", a highly immature and proliferative population of cells, compared to large-EPCs which are believed to represent "definitive EPCs", cells prone to differentiate and promote vasculogenesis**.**

Non-colony Forming EPCs

The widely used "classical EPC culture assay" systems are characterized by the appearance of adhesive endothelial lineage (-like) cells upon conditioning of PB- or BM-derived mononuclear cells with endothelial growth factor supplemented media (Dimmeler et al. 2001; Takahashi et al. 1999; Vasa et al. 2001a). These overall reproducible and standardized assay systems were used for the characterization of a wide range of EPCs, ranging from "cultured EPCs" (Dimmeler et al. 2001; Vasa et al. 2001a; Kalka et al. 2000a; Murohara et al. 2000; Sharpe et al. 2006), "EC-like cells" (Timmermans et al. 2009), "early EPCs" (Sharpe et al. 2006; Gulati et al. 2003; Hur et al. 2007) to so called "circulating angiogenic cells" (Rehman et al. 2003; Shepherd et al. 2006), which generally do not form colonies under conventional endothelial differentiation conditions (Fig. 2.2).

Cultured EPCs are often called "EC-like cells" due to the expression of certain endothelial features such as: (1) the expression of certain endothelial lineage marker genes/proteins, like CD31, Flk-1/KDR, Flt-1, VE-cadherin, Tie-2, vWF, (2) an EC-like bioactivity, characterized by their capacity to migrate towards an angiogenic growth factor gradient or to support the formation of or incorporate into tube-like structures, and (3) their direct/indirect contribution to the formation of new blood vessels in ischemic tissues after in vivo transplantation. Other characteristics of these cells cover also non-endothelial features like (1) hematopoietic cell marker expression, e.g. CD45 or CD14 up to 2 weeks in culture, (2) loss of EC monolayer formation and (3) reduction of their proliferative activity in culture similar to cultured human endothelial cells, e.g. HUVEC (Dimmeler et al. 2001; Kalka et al. 2000a; Sharpe et al. 2006). The obvious discrepancies between differentiating EPCs and differentiated ECs characterized by a lack of certain endothelial specific markers and properties of EC-like cells and the obvious diminished EPC differentiation capacity into totally differentiated EC phenotype in vitro have been discussed for years and still remain to be clarified.

2.1.2 EPC Mobilization and Kinetics in Peripheral Blood

As described previously, tissue trauma causes mobilization of hematopoietic cells as well as pluripotent stem or progenitor cells from the hematopoietic system (Grzelak et al. 1998). Consistent with the notion that EPCs and HSCs share common surface angigens, our recent data has shown that mobilization of BM-derived EPCs constitutes a natural response to tissue ischemia. The murine BMT model also provided direct evidence of enhanced BM-derived EPC incorporation into foci of corneal neovascularization following the development of hindlimb ischemia (Takahashi et al. 1999), indicating that circulating EPCs are mobilized endogenously in response to tissue ischemia and can incorporate into neovascular foci to promote tissue repair. These results in animals were recently confirmed by human studies illustrating EPC mobilization in patients following burns, CABG, or acute myocardial infarction (Shintani et al. 2001).

In the pathophysiological events that require neovascularization in vivo, a variety of cytokines, growth factors, or hormones released from the jeopardized tissue affect BM remotely and cause EPC mobilization from BM. For instance, granulocyte macrophage colony-stimulating factor (GM-CSF) is well known to stimulate hematopoietic progenitor cells and myeloid lineage cells, but has recently been shown to exert a potent stimulatory effect on EPC kinetics. The delivery of this cytokine induced EPC mobilization and enhanced neovascularization in severely ischemic tissues and de novo corneal vascularization (Takahashi et al. 1999). Vascular endothelial growth factor (VEGF), critical for angio/vasculogenesis in the embryo (Ferrara et al. 1996; Carmeliet et al. 1996; Shalaby et al. 1995), has also been shown to be an important stimulus of adult EPC kinetics recently. Our studies performed first in mice (Asahara et al. 1999a) and subsequently in patients undergoing VEGF gene transfer for limb or myocardial ischemia (Kalka et al. 2000b) revealed a previously unappreciated mechanism by which VEGF contributes to neovascularization in part by mobilizing BM-derived EPCs. Similar modulation of EPC kinetics has been observed in response to other hematopoietic stimulators; granulocyte-colony stimulating factor (G-CSF) and stromal-derived factor-1 (SDF-1) (Moore et al. 2001), growth factors; platelet derived growth factor -CC (PDGF-CC) (Li et al. 2005), brain derived neurotropic factor (BDNF) (Kermani et al. 2005), insulin-like growth factor 2 (IGF-2) (Maeng et al. 2009) and placental growth factor (PlGF) (Hattori et al. 2002), and hormones; estrogen (Iwakura et al. 2003) and erythropoietin (Heeschen et al. 2003) (Fig. 2.1). The distinct mechanism by which EPCs are mobilized to the peripheral circulation remains unknown, but may mimic aspects of embryonic development.

EPC mobilization has recently been implicated not only by natural hematopoietic or angiogenic stimulants but also by pharmacological agents. For instance, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are known to rapidly activate Akt signaling in ECs, thereby stimulating EC bioac-

tivity in vitro and enhancing angiogenesis in vivo (Kureishi et al. 2000). Recent studies by Dimmeler et al. and our laboratory have demonstrated a novel function of statins by mobilizing BM-derived EPCs through the stimulation of the Akt signaling pathway (Dimmeler et al. 2001; Vasa et al. 2001b; Urbich et al. 2002; Llevadot et al. 2001). Also, emerging evidences indicated that AMD3100 which is an antagonist of CXCR4 receptor for SDF-1 exhibited therapeutic effects on wound healing (Nishimura et al. 2012), diabetic neuropathy (Kim et al. 2013), and cardiovascular diseases (Jujo et al. 2010, 2013). One concrete mechanism for these favorable effects is due to EPC mobilization by blocking SDF-1/CXCR4 axis in EPC retention in BM, however, since high dose AMD3100 blocks the SDF-1/CXCR4 axis not only in BM but also inhibits circulating or mobilized EPCs' homing capacity to sites of ischemia or target, the therapeutic dose of AMD3100 should be determined carefully before any application for EPC mobilizer.

Therefore these newly appreciated role of statins and that of AMD3100 suggest that they can be beneficial in treating various forms of vascular diseases expecting endogenous EPC contribution.

2.1.3 Role of EPCs in Post-natal Neovascularization

Post-natal neovascularization was originally recognized to be constituted by the mechanism of "angiogenesis", which is new vessel formation, operated by in situ proliferation and migration of preexisting ECs as previously described (Folkman and Shing 1992). However, the discovery of EPCs resulted in the addition of the new mechanism for vascular formation in adults, "vasculogenesis", which is frequently observed during embryogenesis. "Vasculogenesis" is de novo vessel formation by in situ incorporation, differentiation, migration, and/or proliferation of BM-derived EPCs (Asahara et al. 1999a). The incorporation of BM-derived EPCs into foci of physiological and pathological neovascularization has been demonstrated in various animal experiments. One well-established model that allows us to detect BM-derived EPCs utilizes wild-type mice with BM cells transplanted from transgenic mice in which LacZ expresses under the regulation of an EC lineage-specific promoter, flk-1 or Tie-2 (Flk-1/LacZ/BMT, Tie-2/LacZ/BMT). Using these mice, Flk-1- or Tie-2-expressing endothelial lineage cells derived from BM (EPCs) have been shown to localize to vessels during tumor growth, wound healing, skeletal and cardiac ischemia, corneal neovascularization, and endometrial remodeling following hormone-induced ovulation (Asahara et al. 1999a) (Figs. 2.1 and 2.2). On the other hand, tissue specific stem/progenitor cells with the potency of differentiation into myocytes or ECs was also isolated in skeletal muscle tissue in murine hindlimb later on, although the origin of the cells remains to be cleared (Tamaki et al. 2002).This finding suggests that the origin of EPCs may not be limited to BM, e.g., tissue specific stem/progenitor cells possibly provide "in situ EPCs" as other sources of EPCs than BM. Regardless of the origin of EPCs, they certainly play a significant role contributing to neovascularization directly via vasculogenesis in the tissue.

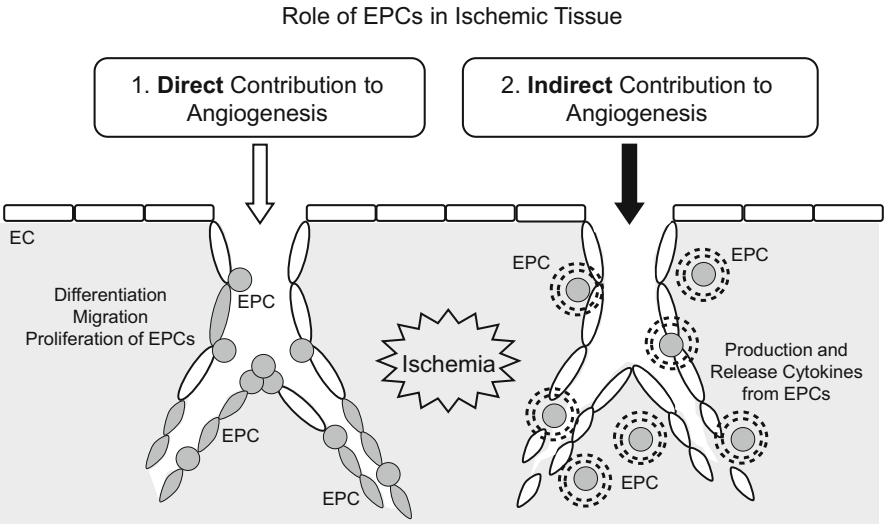

Fig. 2.3 Two different roles of EPCs in ischemic tissue. In the case of new vessel formation, one role of EPCs is the direct participation in neovascularization accompanying preexisting EC proliferation and migration (*left in the figure*). The other role of EPCs is the indirect effect on angiogenesis with production and release of pro-angiogenic cytokines/growth factors. The recruited EPCs remain in the site without participating in neovasculature exhibiting so-called "paracrine effect" (*right in the figure*)

Apart from the established role of EPCs in neovascularization, namely "direct participation in neovasculature via vasculogenesis", recruited EPCs to the jeopardized tissue that requires vessel regeneration do not always participate in the neovasculature but rather stay in interstitial tissue along with neovascularization (Fig. 2.3). These 'resting' EPCs in the tissue produce a variety of cytokines/growth factors, specifically pro-angiogenic ones, and promote pre-existing EC proliferation and migration resulting in angiogenesis. This paracrine effect of EPCs represents indirect contribution to neovascularization. As far as we and others confirm the cytokines/growth factors produced from EPCs, EPCs will release VEGF, hepatocyte growth factor (HGF), angiopoietin-1 (Ang-1), endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), SDF-1α, and insulin-like growth factor-1 (IGF-1), etc. Both VEGF and HGF promote EC proliferation leading to angiogenesis, and Ang-1 may play a role for stabilizing pre-matured vessels in ischemic tissue. Nitric oxide (NO) synthase, by ether eNOS or iNOS, maintains tissue blood perfusion in microcirculating systems acting as a vasodilator. Since little eNOS expression is observed in cardiac capillaries except for ECs in coronary arteries, 'imported' eNOS produced from the recruited EPCs is thought to be a major source of eNOS and important in short term ischemia, specifically inchemia-reperfusion injury (Ii et al. 2005). iNOS is also produced from the recruited EPCs, however, the expression is prominent only when the tissue hypoxia is sustained for a long time, i.e. in the case of chronic myocardial ischemia rather than ischemia-

reperfusion injury. BM-derived cell eNOS or iNOS deletion results in the exacerbation of myocardial infarction induced by ischemia-reperfusion injury or permanent vessel occlusion, respectively, suggesting that 'imported' NOS is crucial for preventing ischemic myocardium depending on the type of ischemic injury (Ii et al. 2005). SDF-1α released from recruited EPCs further recruits more EPCs triggering a chain reaction. On the other hand, EPCs will prevent cardiac apoptosis caused by ischemia via a production of IGF-1, a potent anti-apoptotic factor, activating the Akt signaling pathway. Thus, EPCs demonstrate tissue-protective effects producing favorable factors, namely "indirect contribution to neovascularization in ischemic tissue".

2.2 EPC-Based Therapeutic Angiogenesis

Since the discovery of EPCs in 1997, we immediately focused on the regenerative potential of stem/progenitor cells as well as the unique characteristics. In vitro, stem/progenitor cells have the capability of self-renewal and differentiation into organ-specific cell types. In vivo, these cells are then directed by the appropriate milieu that allows them to differentiate and reconstitute target organs. The novel therapeutic strategy for ischemic diseases, EPC transplantation, may therefore be an epoch as a cell therapy involving the classic paradigm of angiogenesis developed by Folkman and colleagues.

2.2.1 EPC Transplantation in Experimental Animals

We and others indicated that cell therapy with culture-expanded EPCs can successfully promote neovascularization in ischemic tissue, even when administered as "sole therapy," i.e., in the absence of angiogenic growth factors. Such a "supply-side" version of therapeutic neovascularization in which the substrate (EPCs/ECs) rather than ligand (growth factor) comprises the therapeutic tool, was first reported by intravenously transplanting human EPCs to immunodeficient mice with hindlimb ischemia (Kalka et al. 2000a). These findings provided a novel insight that exogenously administered EPCs restored impaired neovascularization in a mouse ischemic hindlimb model. A similar study in which human EPCs were transplanted in a myocardial ischemia model of nude rat, demonstrated that transplanted EPCs localized to the area of neovascularization with the differentiation into mature ECs. These findings were consistent with preserved left ventricular (LV) function and reduced infraction size (Kawamoto et al. 2001). Another study in which human cord blood-derived EPCs were transplanted in an ischemic hindlimb model of nude rats also demonstrated similar findings with enhanced neovascularization in ischemic tissue (Murohara et al. 2000).

Recently, other investigators have explored the therapeutic potential of CD34+ Cells as an EPC-enriched fraction. Shatteman et al. transplanted freshly isolated human CD34+ cells into diabetic nude mice with hindlimb ischemia, and showed a blood flow recovery in the ischemic limb (Schatteman et al. 2000). Also, Kocher et al. attempted intravenous infusion of freshly isolated human CD34+ cells into nude rats with myocardial ischemia, and observed preservation of LV function in consistent with the inhibition of cardiac apoptosis (Kocher et al. 2001). CD34+ cell dose-dependent contribution to LV functional recovery and neovascularization in ischemic myocardium has been demonstrated (Iwasaki et al. 2006). The major mechanism for these therapeutic effects are attributed by the paracrine effect rather than direct contribution to neovascularization of CD34+ cells due to its less ability of transdifferentiation to cardiovascular lineage cells. In order to promote the direct contribution of CD34+ cells to therapeutic angiogenesis, we and others focused on one of the morphogens in embryonic stage, sonic hedgehog (SHh), and tested the effect of pretreated CD34+ cells with SHh comparing with non-treated CD34+ cells in mouse ischemic disease models (Kanaya et al. 2015; Mackie et al. 2012). The SHh treatment significantly upregulated gene expressions of angiogenic growth factor and vascular-related marker in vitro, and exhibited high therapeutic outcomes with increased vascularity including endothelial and smooth muscle cell differentiation of the locally transplanted CD34+ cells (Fig. 2.3).

2.2.2 EPC Transplantation in Clinical Trials

Based upon the previous reports regarding the therapeutic effects of EPCs seen in animal models on ischemic diseases, a broad range of cells which are all believed to consist of or contain to a certain extent EPCs and/or pro-vasculogenic/angiogenic cell populations have been evaluated in numerous clinical trials (Table 2.1). Excellent in depth reviews summarizing the cells, methods for cell preparation and administration, and target diseases as well as the therapeutic outcome of the applied strategies are available (Losordo and Kishore 2009; Losordo et al. 2007; Jujo et al. 2008).

We have recently reported a phase I/II clinical trial regarding intramuscular transplantation of autologous and G-CSF-mobilized CD34+ cells in patients with intractable critical limb ischemia (CLI) (Kawamoto et al. 2009). The first-in man trial was conducted as a prospective, multicenter, single-blinded and dose-escalation study since 2003 in our institute. G-CSF was used to efficiently mobilize BM-EPCs to PB, and the mobilized CD34+ cells were isolated as EPC-enriched fraction.

In all subjects, primary endpoint, the Efficacy score at week 12 was positive value indicating improvement of lower limb ischemia after the cell therapy. In addition, both subjective and objective parameters of lower limb ischemia such as toe brachial pressure index (TBPI), transcutaneous partial oxygen pressure ($TcPO_2$), total walking distance (TWD), pain-free walking distance (PFWD), Wang-Baker's pain rating scale and the ulcer size significantly, (Fig. 2.4) and serially improved

Table 2.1 Clinical trials for ischemic diseases with EPCs

Trial name/ author	Disease type	Number of patients (T/C)	EPC type	Study design	Outcome	Reference
TOPCARE-AMI	AMI	30/29	PB-/ BM-derived cultured EPCs	RT	Effective	Schachinger et al. (2004)
Bartunek et al.	AMI	19/16	CD133	RT	Effective	Bartunek et al. (2005)
Li et al.	AMI	35/35	Gm-PB-CD34	Cohort	Effective	Li et al. (2007)
Tatsumi et al.	AMI	36/18	PB-MNC	Cohort	Effective	Tatsumi et al. (2007)
Dobert et al.	AMI	11/15	PB-/ BM- derived cultured EPCs	Cohort	Effective	Dobert et al. (2004)
Stamm et al.	RMI	46/9	CD133	NRT	Effective	Stamm et al. (2003, 2007)
Ahmadi et al.	RMI	18/9	CD133	NRT	Effective	Ahmadi et al. (2007)
Balogh et al.	RMI	8/18	CD34	NRT	Inconclusive	Balogh et al. (2007)
Erbs et al.	OMI	13/13	PB- derived cultured EPCs	RT	Effective	Erbs et al. (2005)
Assmus et al.	OMI	24/23	PB-derived cultured EPCs	RCT	Ineffective	Assmus et al. (2006)
Boyle et al.	OMI	5/0	Gm-PB-CD34	NRT	NA	Boyle et al. (2006)
Losordo et al.	AP	18/6	Gm-PB-CD34	RT	Safe and feasible	Losordo et al. (2007)
Lara-Hernandez et al.	CLI	28/0	Gm-PB-CD34	Cohort	Effective	Lara-Hernandez et al. (2010)
EPOCH-CLI	CLI	17/0	Gm-PB-CD34	Cohort	Effective	Kawamoto et al. (2009)
Kuroda et al.	NUF	4/0	Gm-PB-CD34	Cohort	Effective	Kuroda et al. (2014)

AMI acute myocardial infarction, *OMI* old myocardial infarction, *RMI* recent myocardial infarction, *AP* angina pectoris, *CLI* critical limb ischemia, *NUF* non-union fracture, *PB* peripheral blood, *BM* bone marrow, *Gm* G-CSF (granulocyte colony-stimulating factor) mobilized, *T/C* treatment/control, *RT* randomized trial, *NRT* non-randomized trial, *NA* not available

after transplantation of CD34+ cells. Because this was not a randomized, controlled study, possibility of the placebo effect after CD34+ cell transplantation needs to be evaluated in the large-scaled future trial. As for the safety evaluation, neither death nor life-threatening adverse events were observed in this study. No severe adverse event, for which relation to a series of cell therapy could not be denied, was also observed. Although mild to moderate adverse events were frequent, these events

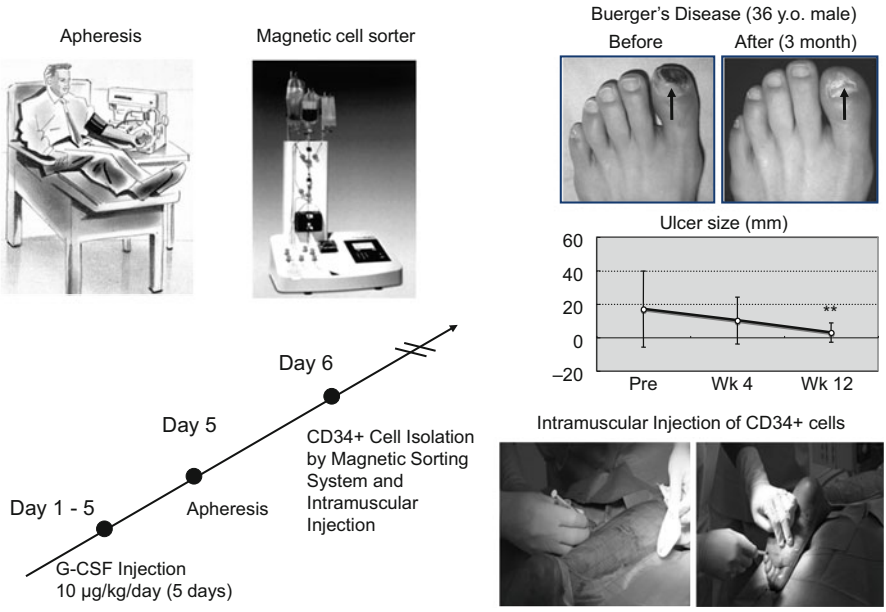

Fig. 2.4 Representative case of autologous CD34+ cell transplantation therapy for CLI in Buerger's disease. Thirty six year old male patient who had toe necrosis due to microcirculation failure received CD34+ cell injection at 40 sites in ischemic limb under lumbar anesthesia. Skin ulcer and necrosis completely healed accompanied with objective evidence of blood flow recovery 3 months after the cell therapy. Quantitative analysis revealed significant decrease of ulcer size 12 weeks post cell therapy (Graph) **, $P<0.01$ vs. Pre (baseline). The improvement could be maintained for more than 1 year without recurrence

were transient and expected. No malignant tumor was also clinically identified during the study period.

In addition to CD34, CD133 is a surface marker of early EPC phenotype. A recent clinical study by Burt et al. showed the safety and feasibility of autologous, GCSF-mobilized CD133+ cell implantation into lower extremity muscles of nine patients with CLI including a patient with Buerger's disease (Burt et al. 2010). PB-MNCs were collected by leukapheresis after GCSF mobilization (10 μg/kg/day for 4–5 days), and CD133+ stem cells were selected using a magnetic separation system. There were no major complications from either leukapheresis or cell injection. The patient with Buerger's disease underwent the procedure twice. After the procedure, rest pain resolved rapidly by day 2, and seven of nine patients including a case of Buerger's disease were able to avoid limb amputation during the 1-year follow-up.

Although these studies were small-sized, non-randomized trials, these initial results suggest the potential effectiveness of the purified EPC population in CLI patients.

As for the EPC therapy for coronary artery disease, Losordo et al. recently reported a phase II, randomized, placebo-controlled and dose-ranging clinical trial

for 167 patients with refractory angina. Autologous CD34+ cells isolated from GCF-mobilized apheresis products were intramyocardially injected into ischemic myocardium under the guidance of NOGA endomyocardial mapping. Six and 12 months later, angina counts and changes in exercise time significantly improved in CD34+ cell group than placebo group (Losordo et al. 2011). These promising outcomes also support the clinical usefulness of EPC transplantation for reduction of tissue ischemia.

2.2.3 Problems in EPC Transplantation

Our animal studies (Kalka et al. 2000a) suggest that heterologous EPC transplantation requires systemic injection of $0.5–2.0 \times 10^4$ human EPCs/g body weight of the recipient animal to achieve satisfactory improvement of hindlimb ischemia. In general, cultured EPCs obtained from healthy human volunteers yields 5.0×10^6 cells per 100 ml of peripheral blood on day 7. Based on these data in human, a blood volume of as much as 12 l will be necessary to obtain enough number of EPCs to treat patients who have critical ischemic hindlimb. Therefore, the background factors in clinical patients such as aging (Heiss et al. 2005), diabetes (Vasa et al. 2001a; Ii et al. 2006), hypercholesterolemia (Vasa et al. 2001a), hypertension (Vasa et al. 2001a; Imanishi et al. 2005) and smoking (Kondo et al. 2004; Michaud et al. 2006) that may reduce the number of circulating/BM EPCs and the function will cause major limitations of primary EPC transplantation. In reality, most of the patients who are going to undergo EPC therapy for the ischemic diseases more or less have background diseases as described above. Considering autologous EPC therapy, certain technical improvements that may help to overcome the malfunction of EPCs should include; (1) local delivery of EPCs, (2) endogenous EPC mobilization i.e. cytokine/growth factor supplements to promote BM-derived EPC mobilization (Asahara et al. 1999a; Takahashi et al. 1999), (3) enrichment procedures, i.e., leukapheresis or BM aspiration, (4) enhancement of EPC functions by gene transduction, or (5) culture-expansion of EPCs from self-renewable primitive stem/progenitor cells in BM or other sources. Unless the quality and quantity of autologous EPCs is obtained by the technical improvements as described above, allogenic EPCs derived from umbilical cord blood or culture-expanded from human embryonic stem cells (Murohara et al. 2000; Levenberg et al. 2002), may be another alternative source supplying EPCs.

2.2.4 EPC as a Biomarker for Ischemic Diseases

Previous clinical studies reported that the number of circulating EPCs defined with cell surface markers, CD34+, CD34+/KDR+, CD133+/KDR, or CD34/CD133/KDR+, inversely correlated with the severity of cardiovascular diseases including

Table 2.2 Correlation between circulating EPC number/function and cardiovascular diseases

Subject disease	Control disease	N	EPC type	EPC number/ function	Reference
AMI (Day 7 after onset)	AMI (Day of onset)	16	CD34+	2X ↑/CFA ↑	Shintani et al. (2001)
Non–ST ↑ AMI (with collaterals)	Non–ST ↑ AMI (without collaterals)	20	CD133+/KDR+	2X ↑/CFA →	Lev et al. (2005)
CAD (cardiac event +)	CAD (cardiac event –)	77	CD34+/KDR+	0.5X ↑/(–)	Schmidt-Lucke et al. (2005)
CAD (cardiac event –)	CAD (cardiac event +)	519	CD34+/KDR+	1.5–2X ↑/(–)	Werner et al. (2005)
Congestive HF	Healthy volunteer	46	CD34+/CD133+/ KDR+	Mild HF 3–4X ↑/ CFA ↑ Severe HF: 0.7–0.5X ↓/CFA ↓	Valgimigli et al. (2004)
Eisenmenger Syndrome (with pulmonary HT)	Healthy volunteer	96	CD34+, CD34+/ CD133+, KDR+	0.3–0.5X ↓/CFA ↓	Diller et al. (2008)

AMI acute myocardial infarction, *CAD* coronary artery disease, *HF* heart failure, *HT* hypertension, *CFA* colony forming activity

congestive heart failure (Shintani et al. 2001; Diller et al. 2008; Lev et al. 2005; Schmidt-Lucke et al. 2005; Valgimigli et al. 2004; Werner et al. 2005) (Table 2.2). Colony forming activity of EPCs analyzed by Hill's method (Hill et al. 2003) has also been known to correlate with the number of circulating EPCs and used for the assessment of EPC function, however, Hill's colony assay is recognized as just a method for detecting EPC aggregation.

Thus, we have recently developed a novel EPC colony forming assay (EPC-CFA) system, capable to address and overcome most of the limitations of the classical assay systems, is challenging several of the predominant classical opinions about EPCs, and enabling an until now missing differential hierarchic view on EPCs. We have reported one of the first examples of such an assay system, initially designed to work with mouse samples. c-Kit$^+$/Sca-1$^+$/Lineage negative (KSL) cells were used as a putative murine hematopoietic EPC-enriched cell population, allowing the identification of two clearly distinguishable types of colonies (small and large colonies) that in turn correspond to two distinct EPC populations, primitive (small) and definitive (large) EPCs, respectively (Kwon et al. 2008; Tanaka and Sata 2008; Kamei et al. 2010) (Fig. 2.3). The concept of an EPC-CFA was recently introduced and further developed for analysis of human EPC samples (Asahara et al. 2011). The EPC-CFA enables hereby not only the EPC-colony formation analysis of single and/or bulk cells from EPC-enriched arbitrary fractions or non-selected cell populations but allows also the cell fate analysis of primary and/or

suspension culture cultivated single and/or bulk cells. It can further be easily combined with a classical HPC colony assay system, thus allowing a direct and comprehensive elucidation of the differences and similarities between EPCs and HPCs via the clarification of the cell fate of each cell type. The use of such an EPC-CFA allows not only the elucidation of a possible but so far elusive differentiation hierarchy of EPCs, but can be further used to identify and characterize the parameters associated with proliferation, commitment, and differentiation of EPCs in vitro and in vivo (Asahara et al. 2011).

Indeed, application of EPC-CFA on human CD34+ or CD133+ stem/progenitor cells enabled the identification of small and large distinct colony types each derived from a single cell, small-EPCs and large-EPCs, respectively. Small-EPCs showed a higher rate of proliferative activity with a higher number of cells being in the S-phase, when compared to large-EPCs. Interestingly, large-EPCs showed a significantly higher rate of vasculogenic activity with overall increased potential for cell adhesion and tube-like structure formation in vitro as well as a high in vivo de novo blood vessel forming activity following transplantation of these cells into a murine ischemic hindlimb model, as compared to small-EPCs. In contrast to small-EPCs, large-EPCs did not form secondary colonies but gave rise to isolated endothelial cell (EC) like cells when reseeded. Due to the observed in vitro (by FACS analysis) and in vivo characteristics of these colony types, small-EPCs were further characterized and believed to represent "primitive EPCs", a highly immature and proliferative population of cells, compared to large-EPCs which are believed to represent "definitive EPCs", cells prone to differentiate and promote vasculogenesis**.**

The advantage of these assessment for the number and colony forming activity of circulating EPCs is a convenient tool for clinical application in terms of a medical regulatory feasibility of sampling from blood cells by antibody targeting isolation, and a potent effectiveness on ischemic diseases through vasculogenic and angiogenic mechanisms by primary cells.

2.2.5 Future Strategy with EPC Transplantation

The possible and feasible strategy that may recover potential EPC dysfunction in ischemic disorders should be considered, given the findings that EPC function and mobilization may be impaired in certain diseases. One of the strategies, genetic modification of EPCs to overexpress angiogenic growth factors, will enhance signaling activity of the angiogenic response and reactivate the bioactivity and/or extend the life span of EPCs.

We have recently shown for the first time that gene-modified EPCs rescue impaired neovascularization in an animal model of limb ischemia (Iwaguro et al. 2002). Transplantation of heterologous EPCs transduced with adenovirus encoding human VEGF165 improved neovascularization and blood flow recovery, reducing the limb necrosis and auto-amputation rate in comparison with controls. The dose of EPCs needed to achieve limb salvage in these in vivo experiments was 30 times

less than that required in the previous experiments involving unmodified EPCs (Kalka et al. 2000a). Other investigators have also demonstrated the therapeutic efficacy of genetically engineered EPCs with a variety of target genes such as adrenomedullin (AM) (Nagaya et al. 2003), eNOS (Kong et al. 2004), tissue plasminogen activator (tPA) (Griese et al. 2003) and integrin-like kinase (ILK) (Cho et al. 2005) in animal models. Thus, genetic modification might overcome the potential problems in the patients' EPCs for EPC transplantation therapy in ischemic diseases as so-called "second generation EPC therapy". Also, combining EPC cell therapy with gene (i.e., VEGF) therapy (Kawamoto et al. 2004) may be another option to address the limited number and function of EPCs that can be isolated from peripheral blood in patients.

2.3 Summary

There is accumulating evidence that BM-derived EPCs have characteristics similar to those of angioblasts demonstrating the potential to promote postnatal vasculogenesis in adults, and clinical applications of EPCs in regenerative medicine are now on going. To acquire optimal quality and quantity of EPCs, however, several issues remain to be addressed, such as the development of a more efficient method of EPC purification and expansion, the methods of administration, and background disease-induced dysfunction or senescence in EPCs in patients. Alternatively, in the case of impossible utility of autologous BM-derived EPCs in patients with impaired BM function, appreciable EPCs isolated from umbilical cord blood or differentiated from tissue specific stem/progenitor or embryonic stem cells need to be optimized for EPC therapy. However, the unlimited potential of EPCs along with the emerging concepts of autologous cell therapy with gene modification suggests that they may soon reach clinical fruition.

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Chapter 3
Heart

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Abstract Coronary heart disease and chronic heart failure are common and have an increasing incidence and morbidity in the Western Society. Although revascularization procedures and conventional drug therapy may delay ventricular remodeling, there is no basic accepted therapeutic regime available for preventing or even reversing this process. Numerous studies within the past few years have demonstrated that cardiac stem cell therapy may be considered as a safe alternative intervention for ischaemic heart disease, in order to regenerate destroyed and/or compromised heart muscle. Different autologous or allogeneic progenitor cell populations have already been assessed to test their therapeutic value for cardiac cell therapy. Several preclinical, as well as clinical trials have shown that transplantation of autologous and allogeneic bone marrow stem cells and cardiovascular precursor cells improve cardiac function after myocardial infarction and in chronic ischaemic heart disease. Further indications for cardiovascular stem cell therapy may include non-ischaemic and dilated cardiomyopathy, as well as heart failure due to an infectious cause, like Chagas heart disease. Other clinical applications like the bioengineering of artificial heart valves and vascular conduits also have been explored. Further clinical development will be geared towards the modification of cardiac inflammation and cardiogenesis by stem cell modification and to test other stem cell sources.

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3.1 Background and Rationale for Stem Cell Therapy in Heart Disease

Stem cells have the important properties of self-regeneration and pluripotent, or even omnipotent differentiation capacity (Allgöwer 1956; Jiang et al. 2002; Krause et al. 2001; Quaini et al. 2002). Thus, they are ideal candidates to promote regeneration of damaged myocardial tissue (Goodell et al. 2001), for example, after myocardial infarction or in patients with congestive heart failure. Following acute myocardial infarction, coronary perfusion is restored by (primary) percutaneous coronary interventions. However, in most cases the cardiac function may be restored incompletely due to ischaemic myonecrosis and reperfusion injury following intervention, so that post infarct adverse remodelling and congestive heart failure mostly cannot be prevented. This adverse remodelling which occurs in approximately 60 % of post infarction patients, may be prevented by myocardial restitution by cell transplantation, which then leads to restoration or normalization of compromised heart function (Kocher et al. 2001).

One possible approach to cardiac regeneration is by the transplantation of cells from a primarily non-cardiac origin. In the first clinical studies of cardiac cell therapy in 2000, the Paris-based group of Menasche (Menasche et al. 2001), applied autologous, cultured, non-cardiac skeletal myoblasts by intramyocardial injections in post-infarct patients during coronary bypass surgery. The origin of skeletal myoblasts from bone marrow stem cells was first described by Ferrari et al. (1998). Bone marrow contains progenitor cells for different lineages, including the cardiomyocytes and endothelial cells (Reyes et al. 2001), that constitutes myocardial tissue and coronary blood vessels, (Orlic et al. 2001; Kocher et al. 2001). Human bone marrow contains e.g. CD34/CD133-positive, haematopoietic and CD34/CD133-negative, mesenchymal stem cells (Reyes et al. 2001), and both these types of stem cells have been shown to contribute to cardiac regeneration. The first experimental studies in myocardial infarction animal models demonstrated (i) new blood vessel formation in the infarct-bed (vasculogenesis) and proliferation of preexisting vasculature (angiogenesis) (Kocher et al. 2001) (ii) decreased apoptosis of hypertrophied myocytes in the peri-infarct region (Kocher et al. 2001); and (iii) de novo expression of cardiac proteins by infiltrating human bone marrow mononuclear cells (Toma et al. 2002). Strauer and co-workers performed one of the first clinical percutaneous stem cell therapy by and intracoronary infusion of bone marrow derived (unfractionated) mononuclear stem cells in patients that had been admitted with a myocardial infarction in March 2001 (Strauer et al. 2001). In July 2001, Steinhoff and co-workers in Rostock treated for the first time patients with chronic myocardial ischaemia during bypass surgery patients by intramyocardial injections of immune-isolated CD133+

bone marrow stem cells (Stamm et al. 2003). The objectives of these procedures, which had not been achieved before, were simply:

1. To use autologous stem cells isolated from bone marrow or from peripheral blood for cardiac tissue repair; that is, (a) for intracoronary (endovascular) application to use all three fractions of mononuclear, bone marrow stem cells: haematopoietic, angioblastic and mesenchymal stem cell fraction; (b) for direct intramyocardial (interstitial) transplantation to use highly purified $CD133^+$ bone marrow stem cells.
2. To facilitate and potentiate cell migration by artificial cardiac ischemia, an effective stimuli for stem cell differentiation and recruitment (Kamota et al. 2009), most probably via the CXCR4 – SDF-1 interrelationship. In the endovascular approach, this has been achieved by repetitive, intracoronary balloon inflations within the infarct-related artery.
3. To enrich and to accumulate bone marrow stem cells within the post-ischaemic infarct border zone, either by intracoronary (endovascular) or intramyocardial (interstitial) administration.

Studies have previously suggested that cardiomyocyte renewal indeed takes place in humans, although this process seems to be of low magnitude (Bergmann et al. 2009). Future therapeutic strategies may aim to stimulate this local process. Stem cell transplantation could offer a therapeutic tool to fully exploit this self-renewing capacity of the adult human heart.

3.2 Cell Types

3.2.1 Adult Tissue-Derived Cells

3.2.1.1 Freshly Isolated Autologous Bone Marrow Mononuclear Cells (BMMNC)

Freshly isolated, autologous bone marrow stem cells consist of a heterogeneous cell population, including hematopoietic stem cells ($CD34^+$, $CD133^+$, $CD117^+$ (c-kit)), endothelial progenitor cells, mesenchymal stem cells, very small embryonic-like stem cells, and supporting cells etc.

In one of the first clinical studies, autologous BMMNC were transferred by intracoronary infusion in patients with prominent heart failure (Strauer et al. 2001). The subsequent randomized, placebo-controlled clinical trials, were able to show that these novel stem cell therapies by intracoronary BMMNC infusion were also safe and effective following the percutaneous coronary intervention in patients with an acute AMI (respectively ASTAMI, BOOST; REPAIR-AMI, and TOPCARE-AMI, Wollert et al. 2004; Schächinger et al. 2004; Lunde et al. 2007; Marenzi and Bartorelli 2007). To date, BMMNC therapy have been assessed in patients with chronic ischaemic heart failure (Assmus et al. 2007; Strauer et al. 2010; Honold et al. 2013), patients with refractory angina ineligible for conventional revascularization

(Tse et al. 2007; van Ramshorst et al. 2009; Hossne et al. 2015), and non-ischaemic dilated cardiomyopathy (Sant'anna et al. 2014). Another indication for stem cell treatment may be to increase the capillary density in ungraftable coronary territories in combination with a CABG procedure (Pätilä et al. 2014; Akar et al. 2009). Also, there have been case reports that found beneficial effects of BMMNC therapy in children with congenital heart disease, or patients with dilated cardio;myopathy (Lacis and Erglis 2011; Rupp et al. 2012).

While the unfractioned BMMNC population may consist of different cell types, some studies have assessed the therapeutic effect of selected subpopulations of bone marrow stem cells, including immune-isolated $CD34^+$ and $CD133^+$ cells which appeared to have a higher proliferative capacity, and angiogenic potential in vitro and in vivo (Quyyumi et al. 2011; Hansen et al. 2014; Bartunek et al. 2005; Yerebakan et al. 2011; Colombo et al. 2011; Bhatia 2001; Quirici et al. 2001; Koutna et al. 2011).

3.2.1.2 Endothelial Progenitor Cells

From adult bone marrow and the circulation, the endothelial committed progenitor cells have been the subject of various studies. Previously, it was hypothesized that neoangiogenesis and reendothelialisation of denuded vascular segments occurred exclusively by dedifferentiation, migration and proliferation of resident endothelial cells in the ischaemic region or damaged vessel. In 1997, Asahara and co-workers first described that circulating endothelial progenitor cells were recruited from the bone marrow, homed into sites of vascular injury or ischemia, and were incorporated in the developing microvasculature (Asahara et al. 1997; Shi et al. 1998). Although there is some debate regarding the appropriate phenotypical markers and function of these endothelial-committed EPCs. EPCs can be generally identified by their capability to express endothelial phenotypical markers, including the expression of CD34, CD133, the vascular endothelial growth factor receptor 2 (VEGFR-2, KDR1, Flk1), and vascular endothelial cadherin (VE-cadherin), as well as some endothelial cell functional characteristics in vitro and in vivo, like acLDL uptake and the formation of endothelial colony forming units. EPCs are mobilized from the bone marrow following ischemia or cardiovascular injury, like vascular trauma or myocardial infarction (Gill et al. 2001; Shintani et al. 2001). In animal models of myocardial infarction, intramyocardial injections of EPCs preserved left ventricular contractile function and reduced fibrosis formation (Jujo et al. 2008; Kocher et al. 2001). These results led to the first phase I–II clinical trials, assessing the safety and feasibility of EPC transplantation therapy in patients with stable angina pectoris and heart failure (NCT00694642, NCT00384514, NCT00221182). Remarkably, EPCs have already found an unique niche in the field of interventional cardiology and bioengineered stents. The HEALING studies assessed the safety and efficacy of the bioengineered Genous stent (OrbusNeich) which captured and sequestered circulating $CD34^+$ cells to the stent by an anti-$CD34^+$ antibody coating, thereby expediting

reendothelialization of the stent, preventing (acute and late) stent thrombosis and reducing in-stent restenosis (Aoki et al. 2005; Co et al. 2008; Low et al. 2011). The HEALING-FIM and the HEALING II study suggested that, provided a normal circulating EPC titer was present (>6.5 EPC/100 μl), the coronary in-stent restenosis was prevented (late luminal loss 0.53) in patients scheduled for elective PCI. Remarkable, patients with low EPC titers (<6.5 EPC/100 μl), had restenosis formation comparable to bare metal stents (late luminal loss 1.02) (Duckers et al. 2007). Low EPC titers were correlated to untreated hypercholesterolemia, whereas HMGcoA reductase (statin) therapy has been associated with EPC recruitment, activation and improved survival, and improved vascular repair following injury. The e-HEALING, a post-marketing registry of 5,000 'all-comers' CAD patients indeed suggested that the EPC capturing Genous stent was indeed associated with reduced clinical MACE events and late stent thrombosis (Silber et al. 2011). In patients with an acute coronary syndrome, EPC capture stent technology showed a reduced binary restenosis rate compared to bare metal stents at 6 months follow up, with a reduced target lesion revascularization rate (JACK-EPC; Wojakowski et al. 2013).

3.2.1.3 Very Small Embryonic-Like Stem Cells

The very small embryonic-like (VSEL) stem cells can be isolated from the bone marrow and have been defined as a non-hemopoietic cells that express the $Sca1^{+/}Lin^{-/}CD45^{-}$ phenotypical markers, as well as the pluripotent stem cell markers, stage-specific embryonic antigen 4 (SSEA4) and octamer-binding transcription factor 4 (Oct-4), the surface markers, CD133, and CD34 (Wojakowski et al. 2011; Zuba-Surma and Ratajczak 2010) as well as receptors for FGF2 (FGF-2R), PDGF (PDGF-R), SDF1 (CXCR4) and KL (c-kit; Shin et al. 2013).

Cultured VSEL are able to differentiate into functional cardiomyocytes (Wojakowski et al. 2010), whereas these cells do express early cardiac markers (GATA-4 and Nkx2.5/Csx). Upon myocardial infarction, VSELs are recruited into circulation (Kucia et al. 2004; Wojakowski et al. 2009) and ischaemic cardiac tissue (Ratajczak et al. 2008, 2009). The mobilization of VSEL stem cells into the circulation inversely correlated with age and presence of diabetes (Wojakowski et al. 2009). The VSEL-CAD clinical trial is currently assessing the correlation between VSEL levels and clinical events and prognosis of CAD patients administrating Atorvastatin ("Association between VSEL and the prognosis of coronary artery disease patients"; NCT01633359).

Intramyocardial injection of VSEL stem cells in a murine AMI model showed improvement in global systolic function compared to the control group (Dawn et al. 2008). In the REGENT trial assessed the effect of intracoronary infusion of immune-isolated CD34+/CXCR4+ cells, enriched with VSELs in 80 patients with acute myocardial infarction and reduced <40 % LVEF, CD34+/CXCR4+/VSEL therapy resulted in improved LVEF (by 3 %) as measured by magnetic resonance imaging as compared to the control group (Tendera et al. 2009).

3.2.1.4 Aldehyde Dehydrogenase-Bright Stem Cells (ALDH^{br})

Aldehyde dehydrogenase-bright stem cells is a subpopulation of hematopoietic progenitor cells, which express high levels of the intracellular enzyme, aldehyde dehydrogenase. One percent of the bone marrow mononuclear stem cells are ALDH^{br}. These cells highly express the phenotypical markers CD34, CD117, CD105, CD127, CD133, CD166, and in primitive CD34^{+} CD38 (lo/−) and CD34^{+} CD133^{+} progenitors (Storms et al. 1999; Keller 2009). ALDH^{br}cells were found in neonatal as well as adult hearts, and more frequent in atria than in ventricle (Roehrich et al. 2013). A small phase I clinical study to assess the safety effect ALDH^{br} BMMNC in patients with ischaemic heart failure showed safety and suggested a significant decrease in left ventricular end-systolic volume at 6 months follow-up (Perin et al. 2012).

3.2.1.5 Circulating Peripheral Blood Progenitor Cells Stimulation by G-CSF

Granulocyte colony-stimulating factor (G-CSF) mobilizes progenitor cells from bone marrow into the circulation. It was proposed that mobilized peripheral blood cells have improved homing and motility compared to steady-state (unstimulated) bone marrow stem cells (Bonig et al. 2007). G-CSF was assessed as a stand-alone therapy as well as G-CSF stem cells mobilization combined with CD34^{+} plasmopheresis and intramyocardial injection of the isolated CD34^{+} cells (Ince et al. 2005; Theiss et al. 2010; Hibbert et al. 2014). Initial clinical studies as well as multicenter, double-blind, randomized placebo-controlled studies suggested safety and functional efficacy of GCSF-recruited circulating peripheral blood progenitor cell therapy in patients with a subacute myocardial infarction (Kang et al. 2006; Pasquet et al. 2009), refractory angina (Losordo et al. 2011), dilated cardiomyopathy (Vrtovec et al. 2013a; Lezaic et al. 2015) and ischaemic heart failure (Mozid et al. 2014b; Poglajen et al. 2014). In one late stage clinical trial, cell-mobilization and -collection procedure however was associated with bone pain in 20.1 %, angina in 17.4 %, congestive heart failure in 1,2 % and cardiac enzyme elevations in 4,6 % of the study patients (Losordo et al. 2011). However, the meta-analysis of stand-alone G-CSF therapy (2.5–10 μg/kg/day) on cardiovascular outcomes in patients with acute MI showed safety (no differences in the incidence of death, recurrent myocardial infarction or in-stent restenosis between G-CSF–treated patients and controls), but didn't revealed improvement of left ventricle ejection fraction and dimensions (Moazzami et al. 2013), nevertheless, in studies that enrolled patients with left ventricle dysfunction (EF <50 %) or when G-CSF was administered relatively early (≤ 37 h) after the acute event, significant increase in ejection fraction was observed (+4.73 %, $P < 0.0001$) (Abdel-Latif et al. 2008).

3.2.1.6 Mesenchymal Stem Cells

Mesenchymal stem cells (MSC) have been first described in 1991 by Caplan and co-workers (Caplan 1991). These MSC can be detected in the adult in multiple organs, including bone marrow and subcutaneous fat, and can differentiate into osteoblasts, chondrocytes, adipocytes and myocytes (Alhadlaq and Mao 2004; Jiang et al. 2002). They can be separated from hemapoietic stem cells by their ability to adhere to plastic (Alhadlaq and Mao 2004). In vitro, MSC are able to differentiate into cardiomyocytes under appropriate culture conditions (Makino et al. 1999; Li et al. 2007). Today, more than 20 clinical trials assessing MSC in the treatment of CAD are registered. Published randomized, double-blind, placebo-controlled trials have confirmed the safety and efficacy of autologous MSC injections in patients with chronic ischaemic heart failure (MSC-HF Trial, Mathiasen et al. 2012, 2015; C-CURE Trial, Bartunek et al. 2013, TAC-HFT Trial, Heldman et al. 2014). In patients with severe stable coronary artery disease and refractory angina, autologous MSC therapy resulted in improvements in angina class, number of angina attacks and anti-anginal medication (Mathiasen et al. 2013). MSC-treated patients showed a significant increase in left ventricle ejection fraction as measured by SPECT (Lee et al. 2014). MSC transplantation combined with coronary bypass surgery, improved myocardial perfusion and contractile function in MSC-injected segments (Pätilä et al. 2014; Karantalis et al. 2014) and showed safety in combination with left ventricular assist devices (Ascheim et al. 2014). The intramyocardial MSC injection in nine patients, shortly after AMI, demonstrated safety and improved left ventricle ejection fraction (by 9 %) 12 months follow-up compared to controls (Rodrigo et al. 2013). Ongoing studies assess the effect of MSC treatment in patients with idiopathic dilated cardiomyopathy as well (NCT01957826, NCT01392625).

Alternatively, allogeneic stable MSC cell lines have also been generated. Several studies have shown that cardiovascular risk factors, like smoking, diabetes, aging, hypercholesterolemia and hypertension all are associated with reduced stem cell numbers and function. Moreover as the isolation of the autologous stem cells for each individual patient is laborious, cost-intensive and potentially prone for contamination and graft failure, one could argue that a stable MSC cell line generated from a healthy and young donor could generate more effective MSC in a more cost effective manner. Moreover, generation of stable MSC cell lines enables appropriate quality control analysis before batches are released for patient care (for instance for contamination, tumorigenecity, functional effect (in vitro and in vivo) and reproducibility). Initial in vivo studies in large animal models indeed suggested that allogeneic is safe and more effective than autologous MSC in a large myocardial infarction and congestive heart failure models (Houtgraaf et al. 2013; Amado et al. 2005). In the POSEIDON-DCM, cultured allogeneic and autologous MSC were compared in the treatment of 30 patients with ischaemic cardiomyopathy in a pilot study. This first study suggested that allogeneic MSC is at least equally effective as autologous MSC, whereas incidence of hard clinical end points were consistently more reduced in the allogeneic MSC treated patients (POSEIDON – DCM trial, Mushtaq et al. 2014).

In the more recent TEVA-CHF study, 60 patients with ischaemic and non-ischaemic heart failure patients (NYHA class II–III) were treated with two doses of mesenchymal progenitor stem cells (MPC/MSC) versus placebo control treatment (N = 20vs20vs20). Mesenchymal progenitor stem cells are isolated from donor bone marrow using Stro3 immune-isolation and expanded in cell culture, resulting in a selected MSC population with a higher colony forming unit assay activity (fibroblast CFU assay) and higher SDF-1 release (32-fold) as compared to Stro-BMMNC. In the single-blinded phase I–II TEVA-CHF study, MPC/MSC treatment resulted in a marked reduction of clinical events in CHF patients, including reduction in cardiac death, second AMI and admission due to an exacerbation of heart failure at 12 month follow-up. The serious adverse cardiac event rate was reduced by 48 %, whereas MACE rate was reduced by 83 % (Perin et al. 2015). The subsequent phase III DREAM-CHF study will assess the safety and efficacy of intramyocardial delivery of MPC in the treatment of 1,700 patients with (advanced ischaemic and non-ischaemic) congestive heart failure and has been initiated in EU and US clinical centers.

3.2.1.7 Adipose-Derived Stem Cells (ADSC)

Human adipose-derived stem cells (ADSC), also referred to as vascular stromal fraction, are a pleiotrophic cell population derived from the subcutaneous lipoaspirate by a simple cell digestion (using enzyme digestion with a protease and collagenase) and centrifugation, thereby separating the buoyant fraction of irrelevant adipocytes and free fat, and the non buoyant fraction which contain the ADSC, composed of endothelial progenitor cells, immune competent cells and mesenchymal-like stem cells. Since these minimal manipulations of lipoaspirate only requires a simple digestion and centrifugation steps, ADSC can be prepared for transplantation in therapeutically relevant amounts, in just 1–2 h after the harvest of the fat, and requires thus not the use of a clean room facility. The mesenchymal-like stem cells are phenotypically and biologically similar to mesenchymal stem cells isolated from the bone marrow (ADSC do not express CD106) (Rodriguez et al. 2005; Fraser et al. 2008). Cultured ADSC have been shown to differentiate into multiple cell lineages, including cardiomyocytes and endothelial cells under appropriate growth factor stimulation (Planat-Bénard et al. 2004; Miranville et al. 2004). In vivo, ADSC differentiation into cardiomyocytes is a rare event. In porcine models of acute myocardial infarction, chronic heart failure and dilated cardiomyopathy ADSC therapy resulted consistently in improved left ventricle contractility with improved myocardial perfusion due to stimulated neoangiogenesis (Ishida et al. 2015; Li and Xia 2014; Araña et al. 2014; Chi et al. 2015). The APOLLO study was the first randomized, placebo-controlled, double-blind trial to assess the safety and efficacy of treatment of autologous ADSC in patients with an acute myocardial infarction with a residual left ventricle ejection fraction of <50 %. This trial suggested that a liposuction procedure and intracoronary infusion of these freshly isolated ADSC/MSCs was well tolerated, safe and did not result in microvascular

obstruction. ADSC therapy resulted in markedly improved myocardial perfusion (SPECT) and reduction in myocardial loss (i.e. a reduction of infarct size by 59 % at 6 months follow-up, or an increase of 14 g muscle tissue). Reduced damage and improved myocardial perfusion resulted in preserved cardiac function at short and long term follow-up and prevention of adverse post-AMI remodeling. Remarkably treatment with ADSC in the acute phase of myocardial infarction also resulted in a marked reduction of ventricular ectopy by 90 % and reduction in episodes of non-sustained ventricular tachyarrhythmia as detected by continuous telemonitoring and holter analysis during the first 7 days following the acute myocardial infarction cell transplantation. The phase IIb randomized, controlled multicenter clinical trial, the ADVANCE, sought to define the safety and efficacy of intracoronary infusion of two doses of ADSC in the treatment of 275 patients with an acute anterior myocardial infarction within 24 h after successful primary PCI procedure.

In the PRECISE study the effect of several doses of intramyocardial injected ADSC in 28 patients with ischaemic congestive heart failure and with (SPECT) documented residual ischaemic myocardial segments (LVEF <45 %, NYHA class II–III). Although ADSC did not improve global left ventricle ejection fraction, nevertheless, exercise tolerance as quantified by mVO_2 values were preserved in the ADRC-treated group (from 17.2 to 17.4 mL/[kg min] [$P = 0.8$]) and declined significantly in the control group (from 19.0 to 14.8 mL/[kg min] [$P = 0.001$]) (Perin et al. 2014). No clear dose-response relationship was observed between the two doses of ADSC and clinical or functional outcome data. The ATHENA 1 and 2, phase IIb studies will assess the efficacy of two increasing doses of ADSC in 48 patients (each trial) with ischaemic congestive heart failure and documented myocardial ischemia by nuclear perfusion imaging (NCT02052427).

3.2.1.8 Skeletal Myoblasts

Skeletal myoblasts were among the first cell types considered for cardiac repair. Also called satellite cells, they are found beneath the basal membrane of muscle tissue and muscle tendons and start to proliferate when stimulated by muscle injury or disease (Buckingham and Montarras 2008). Skeletal myoblasts were of special interest for cardiac repair, as they have been shown to transdifferentiate into non-striated 'myocardial-like' muscle cell types upon intramyocardial injection (Arsic et al. 2008). Moreover, striated myoblasts are relatively resistant to ischemia (Pagani et al. 2003), which is an obvious obstacle to the therapeutic effect of other stem cells in injured myocardium. However, skeletal myoblasts do not fully differentiate into cardiomyocytes in vivo after transplantation. The contracting myotubes do not operate in synchrony with the surrounding myocardium, which is most likely due to a lack of expression of the gap-junction protein, connexin 43, resulting in a lack of electromechanical coupling with the resident cardiomyocytes (Reinecke et al. 2002; Leobon et al. 2003). The first pilot studies provided the proof of concept and feasibility of the peri procedural and percutaneous intramyocardial delivery of autologous myoblasts in the treatment of patients with advanced congestive heart failure

(Herreros et al. 2003; Menasche et al. 2003; Pagani et al. 2003; Smits et al. 2003), yet the observed improvements of cardiac contractile function appeared to be only marginal. More importantly, these studies also raised the concern of ventricular tachyarrhythmias and ventricular storm (Fouts et al. 2006; Itabashi et al. 2005; Smits et al. 2003).

In the interim analysis of the first 21 patients with heart failure (NYHA class II–IV and ejection fraction <35 %) enrolled in the phase IIb MARVEL-1 study, authors suggested that tachyarrhythmia may be provoked by myoblast injection, but appears to be a transient and treatable problem if they were adequately pre medicated with an anti arrhythmic drug (i.e. Amiodarone 200 mg qd) (Povsic et al. 2011). The studies, assessing the safety and feasibility of percutaneous myoblast implantation in heart failure patients with implanted cardioverter-defibrillators, did not show an significant improvement of regional or global left ventricle function in treatment group compared to control group as well as ventricular arrhythmias did not differ significantly between groups (Duckers et al. 2011; Menasche et al. 2008). However, a greater propensity for arrhythmias during the early post-operative period cannot still be completely eliminated (Menasche 2008).

3.2.1.9 Resident Cardiac Stem Cells and Cardiospheres-Derived Cells

Several studies have suggested that the resident cardiomyocytes do respond to cardiovascular injury with an increased frequency of cell proliferation, suggesting that the heart is able to self renewal (Beltrami et al. 2001; Bergman et al. 2009). Moreover, Quaini and coworkers described autopsy studies in male recipients of female donor hearts (4–552 days following transplantation). Remarkably, the gender-related Y chromosome could be detected in the resident cardiomyocytes of the female donor hearts (Quaini et al. 2002). Alternatively, Deb and co-workers analyzed the hearts of three female recipients up to 2 years (35–600 days) following bone marrow transplantation from a male donor (Deb et al. 2003). In line, the gender-specific Y-chromosome could be detected in cardiomyocytes and endothelial cell in the heart of the female bone marrow recipient, suggesting that bone marrow-derived male cells had migrated into the female recipient heart to transdifferentiate into cardiomyocytes and endothelial cells. It was hypothesized that cardiac stem cells (CSCs) may reside in specialized niches, which support the growth and maintenance of the local cell pool (Fuchs et al. 2004). These proposed cardiac stem cell niches have been suggested to be localized in the myocardium with a concentration in the atria and apex (Beltrami at al. 2003; Urbanek et al. 2005, 2006). Several different phenotypical markers have proposed to designate the potential residential cardiac stem cell population, including stem cell antigen-1$^+$ (Sca-1)$^+$ C-kit$^+$ and Isl1$^+$. Isl1$^+$ cells can be detected in murine and human hearts, predominantly in the out-flow tracts, right ventricle and atria (Cai et al. 2003; Serradifalco et al. 2011; Weinberger et al. 2015).

In the phase I, randomized SCIPIO trial (Cardiac Stem Cell Infusion in Patients With Ischaemic CardiOmyopathy), transplanted C-kit$^+$, (CD117+)/lineage – (Lin –)

cardiac stem cells, previously isolated and expanded from autologous myocardial auricle material, led to significant improvement in ventricular function (by 8 %) in 16 patients with congestive heart failure at 4 months follow-up (CHF defined as LVEF <40 %) (Bolli et al. 2011).

Alternatively, in the CADUCEUS trial, autologous myocardial biopsies were harvested percutaneously and cultured in suspension to develop three-dimensional "cardiospheres". Stem cells derived from these cardiosphere, designated cardiospheres-derived cells (CDCs) express more than 95 % the marker CD105, suggesting predominantly mesenchymal (like) stem cell component of the cardiospheres. The, randomized, phase I trial (CADUCEUS) sought to define the safety of intracoronary infusion of cells derived from autologous cardiospheres in 31 patients with advanced heart failure (defined as 25 %< LVEF <45 %). At 12 months follow-up, this phase I study suggested that the procedure could be performed safely and showed a decrease in scar size (−11.9 ± 6.8 g myocardial tissue) and a non-significant improvement of global left ventricle ejection fraction compared to routine-care control patients (Malliaras et al. 2014). Notably, these CDCs originate from the heart and do not appear to migrate from the circulation or bone marrow, as evidenced by the analysis of short tandem repeat, FISH imaging for sex chromosomes, and qPCR analysis of recipient-specific HLA-A alleles (White et al. 2013).

CDCs derived from neonates demonstrated a higher ability to proliferate, resulting in a higher capacity, to preserve myocardial function, prevent adverse remodeling, and enhance blood vessel formation when compared with adult-derived CDCs in animal models (Mishra et al. 2011; Simpson et al. 2012). Allogeneic CDCs transplantation was also more potent to reverse adverse remodeling and improve the contractile function, supposedly due to the local immune reaction, although no histological evidence of rejection or systemic immunogenicity was detected (Malliaras et al. 2012; Kanazawa et al. 2015). The subsequent phase I/II randomized, double-blinded, placebo-controlled ALLSTAR trial will assess the safety and efficacy of these allogeneic CDCs in 274 human subjects with post-MI left ventricle dysfunction (NCT01458405). Alternatively, cardiac atrial appendage stem cells (CASCs) isolated from the right atrial appendages have shown to exert better cardiomyogenic differentiation than CDCs, with the ability to express sarcomeric-organized cardiac troponin I (cTnI) (Koninckx et al. 2013). In an animal model of acute myocardial infarction CDC-injected mice had a higher fraction of viable fuchsin-positive tissue within the infarct zone (24.9 %) than fibroblast-injected mice (17.7 %, $P < 0.01$) or PBS-injected mice (13.7 %, $P < 0.01$) (Smith et al. 2007).

3.2.1.10 Induced Pluripotent Stem Cells (iPSs)

iPS are derived from somatic cells by transgenic expression of the transcription factors Oct4, Sox2, Klf4 and cMyc or, Oct4, Sox2, NANOG and Lin2 to induce functional pluripotent stem cells, that express embryonic stem cell specific surface antigens (Takahashi et al. 2007; Yu et al. 2007). It was shown that iPS cells

an able to differentiate into cardiomyocytes in vitro and in vivo (Takahashi et al. 2007; Nelson et al. 2009; Martinez-Fernandez et al. 2009; Mummery et al. 2012; Burridge and Zambidis 2013). To date, iPS are used as an elegant in vitro model of human arrhythmogenic heart disease, including long QT syndrome, catecholaminergic polymorphic ventricular tachycardia, arrhythmogenic right ventricular cardiomyopathy and others (Eschenhagen et al. 2015). In vivo, iPS increased cardiac function after intramyocardial injection following an acute infarction, as well as in ischaemic cardiomyopathy models (Kawamura et al. 2012; Citro et al. 2014; Zhang et al. 2015). However, immunogenicity and the probability of teratoma formation prevent the clinical advancement of iPS technology for now (Araki et al. 2013; Cao et al. 2014).

A modification of this technology is the expression of embryonic sinoatrial node transcription factors in postnatal ventricular cardiomyocytes to generate sinoatrial cells. Adenoviral Tbx18 gene transfer have been shown to convert these ventricular myocytes to functional sinoatrial node pacemaker cells, which have been injected to generate a biological pacemaker in a complete heart block model (Kapoor et al. 2013; Hu et al. 2014b).

3.2.2 Embryonic Tissue-Derived Cells

Embryonic stem cells (ESC), derived from the inner mass of the blastocyst, offer theoretically limitless regenerative capacity, since they are able to give rise to most somatic cell lineages in vivo and in vitro. Furthermore, by culturing in various growth media, differentiation can be driven towards a desired cell type, including cardiomyocytes (Odorico et al. 2001). Implantation of these cells into infarcted cardiac tissue resulted in improvement of LV function in rats model (Min et al. 2002). However, there are some major concerns regarding the use of ESC in humans for regenerative therapy. First, the broad differentiation capacity along endo-, ecto- and mesodermal lineages, considerably increases the likelihood of teratoma formation (Blum and Benvenisty 2008). Second, there is increasing evidence that ESC, once thought to be uniquely immune privileged, express HLA subclasses (Draper et al. 2002). This raises the question how to avoid a possible alloimmune response. Immunosuppression with steroids is known to be harmful for ischaemic myocardium (Silverman and Pfeifer 1987). Nevertheless, new preclinical studies of ESC-derived cardiovascular progenitor cells showed differentiation into cardiomyocytes which cause left ventricle functional improvement without causing teratomas in a rodent infarct model and non-human primates (Blin et al. 2010; Bellamy et al. 2015). These observations led to the clinical trial using autologous human embryonic stem cell (hESC)-derived cardiac progenitors grown on a scaffold in six patients with severely impaired cardiac function scheduled for CABG (Menasché et al. 2014).

3.3 Cardiac Cell Therapy, Regenerative Principles

3.3.1 Stem Cell Isolation and Methodological Prerequisites

Important conditions for an effective clinical stem cell therapy are the precise and careful techniques of bone marrow cell harvest and preparation, availability of therapeutically relevant stem cell concentrations within the area of interest (for instance border zone of infarction), potential migration of resident stem cells into the apoptotic or necrotic myocardial area, and prevention of shedding of transplanted cells to extracardiac organs.

For cardiac stem cell transplantation, adult bone marrow (40–200 ml) is aspirated under local (or general) anaesthesia from the iliac crest. Specific bone marrow stem cell populations may then need to be isolated under good manufacturing practice (GMP) conditions. All microbiological tests of the clinically used cell preparations must prove negative for endogenous (HIV, HBV, HCV) and exogenous viral, fungal and bacterial contamination.

Remarkably, functional side-by-side analysis of the cellular grafts that have been used in clinical studies and cross correlation with clinical outcome, suggested that a significant part of the cellular graft had insufficient functional response on a colony forming unit assay or Boyden cell migration assay, and cross correlated with a poor clinical response to cell therapy (Bartunek et al. 2013; Assmus et al. 2014). At this time, it is unclear what the appropriate action should be when a graft does not meet the pre specified functional criteria, including exclusion of the patient from treatment, isolation of a new graft or treatment of the graft in vitro or combination of the cell graft with a correcting growth factor.

Once each individual stem cell preparation has passed all quality control analyses, the batch may then be released by the appropriate local qualified person. However, to date there is no consensus regarding the mandatory release criteria and quality control analyses for autologous (and allogeneic) grafts for cardiovascular purposes.

3.3.2 Stem Cells Distribution and Retention

After intramyocardial injection, a marked stem cells loss and pulmonary cell accumulation were found (Hou et al. 2005; Hong et al. 2013; Martens et al. 2014). A mere 16–37 % of the initial injected autologous stem cells could be detected at the injection site, 10 min post delivery, and only 7.6 % of stem cells could be still found after 7 days post transplantation (Zhang et al. 2012a; Hong et al. 2013). Intracoronary injected, labeled bone marrow progenitor cells were observed in the infarct border, rather than within the infarct as assessed by cardiac magnetic resonance imaging (Graham et al. 2010).

Stem cells distribution can be traced by different imaging methods. Cardiac magnetic resonance imaging (CMR) is potentially a better tool for high-resolution visualization compared to fluorescence imaging in the acute phase (Jasmin et al. 2012). However, for long-term cell tracking studies luciferase-based bioluminescence imaging of lentivirally labeled stem cells expressing green fluorescence protein may be preferred as this analysis of transgenic bioluminescence was able to successful track cells for up to 10 weeks after transplantation (Bai et al. 2011). In clinical studies, stem cells were traced by radioactive ^{111}Indium labeling and visualization by MIBI-SPECT in CAD patients (Kurpisz et al. 2007; Caveliers et al. 2007). SPECT revealed that only ±10 % of the cells were retained within the myocardium, while the majority sheded to the spleen, lungs and liver. Alternatively, the new imaging agent, 99mTc-NC100692 has a high affinity for αvβ3 Integrin, which is upregulated on proliferating endothelial cells. This may thus provide a new opportunity to analyze neoangiogenesis after stem cells therapy in clinical studies (Mozid et al. 2014a).

Although several studies demonstrated higher retention of stem cells at the treated region following intramyocardial, compared to intracoronary delivery (Hackett et al. 2000; Hou et al. 2005; Perin et al. 2008; George et al. 2008; Sun et al. 2008; Vrtovec et al. 2013b), comparison between intramyocardial and intracoronary delivery revealed no significant differences in safety (30 days MACE), feasibility, and outcome of the procedure (Mozid et al. 2014a). Also stem cells injection in the arrested, non-beating heart was superior with regard to cell retention as compared to cell injections into a beating heart (Zhang et al. 2007).

New injection methods have been explored to increase stem cell myocardial retention and cell viability. One of these options would be the injection of stem cells mixed with a hyaluronan-gelatin hydrogel, PEG-based hydrogels or a fibrin scaffold (Zhang et al. 2008a, 2010; Smith et al. 2013). Various studies have verified the viability of the stem cells, ability to proliferation, migration, and growth factor secretion in these polymers (Fang et al. 2004; Almond 2007; Shapira-Schweitzer et al. 2009; Guo et al. 2011; Ferriera et al. 2012; Wu et al. 2012; Kim et al. 2013). In vivo, injection of stem cells suspended in fibrin glue showed an enhanced neoangiogenesis response and further improvement of left ventricle function as compared to treatment with stem cells or fibrin glue alone (Christman et al. 2004; Li and Gao 2007).

Ischaemic preconditioning of stem cells is another simple option technique to increase stem cells viability and retention. Ischaemic preconditioning of the heart has been shown to limit infarct size and decrease myocardial cell death in an AMI model (Murry et al. 1986). In addition, hypoxic preconditioning of the stem cells increased expression of pro-survival and pro-angiogenic factors, promoted stem cells migration, which resulted in attenuated mitochondrial damages, increased capillary density with increased ejection fraction and decreased infarct size (Hu et al. 2008, 2014a; Wang et al. 2009a; Yan et al. 2012; Jaussaud et al. 2013). These effects are mediated via intracellular mechanisms, including the increase in hypoxia-inducible factor-1 (HIF- 1), cytoprotection against reactive oxygen species (ROS)

produced in mitochondria, upregulation of Connexin-43 expression, and SDF-1 and/or CXCR4 expression (Yu et al. 2013; Sart et al. 2014). Following the hypoxic insult, upregulation of miR-22 in stem cells after ischaemic preconditioning can transferred from stem cells to cardiomyocytes and attenuate apoptosis (Feng et al. 2014).

Pharmacological preconditioning by co-incubation with various growth factor and cytokines likewise resulted in the improvement in stem cell viability and inhibit apoptosis, including vascular endothelial growth factor (VEGF) (Pons et al.2008), insulin-like growth factor 1 (IGF-1) (Guo et al. 2008), TGF-beta (Herrmann et al. 2010), bradykinin (Sheng et al. 2013), angiotensin II type 2 receptor (Xu et al. 2013), and oxytocin (Noiseux et al. 2012).

3.3.3 Delivery Techniques

3.3.3.1 Intracoronary Application

Initially interventional cardiology, selective intracoronary delivery route was used with the intention to minimize shedding to non-targeted organs (Strauer et al. 2001, 2002). To facilitate transendothelial passage and migration into the infarcted zone, an angioplasty balloon was inflated in the proximal segment of coronary arteries and cells were infused in the culprit vessel (stop-flow technique) (Strauer and Steinhoff 2011). The balloon was kept inflated for 2–4 min to prevent washing away of grafted cells, and repeated four to five times. The balloon occlusion prevented backflow of cells, and at the same time produced a stop-flow beyond the site of balloon inflation to facilitate adhesion and transmigration of the cells into the infarcted zone. This transmigration process is facilitated in injured and ischaemic viable tissue (Szilvassy et al. 1999), as local myocardial ischemia is a potent stimulus for chemokinesis of stem cells due to SDF-1 and CXCR4 signalling (Kamota et al. 2009; Elmadbouh et al. 2007; Jiang et al. 2013).

Therefore, the ischemia-producing stimulus due to stop flow technique may actually promote the cell adhesion and extravasation into the myocardial tissue (Sussman 2001; Loffredo et al. 2011). Delivery of stem cells to the injured or failing myocardium by a simple intracoronary administration seemed to be sufficient to promote myocardial repair (Fuchs et al. 2001; Galinanes et al. 2004). The intracoronary approach, however, should be reserved for the smaller, mononuclear bone marrow stem cells, since intracoronary application of cultured cell types, like mesenchymal stem cells or skeletal myoblasts, was associated with microembolisation and significant microvascular obstruction (Furlani et al. 2009). Due to their size, flexibility and shape, these cells are more prone to embolisation than bone marrow mononuclear cells. To date, thromboembolisation due to the clinical application of BMMNC has not been reported.

3.3.3.2 Intramyocardial Application

In contrast to intracoronary application, the intramyocardial injection of stem cells results in the direct delivery into the myocardial target area, without dependence on vascular access or sufficient cell migration across the endothelial barrier.

Endoventricular Intramyocardial Injection of Stem Cells

Percutaneous endoventricular injection of cell preparations are generally guided using routine fluoroscopic ventriculography or by electromechanical mapping using the NogaStar mapping catheter (Biologics Delivery Systems, Diamond Bar, CA).

The percutaneous transendocardial delivery catheter Helix® (BioCardia) uses direct fluoroscopic imaging and has a corkscrew needle, which ensures fixation of the catheter to the beating heart wall during injection of the cells (de la Fuente et al. 2007; Trachtenberg et al. 2011). The C-Cath® transendocardial injection catheter as well uses simple fluoroscopic guidance, has a 75°-curved, deflectable nitinol needle to secure the appositioning of the catheter to the wall during the cardiac cycle (Behfar et al. 2013). The myocardial cell retention of these catheters typically varies between the 20 and 35 %.

The Myostar® is a steerable injection catheter interfaced with the NOGA© three-dimensional electromechanical endoventricular mapping system. Based on the structural reconstruction of unipolar voltage mapping in combination with the mapping of linear local shortening, the operator can distinguish in real time viable myocardium (generation of local unipolar voltage of ≥ 6.9 mV, and local shortening), from non-viable, scar tissue (absence of unipolar voltage and local shortening). Hibernating myocardium in the ischaemic (infarct border) zone is characterized by the local generation of an unipolar voltage signal (suggesting viable myocardial tissue), in the absence of local contraction. It is hypothesized that the ischaemic, hibernating myocardium would be optimal myocardial target segment for cell therapy, as locally improved myocardial perfusion may reinstate myocardial contraction. Clinical studies have established safety and feasibility of the transendocardial intramyocardial injection in the setting of chronic heart failure (Perin et al. 2003), refractory angina (Losordo et al. 2007), as well as in subacute myocardial infarction (Heeger et al. 2012). However, electromechanical mapping and injection is still laborious, technically demanding, and requires two interventional operators. In addition, cell loss and shedding to non-targeted organs is still considerable for all percutaneous injection catheters.

Epicardial Application

Stem cell implantation during open heart surgery is generally performed into well exposed epicardial ischaemic areas allowing for multiple injections within, and principally, around the infarct area with a thin needle. The first clinical studies

performed stem cell injections in combination with coronary artery bypass grafting (CABG). Once the graft-coronary artery bypasses have been completed the residual ischaemic area is visualized, and the cells can be injected (Dib et al. 2005; Stamm et al. 2007). This procedure is limited to certain areas of the left ventricle, and cannot be used for the septal myocardial segments. This method also has been applied successfully during off-pump coronary artery bypass grafting, as well as stand-alone minimally invasive procedures. However, the therapeutic effect of intramyocardial stem cell therapy is difficult to be unequivocally interpreted when performed together with a revascularisation procedure. Therefore, recent reports about surgical "stand alone" stem cell therapy are of particular interest (Klein et al. 2007; Pompilio et al. 2008). Herein, myocardial perfusion and clinical symptoms improved as a result of stem cell injection only via lateral minithoracotomy. Besides distinguishing between the effect of stem cells and revascularization effects on cardiac function, this approach could help to further minimize perioperative risks in the context of surgical stem cell therapy.

Epicardial delivery of bioengineered composite sheets harboring stem cells is a new method of stem cell delivery. Stem cells sheets adhere to the epicardial surface spontaneously, or as collagen-based patches (Bel et al. 2010; Hamdi et al. 2014). Adipose-derived stromal cell sheets resulted in significant improved survival and left ventricular remodeling in an infarct model in rats compared to intramyocardial injections (Hamdi et al. 2011).

Transvascular Delivery

The transvascular delivery of stem cells occurs via transvenous or transarterial delivery under intravascular ultrasound (IVUS) imaging. Transvenous intramyocardial delivery of a cell-hydrogel by the TransAccess® delivery catheter (Medtronic Vascular, Santa Rosa, CA, USA) was first described in 2003 (Thomson et al. 2003). Later, the safety of procedure and superior cell retention compared to intracoronary delivery was demonstrated in a pig infarct model (George et al. 2008). The Mercator Cricket® and Bullfrog® microinfusion devices (Mercator MedSystems, Emeryville, CA) were developed for the delivery of therapeutic agents specifically into the perivascular space. Upon inflation of the balloon on these delivery devices, a perpendicular-positioned microneedle penetrates the coronary artery wall and allows the user to inject the stem cells directly into the perivascular space (tunica adventitia of the coronary artery). In preclinical phase the method has shown safety and efficacy in a pig infarct model (Wang et al. 2009b; Medicetty et al. 2012). In a phase I clinical trial procedural safety and efficacy was suggested as ventricular function was improved with no detectable arrhythmias at 6 months follow-up in ten patients with heart failure after percutaneous transvenous transplantation of autologous skeletal myoblasts (Siminiak et al. 2006).

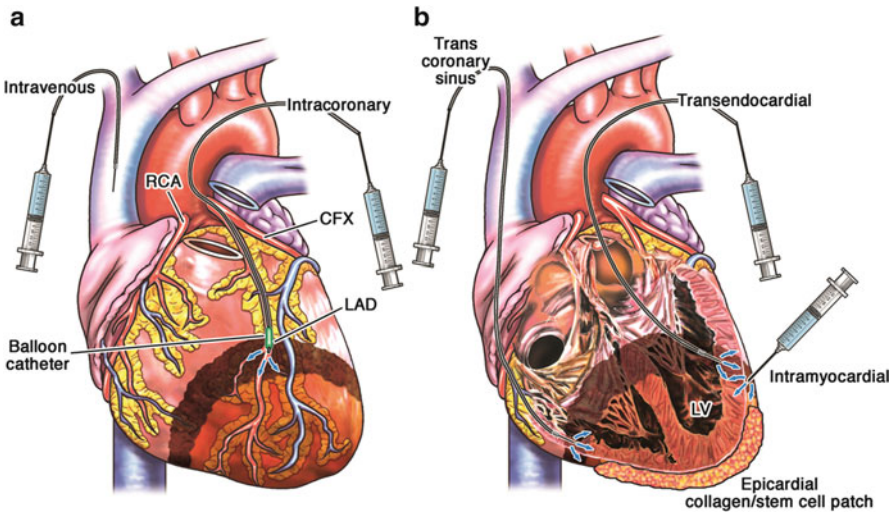

Fig. 3.1 Illustration of stem cell delivery techniques (Strauer and Steinhoff 2011)

3.3.3.3 Retrograde Coronary Sinus Infusion of Stem Cells

Retrograde coronary sinus (RCV) infusion is performed by femoral venous access and the positioning of a conventional angioplasty balloon into the mid portion of the coronary sinus followed by stem cell infusion. Clinical studies demonstrated safety and efficacy of delivering stem cells via the coronary sinus (Patel et al. 2015; Tuma et al. 2011). At 1 day post-RCV infusion, the highest level of stem cells were observed in the left ventricle, compared with the remaining myocardial chambers and scar area in AMI murine model, as well as RCV-delivered stem cells retained in the CHF heart as minimum for 21 days (Zakharova et al. 2014).

Coronary sinus delivery could be recommended in cases of severe aortic stenosis, severe peripheral artery disease or intraventricular thrombus formations which precludes percutaneous endoventricular injection. The method is also possible for patients with a resynchronization device. Potential complications are coronary sinus rupture and embolisation (Dib et al. 2011; Hong et al. 2014).

3.3.3.4 Intravenous Delivery of Stem Cells

Intavenous infusion is the most safe and cost-effective stem cell delivery method. Its safety and feasibility have been shown in swine model of myocardial infarction (Halkos et al. 2008) and later in a phase I clinical study in reperfused myocardial infarction patients (n=53) after delivery of allogeneic mesenchymal stem cells (O'Hare et al. 2009). The study resulted significant improvement of injection fraction in treated group versus placebo at 12 months follow-up, although the myocardial retention following intravenous injections is mere 0.5% (Wang et al. 2011) (Fig. 3.1).

3.3.4 Cardiac Tissue Engineering

The purpose of cardiac tissue engineering is to replace or repair injured heart muscle or vasculature effectively. It comprises a biomaterial based 'vehicle', either a porous scaffold or dense patch, made of either natural or synthetic polymeric materials, to aid transportation of cells into the diseased region in the heart. Such scaffolds serve several purposes: they allow cell attachment and cell migration, they deliver and retain cells and biochemical factors and they can exert certain mechanical and biological influence to modify the behaviour of the cell phase (Christman and Lee 2006). To achieve sufficient tissue reconstruction, scaffolds have to meet some specific requirements. They should provide high porosity and an adequate pore size to facilitate cell seeding and migration throughout the whole structure. Furthermore, biodegradability is essential, as implanted scaffolds should be absorbed by the surrounding tissue, without the need for surgical removal, and the rate of degradation should match the rate of tissue formation as much as possible. This means the scaffold will provide structural integrity as long as the seeded cells produce their own natural matrix structure (Eschenhagen and Zimmermann 2005). The final goal is to allow- by the use of specific biomaterials- the creation of a microenvironment, where exogenous and endogenous cells find the optimal conditions for myocardial repair with low scar formation (Chachques 2009). One should also think of standalone implantable, exogenously cellularized matrixes as a supplement of intramyocardial stem cell therapy. This principle has been successfully demonstrated for tissue generated from neonatal cardiomyocytes (Shimizu et al. 2002; Zimmermann et al. 2006). The exogenous matrix could, for example, help to adjust modified collagen proportions within the scar zone and thereby contribute to the regenerative process (Kutschka et al. 2006). The first clinical studies with alginate injections after myocardial infarction and congestive heart failure are underway (PRESERVATION1 study, NCT01226563; AUGMENT HF, NCT01311791) (Hirt et al. 2014).

Some bioengineering studies focussed on specific cardiac structures, such as heart valves and the conduction system. Tissue bioengineering was started on the basis of seeding biodegradable polymer scaffold with autologous vascular cells in 1995 (Shinoka et al. 1995). These autologous vascular graft was further developed and successfully introduced into cardiac surgery as an extracardiac cavopulmonary conduit in patients with single ventricle physiology (Shinoka and Breuer 2008; Hibino et al. 2010). Enzymatically decellularized biological heart valves, which were reseeded with autologous myofibroblasts and/or endothelial cells were developed by the Hannover group (Steinhoff et al. 2000; Tudorache et al. 2013) and introduced in patients with pediatric heart valve surgery with pulmonary conduit replacement (Cebotari et al. 2006).

Bioengineering of pacemaker cells was only performed experimentally. Transplantation of fetal cardiac cells including conduction tissue was successfully applied in an experimental atrioventricular block canine model (Ruhparwar et al. 2002), ex vivo engineered conduction tissue was successfully applied in a rat of conduction block (Choi et al. 2006; Cingolani et al. 2014). As of yet clinical applications have not been performed with tissue engineered conduction tissue.

3.3.5 Pharmacological Strategies

Pharmacological strategies have been aimed to (i) intensify stem cell action by promoting homing into heart muscle and survival of the stem cells, and (ii) by stimulating recruitment of stem cells from bone marrow. Intravenous dobutamine during and up to 24 h after intracoronary stem cell transplantation was effective to promote cell homing, likewise, dipyridamole (0,5 mg/kg intracoronarily over 10 min) has been used prior to stem cell application to stimulation uptake (Strauer et al. 2008, 2009). Pharmacologically, recruitment of mononuclear bone marrow cells to the circulation can be induced by G-CSF cytokine injection (e.g. granulocyte colony stimulating factor). Recent combination of GM-CSF with SDF-1/CXCR-4 inducing substances, like erythropoietin (Klopsch et al. 2009; Brunner et al. 2009), parathyroid hormone (Huber et al. 2010, 2011) or sitagliptin (Theiss et al. 2011), show promising effects on post infarction recovery in experimental animal models and are now introduced in clinical trials in patients admitted for an acute myocardial infarction. Clinical studies employing erythropoietin treatment immediately after primary PCI in patients with an acute myocardial infarction showed reduction of infarction size by 30 % (Ferrario et al. 2011), but also showed microvascular obstruction (Prunier et al. 2012). Further studies are needed to test clinical safety and efficacy.

3.4 Indications and Clinical Principles

The most studied indications for cardiovascular stem cell therapy have been myocardial infarction with a large infarct area and reduced residual left ventricle ejection fraction, as well as ischaemic congestive heart failure. Further indications may also include non-ischaemic cardiomyopathy, like diabetic cardiomyopathy, dilating idiopathic cardiomyopathy and Chagas disease. The common inclusion criterion for cardiac cell therapy was the reduction of the ventricular function with an akinetic, viable myocardial target segment (which offered no target for conventional revascularization procedures). Prior to cell transplantation, the patient's heart failure symptoms (and NYHA classification), neurohumoral status, and baseline myocardial function, viability (and perfusion) should be assessed. If cell therapy will be delivered by intramyocardial (endoventricular) injection, a transthoracic echocardiography should be performed to exclude an intracardial thrombus and to determine the thickness of the target myocardial segments (minimal thickness should exceed 8 mm), and to exclude significant aortic valvular stenoses. Some of the cell therapy studies have been geared to get the patient of the transplantation list (VO2 max >14) or to wean them off the left ventricle assist device (end therapy).

3.4.1 Ischaemic Cardiomyopathy

Cardiac stem cell therapy is indicated in patients with significantly impaired left ventricular ejection fraction (between 20 and 40 %) due to prior myocardial infarction, leading to symptoms of NYHA heart failure class II–IV, with or without angina. Preferably, the target myocardial segment(s) should be hibernating myocardial segments (viable, non contractile tissue) with objectified local ischemia The wall thickness of the target segment should be minimally 8 mm on echocardiographic evaluation in order to avoid potential ventricular wall perforation, pericardial effusion and cardiac tamponade (Chachques 2009). Although, early injection after acute myocardial infarction could be beneficial, acute heart failure (and/or cardiogenic shock), rhythmic instability and necrotic (target myocardial segment precluded the acute treatment of the patients. In addition, it was hypothesized that cell transplantation would be more advatagious in the subacute phase of AMI, at day 8–30 post-AMI, as the post-ischaemic inflammatory reaction would have subsided and the cells would be transferred into a less hostile microenvironment, thereby promoting the cell viability and efficacy (Chachques 2009; Kaminski and Steinhoff 2008). Stem cell transplantation into an inflammatory ischaemic zone may lead BMMNC to take part in the inflammation cascade, rather than to promote the myocardial and vascular repair response (Soeki et al. 2000). Clinical trials reported benefits in patients with ischaemic cardiomyopathy from BMMNCs (Strauer et al. 2010; Honold et al. 2013), autologous MSCs (Mathiasen et al. 2012; Heldman et al. 2014), ADSC (Houtgraaf et al. 2013), CDCs (Makkar et al. 2012; Malliaras et al. 2014) and peripheral mobilized SCs (Poglajen et al. 2014; Mozid et al. 2014b). Combined coronary artery bypass surgery with BMMNC application resulted in an increase in left ventricular ejection fraction of ±10 %, and improvement of wall motion caused by enhanced myocardial perfusion (Ahmadi et al. 2007; Stamm et al. 2007; Zhao et al. 2008). Studies combining stem cell transplantation with off-pump CABG surgery reported similar results (Patel et al. 2005), implicating that cardiac cardioplegia is not mandatory for safe and efficient stem cell implantation.

3.4.2 Refractory Angina Pectoris

A relative new indication for stem cell therapy are patients with severe refractory angina not suitable for conventional revascularization. Usually, these patients have viable ischaemic myocardium without ventricular dysfunction. Stand alone intracoronary or intramyocardial infusion of bone marrow or G-CSF-mobilized $CD34^+$ stem cell (Wang et al. 2010; Povsic et al. 2013), as well as intramyocardial injection of BMMNC (Tse et al. 2007; Hossne et al. 2015), CD 133^+ SC

(Jimenez-Quevedo et al. 2014) or MSC (Mathiasen et al. 2013) resulted in a decrease of angina frequency, nitroglycerine usage, the Canadian Cardiovascular Society angina classification, increase in exercise tolerance, due to improved myocardial perfusion as assessed by single-photon emission computed tomography or magnetic resonance imaging (Wang et al. 2010; Hossne et al. 2015). In one study, patients with multivessel CAD and incomplete CABG were enrolled. CMR analysis showed a similar reduction in the ischaemic score in patients who received complete versus patients with an incomplete CABG combined with BMMNC injection (Gowdak et al. 2011).

3.4.3 Acute Myocardial Infarction

A variety of phase I and II randomized clinical trials have been able to establish the short and long term improvement of ventricular function after using stem cell therapy. Unfortunately, there has been considerable heterogeneity of study protocols in the initial cardiovascular cell therapy trials, that precludes the appropriate interpretation of the study results, including a variety of patient selection, cardiac imaging and cell transplantation protocol, including the timing of cell transplantation, different methods of cell harvest, preparation, cell storage (Ficoll, heparinization in storage solution), BMMNC composition (and number of CD34 or CD133 cells), BMMCN dose and cell delivery. On meta analysis (of 22 randomized controlled trials (RCTs)), autologous BMMNC therapy led to an unequivocal improvement of left ventricle function of 2.1 % and infarct size reduction by −2.69 % as well as had is no effect on cardiac function, volumes, or infarct size, when only RCTs (n = 9) that used MRI-derived end points were analyzed (de Jong et al. 2014). This improvement is comparable to the benefit on post-infarct left ventricle function of standard primary PCI and optimal pharmacotherapy, which conventionally leads to 4 % left ventricle ejection fraction improvement (Assmus et al. 2006; Schachinger et al. 2006). Moreover, no beneficial effect could be detected on major adverse cardiac and cerebrovascular event (MACCE) rates after BMMNC infusion after 6 month follow-up (de Jong et al. 2014) and specifically at 5 year follow-up of the REPAIR-AMI (Assmus et al. 2014). Whereas, MSC transplantation in patients with subacute AMI resulted in an increase of ejection fraction +6.28 % as well as reduction in ventricular fibrillation/ventricular tachycardia (OR, 0.08) and implantable cardioverter defibrillator implantations (OR, 0.08) compared to control group (de Jong et al. 2014).

Phase III ongoing trials are of particular interest, since they could clarify the effect of autologous BMMNCs (RACE-STEMI (NCT02323620), BAMI (NCT01569178)) and MCSs (RELIEF (NCT01652209), ESTIMATION (NCT01394432)) infusion into the setting of myocardial infarction. The main inclusion criteria are acute myocardial infarction treated with PCI and reduced ejection fraction (EF <45 %). Primary and secondary end points include changes in ejection fraction and left ventricle dimension as well as cardiac death and MACCEs. The trials are expected to report their final data in 2016 year.

3.4.4 Non Ischaemic Cardiomyopathy

Also patients suffering from non-ischaemic cardiomyopathy (CMP) may benefit from cardiac stem cell therapy. In preclinical animal models of non-ischaemic CMP, including the rabbit model of idiopathic dilated cardiomyopathy and doxorubicin-induced heart failure model, BMMNC treatment benefited in left ventricle contractility testing with Millar catheter as well as BMMNC transplantation led to significantly less beta-adrenoreceptor down-regulation in septum and left ventricle in comparison with doxorubicin-treated animals and sham-operated rabbits (Dhein et al. 2006). In the first randomized controlled studies intracoronary admission of autologous BMMNC in 44 and 20 patients with idiopathic (coronary disease had been excluded by coronary angiography, whereas myocarditis had been excluded by an endomyocardial biopsy) dilated cardiomyopathy resulted in a decrease of the NYHA Functional Classification in cell treatment patients, as well as an improvement of left ventricle ejection fraction by 5.4 % in first-in-man ABCD and 8 % in Düsseldorf ABCD Trial. Furthermore, data from the First-in-Man ABCD trial suggested that the benefit of stem cell therapy was mediated be a paracrine effect resulting in improved vascular density (Seth et al. 2006). At 5 years follow-up the intracoronary administration of $CD34^+$ stem cells in 40 patients with non-ischaemic dilated cardiomyopathy sustained improvement of left ventricle function (EF +8.1 %) and exercise activity (6-min Walk Test +125 m) (Vrtovec et al. 2013a). In two paediatric patients suffering from idiopathic dilating cardiomyopathy showed a clear improvement in left ventricular ejection fraction (from 16 to 39 % and from 34 to 51 %) 8 weeks follow-up after intracoronary transplantation of circulating peripheral blood progenitor cells (Olgunturk et al. 2009). The INTRACELL trial was a prospective randomized controlled trial to determine the effect of intramyocardial injection of unfractionated BMMNC in 30 patients with non-ischaemic dilated cardiomyopathy (LVEF <35 %). At 9 month follow-up no improvement of left ventricle ejection fraction was detected as measured by MRI (Sant'Anna et al. 2014). The authors suggested that high mortality could be attributed to very low LVEF and proposed to include patients with LVEF >25 %.

In the MesoBlast-CHF phase I–II, single blinded, controlled study, 60 patients both with non-ischaemic and ischaemic cardiomyopathy were treated with intramyocardial injected allogeneic mesenchymal precursor cells. At 36 months follow-up, a significant reduction in major adverse heart failure events had been noted in allogeneic high-dose MSC/MPC-treated group in comparison to placebo treated patients, whereas no significant effect had been noted on left ventricle function at 6 and 12 month follow-up (Perin et al. 2015).

3.4.5 Diabetic Cardiomyopathy

It is well known that diabetic patients have an increased risk of developing heart failure due to diabetic cardiomyopathy, which is characterized by microvascular and macrovascular angiopathy and interstitial fibrosis. Diabetic cardiomyopathy

animal models have shown that transfer of mesenchymal stem cells and induced pluripotent stem cells prevent apoptosis of cardiomyocytes via upregulation of Akt, and inhibition of myocardial fibrosis by decreasing the transcriptional level of the matrix metalloproteinase MMP-9 (Zhang et al. 2008; Yan and Singla 2013). Furthermore, mesenchymal stem cell transplantation suppressed the inducibility of ventricular arrhythmias (Wang et al. 2013), as well as significantly increase myocardial arteriolar density in diabetic myocardium, leading to an improved cardiac function (Zhang et al. 2008b).

3.4.6 Chagas Heart Disease

Chagas disease, also called American Trypanosomisasis, is caused by the protozoan Trypanosoma cruzi and remains one of the major health problems in Latin America. In chronic cases of Chagas disease, 10–30 % of the patients will eventually develop cardiomyopathy. In its final stages, no other treatment options exist aside heart transplantation. Three main pathogenetic mechanisms characterize the Chagas heart disease: derangements of the autonomic nervous system, microvascular disturbance and immune-mediated myocardial injury (Chachques 2009). On the long run, patients develop severe cardiac arrhythmias, dilated cardiomyopathy with manifest heart failure. Since the number of available organs for transplantation is very limited, and in addition, late reactivation of the Chagas disease in the heart have been reported after heart transplantation due to isolated organ lesions, cardiac cell therapy is an important option for patients with secondary heart failure caused by Chagas disease. In the pilot Phase I clinical study BMMSC transplantation in patients with Chagas disease resulted in improvement of LVEF by + 2.9 %, NYHA functional classification by – 1.3 class and distance walked in six minutes by + 88 meters (Vilas-Boas et al. 2006). Regrettably, the large clinical trial in 183 patients (90 stem cell and 93 control) with chagasic cardiomyopathy (NYHA class II to IV, LVEF <35 %) did not reveal significant difference between stem cell and control group in cardiac functions and quality of life after selective intracoronary infusion of BMMSC (Ribeiro Dos Santos et al. 2012). However, clinical studies with different types of cells should be continued.

3.4.7 Right Ventricular Heart Failure

Preclinical studies in a murine model of pressure-overloaded right ventricle heart failure showed efficacy of myoblast sheet transplantation as suggest by a decrease in the right ventricle end diastolic pressure and improvement of isovolumic relaxation (Hoashi et al. 2009). Attenuation of pulmonary arterial hypertension and right ventricle hypertrophy were also noticed after intravenous stem cells injection of allogeneic MSCs, ADSC and ESC in rats, potentially by stem cells colonization and

differentiation into endothelial cells thereby reinstating endothelial function and prevention of pulmonary arterial remodeling (Umar et al. 2009; Zhang et al. 2012b; Luo et al. 2014).

In patients with hypoplasic left ventricle syndrome, patients have aside from hypoplasia of the left ventricle, also hypoplasia of the aorta, and related valvular components. The systemic flow is generated by the right ventricle with a right-left shunt via the ductus arteriosus. Although palliative operations, including Norwood, Glenn, and Fontan corrections, prevent early mortality, children nevertheless die due to progressive right ventricular dysfunction. Intracoronary administration of autologous CDCs improved right ventricular ejection fraction and somatic growth resulting in reduced heart failure in 14 patients with hypoplastic left heart syndrome after staged surgery (Ishigami et al. 2015).

A decrease in pulmonary peak pressure was also observed in STEMI patients treated with a combination of intracoronary BMMC therapy and thrombolysis compared to patients with thrombolytic therapy alone in the FINCELL trial (Miettinen et al. 2010).

Intravenous infusion of autologous endothelial progenitor cells also ameliorated 15 patients with idiopathic pulmonary arterial hypertension as suggested by a decrease of 4 mm Hg mean pulmonary artery pressure, and an increase of 40 m on the 6 min walk distance at 12 weeks follow-up compared to control patients (Wang et al. 2007).

3.4.8 Atherosclerosis

Recent meta-analyses of adult bone marrow cell transplantation in 2,625 and 1,255 patients with chronic ischaemic heart disease revealed significantly decrease of cardiac mortality, recurrent AMI, angina frequency and Canadian Cardiovascular Society angina class after stem cells transplantation (Jeevanantham et al. 2012; Fisher et al. 2014). These indices could be explained by positive influence of stem cells therapy on the progression of atherosclerosis and atherosclerotic plaque stabilization. This may be due to the reendothelialisation of atherosclerotic plaques, thereby replacing dysfunctional endothelial cells en reinstating the endothelial, quiescent function. The positive effect of stem cells on atherosclerosis formation and stabilization was further reported for MSCs. Human MSCs co-cultured with endothelial cells restored Akt/eNOS activity, increased eNOS level and NO production (Lin et al. 2015). Injection of MSCs decreased the size of atherosclerotic plaques by increasing the number and function of T-regs and inhibiting the formation of macrophage foam cells in Apolipoprotein E(−/−) (ApoE-KO) mice (Wang et al. 2015). G-CSF treatment was also associated with smaller atheromatous plaque, supposedly by mobilization of bone marrow stem cells in the circulation (Sinha et al. 2014).

Interaction of hyperlipideamia and stem cells have also been reported: It was noticed that low density lipoprotein inhibited EPC survival and function due to

their inhibitory effect on eNOS activity and expression (Ma et al. 2006; Carracedo et al. 2011). Moreover, the number of circulating EPCs correlated with artery elasticity indices (Tao et al. 2006). Intravenously bone marrow stem cells or spleen cell-derived EPCs transplantation resulted in a normalization of plasma cholesterol level and substantial reduction of atherosclerotic lesions in animal models (George et al. 2005).

3.4.9 Exclusion Criteria for Cardiac Cell Therapy

Patients presenting with a prior history of ventricular arrhythmias, as well as patients with an implanted cardiac defibrillator (ICD) or candidates for an implantation of an ICD should be carefully monitored following cell transplantation to exclude a potential arrhythmia as a complication of cell transfer, although arrhythmia have only been associated with myoblast injections (Villa et al. 2007). Furthermore, patients with hematologic disease should be excluded from bone marrow stem cell transplantation. In addition, subjects with a history of drug abuse, cancer, or with active infectious disease (HIV, viral hepatitis) or positive viral test are also less likely candidates for stem cell therapy (Chachques 2009).

3.4.10 Stem Cells Arrhythmogenicity

Stem cells therapy in heart failure is aimed to improve ventricular geometry and function, thereby indirectly reducing ventricular arrhythmias, by reduction of its provoking determinants (prevention of left ventricle remodelling, reducing wall stress, reducing cardiac ischemia, ect.). Studies concerning the arrhythmogenicity of different stem cells have to consider that the diseased heart muscle is prone to ventricular arrhythmias and may alter its electric instability parallel to remodelling. The arrhythmogenic risk in the course of stem cell transplantation depends on the degree of myocardial damage, the specific ability of the stem cell type itself to create arrhythmias (for instance fail to generate electro-mechanical coupling with resident cardiomyocytes), and the mode of delivery. Skeletal myoblasts lose its capacity to create connexion 43 over time which results in insufficient gap junction formation (Macia and Boyden 2009). The functional integration of skeletal myoblasts in the resident cardiomyocyte population is therefore poor and increases arrhythmogenicity (by re-entry tachyarrhythmias). Mesenchymal stem cells have an inhomogenously increased arrhythmogenicity, despite good coupling with cardiomyocytes, presumably because of increased tissue heterogeneity induced by unexcitable mesenchymal cells (Ly and Nattel 2009). In the majority of studies, bone marrow stem cells do not appear to provoke arrhythmias. In contrast, anti-arrhythmic effects have been observed after BMMNC and MSC therapy in patients with an acute myocardial infarction and in patients with chronic heart failure (Wollert et al. 2004; Yousef

et al. 2009). The significant reduction of non-sustained ventricle tachycardia and ventricular ectopy (PVCs) was observed in APOLLO study in ADCS-treated patients post AMI (Houtgraaf et al. 2012). Stem cell therapy prevented ventricular adverse remodelling and decrease wall stress, both know to be important pro-arrhythmic factors. In advanced cardiac failure a significant decrease in premature beats and the occurrence late potentials have been found, which is compatible with an anti-arhythmic effect of stem cells. Moreover, after stem cell transplantation, left ventricular synchrony was improved, a result, which may potentially render BMC therapy suitable for resynchronisation interventions. In brief, it seems reasonable to assume that the type of stem cells, may determine the arrhythmic fate of the heart: bone marrow stem cells seem to be neutral with regard to arrhythmogenity and may even exert anti-arrhythmic properties, whereas myoblasts, mesenchymal and preferably embryonic stem cells are prone to inducible arrhythmias.

3.5 Clinical Trials

More then 600 clinical trials in different phases, studying stem cells therapy in cardiovascular disease, have been registered in www.clinicaltrials.gov. The extent to which stem cell transplantation can improve the patient's outcome, however, is debated, as there is no unity of primary (and secondary) relevant endpoints in the different clinical trials, and the methodology of cell transplantation, and in- and exclusion criteria used have been quite heterogeneous. Here, we tried to provide an overview of the major randomized, placebo-controlled, phase II–III clinical trials, and the most recent meta-analyses of stem cells cardiovascular therapy.

3.5.1 Current and Future Cardiovascular Stem Cell Therapy Clinical Trials in Patients with Acute Myocardial Infarction

Nowadays, a variety of studies have demonstrated safety and efficacy of MCSc or BMMNCs transplantation in patients with AMI. The completed phase III studies (64 and 59 patients each) resulted in an improved ejection fraction by 2.8 % at 12 month follow-up and by 6.5 % at 24 month follow-up as assessed by MRI in BMMNC-treated group compared to control (Dill et al. 2009; Assmus et al. 2010). Otherwise, the Late TIME trial did not show any preferences in functional improvement in the BMMNC-treated group treated 2–3 weeks after myocardial infarction compared to control patients receiving optimal medical care (Traverse et al. 2011). In phase III trial determined an effect of BM-derived MSCs in 58 patients with acute myocardial infarction, left ventricle ejection fraction by SPECT was greater in the treated group than in the control group (5.9 % vs 1.6 %, P=0.037) at 6 month

follow-up (Lee et al. 2014). Autologous intramyocardial bone marrow derived $CD133^+$ delivery during CABG in patients with a recent myocardial infarction resulted in significant improvement in myocardial viability and perfusion compared to the control group as measured on 18 and 9 patients (Ahmadi et al. 2007).

Several large meta-analyses analysed the effect of stem cell therapy in patients with acute myocardial infarction that were reported in randomized controlled trials (with/without registry data). Clifford and co-authors compiled data from 33 studies with a total 1,765 participants treated with adult bone marrow-derived stem cells after acute myocardial infarction. Stem/progenitor cell treatment was not associated with statistically significant changes in the incidence of mortality or MACCE. Nevertheless, stem cell treatment was observed to improve left ventricular ejection fraction by +2.8 % (95 % CI 2.00–3.73) in short-term and by +3.7, (95 % CI 2.57–4.93) in long term follow-up (Clifford et al. 2012). In another recent meta analysis, de Jong and co-workers compiled the data of solely randomized controlled trials (total of 22 trials) of BMMNC therapy in 2,037 patients with an acute myocardial infarction, and likewise described an improvement of left ventricle ejection fraction by +2.1 % (95 % CI, 0.68–3.52; P=0.004), a reduction of left ventricle end systolic volume by 4.1 mL; 95 % CI, −6.91 to −1.18; P=0.006) and a reduction in infarct size by 2.7 % (95 % CI, −4.83 to −0.56; P=0.01) in the BMMNC group (1,218 patients), as compared with control patients (819 patients). However, no beneficial effect of BMMNC was detected on the incidence of MACCE at 6 months follow-up (de Jong et al. 2014).

Regarding the effect of autologous stem cell infusion of subacute myocardial infarction, ongoing phase II–III trials like the RACE-STEMI (NCT02323620) and BAMI (NCT01569178) studied BMMNCs, as well as RELIEF (NCT01652209) and ESTIMATION (NCT01394432) studied effect of MCSs, are of particular interest. A double blind, randomized, placebo controlled phase II study was established to determine the safety and feasibility of intracoronary delivery of allogeneic MSCs after undergoing PCI in 225 subjects with de novo anterior myocardial infarction due to a lesion of the left anterior descending coronary artery versus only PCI (AMICI, NCT01781390). The purpose of ALL STAR phase II study is to show whether allogeneic cardiosphere-derived cells (CAP-1002) are safe and effective in decreasing infarct size assessed by MRI at 6 and 12 months post-infusion in patients with a myocardial infarction (NCT01458405).

3.5.2 Current and Future Cardiovascular Stem Cell Therapy Clinical Trials in Patients with Ischaemic and non-Ischaemic Heart Failure

Fewer clinical trials have addressed the effect of stem cell therapy in the treatment of patients with advanced congestive heart failure. Fisher et al. compiled data from 23 studies with a total 1,255 participants with chronic ischaemic heart disease and

heart failure treated with autologous adult bone marrow-derived stem cells. The meta-analysis suggested that BMSC treatment reduced the incidence of mortality (risk ratio (RR) 0.28, 95% confidence interval (CI) 0.14–0.53, P=0.0001) and rehospitalisation (RR 0.26, 95% CI 0.07–0.94, P=0.04), as well as reduced left ventricular end systolic volume (−14.6 ml, 95% CI −20.88 ml to −8.39 ml, P<0.00001) and an improved left ventricular ejection fraction (+2.6%, 95% CI 0.50–4.73%, P=0.02 at long-term follow-up (>12 months). Overall, BMSC treatment benefited in NYHA functional class (−0.63, 95% CI −1.08 to −0.19, P=0.005) as well as an angina class (−0.81, 95% CI −1.55 to −0.07, P=0.03) compared to controls (Fisher et al. 2014). Most recently, Afzal and co-workers compiled data from 48 studies (both randomized controlled trials and registries) with a total of 2,602 patients with ischaemic heart disease not specified to AMI or chronic. Meta-analyses indicated that BMMNC transplantation improved left ventricle ejection fraction by 2.9% (95% confidence interval [CI], 1.91–3.92; $P<0.00001$), left ventricle end-systolic volume by 6.4 mL (95% CL, −8.95 to −3.80; $P<0.00001$) as well as reduced infarct size by 2.3% (95% CL, −3.55 to −0.95; $P=0.0007$). The incidence of all-cause mortality (OR, 0.55; 95% CI, 0.34–0.89; $I^2=37\%$; $P=0.01$), recurrent MI (OR, 0.50; 95% CI, 0.27–0.92; $I^2=21\%$; $P=0.03$), ventricular tachycardia/fibrillation (OR, 0.45; 95% CI, 0.22–0.93; $I^2=10\%$; $P=0.03$), and transient ischaemic attacks (OR, 0.25; 95% CI, 0.08–0.81; $I^2=0\%$; $P=0.02$) were all reduced in BMMNC-treated group (Afzal et al. 2015).

The meta-analysis of the studies that assessed combined coronary artery bypass graft surgery with BMMNC stem cell transplantation compiled data of nine studies that enrolled a total of 305 patients with ischaemic heart disease and impaired heart function. This analysis revealed that patients treated with combined revascularization and BMMNC (n=158 patients) showed an improvement of left ventricle ejection fraction by +5.8% (95% CI, 3.0–8.52; P<0.0001), a reduction of left ventricle end-diastolic volume by −11.7 ml (95% CI, −19.36 to −4.04; P=0.003) as well as left ventricle end-systolic volume by −11.6 ml (95% CI, −17.4 to −5.84; P<0.001) at mean 7 month follow-up, as comparison to patients treated with CABG alone (n=147) (Qin et al. 2015). The meta-analysis in 244 patients with non-ischaemic cardiomyopathy resulted in left ventricle ejection fraction (+4.8%, 95% CL, 1.32–8.43%, P=0.01) and left ventricle end-diastolic diameter (−2.19 mm, 95% CL, −5.69 to 1.30, P=0.22) in stem cell treated group compared to control (Marquis-Grave et al. 2015).

The randomized placebo-controlled, double blinded, multicentre ongoing study the PERFECT trial (NCT00950274) has investigated the effect of $CD133^+$ stem cell injection combined with CABG surgery in 142 patients with ischaemic heart failure. The trial was initiated in 2009 and the intermediate analysis is expected soon. Fifty-one patients suffered from idiopathic dilated cardiomyopathy and heart failure (LVEF <40% or LVEF 40–50% if left ventricular end-diastolic volume >110 ml/m2) are enrolled in multicenter phase II–III ongoing trial (NCT02033278) evaluating intracoronary injection of bone marrow progenitor cells. The CHART 1 and 2 trials investigate efficacy and safety of autologous BM-derived MSCs (C3BS-CQR-1) for the treatment of chronic advanced ischaemic heart failure (LVEF

>35 %) in patients who are not suitable for revascularization (NCT01768702, NCT02317458). The first results are expected in August 2016. In a particular interest there is a multicenter Dream CHF clinical trial investigated heart failure-related major adverse cardiac events after injection of allogeneic MSC in 1,730 patients with ischaemic and non-ischaemic congestive heart failure. The trial is projected to render final data in the middle of 2018 (NCT02032004). Improvement in myocardial perfusion/viability is primary outcome for trial enrolled patients with end-stage heart failure and left ventricular assist device treated with allogeneic mesenchymal stem cells implantation (NCT01759212).

3.5.3 Current and Future Cardiovascular Stem Cell Therapy Clinical Trials in Patients with Refractory Angina

The first large phase III study investigated intramyocardial injections of G-CSF-mobilized $CD34^+$ stem cell therapy for the treatment of patients with refractory angina is called RENEW (NCT01508910). A total 291 patients will be enrolled to estimate changes in exercise time on exercise tolerance test and angina frequency (episodes per week) after treatment.

3.6 Future Perspectives

Since the first description of the use of myoblasts for treatment of heart disease in 2001, a large number of clinical studies have been able to demonstrate the effectiveness of cell therapy in various cardiovascular indications, but with very different preparation techniques. The use of non-standardized cell transplantation procedures is common with large variation in (i) type of transplanted cells, (ii) number of transplanted cells (iii) additive preconditioning measures (iv) timing of cell therapy, (v) selection of patients and (vi) surrogate end point markers. Therefore, future studies should be aimed to optimise the technique of cell preparation, to discover the best cell type for myocardial regeneration, to analyse their homing characteristics to the cardiac niche and to other extra cardiac organs, to improve cell delivery techniques and to try to establish the specific indications for cell therapy in various heart diseases. In realising these perspectives, joint and cooperative studies between preclinical and clinical research are essential. The mechanisms of stem cell related cardiac repair need to be investigated in depth and alternative modes. The immune modulatory capacity of mesenchymal stem cells, for example, could offer new options to supplement the established immunosuppressive therapies in the setting of solid organ transplantation. Basic principle in clinical studies is safety of stem cells therapy. Thus far, meta-analysis have underscored the safety of stem cell therapy, even in the vulnerable advanced heart failure population, and patients with a significant acute myocardial infarction. Clinical trials should be carried on according

Good Clinical Practice (GCP) rules. Trials should monitor and compile clinical data in national/international registries of MACCE data, including all-cause mortality, cardiac death, myocardial reinfarction, rehospitalization and intensive care stays due to cardiac events, and percutaneous or surgical revascularization, episodes of acute heart failure, ventricular arrhythmias, postoperative implantation of defibrillators and pacemakers, and apoplexies, as well as malignancies and pericarditis. A list of monitor dates should be opened and include all adverse events and reactions, presumed related and non-related to the stem cells therapy procedure, to monitor for unexpected side effects (SUSAR). The criteria of adequate response to the cell therapy should be better defined and should be the basis of the latest diagnostic protocols, and to unravel the confounding factors that determine cardiovascular cell therapy efficacy.

Furthermore, attempts to create dynamic "multi-lineage" cardiac regeneration by combining cell therapy with tissue engineered scaffolds or cardiac resynchronisation therapy should be further explored, since they offer a realistic perspective to come to an integrated regenerative approach. Transgenic cell reprogramming for instance via viral vector mediated transfection, or genetic modification of allogeneic cell banks open new avenues in stem cells treatment. For example, adenoviral Tbx18 gene transfer converted postnatal ventricular myocytes or pluripotent stem cells into sinoatrial node pacemaker cells (Kapoor et al. 2013; Hu et al. 2014). Alternatively, transcription factors, including Gata4, Hand2, Mef2C, and Tbx5 (Ghmt) converted cardiac fibroblasts into beating cardiac-like muscle cells (Song et al. 2012).

Much attention should be given to miRNA, which plays a critical role in proliferation and differentiation of stem cells. Moreover, miRNA, located in resident cardiac progenitor cells or invading stem cells transfer via exosomes into cardiomyocytes, and modify the cardiomyocyte properties, for instance cell survival, contractility, hypertrophic response or pacemaker properties (Barile et al. 2014). This knowledge might open a new and exciting opportunities in stem cell treatment.

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Chapter 4
Myocardial Pharmacoregeneration

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Abstract Novel pharmacological approaches addressing the underlying cause of heart failure progression, namely cardiomyocyte loss, are emerging. The main therapeutic aims are to either protect cardiomyocytes from ischemia-associated stressors or to facilitate endogenous regeneration. The latter may be achieved by (1) induction of cardiomyocyte proliferation, (2) activation of resident cardiac progenitor cells, or (3) non-myocyte-to-cardiomyocyte conversion. The development of pharmacological approaches to enhance these under normal circumstances ineffective self-repair mechanisms may enable the regeneration of what we consider today the irreversibly damaged heart. This chapter provides an overview of the current state-of-the-art in myocardial pharmacoregeneration.

Keywords Heart • Regeneration • Stem cells • Pharmacology

4.1 Introduction

The observation that bone marrow contains progenitor or stem cell populations with cardio-regenerative potential (Orlic et al. 2001a, b) quickly led to the initiation of several clinical trials (Fisher et al. 2015). Most of these trials showed no or only modest benefits. Evidence for paracrine mechanisms rather than functional integration of exogenous stem cells and their transformation into cardiomyocytes is accumulating (Mirotsou et al. 2011). Deciphering the postulated paracrine mechanisms and enhancing them pharmacologically would be clinically extremely attractive.

The investigation of the regenerative capacity of the adult mammalian heart has generated ambiguous results (Soonpaa and Field 1998; Bergmann et al. 2009; Anversa et al. 2007; Kajstura et al. 2010). Despite the contention in the field, there

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is agreement that endogenous myocardial regeneration is insufficient to stop disease progression towards end-stage heart failure. Guideline-based drug therapy can reduce symptoms, shield the heart from neurohumoral overstimulation, and control pathological remodeling, but fails to regenerate the heart. Insufficient myocardial regeneration in the mammalian heart is in clear contrast to the remarkable regeneration capacity in lower vertebrates, such as zebrafish and newts (Poss et al. 2002; Lepilina et al. 2006; Oberpriller and Oberpriller 1974). Here, little residual injury can be observed after removal of up to 20 % of the heart. The phylogenetic distance between lower vertebrates and humans has so far been a barrier for translating mechanistic insight into clinical therapy. Observations in mice confirmed that the mammalian heart can regenerate postnatally, albeit only during the first days after birth (Porrello et al. 2011). Clinical data from intrauterine surgery documented that the immature human heart is also capable of regeneration (Herdrich et al. 2010). Recent data from an elegant carbon dating study demonstrated that cardiomyocyte proliferation drops dramatically to <1 % annually within 10 years after birth; conversely, endothelial cells (20 %) and mesenchymal stroma cells (<5 %) continue to self-renew annually (Bergmann et al. 2015). Collectively, these data form the rationale for the continuous search for drugs that may enhance or activate cardiomyocyte proliferation in the setting of heart failure.

Cardiac stem or progenitor cells (the term cardiac progenitor cells or CPCs will be used to describe this cell population throughout this chapter) have been identified in the mouse and human heart based on surface marker expression and in vitro growth characteristics (Beltrami et al. 2003). CPCs may represent remnants of embryonic heart development, but their origin and postnatal relevance remain a matter of debate (Sussman and Murry 2008). Recent data underscores that a CPC contribution to normal postnatal heart growth is rather unlikely (Bergmann et al. 2015). Whether CPCs can be activated in the diseased heart remains unclear. After isolation from the heart, these cells exhibit the potential to differentiate into most cellular components of the heart, including endothelial cells, smooth muscle cells, and cardiomyocytes. Genetic lineage tracing in mice provided data in support of a CPC contribution to the remodeling of the adult heart after injury (Hsieh et al. 2007). To define means for pharmacological activation of CPCs or other repair mechanisms, in vitro studies under defined conditions are particularly useful as they may help to identify distinct paracrine factors secreted upon a simulated pathological stimulus (Tang et al. 2010; Psaltis et al. 2010; Urbich et al. 2005; Amado et al. 2005). This will also help to delineate components of signaling pathways as potential drug targets for the induction of cardiomyocyte regeneration (Mirotsou et al. 2007; Zelarayan et al. 2008; Oerlemans et al. 2010; Oikonomopoulos et al. 2011), and to effectively screen small molecules for their capacity to regulate for example CPC activity (Sadek et al. 2008; Russell et al. 2012).

Coming from the "historical" perspective of bone marrow cell activation as means to regenerate the heart, this chapter will (1) elaborate on the putative target cells for myocardial pharmacoregeneration and (2) summarize available data on specific pharmacological approaches to enhance endogenous myocardial repair mechanisms.

4.2 Bone-Marrow Cells in Heart Regeneration

The bone marrow is a reservoir for hematopoietic (HSCs), mesenchymal (MSCs), and endothelial (EPCs) stem/progenitor cells. These stem and progenitor cells can be readily isolated from the bone marrow's mononuclear cell (BMNC) fraction (Fernandez-Aviles et al. 2004). Initial efforts to translate findings from animal models into the clinic were inspired by the observation of the regenerative capacity of bone marrow derived cells in mice (Orlic et al. 2001a, b; Yoon et al. 2005). Despite the attractive abilities of these cells, concerns were raised by others who could not replicate these experimental findings and further demonstrated that bone marrow-derived cells stem cells do not form cardiomyocytes, but instead adopt characteristic haematopoietic fates after transplantation in ischemic hearts (Laflamme and Murry 2011; Balsam et al. 2004; Murry et al. 2004). Notably, an increasing body of clinical trials now provides hints that BMNCs have the capacity to improve myocardial performance post-myocardial infarction (Wei et al. 2009; Dawn et al. 2009; Abdel-Latif et al. 2007). The large multicenter phase III BAMI trial (NCT01569178), is anticipated to provide definitive answers as to the utility of BMNC in heart repair after acute myocardial infarction (Traverse et al. 2009, 2011). Whether more specific subsets of bone marrow stem cells, such as MSCs (Hare et al. 2009), will be less, equally or more effective is also under investigation (e.g., PROMETHEUS trial: NCT00587990; POSEIDON-DCM trial: NCT01392625; TRIDENT trial: NCT02013674). MSCs appear particularly interesting given their documented potential for differentiation into various mesodermal cell types, including myocytes (Pittenger et al. 1999). Finally, the mechanism(s) of action of bone marrow cell-based cardiac repair may be manifold (e.g., pro-angiogenesis, anti-apoptosis, re-muscularization) and it is fair to concede that additional research is needed to define the therapeutically meaningful biological activity of cell grafts in general.

One mechanism of action may be the activation of endogenous CPCs. This is supported by studies in mice showing activation of pro-regenerative processes in resident CPCs after bone marrow-derived c-kit cell application (Loffredo et al. 2011). This effect was not observed when bone marrow-derived MSCs (c-kit negative) were employed. Notably, transdifferentiation of exogenously delivered cell was not detected, indicating that the c-kit cells elicited a regenerative response via the release of so far undefined factors. Candidates for therapeutic paracrine factors include fibroblast growth factors, stromal cell-derived factor 1, hepatocyte growth factor, and insulin-like growth factor-1 (Urbich et al. 2005; Gnecchi et al. 2006).

4.3 Cardiac Target Cells for Pharmacoregeneration

Two different mechanisms of endogenous cardiomyocyte generation have been suggested. These include (1) proliferation of pre-existing cardiomyocytes which may necessitate initial cardiomyocyte dedifferentiation and (2) provision of new

cardiomyocytes from endogenous progenitor cell pools (Steinhauser and Lee 2011). The following sections introduce the different cell types contained in the heart as targets for pharmacoregeneration.

4.3.1 Cardiomyocytes

Cell cycle activity in the adult heart has been reported by several groups (Bergmann et al. 2009, 2015; Kajstura et al. 1998, 2010; Soonpaa et al. 1996), ranging from annual proliferation rates of <1–20 %. The quantitative disagreement could at least in part be attributed to the difficulty to unambiguously distinguish cardiomyocyte from non-myocyte nuclei and karyokinesis from cytokinesis. Despite this discrepancy, there is clear evidence that cardiomyocytes can in principle be coaxed into cell cycle progression for example by overexpression of large T antigen (Field 1988) and cyclin D2 (Pasumarthi et al. 2005). The latter study is particularly interesting because it demonstrated that cardiomyocyte restricted cyclin D2 overexpression would only enhance cardiomyocyte proliferation after infarction in the infarct border zone. This disease specific and spatially restricted activity of the cell cycle regulator cyclin D2 suggests that myocardial infarction and/or post-infarct remodeling create a unique myocyte growth supporting environment.

In mice, a combination of multi-isotope imaging mass spectrometry and a double-transgenic MercreMer/ZEG animal model was used to detect nuclear incorporation of [15N]thymidine as indication for cell cycle progression. In this system, cardiomyocytes express constitutively β-galactosidase (β-gal) whereas Cre-mediated recombination induced by 4-OH-tamoxifen treatment results in irreversible expression of green fluorescent protein (GFP) in these cells (Hsieh et al. 2007). An increase of the β-gal cardiomyocyte pool would indicate de novo cardiomyogenesis; whereas an augmented GFP cardiomyocyte pool would be a result of cardiomyocyte proliferation from pre-existing recombined cardiomyocytes. This model confirmed that new cardiomyocytes can be "generated" by proliferation of pre-existing cardiomyocytes at a yearly rate of 5.5 % in young and 2.6 % in old mice under physiological conditions; proliferation was enhanced fourfold after myocardial injury in the border region. Given that cardiomyocytes are known to undergo DNA replication without completing the cell cycle, these calculations represent the upper limit of cardiomyocyte generation. Moreover, [15N] incorporation was mainly detected in GFP positive (pos) cells, which would argue against a considerable CPC contribution to myocardial homeostasis. However, dilution of GFPpos cardiomyocytes was observed after injury, which could be attributed to preferential loss of GFPpos cardiomyocytes, cardiomyocyte formation from activated CPCs, or proliferation of non-myocytes (Senyo et al. 2013).

Recent experimental studies suggest that cardiomyocyte proliferation is preceded by cardiomyocyte dedifferentiation. For example, in zebrafish resection of cardiac tissue caused sarcomere disassembly and re-expression of so called fetal genes, followed by DNA synthesis leading to cytokinesis and re-differentiation

toward mature cardiomyocyte (Kikuchi et al. 2010; Jopling et al. 2010). Interestingly, similar observations were made in the early neonatal mouse heart (Porrello et al. 2011). In agreement with the data mentioned above, these finding suggest that cardiomyocytes can undergo division under defined circumstances. Oncostatin M has recently been suggested as a specific paracrine mediator of cardiomyocyte dedifferentiation post myocardial injury (Kubin et al. 2011). Collectively, these data suggest that cardiomyocyte dedifferentiation and proliferation are closely interrelated. Controlling these processes may help to enhance the cardiomyocyte content in the diseased heart.

4.3.2 Stem Cell Antigen 1 (Sca1 or Ly-6A/E) Positive CPCs

Sca1 is a glycosylphosphatidylinositol-anchored membrane protein, which is expressed by immature hematopoietic progenitor cells and also found in a small number of cardiac cells (Oh et al. 2003). Sca1 cells are negative for c-kit and do not express hematopoietic or stem or endothelial progenitor cell markers, but they exhibit high telomerase activity and express cardiogenic factors suggesting a cardiac pre-determination. Accordingly, in vitro differentiation of Sca1 cells towards cardiomyocyte-like cells has been documented upon treatment with the DNA methyltransferase inhibitor 5′azacytidine (5aza) (Oh et al. 2003). Cardiac differentiation of Sca1 cells depends at least in part on the activation of bone morphogenetic protein receptor (Bmpr)1a. In vivo these cells home to injured myocardium, integrate and differentiate when administered intravenously following ischemia-reperfusion injury (Oh et al. 2003). In this model, differentiation was found to be a result of two events: direct donor differentiation and cell fusion with the host tissue. Notably, the Sca1 epitope does not exist in the human. Despite this, Sca1-antibody selection has been employed to isolate a cardiogenic cell type from fetal and adult human heart with similar cardiogenic properties as mouse Sca1 cells (van Vliet et al. 2008; Smits et al. 2005). Moreover, these cells also differentiate into functional cardiomyocytes in vitro in response to 5-aza-demethylation and TGFbeta1 supplementation (Smits et al. 2005; Goumans et al. 2007).

A subgroup of cells within the Sca1 cell fraction, also known as side population (SP), can be defined by their propensity to actively export Hoechst 33342 via verapamil sensitive Abcg2 efflux pumps (Bunting 2002; Zhou et al. 2001). SP cells can be isolated from multiple adult tissues including skeletal muscle, bone marrow, liver, lung, kidney, and brain. SP cells could also be identified in the early developing and adult heart tissue where they appear to contribute to organogenesis and tissue maintenance, respectively (Martin et al. 2004). A Sca1 positive SP subpopulation, negative for the endothelial marker CD31, was identified to have a unique potential to differentiate into functional cardiomyocytes (Pfister et al. 2005, 2010). This appeared to depend on direct coupling with adult cardiomyocytes, although the exact mechanism remains unclear. Recently, Sca1/PDGFRα cells negative for CD31 were purified, expanded in vitro and injected in mouse hearts with and

without infarction. In both settings, engraftment and co-expression of α-sarcomeric actin were confirmed at 2 weeks (Noseda et al. 2015). This study confirmed the earlier work on Sca1 and highlighted the need for a better definition of Sca1-CPCs as potential target for pharmacoregeneration.

4.3.3 Tyrosine-Protein Kinase Kit (c-kit or CD117) Positive CPCs

c-Kit is a tyrosine-kinase receptor found in circulating hematopoietic progenitors as well as in the bone marrow, telocytes, thymic epithelium, mast cells, and embryonic stem cells (Reber et al. 2006; Yasuda et al. 1993). Similar to Sca1-CPCs, c-kit-CPCs do not co-express hematopoietic markers; they are moreover clonogenic, self-renewing, and multipotent (Beltrami et al. 2003). The cardiomyogenic capacity of c-kit cells remains highly disputed. Some groups reported the ability of c-kit cells to regenerate adult myocardium and vessels along with functional improvement after cardiac infarction of the adult heart (Beltrami et al. 2003; Bearzi et al. 2007); others reported poor cardiomyogenic differentiation only of neonatal c-kit cells and no transdifferentiation of adult cardiac c-kit cells (Zaruba et al. 2010). Data from a transgenic mouse model, which expressed GFP under the transcriptional control of the endogenous genomic c-kit locus, confirmed that cardiac c-kit cells can be identified in a mixed developmental state in the developing as well as the neonatal heart; moreover, a transcriptional reactivation of c-kit in adult cardiomyocytes was observed following injury (Tallini et al. 2009). This study also showed the potential role of c-kit in vascular repair and is in agreement with studies showing the involvement of in vitro activated cardiac c-kit cells primarily in vasculogenesis post-infarction (Tillmanns et al. 2008). The comprehensive data in support of the utility of c-kit CPCs in heart regeneration provided the rationale for the SCIPIO trial (NCT00474461), designed to investigate the safety of intracoronary autologous cardiac stem cell therapy as an adjunct treatment for patients with ischemic cardiomyopathy (Bolli et al. 2011). In the SCIPIO trial patients with heart failure after myocardial infarction (MI) were subjected to right atrial biopsy for c-kit CPC isolation and expansion. Finally, 0.5–1 million CPCs were perfused through the heart via intracoronary injection. 3D echocardiography or cardiac MRI 4 and 12 months after CPC administration provided evidence for a reduction in infarct size in a subset of patients. Follow-up studies are needed to validate those early clinical findings. In a similar approach allogeneic CPCs are tested in the CAREMI trial (NCT02439398).

4.3.4 Cardiosphere-Derived Cells (CDCs)

From myocardial biopsies, cells with the capacity to form spherical aggregates, also known as cardiospheres, can be derived (Messina et al. 2004). Cardiospheres can be generated from embryo, fetal, and postnatal mouse as well as explanted human atrial or ventricular biopsy specimens (Messina et al. 2004; Johnston et al. 2009).

From cardiospheres self-renewing and clonogenic cells can be derived. Cardiosphere-derived cells (CDCs) are Flk-1 positive, which is expressed in early cardiac mesodermal cells and hemangioblasts, along with the endothelial markers CD31 and CD34 as well as c-kit and Sca1. The origin of CDCs, i.e., either from dedifferentiated proliferative cardiomyocytes or CPCs, is unclear (Rasmussen et al. 2011). CDCs injected into the adult injured pig heart induced tissue repair and regeneration, attenuating adverse remodeling post-infarction. This effect was attributed to the ability of CDCs to engraft and form mature cardiac cells (Johnston et al. 2009; Barile et al. 2007). In addition, CDCs appear to exhibit a unique paracrine activity, with the capacity to produce for example fibroblast and hepatocyte growth factors (Barile et al. 2007). This may have contributed to their more pronounced benefit on cardiac function when compared to bone-marrow derived cell grafts (Zhou et al. 2012). These results led to the initiation of the CADUCEUS trial to test the feasibility and safety of intracoronary injection of CDCs after myocardial infarction (Makkar et al. 2012). Right ventricular biopsies from patients with a mean ejection fraction of <40 % undergoing primary angioplasty 2–4 weeks after myocardial ischemia were harvested. Predominantly endoglin (CD105) expressing cells (Smith et al. 2007) were expanded 1–3 months to finally deliver 25 million CDCs via intracoronary infusion. Although the primary endpoint was safety, cardiac MRI at 6 and 12 months after cell delivery revealed a reduction in infarct size and an increase in viable myocardium (Makkar et al. 2012; Malliaras et al. 2014), leading the authors to conclude that myocardial regeneration occurred.

4.3.5 Epicardium-Derived Cells (EPDCs)

The epicardium has recently moved into the focus of myocardial regeneration as a putative source for multiple cell types in the developing and adult heart (Smart et al. 2007, 2011). Its embryonic origin is the proepicardial organ (PEO) or proepicardium primordium. The PEO is a transient mesothelial cell cluster located near the venous pole of the embryonic heart that migrates and expands onto the myocardium to cover almost the whole surface of the ventricle (Ratajska et al. 2008). It is marked by the expression of the Wilm's tumor (WT-1) suppressor gene (Perez-Pomares et al. 2002). The epicardium comprises of epicardial cells that proliferate, undergo epithelial-to-mesenchymal transformation, and differentiate into endothelial and smooth muscle cells that constitute the coronary vasculature, interstitial fibroblast as well as the Purkinje fiber network (Ratajska et al. 2008; Winter and Gittenberger-de Groot 2007). Using reporter mice, WT-1 cells were found to contribute to cardiomyocyte formation during normal heart development (Zhou et al. 2008). Collectively, these studies showed the high plasticity of EPDCs, making them an attractive target for pharmacoregeneration of the heart. Moreover, the epicardium is also a source of diffusible factors required for development (Limana et al. 2011). Interestingly, epicardium-based myocardial repair activity in the mouse appears to be present only after preconditioning with thymosin beta 4 (Smart et al. 2011) and absent under "normal" pathological conditions (Zhou et al. 2012). EPDCs massively differentiate into

fibroblasts after MI but not upon angiotensin II–induced pressure overload, indicating a disease specific response. Epicardium-derived fibroblasts display stromal properties and contribute to the sustained recruitment of circulating cells to the damaged zone and the cardiac persistence of hematopoietic progenitors/stem cells after MI. These data indicate the importance of this cell population in the course of acute ischemic cardiac remodeling (Ruiz-Villalba et al. 2015; Qian and Srivastava 2013).

4.3.6 Myocardial Fibroblasts

Fibroblasts comprise the majority of the non-myocytes types in the adult heart. They play a major role for structural and paracrine support of the adjacent cardiomyocytes. In the injured heart, cardiac fibroblasts become activated and contribute to scar formation in order to maintain structural stability. Conversion of cardiac fibroblasts into cardiomyocytes by transcription factor reprogramming has recently been demonstrated in vitro and in vivo (Qian et al. 2012; Ieda et al. 2010; Song et al. 2012). The first set of transcription factors reported to induce direct lineage conversion of fibroblasts into cardiomyocytes was *Gata4*, *Mef2c*, and *Tbx5*, also referred to as GMT (Ieda et al. 2010). Subsequently, GMT with additional factors (Mesp1, Hand1, Hand2, Nkx2.5, Myocd, Smarcd3, SRF, miR-1, miR-133) was reported with an enhanced reprogramming efficiency (Chen and Qian 2015; Doppler et al. 2015). A common observation from these studies is that in vivo cardiac reprogramming showed significantly greater reprogramming efficiencies than in vitro experiments, demonstrating the crucial role of the cardiac microenvironment for fibroblast conversion into functional cardiomyocytes (Chen and Qian 2015). As to the application of the reprogramming technology to human fibroblasts it appears that GMT alone will not suffice to achieve the anticipated outcome unless supplemented with additional factors such as Hand2, Myocd, Mesp1, Essrg, Zfpm2, miR-1, miR-133 (Nam et al. 2013; Fu et al. 2013; Wada et al. 2013). Further work is needed to optimize the use of transcription factors, miR, or small molecules for specific targeting of (myo)fibroblasts within the myocardial scar.

4.4 Pharmacological Activation of Endogenous Cardiac Regeneration

Induction of cardiomyocyte proliferation, activation of CPCs or conversion of fibroblasts into cardiomyocytes could ideally be achieved pharmacologically. Figure 4.1 summarizes the potential pharmacological interventions discussed below in more detail. We would like to emphasize that the mechanisms of the regenerative action of the discussed growth factors/peptides, small molecules and non-coding RNAs (ncRNAs) are only incompletely understood and a matter of investigation by many groups.

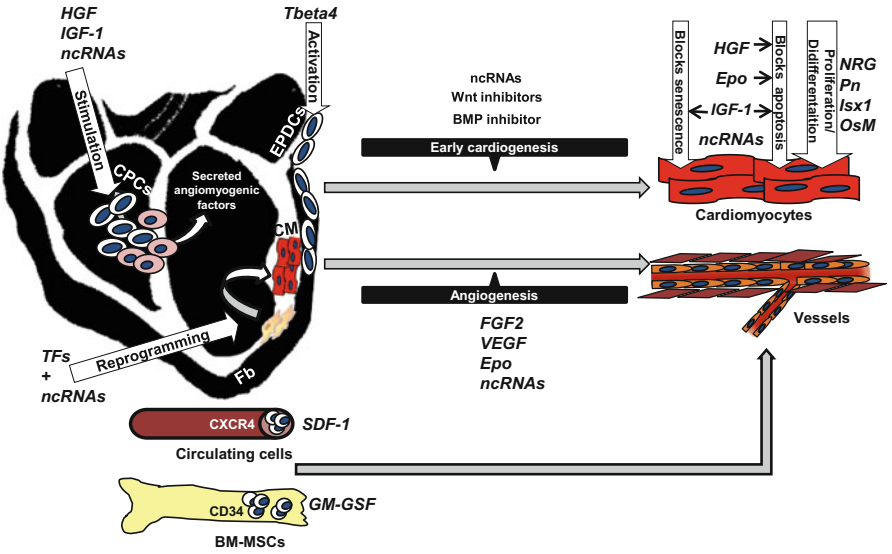

Fig. 4.1 Overview of proposed pharmacological interventions for myocardial regeneration

4.4.1 Growth Factors/Peptides

Paracrine mechanisms are key regulators of myocardial homeostasis. These are typically mediated by growth factors released from resident myocardial cells. In addition, myocardial damage causes leukocytes to home to the heart and secrete a wide spectrum of biologically active chemo- and cytokines acting in a paracrine manner on cardiomyocytes and the cardiac stroma cells, including CPCs. The following paragraphs will discuss the administration of peptides to therapeutically alter the paracrine milieu of the heart:

4.4.1.1 Granulocyte and Macrophage Colony Stimulating Factor (GM-CSF)

GM-CSF is a cytokine responsible for the mobilization of hematopoietic stem cells. GM-CSF is produced by a variety of tissues and its well-known function is to drive the proliferation of BMCs via activation of its canonical receptor (GM-CSFR). This is followed by activation of JAK/STAT, Ras/MAPK, and PI3K/AKT signaling cascades. GM-CSFR has also been detected in adult cardiomyocytes and its activation under stress seems to promote cell survival (Harada et al. 2005). Early studies explored the systemic application of GM-CSF after myocardial infarction in a mouse model (Orlic et al. 2001a, b). They found recruitment of c-kit-BMCs to the infracted heart and attributed the observed reduction in infarct size in the GM-CSF treated group to stem cell transdifferentiation into myocytes and endothelial cells.

This mechanism of action has been clearly refuted by a number of elegant studies (Balsam et al. 2004; Murry et al. 2004), but the therapeutic potential of GM-CSF (not its originally postulated mechanism) has been further substantiated (Hasegawa et al. 2006; Sugano et al. 2005). In line with these data, the first clinical trial using GM-CSF in combination with intracoronary infusion of peripheral blood mononuclear cells (PBMCs; MAGIC trial) showed an improvement in cardiac function and promotion of angiogenesis post-infarction (Kang et al. 2004). However, aggravated coronary restenosis observed in the patients receiving GM-CSF raised safety concerns and the patient enrollment was consequently terminated. In contrast to MAGIC, no coronary restenosis was observed in a follow-up trial (FIRSTLINE-AMI), where GM-CSF alone was administered to patients with myocardial infarction (Ince et al. 2005); mobilization of CD34-BMCs along with significant improvement in cardiac contractility were observed. The results from FIRSTLINE-AMI encouraged subsequent trials with a higher number of patients as well as placebo controls (STEMMI, REVIVAL-2, G-CSF-STEMI). These trials, however, did not show any beneficial effect of GM-CSF post infarction (Ripa et al. 2006; Zohlnhofer et al. 2006; Engelmann et al. 2006). A meta-analysis of these trials revealed that a significant amelioration in cardiac function ($\Delta EF \approx 4.7\,\%$) was observed when GM-CSF was administered within 37 h post ischemia (Abdel-Latif et al. 2008). In conclusion, GM-CSF seems to exert a modest therapeutic effect if administered shortly after acute ischemia. The GM-CSF effect in acute phases of myocardial injury argues for a cell protective effect, which could be mediated via anti-apoptotic AKT-signaling. Activation of myocardial regeneration by induction of cardiomyocyte proliferation and/or CPC activation may also have contributed to the observed effects.

4.4.1.2 Stromal Cell-Derived Factor 1 (SDF-1)

SDF-1 is a small chemokine of 8 kDa that by binding to C-X-C chemokine receptor type 4 (CXCR4) has originally been identified to chemoattract CD34-BMCs (Aiuti et al. 1997). Embryos lacking either SDF-1 or its receptor die due to impaired myelopoiesis (Zou et al. 1998). In addition, SDF-1 null embryos show cardiac ventricular septal defects suggesting that SDF-1/CXCR4 may play a distinct role during cardiogenesis (Nagasawa et al. 1996). In line with this, gene knock down of CXCR4 in pluripotent stem cells abrogated their spontaneous differentiation into functional cardiomyocytes (Chiriac et al. 2010). Together, these data suggest that the SDF-1/CXCR4 axis does not only induce progenitor cell migration in the heart, but also promotes lineage-specific differentiation. Under the hypothesis that SDF-1 could promote cardiogenesis after myocardial infarction in a similar way as in development, Askari et al. transplanted stably over-expressing SDF-1 fibroblasts in the ischemic rat heart (Askari et al. 2003). Here, high levels of SDF-1 resulted in a higher vessel density and improved cardiac function. SDF-1 is endogenously expressed by endothelial cells in the ischemic heart under the control of hypoxia-inducible factor-1 (Ceradini et al. 2004). SDF-1 expression increased migration and

homing of circulating CXCR4-positive progenitor cells to ischemic tissue whereas in the absence of SDF-1 or CXCR4 no progenitor cell recruitment was observed. Although, SDF-1 is activated under hypoxia, its clinical potential against cardiac remodeling is limited by its rapid degradation by proteases present in the ischemic heart such as matrix metalloproteinase MMP-2 (McQuibban et al. 2001) and dipeptidylpeptidase IV (DPP IV/CD26) (De La Luz Sierra et al. 2004). In an initial approach to overcome this problem, a cleavage protected variant of SDF-1, called SSDF-1(S4V), was designed. Delivery of SSDF-1(S4V) with self-assembling peptide nanofibers achieved high and locally contained concentrations; attributed to this was increased cardiac function and vessel density in a rat model of cardiac ischemia (Segers et al. 2007). An alternative approach to increase SDF-1 is by DPP IV inhibition. This results in CD34-BMCs recruitment to the infarct border zone and enhanced micro-vascularization; both may have contributed to the observed reduction in infarct scar size (Zaruba et al. 2009). A combination with GM-CSF-based stem cell mobilization further enhanced survival and cardiac function (Zaruba et al. 2009). Non-viral gene transfer of naked plasmid DNA encoding for human SDF-1 promoted angiogenesis and improved cardiac function in rats with ischemic heart failure (Sundararaman et al. 2011). These studies demonstrated collectively that SDF-1 attenuates the progression of chronic ischemic heart failure primarily by increasing vasculogenesis, reducing scar formation and fibrosis. A first-in-patient trial (NCT01082094) demonstrated an improvement in heart failure symptoms if administered via plasmid encoding DNA in patients with reduced ejection fraction after myocardial infarction (Penn et al. 2013). A Phase I/II trial (RETRO-HF; NCT01961726) has been initiated to follow-up on the initial encouraging observations.

4.4.1.3 Hepatocyte Growth Factor (HGF)

HGF was originally identified as a cytokine acting in hepatocytes (Nakamura et al. 1989) by binding with high affinity to its receptor c-met. c-Met is expressed in a variety of cells, which upon HGF challenge can proliferate, migrate, and form tubules (Derman et al. 1995). Interestingly, c-met is expressed on different populations of progenitor cells including c-kit-cells (Beltrami et al. 2003; Miyazaki et al. 2004), Sca1-cells (Iwasaki et al. 2005) and mesenchymal stem cells (MSCs) (Forte et al. 2006). Following ischemia/reperfusion, c-met expression is induced in the ischemic rat heart (Nakamura et al. 2000). Recombinant HGF has been shown to reduce scar size by protecting myocytes from apoptosis whereas HGF neutralization resulted in an unfavourable outcome. Besides its anti-apoptotic role, HGF in combination with insulin like growth factor-1 (IGF-1) has been shown to recruit c-kit-positive CPCs in the ischemic heart (Urbanek et al. 2005). The observed amelioration in cardiac function was attributed to the differentiation of c-kit-positive cells into cardiomyocytes as well as endothelial cells in the infarct border zone and epicardium (Wang et al. 2004). The anti-apoptotic properties of HGF seem to be mediated via the activation of PI3-kinase/Akt pathway. A similar property is

attributed to IGF-1 and PDGF-BB (Vantler et al. 2010). Since HGF administration in preclinical trials provided promising results, a clinical study that assesses the safety and efficiency of adenovirus-hepatocyte growth factor (Ad-HGF) treatment in ischemic heart disease is currently recruiting (NCT01925352) with an estimated completion in December 2015.

4.4.1.4 Insulin-Like Growth Factor-1 (IGF-1)

IGF-1 is typically released from a variety of cells after growth hormone stimulation. A surplus in IGF-1 is associated with acromegaly, which may cause cardiac hypertrophy (Bogazzi et al. 2008). Approximately 98 % of IGF-1 is bound to one of the seven binding proteins (IGF-BP). IGF-1 binds to its tyrosine kinase receptor, the insulin like growth factor 1 receptor (IGF1R), and thereby triggers PI3K/AKT activation with its well-known supportive effects on cell growth, i.e. hyperplasia and hypertrophy, and cell survival. The hypertrophy inducing effects of IGF-1 formed the rationale for clinical trials testing IGF-1 administration in patients with dilated cardiomyopathy (Fazio et al. 1996). These early trials ended, however, neutral and thus further testing was abandoned. Additional academic interest was raised by the in vivo evidence of the IGF1/IGF1R system being activated in cardiomyocytes shortly after myocardial infarction (Anversa et al. 1995). Moreover, constitutive overexpression of IGF-1 reduced apoptosis at the infarct border zone while limiting adverse cardiac remodeling (Li et al. 1997). Aside from its anti-apoptotic effects, IGF-1 was also implicated in angiomyogenic regeneration (Hynes et al. 2011). More recently, IGF-1 was identified as a key regulator of cellular senescence (Torella et al. 2004). Accordingly, in IGF-1 overexpressing mice transcription of senescence genes p27Kip1, p53, p16INK4a, and p19ARF was repressed. Moreover, IGF-1 overexpressing mice exhibited high nuclear phospho-Akt and telomerase activity particularly in cardiomyocytes and c-kit-positive cells in the heart, favoring cardiac regeneration (Torella et al. 2004). Interestingly, also in humans an age-related inverse relationship between senescence and IGF could be identified (Reeves et al. 2000). It is consequently intriguing to speculate that there is a causal link between loss of IGF-1 and CPC quantity as well as activity during aging and that reintroduction of “juvenile” IGF-1 levels would have the capacity to antagonize age-associated myocardial deterioration. The link between IGF-1 and CPCs was further substantiated by studies showing that c-kit-positive CPCs express IGF-1R and also synthesize IGF-1, suggesting an autocrine mechanism (D’Amario et al. 2011). Full activity of this putative positive feedback mechanism may however depend on the availability of HGF (Rota et al. 2008; Padin-Iruegas et al. 2009). In addition to its direct effects on CPC fate, IGF-1 may also protect/regenerate the heart by inducing the secretion of other protective or regenerative factors such as SDF-1 (Haider et al. 2008) or modulation of micro RNA processing (e.g. miR-34a), which can confer anti-apoptotic effects (Iekushi et al. 2012). Finally, a clinical trial (RESUS-AMI; NCT01438086) testing the safety and preliminary efficacy of IGFR activation in the heart by applying a synthetic IGF-1 analog (i.e. mescasermin) in

acute ischemia is currently recruiting with an estimated study completion date in August 2016.

4.4.1.5 Fibroblast Growth Factor (FGF)/Vascular Endothelial Growth Factor (VEGF)

Angiogenesis is closely linked to myocardial repair or regeneration. Growth factors with well documented angiogenic activity include fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF). Their role in direct cardiomyocyte regeneration is less well defined. In embryonic development, FGFs have a pivotal role in the control of early cardiogenesis (Watanabe et al. 2006; Grego-Bessa et al. 2007). Similarly, VEGF is essential for embryonic vessel formation, stabilization, and remodeling via regulation of NOTCH signaling (Lin et al. 2007). In the adult heart, a combination of FGF-2 and VEGF induced endothelial cell proliferation and angiogenesis, resulting in attenuation of post infarction injury (Pearlman et al. 1995; Watanabe et al. 1998; Yanagisawa-Miwa et al. 1992). Despite the promising results obtained in preclinical studies, safety concerns about the mitogenicity of these factors and the broad expression of their receptors restricted their systemic administration in humans. To circumvent systemic delivery of angiogenic growth factors, which would facilitate the risk of tumor growth, a mutated non-angiogenic form of FGF-2 (S117A-FGF-2 (Jiang et al. 2004)) and targeted VEGF delivery via P-selectin coated liposomes (Scott et al. 2009) were tested and demonstrated protection of infarcted myocardium from myocyte loss and adverse remodeling (Jiang et al. 2004). Despite these encouraging data, it has become apparent that for functional vascularization not only endothelial cells, but also smooth muscle cells and pericytes are required. Thus, additional growth factors may have to be administered to ensure proper vessel formation. Despite these caveats, clinical tests with combined VEGF/FGF administration via intramyocardial injection of plasmid-DNA (pVIF) in patients with refractory cardiac ischemia (VIF-CAD; NCT00620217) have been performed (Kukula et al. 2011). Although the primary study endpoint, i.e. enhancement of myocardial perfusion, was not reached, patients did show better exercise tolerance. Using an alternative approach, the recently completed ALCADIA trial evaluated the safety and efficacy of autologous human CPCs transplantation in combination with controlled release of FGF-2 in a gelatin hydrogel sheet in patients with chronic ischemic cardiomyopathy (NCT00981006). No results from this study are made publically available yet.

4.4.1.6 Erythropoietin (Epo)

Epo is a 34 kDa growth hormone, widely known for its role in erythropoiesis. It was originally identified to induce the proliferation of the late erythroid progenitors (Gregory and Eaves 1978). However, recent data on the broad expression of erythropoietin receptor (EpoR) in adult organs (Suzuki et al. 2002) suggested a

comprehensive role for Epo beyond erythropoiesis. Specifically in the heart, EpoR is detected around embryonic stage E10 and although significantly lower it persists through adulthood. Erythroid rescued EpoR knock-out mice appear to have less immature proliferating myocytes during embryogenesis, but reach adulthood without apparent morphological defects (Suzuki et al. 2002). However, upon ischemia reperfusion, these animals show a greater infarct size suggesting that a deficient Epo/EpoR system in the non-hematopoietic lineage may deteriorate left ventricular remodeling (Tada et al. 2006). Epo expression is controlled by HIF-1alpha, suggesting a central role in ischemic tissue (Makita et al. 2005). In agreement with this, several preclinical studies showed that Epo administration post infarction improves cardiac function via neo-angiogenesis, anti-apoptotic mechanisms, and/or CPC activation (Xu et al. 2005; Ye et al. 2005; Hirata et al. 2005; Moon et al. 2003; Calvillo et al. 2003; Zafiriou et al. 2014). Owing to its approved availability for anemia therapy, Epo was quickly tested clinically for its utility in post myocardial infarction repair. In the first pilot study, a single high i.v. dose of Epo was able to induce endothelial cell proliferation, but had no effect in cardiac function (Lipsic et al. 2006). Subsequent studies with larger number of patients failed to show a significant amelioration in cardiac function (Ott et al. 2010). Nevertheless, a smaller pilot study where small amounts of Epo were administered subcutaneously once a week in patients for a prolonged period of time demonstrated that Epo had a beneficial effect on cardiac remodeling and function (Bergmann et al. 2011). These data suggest that the discrepancy between the promising pre-clinical results of Epo and the human trials may be due to differences in dosing and mode of delivery (intravenously vs. subcutaneously). Moreover, the biological half-life of Epo was found to be markedly different if the same dose of Epo was administered intravenously and subcutaneously, i.e. ~5 vs. ~25 h, respectively (Salmonson et al. 1990). Collectively, these data suggest that a slow release application of Epo could be advantageous. The high receptor affinity (Kd 160 pM) would moreover only necessitate low doses to activate the EpoR effectively (Syed et al. 1998). Thus, it appears timely to test the potential role of subcutaneous low dose of Epo in ischemia and define in more detail its role not only in angiogenesis, but also in CPC activation or cardiomyocyte proliferation.

4.4.1.7 Thymosin Beta 4 (Tbeta4)

Tbeta4 is a highly conserved 5 kDa peptide initially isolated from bovine thymus (Low et al. 1981) that forms a complex with muscle G-actin and inhibits its polymerization (Safer et al. 1991). Tbeta4 ability to induce both ectoderm and mesoderm commitment of P19 embryonic cells and its localization in blood vessels and heart of developing embryos, suggested a role in stem cell biology and angiogenesis (Gomez-Marquez et al. 1996). Indeed, Tbeta4, which is also a direct downstream target of Hand 1 (Smart et al. 2010), was shown to promote myocardial and endothelial cell migration in the embryonic and early postnatal heart (Bock-Marquette et al. 2004). In the first preclinical trial, Tbeta4 treatment of infarcted mice led to an

improvement in cardiac function as well as cardiomyocyte survival. This action was mediated by integrin-linked kinase (ILK) and subsequent Akt activation (Bock-Marquette et al. 2004). Further studies revealed that Tbeta4 is a prerequisite for coronary vessel development in mice and that upon injury it may partially restore the lost vasculature not only by activating quiescent epicardial progenitor cells, but also by inducing their differentiation into fibroblasts, smooth muscle, and endothelial cells (Smart et al. 2007). In a large study, Tbeta4 was identified as a paracrine factor that mediated cardioprotection when embryonic endothelial cells where injected into porcine infarcted myocardium (Hinkel et al. 2010). Finally, a recent study showed that upon myocardial injury WT1-positive EPDCs primed with Tbeta4 were able to transdifferentiate into and integrate with functional cardiomyocytes (Smart et al. 2011). However, being an apparently mitogenic peptide Tbeta4 administration in humans needs to be carefully performed to not cause tumor formation or unknown tumor aggravation (Jo et al. 2011). Therefore novel injectable hydrogels are being designed that are able not only to target Tbeta4 to the heart, but also ensure its sustained delivery (Kraehenbuehl et al. 2011; Chiu and Radisic 2011). Collectively, these promising results about Tbeta4-based cardioprotection led to the first placebo controlled clinical trial, testing intravenous administration of Tbeta4 in patients with acute myocardial infarction. Phase I testing has recently been completed with encouraging results (Crockford 2007). A follow-up phase II trial (NCT01311518) was suspended apparently because of issues with GMP compliance in the drug manufacturing process.

4.4.1.8 Myocyte Mitogens: Periostin and Neuregulin

One of the first exogenous proteins used to mitotically activate myocytes was *periostin* (Pn). Pn is a 90 kDa protein that typically controls cellular and extracellular matrix organization during cardiac development (Butcher et al. 2007). Pn levels decrease substantially during aging and are up-regulated upon injury (Stanton et al. 2000). Pn knockout mice are more sensitive to cardiac rapture and fibrosis, but in the long-term exhibit hypertrophy and a better cardiac function (Oka et al. 2007). In vitro, Pn could be employed to stimulate mononucleated cardiomyocytes to proliferate, and in a rat model of myocardial ischemia delivery of a Pn foam gel resulted in neoangiogenesis, reduced infarct size and fibrosis, and improved cardiac function (Kuhn et al. 2007). However, later studies where Pn was transgenically overexpressed in mouse myocytes failed to validate these results (Fuller et al. 2008; Lorts et al. 2009). Whether the discrepancies between these studies can be explained by the different species used (mouse vs. rat), the mode of delivery of Pn, or the different experimental protocols remains to be clarified.

Neuregulin 1 (NRG) is another "myocyte mitogen" that has raised a lot of attention. The importance of NRG for proper cardiac development was demonstrated in mice with a NRG null mutation, which display heart malformations (Meyer and Birchmeier 1995). Alternative splicing of NRG1 results in a number of different isoforms, including heregulins (HRGs), glial growth factors (GGFs), and sensory

and motor neuron-derived factors (SMDF). Most of these isoforms have been confirmed as inducers of proliferation and/or differentiation of cells, including myocytes, expressing members of the epidermal growth factor receptor family ErbB1-4 (Fuller et al. 2008; Zhao et al. 1998; Peles et al. 1992). NRG1- or ErbB-2 and -4 knockout mice die at mid-gestation due to myocardial hypotrophy (Meyer and Birchmeier 1995; Lee et al. 1995; Gassmann et al. 1995), demonstrating the significance of these genes in cardiac development. Postnatally, both ErbB2 and ErbB4 receptors were found in neonatal and adult ventricular myocytes (Fuller et al. 2008). They could be activated with recombinant human GGF 2 (Zhao et al. 1998). This activation promoted the survival of stressed myocytes and further led to their hypertrophy. Finally, the same authors identified that the source of NRG1 in the rat ventricle is endothelial cells of the coronary microvasculature. Recently, NRG1 was shown to play a mitogenic role in adult mononucleated cardiomyocytes in vitro and in vivo, which apparently contributed to improved cardiac function post myocardial infarction (Bersell et al. 2009). Clinical trials to evaluate the safety and efficacy of NRG-1 have shown improved cardiac function and reversed remodeling in chronic heart failure (Xu et al. 2010). A follow-up clinical trial tested the safety and efficacy of NRG1 (or GGF2) in heart failure patients (NCT01439789 was replaced by NCT01251406); we are not aware that data from this study has been published so far.

4.4.1.9 Cardiomyocyte De-differentiation Agent Oncostatin M

The heart upon stress undergoes cardiac remodelling partially characterized by fetal gene re-expression. As a consequence, cardiomyocytes dedifferentiate and that is believed to be a defence mechanism against cell death and progression to heart failure (Hein et al. 2003; Ausma et al. 1998). Examination of remodelled canine heart tissue upon Ischemia/reperfusion injury revealed the prominent expression the cytokine Oncostatin M (OSM) (Gwechenberger et al. 2004). In line with this observation, DCM patient hearts undergoing cardiac remodelling showed high levels of OSM and its receptor (Oβ) which induced fetal gene re-expression and resulted in cardiomyocyte dedifferentiation (Kubin et al. 2011). Genetic deletion of Oβ in a mouse model of ischemia abrogated the protective effects mediated by OSM, resulting in exacerbated remodelling and lower survival rates (Kubin et al. 2011). In contrast to its initial cardioprotective effects, chronically enhanced OSM activity due to extensive inflammation in DCM mice resulted in higher lethality. These data suggest that upon acute stress OSM, secreted by infiltrating macrophages induces cardiomyocyte de-differentiation as coping mechanism. However, sustained OSM activation due to chronic inflammation impairs cardiac function and leads to death. In that line of thought, a recent pre-clinical study evaluated the therapeutic potential of Oβ inactivation. Administration of an antibody against the extracellular domain of Oβ or genetic inactivation of a single allele of the Oβ gene reduced cardiomyocyte dedifferentiation resulting in improved cardiac function and increased survival (Poling et al. 2014). These data suggested that inhibition of persistent Oβ signalling

is a possible strategy for the treatment of heart failure that is accompanied by inflammatory cell infiltration. A first in human study in healthy subjects is underway to evaluate safety, tolerability, pharmacokinetics, pharmacodynamics and immunogenicity profile of GSK2330811, a humanised monoclonal antibody that blocks OSM (NCT02386436); completion of the study is anticipated in April 2016.

4.4.2 Small Molecules

The introduction of small molecules with regeneration inducing activity would be highly advantageous over peptide based therapeutics, because of their defined chemistry and thus reproducibility under highly defined conditions. Moreover, small molecules are cheaper to produce in bulk quantities and require less regulatory considerations. Small molecules are usually developed based on identified therapeutic targets. This is followed by compound screens and chemical engineering based on for example the crystal structure of a putative therapeutic target. In early compound screens it is essential to use adequate model systems, which simulate as closely as possible the target organ physiology and pathology. Here compromises are often made to achieve high throughput at low costs, e.g. by making use of transformed cell lines (P19CL6, HL-1) with little resemblance to *bona fide* cardiomyocytes. More recently human embryonic stem cell or induced pluripotent stem cell based models have been introduced to provide more realistic models (Davis et al. 2011; Schaaf et al. 2011). However, the validity of stem cell-based assays still needs to be confirmed. Despite these assay limitations, there is some evidence for the capacity of specific small molecules to facilitate cardiac regeneration. These compounds typically modulate signaling pathways involved in embryonic cardiogenesis, cell cycle control, myocardial remodeling, and survival. Some examples are described in the following paragraphs:

4.4.2.1 Sufonylhydrazone (Shz)

Shz was identified in a small-molecule library screen in P19 cells for chemical activators of Nkx2.5 (Sadek et al. 2008). It also activated the expression of the panmesodermal marker brachyury-T along with a cardiac muscle- and smooth muscle- transcriptional activator, myocardin, in pluripotent stem cells. In PBMCs Shz enhanced cell attachment and survival in vitro. Human PBMCs, isolated from healthy subjects and treated for 3 days with Shz, normalized contractile function 21 days after injection into the border zone of a cryo-injured rat heart. The mechanism through which Shz regulated activation of a cardiac regeneration remains so far undiscovered. Contribution of Wnt, BMP, and FGF signaling was excluded, suggesting a non-redundant potentially unique regenerative pathway.

4.4.2.2 Isoxazoles (Isx)

Using the same high-throughput screening system as described above, the cyclopropyl-amide analogue called Isx1, which belongs to the 3,5-disubstituted isoxazoles, was identified as "cardiogenic" (Russell et al. 2012). The effect of Isx1 was tested in a multipotent stromal cell population that dynamically responds to injury and participates in fibrosis repair of the adult heart. These cells were identified as non-hematopoietic ($CD45^-$) and non-endothelial ($CD31^-$) Notch-activated epicardial-derived cells (NECs) (Russell et al. 2011). Daily intraperitoneal administration of Isx1 in mice resulted in robust activation of cardiac gene programs in multipotent NECs and cell cycle activity in myocardial cells. After myocardial ischemia, Isx1 administration initially improved ventricular function, but this effect was not sustained 21 days after injury. In this model Isx1 induced a distinct transcriptional program in NEC, which included activation of genes implicated in angiogenesis, but excluded muscle gene activation. The authors of this study argued, that very strong fibrosis induced upon cardiac remodeling may have acted as a barrier for the Isx1-based cardiogenic effect. Further validation of the specific activity of Isx1 and optimization of its bioavailability are essential for its further exploitation in pharmacoregeneration.

4.4.2.3 Wnt/β-Catenin and BMP Signaling Inhibitors

Regulation of Wnts and BMPs in a highly temporally controlled manner is essential for proper specification and differentiation of cardiogenic cells during embryogenesis (Mercola et al. 2011). Wnts promote cardiogenesis during mesoderm induction, but act as inhibitors of committed cardiac progenitors (Schultheiss et al. 1997; Gessert and Kuhl 2010). In contrast, BMP2 and 4 inhibit cardiogenesis during mesoderm formation while reduced BMP signaling enhances dorsal cardiogenic mesoderm (Yuasa et al. 2005; Hao et al. 2008). Given this background, Wnt and BMP modulation may turn out to be an attractive approach to promote tissue regeneration via cell cycle control or activation of resident CPCs.

Using high-throughput screening the FDA-approved small molecule, pyrvinium, was classified as a Wnt signaling inhibitor. This inhibition is achieved by directly acting on a downstream casein kinase-1, which phophorylates β-catenin to target it for ubiquitination and subsequent degradation (Saraswati et al. 2010). The therapeutic effect of pyrvinium was tested in a mouse model of cardiac ischemia. A single intracardiac injection of pyrvinium reduced adverse cardiac remodeling along with stimulation of proliferation of pre-existing cardiomyocytes in peri-infarct and distal myocardium. Follow-up studies are needed to address potential caveats associated with the unspecific modulation of Wnt signaling (i.e. mainly tumor formation).

Using a chemical screen, the small molecule *XAV939* was identified as a selective inhibitor of β-catenin-mediated transcription. XAV939 stimulates β-catenin degradation by stabilizing axin, which is part of the β-catenin destruction complex.

This stabilization occurs via inhibition of the enzymes tankyrase 1 and 2 (Huang et al. 2009). The potential of this molecule to influence cardiogenesis was tested in mouse embryonic stem cells (mESCs). Application of XAV939 in mESCs resulted in robust stimulation of cardiomyogenesis in a time window coinciding with the initiation of mesoderm formation and specification (Wang et al. 2011). This stimulation occurred at the expense of other mesoderm derived lineages, including endothelial, smooth muscle, and hematopoietic lineages. The bioavailability of XAV939 or its structural analogous are expected to facilitate the exploration of its regenerative capacity also in vivo (Wang et al. 2011).

Dorsomorphin was identified as the first known small-molecule selectively inhibiting BMP type I receptors and therefore, blocking BMP-mediated SMAD1/5/8 phosphorylation (Yu et al. 2008). Similar to XAV939, pharmacological inhibition of BMP signaling during the initial stages of ESC differentiation appears to stimulate pre-cardiac mesodermal cells at the expense of endothelial, smooth muscle, and hematopoietic lineages (Hao et al. 2008). Dorsomorphin treatment in the initial 24 h of cell differentiation was sufficient to significantly boost cardiac induction, indicating its role on very primitive pluripotent cells. However, timing of BMP-blockade is likely crucial because of its known supportive role in cardiomyogenesis.

Pyrvinium, XAV939, and dorsomorphin are examples for compounds that modulate the developmentally extremely important Wnt/β-catenin and BMP signaling pathways. The main challenge is to better understand the activity of these pathways in the adult heart and develop highly specific, efficient, and safe modulators of Wnt/β-catenin and BMP signaling in the heart.

4.4.3 Non-coding RNA (ncRNA)

Only 1.2 % of the human genome comprises protein encoding sequences (Mattick 2011). A large part of the residual genomic "dark matter" is actively transcribed to non-coding RNAs (ncRNAs) of different length and diverse biological roles (Mattick 2011). While small molecules can control a specific signaling pathway, ncRNAs have the ability to regulate a cellular process in a more comprehensive manner by influencing apparently the translation of gene families implicated in physiological and disease processes (Brown and Naldini 2009). ncRNAs of current interest in cardiac regeneration include micro-RNAs (miRNAs) and long non-coding RNAs (lncRNAs):

4.4.3.1 miRNA (miR)

miRs comprise 18–24 bp long ncRNAs which regulate gene expression by post-transcriptional silencing. Primary miRNAs (pri-miRs) are processed by Drosha to the precursor miRs (pre-miRs) for subsequently translocation into the cytosol (Pasquinelli 2012). Upon processing by Dicer the mature miRs bind to Argonaute

to form the miRNA-induced silencing complex (miRISC) and separate into the guide (miR) and the passenger (miR*). miR guides the miRISC complex to the region of interest while miR* was until recently considered as targeted for degradation without biological activity (Pasquinelli 2012). However, a recent study showed a high amount of miR* in cardiac fibroblast-derived exosomes and particularly identified a cardiomyocyte hypertrophy inducing activity for miR-21* (Bang et al. 2014). Modulation of miRNA activity is achieved either by miR-mimics or anti-miRs of various chemistries (Thum et al. 2011). miR-mimics are typically introduced to the cells by infection with cardiotopic adeno-associated virus (AAV) serotype 6 or 9 (Geisler et al. 2011).

Several miRs have been implicated in cardiac regeneration, by for example inducing cardiomyocyte proliferation, enhancing CPC maturation, and/or direct cardiac reprogramming. miR-590 and miR199a were identified by an elegant screen in neonatal rat cardiomyocytes as inducers of cardiomyocyte proliferation (Eulalio et al. 2012). This effect could also be observed by AAV transduction of these miRs in mouse hearts compromised by myocardial ischemia. Several other miRs have been identified as inhibitors of angiogenesis and thus as targets for anti-miR strategies especially after myocardial infarction (Iekushi et al. 2012; Fiedler et al. 2011; Bonauer et al. 2009; Hinkel et al. 2013). Another exciting application of miRs is the direct cellular reprogramming of fibroblasts to cardiomyocyte-like cells in vitro (Jayawardena et al. 2012) and in vivo upon myocardial infarction by a miR-combo (miR-1, -133, -208, -499) (Jayawardena et al. 2015).

Although targeting miR seems highly attractive, there are some specific caveats associated with this approach: (1) miRs come in families with redundant function; (2) the same miR may have different functions in different organs or cell types; (3) a consensus on the optimal chemistry of miR modulators does not exist yet (van Rooij and Olson 2012). Targeting specific organs may also be achieved by nanoparticle-mediated delivery as already successfully used in cancer cells (Babar et al. 2012; Cheng et al. 2015). Encouraging data stems from a phase II clinical trial in patients with hepatitis C (NCT01200420). In this trial a Locked Nucleic Acid (LNA)-based anti-miR122 strategy (miravirsen) was well tolerated and effective in the reduction of hepatitis C virus RNA (Janssen et al. 2013). Similar chemistries may be applicable in patients with heart failure.

4.4.3.2 Long Non-coding RNA (lncRNA)

lncRNAs are typically more than 200 bp in size and transcribed from intergenic or intragenic regions of the genome (Guttman and Rinn 2012). lncRNAs form complexes with or act as molecular decoys for other nucleic acids, influence pre-mRNA splicing, act as scaffold for protein-protein interactions (Mercer and Mattick 2013), and as sponges for miRNAs (Tay et al. 2014). Although little is known about these macromolecules, an interesting class of lncRNAs originating from enhancer regions of the genome (enhancer-associated lncRNAs, elncRNAs) act as potent epigenetic regulators involved in cardiac development and disease:

Myheart (*Mhrt*) is an elncRNA transcribed from an intergenic enhancer of the beta-myosin heavy chain (Myh7) gene. *Mhrt* was found to be strongly downregulated during remodeling in adult mouse and human hearts (Han et al. 2014). It acts as a molecular decoy for Brg1 which in its absence binds DNA and initiates transcription of fetal genes (Hang et al. 2010). *Mhrt* re-expression upon TAC reduce cardiac hypertrophy and partially restores cardiac function (Han et al. 2014). Another interesting elncRNA for cardiac regeneration is the epigenetic regulator *Fetal-lethal noncoding developmental regulatory RNA* (*Fendrr*) that induces fetal cardiomyocyte proliferation and mesodermal gene expression (Grote et al. 2013). Although very little is known about lncRNAs in comparison to miRs, high-throughput RNAseq approaches are continuously revealing so far unknown patterns of lncRNA transcription with an association to heart disease (Ounzain et al. 2015). A specific property of lncRNA is their tissue and cell type specificity (Ounzain et al. 2015; Francescatto et al. 2014). lncRNA therapeutics may thus become useful tools for the specific targeting of cellular disease processes. Recent technological advances showed that antisense oligonucleotides (ASOs) are promising for specific knockdown of nuclear lncRNAs (Wheeler et al. 2012).

4.4.4 Extracellular Vesicles

Extracellular vesicles (EVs) are released by several cell types with a complex biologically active cargo, including peptides and RNA. Current research has focused on microvesicles and exosomes (Gyorgy et al. 2011). Microvesicles, initially described in blood with a size between 0.1 and 1 μm are detachments of cytoplasmic protrusions (Wolf 1967). Exosomes with an average diameter of 40–100 nm form intracellularly in multivesicular endosomes (MVE) and are released upon fusion of the MVE with the plasma membrane (Raposo and Stoorvogel 2013). Several studies suggest that MSC-dependent paracrine function inside the heart is mediated dominantly by exosome secretions containing large amounts of peptides or other molecules (Gallina et al. 2015). Exosome cargo also comprises miR, such as for example miR-19a or miR-22, mediating cardioprotective effects at least in part via Akt/ERK signaling (Yu et al. 2015; Feng et al. 2014). Other studies showed enhanced angiogenesis (Lopatina et al. 2014; Zhu et al. 2012), oxidative stress inhibition (Arslan et al. 2013), and hypoxic signal pathway inhibition (Lee et al. 2012) by exosomes and their respective cargo. A main physiological role of EVs may be to stabilize the intercellular communications in a tissue context, because peptides and RNA in EV are protected from degradation and thus “securely” delivered to target cells. This property makes EV attractive, albeit highly complex, biologics. Clinical testing is on the way for non-cardiac applications (MSC-exosomes for immune modulation in type 1 diabetes mellitus: NCT02138331). Soon, there will likely be clinical trials investigating cell-free regeneration with EV from the various (stem) cell types under scrutiny today.

4.5 Conclusion

Pharmacoregeneration of the heart is an emerging concept. Specifically targeting the disease affected or regenerative cell types in the heart would be desirable. This may ultimately allow cell-free cardiac regeneration. Chemistry, delivery, and timing in addition to the use of human in vitro model systems for a more realistic validation of findings from animal models will be the key to optimal results. Targeting acute rather than chronic myocardial injuries with the aim to limit myocardial damage and delay disease progression seems a realistic goal. Fibroblast conversion appears to be a particularly attractive avenue but requires targeted delivery and better control of the fibroblast reprogramming process. These are, despite the many foreseeable and unforeseeable hurdles in the drug development process, certainly exciting times for refocusing heart failure therapy from pharmacological protection (from neurohumoral overstimulation) to pharmacological regeneration.

4.6 Footnote

We certainly failed to include all outstanding contributions to the emerging field of pharmacoregeneration and wish to apologize to the authors that we have undeliberately missed. Please, contact us to improve any follow-up versions of this book chapter. The authors are supported by the Deutsche Forschungsgemeinschaft (DFG; SFP1002 C04, SFP937 N18), the Bundesministerium für Bildung und Forschung (BMBF), the European Union (EU FP7; CAREMI), and the Fondation Leducq as well as the German Center for Cardiovascular Research (DZHK).

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Chapter 5
Liver

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Abstract The liver has adapted to the inflow of ingested toxins by the evolutionary development of unique self-regenerative properties and responds to injury or tissue loss by rapid restoration of the original organ mass. This high regenerative capacity is sufficient to restore normal volume and function in most forms of acute liver injury and medical interventions are not required. Regenerative therapies in hepatology rather aim to enhance repair mechanisms of the liver in situations, where the capacity to regenerate is severely impaired. Alternatively, regenerative technologies are applied to solve so far unmet medical needs. The development of such therapies requires a fundamental understanding of the (patho-) physiology of the liver. In this chapter we discuss the emerging medical approaches for acute liver failure, chronic liver failure and hereditary liver diseases, which are based on technologies such as (stem) cell therapy, tissue engineering, bio-artificial devices or

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gene therapies. Translation from the laboratory bench into routine clinical applications will also require consideration of the legal framework for "advanced therapy medicinal products" (ATMP) as well as "state of the art" manufacturing (GMP) and good clinical practice (GCP) guidelines.

Keywords Liver regeneration • Stem cells • Cell therapy • Tissue engineering • Gene therapy • Liver support devices

5.1 Regenerative Medicine in Hepatology

Regenerative therapies in hepatology are being developed in three major disease categories: (1) "acute" and "acute on chronic" liver failure, (2) chronic liver disease and (3) hereditary (mostly monogenetic) liver disease.

(1) The acute and self-limiting liver diseases (e.g. due to acute viral disease, toxins or transient ischemia) normally result in complete regeneration and "*restitutio ad integrum*". More massive injuries may temporarily exhaust the regenerative capacity of the liver and result in "acute liver failure", a clinical syndrome, which is characterized by progressive loss of hepatic functions, multiorgan failure and high mortality.
(2) Persistent injury of the liver also induces regenerative tissue responses but eventually results in scarring and excess deposition of extracellular matrix components including collagen. Fibrosis and cirrhosis of the liver are the final result of chronic injuries, which can be caused by a variety of stimuli including metabolic disorders, persistent infections, autoimmune reactions, allergic responses, chemical insults or radiation. Although current treatments for liver fibrosis and cirrhosis typically target the inflammatory response, there is accumulating evidence that the mechanisms driving liver fibrogenesis are distinct from those regulating inflammation. The key cellular mediator of fibrosis is the myofibroblast, which, once activated, serves as the primary extracellular matrix protein producing cell. Myofibroblasts are generated from a variety of sources including resident stellate cells, mesenchymal cells, epithelial and endothelial cells in processes termed epithelial/endothelial-mesenchymal (EMT/EndMT) transition, as well as from circulating fibroblast-like cells called fibrocytes that are derived from bone marrow. Myofibroblasts are activated by several mechanisms including paracrine signals derived from lymphocytes and macrophages, autocrine factors secreted by myofibroblasts, and pathogen-associated molecular patterns (PAMPS) produced by pathogenic organisms that interact with pattern recognition receptors (i.e. TLRs) on target cells.
(3) The liver is central to many metabolic activities with hundreds of genes involved in their regulation. In recent years the genetic basis for more than 100 liver diseases has been discovered. Hereditary liver diseases usually result from point mutations or deletions in single or multiple genes, which are normally

expressed in the liver, and can cause acute or chronic liver diseases. The liver also secrets many proteins, which contribute to functions of other organ systems and, a particular state of protein deficiency may not affect the liver function itself. For most of the hereditary liver diseases liver organ transplantation cures the disease or the state of protein deficiency and has become the most important therapeutic approach. Conceptually, many of these disorders, for which organ transplantation is effective, can be principally cured by cell therapy, gene therapy or tissue engineering approaches.

5.2 Physiology and Pathophysiology of Liver Regeneration

The liver, the largest internal organ of the body, comprises about 1/50th of the total adult body weight (Sherlock and Dooley 2002), receives approximately 25 % of cardiac output (Schiff et al. 2007) and consists of exceptional anatomical structures in both biliary system and vasculature. The biliary system, an exocrine system in the liver, connects the apical surface of every single hepatocyte to the duodenum through bile cannaliculi, which drain into the canals of Hering and finally into bile ducts (Burt et al. 2007). The terminal branches of the hepatic portal vein and hepatic artery enter the liver sinusoids, which are characterised by fenestrated and discontinuous endothelium (Sherlock and Dooley 2002). No basement membrane lines the sinusoid, which allows higher permeability and direct transfer of particles less than 100 nm from the vessels to the basolateral surface of the hepatocytes.

In the absence of injury the adult liver is a "quiescent" organ and as few as 1 out of 3000 hepatocytes divides at a given time point to maintain the physiological liver mass. In situations of acute liver damage or surgical removal of liver mass, however, cell proliferation is extensively stimulated until the original tissue mass is restored (Fausto et al. 2006). In 1 week up to 75 % of a surgically removed liver mass can be regenerated in rodents (Michalopoulos and DeFrances 1997). The parenchymal regeneration after necrogenic or surgical loss of liver tissue originates from extensive proliferation of the mature parenchymal liver cells (hepatocytes and cholangiocytes). In a young adult rat or mouse, approximately 95 % of hepatocytes enter cell cycle during the first 3 days after extensive hepatectomy. Although the term "liver regeneration" is commonly used, restoration of the liver mass after partial hepatectomy is actually a form of compensatory growth of the remaining liver.

In the regenerative phase after acute liver injury or tissue loss the liver immediately induces more than 100 genes, which are not expressed in normal liver (Taub 1996, 2004). The functions served are several and many of these genes appear to play an essential role, however, the precise tasks of the many genes expressed early in liver regeneration are not yet fully understood. The changes in gene expression reflect both the entry of hepatocytes into the cell cycle as well as the orchestration of specific adjustments that hepatocytes have to make, so that they can deliver all essential hepatic functions while going through cell proliferation. The extensive "reprogramming" of hepatic gene expression requires activation of multiple signal-

ling pathways involving matrix remodelling proteins, growth factors, cytokines, paracrine signals, and neuroendocrine factors.

Small RNAs, mainly microRNAs (miRNAs), provide an additional level of regulation in liver regeneration. Global loss of miRNAs leads to the impairment of hepatocyte proliferation at the G1-S stage of cell cycle. In particular, miR-21 and mir-221, one of the upregulated miRNAs in HCCs, has been shown to increase the proliferation of hepatoma cells by targeting *PTEN* and *BTG2* (Song et al. 2010; Yuan et al. 2013). As of now, data are limited and mainly restricted to the early phases of liver regeneration. Importantly, in vivo functions of individual miRNAs during liver regeneration have not yet been identified and more work is required to further elucidate the functional role of known and novel miRNAs in all phases of liver regeneration including the termination process and to examine the effect of inhibition or over-expression of these miRNAs on liver regeneration.

The newborn liver contains mostly diploid hepatocytes, but polyploidization and binuclearity occur rapidly after birth. In perivenous areas the hepatocytes are more often polyploid and serve different liver functions when compared to cells of the periportal region ("metabolic zonation") (Gorla et al. 2001; Jungermann and Kietzmann 2000). The gradient of less complex cells with higher proliferation potential (in vitro) in periportal areas and more "mature" hepatocytes in perivenous areas has been interpreted as evidence for the existence of a physiological niche for cell renewal (Sigal et al. 1995; Fellous et al. 2009) in the periportal region.

Regenerative responses and cell types differ depending on severity and duration of liver injury. Although mature hepatocytes and cholangiocytes represent the first and most important resource for parenchymal tissue repair (Quante and Wang 2009; Duncan et al. 2009) and restore liver mass after acute toxic injury or surgical removal of liver mass, a liver stem/progenitor cell compartment has been postulated to be involved in the repair of chronically injured livers. The first evidence for the existence and activation of a resident hepatic stem/progenitor cell compartment was observed in various murine animal models of "oval cell" proliferation (Alison et al. 1997; Fausto 2004; Thorgeirsson 1996). The general principle underlying "oval cell" activation is based on a combination of liver injury and inability of the hepatocytes to proliferate in response to the damage. Those "oval cells" thus play a facultative role in liver regeneration, i.e., they contribute to tissue regeneration only in cases, where adult hepatocyte proliferation is inhibited or exhausted (Fausto and Campbell 2003). Cells with stem/progenitor phenotypes have been localized at or near the Canals of Hering, in periductular glands and in extrahepatic bile duct structures (Sigal et al. 1992; Lanzoni et al. 2013; Cardinale et al. 2012).

Some researchers found proof that the stem/progenitor cells countinuously generate hepatocytes and bile duct cells and maintain the normal turnover of parenchymal liver cells (Cardinale et al. 2011). According to the above mentioned streaming concept the stem/progenitor cell derived hepatocytes continuously migrate from the periportal areas towards central vein structures, mature and express position dependent differential metabolic activities. Cell fate tracing experiments have attributed Sox9 gene expressing cells with stem/progenitor cell properties in the liver as well as in several other epithelial organs (Furuyama et al. 2011; Sackett et al. 2009; Shin

et al. 2011). More recent data challenge this view and provide evidence for a more complex situation in normal and injured livers.

Following acute injury of the liver CK19$^+$, Sox9$^+$ and LGR5$^+$ cells emerge in the liver and generate hepatocytes (Huch et al. 2013). These cells were identified randomly throughout the liver and could not be traced in the unperturbed liver. Although these cells could still be derived from a (not identified) stem cell niche in the liver another study indicates that mature hepatocytes can de-differentiate and acquire properties of a liver stem/progenitor cell including the expression of progenitor cell and biliary lineage associated cell surface markers. "Knock down" of the Hippo/YAP signalling pathway in hepatocytes resulted in emergence of cells with liver stem/progenitor phenotype. Upon restauration of YAP expression at least some progenitor cells reverted back to the hepatocyte phenotype indicating a bidirectional role of the Hippo/YAP pathway. Isolated mature hepatocyte derived stem/progenitor cells in this study demonstrated self-renewal and engraftment capacity at a single cell level (Yimlamai et al. 2014).

Regardless of nature and phenotype of the stem/progenitor cells the overall contribution of a stem cell niche in normal cell turnover and in various injury models has been questioned (Malato et al. 2011; Schaub et al. 2014; Yanger et al. 2014). The data presented in these studies show that non-hepatocytes in the liver only marginally contribute to hepatocyte regeneration in acute as well as various chronic liver injury models, which have previously been found to induce stem/progenitor cells. The results have recently been confirmed in a chimeric human/mouse liver transplantation model (Tarlow et al. 2014). Although cells with characteristic stem/progenitor markers were induced in chronic liver injury the contribution to hepatocyte regeneration was neglectable. Additionally, all of these studies did not find evidence for stem/progenitor contribution to hepatocyte maintenance in normal livers.

In parallel to what we know from rodent models, the inhibition of mature hepatocyte replication seems to favor the proliferation of cell populations with stem/progenitor phenotypes in human liver. Activation of these cells has been associated with a variety of liver diseases, and, the numbers have been related to severity of the disease (Roskams et al. 2003; Roskams 2006). It has recently been shown that hepatocytes become senescent, owing at least partially to telomere shortening, in the cirrhotic stage of a wide variety of chronic human liver conditions (Marshall et al. 2005; Wiemann et al. 2002). Replicative exhaustion and senescence of the mature hepatocytes as a result of ongoing proliferation during 20–30 years of chronic liver disease has been linked to the emergence of these stem/progenitor cells and finally to the evolution of hepatocarcinoma and cholangiocarcinoma (Alison and Lovell 2005; Mishra et al. 2009).

5.3 Cell-Based Technologies for Regenerative Hepatology

Many of the regenerative technologies for liver diseases, which have been developed, established or envisioned, are based on cellular substrates, which are either transplanted/injected into recipients or utilized in extracorporeal devices and tissue

engineering. The primary hepatocyte, which can be isolated from adult liver organs, is still the most important cellular resource in situations, in which parenchymal liver functions need to be reconstituted. Hepatocytes from pig and human livers as well as immortalized human hepatocytes have been tested in extracorporeal liver devices (see Sect. 5.6.4). Immortalized hepatocytes derived from adult and fetal tissues are restricted to ex vivo applications and have been applied in extracorporal liver devices (see Sect. 5.6.4). Human fetal liver derived hepatoblasts have been transplanted in a small number of patients with acute liver failure (Habibullah et al. 1994) and more recently in one patient with hereditary bilirubinemia (Khan et al. 2008). These cells are also being tested as a cellular substrate for bioartificial liver devices (Poyck et al. 2008). Clinical grade isolated stem/progenitor cells from human adult livers are currently being applied in patients with hereditary liver disease (see Sect. 5.6.8.2).

Transplanted human hepatocytes have been shown to engraft in the recipient mouse and human liver and to respond to growth stimuli in vivo (Dandri et al. 2001; Bissig et al. 2007; Haridass et al. 2009). Despite the high proliferative capacity of hepatocytes, which can undergo more than 60 cell doublings or a 7.3×10^{20}-fold expansion in vivo, the proliferation capacity in vitro is very restricted (Overturf et al. 1997a, b). This lack of in vitro expansion protocols has stimulated the search for alternative cell sources, which can either be expanded in cell culture or easily be harvested from the body in large quantities.

High expectations have been attributed to embryonic stem (ES) cells and more recently to induced pluripotent stem (iPS) cells. These cells can be maintained in a state of pluripotency for long periods of time, grown in large quantities (Evans and Kaufman 1981; Rathjen and Rathjen 2001; Thomson et al. 1998; Boiani and Scholer 2005; Takahashi and Yamanaka 2006; Takahashi et al. 2007) and be differentiated into virtually all cell types of the body. In consequence of his pioneering work, which has paved the way for generation of pluripotent stem cells from almost any postnatal organ such as skin, liver and blood, Shinya Yamanaka was awarded with the Nobel Prize in 2012. These induced pluripotent stem (iPS) cells are generated from somatic cells by transduction with retroviral vectors expressing the stem cell genes *oct4*, *sox2*, *c-myc* and *klf4* (Takahashi and Yamanaka 2006; Takahashi et al. 2007). The combination of these four transcription factors was identified from initially 24 different transcription factors. Later, it was shown that these four factors were also sufficient for human somatic cell reprogramming. Subsequently, iPS cells were generated without genomic integration (Stadtfeld et al. 2008) of the transcription factors by viral vector applications as well as protein transduction methods and by direct transfection of the respective mRNAs. Additionally, Sendai virus based expression systems are available, which would allow "transgen-free" generation of iPS cells suitable for human applications (Fusaki et al. 2009). The iPS cells resemble ES cells as they possess self-renewal capacity, the ability to differentiate into cells of ectoderm, mesoderm and endoderm and form teratomas after transplantation in mice. Similar to mouse ES cells, hepatocyte differentiation of mouse and human iPS cells has been documented (Sullivan et al. 2010). Notably the hepatocytes derived from mouse iPS cells had hepatocyte marker expression and metabolic activity similar to hepatocyte-like cells derived from mouse ES cells (Li et al.

2010; Si-Tayeb et al. 2010; Song et al. 2009; Sullivan et al. 2010; Zhu et al. 2014). Also, iPS cells derived from fumarylacetoacetatehydrolase-deficient ($FAH^{-/-}$) mice could be subjected to lentiviral gene correction and subsequently generated healthy mice using a tetraploid embryo complementation approach (Wu et al. 2011).

In contrast to reprogramming somatic cells into iPS cells followed by applying hepatic differentiation protocols, a direct reprogramming approach attempts to generate cells exhibiting a hepatic phenotype by over-expression of combinations of liver-enriched transcription factors. Several groups have now demonstrated the induction of a mature hepatic phenotype in mouse and human fibroblasts and shown transplantability of the cells in liver repopulation animal models (Huang et al. 2011, 2014; Sekiya and Suzuki 2011; Du et al. 2014). Although many functional features of primary hepatocytes could be demonstrated, fidelity of global gene expression of the so-called iHeps has been questioned (Cahan et al. 2014; Morris et al. 2014). Furthermore, no data are available on genetic stability and tumorigenicity of the cells in transplanted animals. Thus, it remains speculative, whether direct "trans-programming" of somatic cells such as fibroblasts into the desired phenotype by forced expression of sets of transcription factors represents an alternative approach and may circumvent the state of pluripotency, which is associated with teratoma formation in transplanted recipients.

In contrast to primary hepatocytes isolated from donated organs the hepatocyte-like cells derived from pluripotent stem cells may serve as an unlimited cell source for cell therapies or liver tissue engineering. In order to generate hepatic cells from pluripotent stem cells the various published differentiation protocols usually mimic the events, which occur during embryonic development of the liver. As an example, ES cells can be differentiated into the hepatocyte phenotype by the formation of embryoid bodies, followed by the induction of definitive endoderm using instructive cytokines such as Activin A. The endoderm cell population can be further induced towards the hepatocyte lineage by exposure to bone morphogenetic protein (BMP) 4 and fibroblast growth factor (FGF) 2, both important signals from the cardiac mesoderm in early liver embryogenesis (Gouon-Evans et al. 2006). Assessment of the hepatic phenotype is commonly based on hepatocyte specific gene expression profiles and metabolic activities such as cytochrome p450 activity, glycogen storage or urea synthesis, which determine the efficacy of the differentiation protocol. At the end of a differentiation process it is important to remove contaminating undifferentiated ES cells from the heterogeneous cell culture to minimize the risk of teratoma formation. This can be achieved by various FACS/MACS sorting techniques or by the transfer of cell type specific expression of antibiotic resistance genes (Drobinskaya et al. 2008). Transplantation of ES derived hepatic cells into the liver results in engraftment as both, mature hepatocytes and bile duct epithelial cells (Gouon-Evans et al. 2006; Touboul et al. 2010). The level of liver repopulation obtained with hepatocyte-differentiated ESCs is currently very low, but can be increased somewhat, when the cells are transplanted into MUP–urokinase plasminogen activator/severe combined immunodeficient (SCID) mice (Heo et al. 2006). To date, most published ESC differentiation protocols generate hepatocyte-like cells, but not the fully functional, mature, and transplantable equivalents of hepato-

cytes that are isolated from adult liver. Although differentiation of ES into hepatic lineage cells has been improved over time, the resulting cells resemble early fetal hepatoblasts rather than adult mature hepatocytes. A comparative analysis in a xenograft accepting liver repopulation mouse model has shown that ES/iPS derived hepatic cells show less capacity to generate liver tissue compared to adult hepatocytes after transplantation (Haridass et al. 2009). Inhibition of microRNA199a-5p during hepatic differentiation of human ES cells can improve hepatic differentiation and results in low level engraftment and repopulation in $FAH^{-/-}$ livers (Möbus et al. 2015). This and other improvements should soon result in more mature hepatocyte phenotypes and eventually may provide functional cellular substrates for cell therapy and tissue engineering applications.

5.4 Liver Tissue Engineering

Liver tissue engineering is a new and rapidly emerging field, which aims to create a functional liver system using hepatocytes and/or other cells types to treat liver diseases. Under circumstances, in which small, but functional liver tissue could be engineered to provide the equivalent biological function proportional to a few percent of a normal, well-functioning liver, it would be possible to correct many disease phenotypes that result from various forms of inherited metabolic deficiencies as well as acute and chronic liver failures. It has been demonstrated in animal models, that sheets of liver tissue can be grown under the renal capsule or under the skin (Ohashi et al. 2007, 2009). Alternatively, hepatic tissues could be engineered ex vivo to produce therapeutic effects in ectopic locations such as the peritoneal cavity. A Japanese team recently showed in a mouse model that a combination of human stromal, endothelial and iPS derived hepatocytes spontaneously aggregate and form three dimensional organoid structures with intrinsic vasculature. Those liver buds closely resembled the “liver anlage” during embryonic or fetal development. The scientists could further show that following ectopic transplantation into the peritoneum the vasculature of the engineered tissue connected to the recipient blood system, which was proven by detection of human albumin in the serum of mice (Takebe et al. 2013). Organoids from postnatal tissue stem- and progenitors have been created through self-organisation of the cells in three-dimensional cultures (Lancaster and Knoblich 2014). Murine liver organoids engrafted after transplantation into the liver and rescued mice with fatal liver failure (Huch et al. 2013). Similarly, human Lgr5-positive bipotent human liver progenitor cells could be cultivated and expanded in 3D-organoids, which maintained the potential to differentiate towards hepatocytes and cholangiocytes (Huch et al. 2015). Limited data is available, to which extent such cells may serve as transplant for metabolic liver disorders in patients. Nevertheless, the presented set of data provides evidence that the cells could give rise to albumin-producing cells in a xenograft-accepting mouse model after Retrorsine/CCl_4 injury. Essentially, three dimensional liver organoids may also be considered as a “tissue engineering” approach and their application

might be extended as therapeutic approach by roboter-assisted parallelisation and subsequent implementation in bioreactor devices. A much more complex and not yet realistic task will be the generation of artificial, transplantable liver tissue with a functional blood supply and biliary system.

5.5 Gene Therapies for Hereditary Liver Diseases

The liver is involved in the synthesis of serum proteins, regulation of metabolism and maintenance of homeostasis and thus provides a variety of opportunities for gene therapeutic corrections. Gene therapy is the treatment of an inherited or acquired disease through the manipulation of a patients' genetic status or sequence in selected cells by introducing various types of genetic materials such as virally bound nucleic acids, plasmid DNAs, antisense oligonucleotides and short interference RNAs. Currently, only viral vectors have transduction efficacies needed for liver-based gene therapy of inherited metabolic diseases in humans.

Viral gene delivery employs replication deficient viruses as a carrier to deliver genetic materials into cells through their natural infection mechanism. Viral vectors are created using molecular techniques, by which a portion of the viral genome is replaced with a gene of interest. Major drawbacks of viral vectors are their genetic and immunologic toxicities, which are mainly associated with an arbitrary recombination with genomic DNA of the target cells and immune stimulation, respectively. Because adult humans have already developed immunity against several types of viruses, from which viral vectors are developed, an exposure of the viral vectors to patients often results in strong immunological response, and consequently disables efficient gene delivery and long-term gene expression.

Viral vectors frequently used in gene therapy studies are derived from retroviruses, adenoviruses, and adeno-associated viruses. Retroviruses, enveloped RNA viruses with a particle size of approximately 100 nm, only infect dividing cells and are capable of integrating reverse transcribed DNA into the host genome at unpredictable locations (Sinn et al. 2005). Viral integration has led to leukemia development as revealed by the gene therapy trial on X-linked sever combine immunodeficiency disease (SCID) (Bey et al. 2003). The requirement of hepatectomy (~70 %) to stimulate hepatic proliferation is generally considered as a drawback for retrovirus mediated gene delivery to the liver (Rettinger et al. 1993; Branchereau et al. 1994). Lentiviruses, a subclass of retroviruses including human immunodeficiency virus, can transduce non-dividing as well as dividing cells. The lentivirus pre-integration complex is able to pass the intact nuclear membrane, which allows it to integrate into the host genome without cell division (Amado and Chen 1999).

Adenoviruses are double-stranded DNA viruses with a diameter of approximately 110 nm. Adenoviruses infect both replicating and non-replicating cells, have a relatively large genome, and are unable to integrate into the host genome (Ghosh et al. 2006a, b). These vectors exhibit a broad range of liver tropism with serotype 5 as the most commonly used to date (Jager and Ehrhardt 2007). Adenoviral vectors

are the first proven gene carriers for the treatment of cancer (Peng 2005). Because this virus is a natural human pathogen, pre-existing immunity against adenovirus can cause severe allergic reaction and inactivation of viral vectors (Marshall 1999). The current strategy in avoiding these problems is to use a serotype which the patients have no immunity against (Jager and Ehrhardt 2007). If the immunogenic drawbacks can be overcome in the future, adenoviral vectors will probably find a great diversity of clinical applications.

Adeno-associated virus (AAV) belongs to the Parvoviridae family and is approximately 26 nm in diameter and without envelope (Grieger and Samulski 2005). It requires a helper virus for replication such as adenovirus. It is non-pathogenic and can infect quiescent cells. AAV is currently classified into at least 12 serotypes, and the liver is known to be a preferential target especially for AAV-8 (Wu et al. 2006). It was reported that this virus can insert its genome at a defined site on chromosome 19 termed AAVS1 with nearly 100 % certainty (Samulski et al. 1991) The site-directed integration is controlled by viral Rep proteins (Young et al. 2000), which are, however, often deleted in recombinant AAV vectors in favour of more space for the exogenous gene cassette to be packaged into the tiny viral particle. Co-transfection of plasmids coding for Rep protein was reported to restore capability of the site-directed integration and enable a long-term expression of the transgene without inducing insertional mutagenesis (Howden et al. 2007). Results from a number of animal studies indicate that AAV is less immunogenic when compared to adenoviruses (Coura Rdos and Nardi 2007).

Feasability of gene therapies has been demonstrated in a wide variety of animal models. Long-Evans cinnamon rats are a model of Wilson disease and transfer of the *ATP7B* gene to hepatocytes ameliorates both biochemical and histological pathologies (Merle et al. 2006). Transgene products released into blood circulation after successful gene transfer into the hepatocytes corrected the pathological manifestation both inside and/or outside of the liver in glycogen storage diseases (type Ia, (Ghosh 2006) Ib (Yiu et al. 2007) and II (Yiu et al. 2007), mucopolysaccharidosis type I (Kobayashi et al. 2005), IIIB (Di Natale et al. 2005) and VII (Ponder et al. 2002), hereditary tyrosinemia type I (Overturf et al. 1996), UDP glucuronyltransferase deficiency (Crigler-Najjar type I) (Seppen et al. 2003), and hemophilia (Miao et al. 2001; Herzog et al. 1999; Waddington et al. 2004).

A complete and persistent phenotypic correction of phenylketonuria in mice was reported after hydrodynamic gene delivery of murine phenylalanine hydroxylase cDNA with the help of phiBT1 phage integrase for long-term gene expression (Chen and Woo 2005). Further, the efficacy of adenovirus-mediated in vivo gene therapy for ornithine transcarbamylase deficiency was reported in mice and non-human primates (Raper et al. 1998). Hyperlipidemia was not only effectively treated in the respective genetic mouse models through delivery of apolipoprotein B (Crooke et al. 2005) or E (Kim et al. 2001) genes but also in wild type mice treated with a high-fat diet. A reciprocal pathophysiological condition of hypoalphalipoproteinemia was effectively reversed by adenoviral transduction of human apolipoprotein A-I gene in model mice as well (Oka et al. 2007).

5.6 Regenerative Therapies for Liver Disease

Regenerative therapies or treatments involving regenerative technologies are currently being developed for liver diseases of diverse etiologies. In acute liver failure syndromes, "acute on chronic" liver failure and non-function of transplanted livers the therapeutic approaches aim to substitute liver function (synthesis of proteins, metabolism and detoxification) either by extracorporal support devices, transplantation of liver cells or by engineering and transplantation of functional liver tissue. In chronic liver diseases conventional drugs, cytokines, stem cell therapy and gene transfer techniques are being employed to specifically interfere with the inflammatory and profibrotic pathways. In hereditary metabolic liver disease the experimental and clinical approaches focus on substitution of defective genes and proteins through allogeneic transplantation of hepatocytes or gene therapy. Therapies involving regenerative technologies such as (stem) cell therapies and gene transfer protocols, which emerge for liver cancer, viral infections and immune mediated liver diseases, are beyond the scope of this book chapter and have been reviewed elsewhere.

5.6.1 Acute Liver Failure

Acute liver failure (ALF) is a syndrome of diverse etiology, in which patients without previously recognized liver disease sustain a liver injury that results in rapid loss of hepatic function. Depending on the etiology and severity of the insult, some patients undergo rapid hepatic regeneration and spontaneously recover. However, nearly half of the patients with ALF require and undergo orthotopic liver transplantation or die. Even with optimal early management many patients with ALF develop a cascade of complications often presaged by the systemic inflammatory response syndrome, which involves failure of nearly every organ system. For those patients no satisfactory treatment exist other than liver transplantation. However, the number of donor livers available is limited and the outcome of liver transplantation for ALF is significantly lower than transplantation for chronic liver disease. Furthermore, many ALF patients are not placed on the transplant list due to exclusion criteria such as sepsis, psychiatric illness, and multi-system organ failure. Specialised treatment algorithms for the intensive care of patients with liver failure and the introduction of antioxidative drug treatments have already significantly improved the survival of affected patients in the past. Trials of plasmapheresis and hypothermia from European consortia are near completion and drugs that facilitate the excretion of ammonia, such as L-ornithine phenylacetate (Jalan et al. 2007), may provide a neuroprotective bridge to orthotopic liver transplantation.

5.6.2 *Extracorporeal Liver Support in Acute and Acute-on-Chronic Liver Failure Patients*

Future therapies for ALF would ideally maintain the patient's clinical stability long enough to allow liver regeneration to occur, which would obviate the need for orthotopic liver transplantation. Realistically, however, the goal of such therapies will be to serve as a bridge to orthotopic liver transplantation. Extracorporeal liver support devices have been developed to achieve the goal of "bridging" by temporarily supporting liver detoxification function. Artificial liver support refers to purely mechanical devices including albumin dialysis, while bioartificial liver support refers to devices with a cellular component. Artificial systems remove toxins by filtration or adsorption while bioartificial liver systems perform these functions along with biotransformation and protein synthetic functions of biochemically active hepatocytes.

5.6.3 *Non-Cell Based Liver Support Devices*

The molecular adsorbent recirculating system (MARS™; Gambro, Lund, Sweden) is the most frequently used type of albumin dialysis and most studied non-cell based liver-support technique (Mitzner et al. 2009). The key feature to the function of albumin dialysis is the concentration gradient of low-molecular-weight substrates between the patient's blood and the 20 % albumin in the secondary circuit. This concentration gradient allows diffusible low-molecular-weight substrates to flow down their gradient over the membrane where they are transiently bound by albumin in the secondary circuit (Steiner et al. 2004). The low-molecular-weight substrate is then removed from the system by conventional dialysis and hemodiafiltration within the secondary circuit.

The initial clinical study described a series of 13 patients who underwent treatment after failure of response to best medical therapy for acute-on-chronic hepatic failure. In this series, the overall survival was 69 % and the authors cited that all patients showed a positive response to therapy (Stange et al. 1999). Other encouraging case reports and small studies eventually led to more widespread use of the system. To date, roughly 7,500 patients have been treated with MARS for various hepatic diseases, including acute liver failure patients. A meta-analysis assessing the use of MARS looked at four randomized controlled trials including a total of 67 patients and two selected nonrandomized trials including 61 patients (Khuroo et al. 2004). Patients had either acute or acute-on-chronic liver failure. Primary meta-analysis did not show a statistically significant survival benefit. Subgroup analysis for both acute and acute-on-chronic liver failure again failed to show a significant survival benefit. However, explorative analysis of the two nonrandomized trials did show that survival was significantly improved with MARS treatment. The authors concluded that this benefit was possibly related to bias in patient selection. Recently,

the results of a large multicenter randomized trial of MARS in patients with ALF fulfilling high-urgency liver-transplant criteria in France were presented (Saliba et al. 2009). The data show a trend toward better survival in the MARS treatment group, but the difference did not reach significance. The transplant-free 6 month survival, however, was significantly prolonged in those patients treated with at least three sessions of MARS. Although a relatively large number of patients have been treated with MARS and improvements in biochemical and physiologic parameters have been demonstrated, MARS must still be considered experimental, as survival benefit has not been reproducibly shown for the various indications.

"Prometheus™, which employs fractionated plasma separation, is a close variant of MARS. While MARS is a two-circuit system separated by an albumin impermeable membrane, Prometheus utilizes a membrane with a 250 kDa cutoff between circuits, thereby making the membrane permeable to albumin and hence albumin-bound toxins. While a large portion of the toxins, which accumulate during liver failure are water soluble, many are still bound by albumin. Therefore fractionated plasma separation may be advantageous in regard to toxin removal. Other factors that distinguish Prometheus from MARS include the fact that while MARS is prefilled with 120 g of exogenous human albumin, the patient's endogenous albumin loads the secondary circuit in Prometheus. Because Prometheus is loaded with the patient's albumin, there may be a drop in the patient's albumin levels during treatment (Rifai et al. 2003; Santoro et al. 2006).

Most of the clinical data involving Prometheus are either uncontrolled or retrospective. A controlled trial, published as an abstract, looking at the effect of fractionated plasma separation on hepatic encephalopathy, demonstrated that a 6-h treatment course improved clinical grade and sensory-evoked potentials (Kramer 2000). Multiple case series describe both acute and acute-on-chronic liver failure patients being treated with Prometheus. Only recently, the results of a controlled randomised multicenter clinical trial in 145 patients with acute on chronic liver failure were reported (Kribben et al. 2012). Survival rates after 1 and 3 month were not significantly different in the treated versus the control group. However, patients with hepatorenal syndrome type I and MELD score of >30 showed a significant survival benefit. Currently available data thus illustrate a need for new prospective randomized controlled trials to clarify indications and clinical impact of extracorporeal artificial liver support devices.

5.6.4 Cell-Based Liver Support Devices

It is unlikely that the complex mechanism, by which the liver ensures homeostasis, can be replaced by means of non-biologic detoxification alone. A bioartificial liver, which incorporates hepatocytes from various sources, has the theoretical advantage of not only providing blood purification through dialysis, but also providing the hepatocyte-specific functions which are lost with ALF. These include protein synthesis, ureagenesis, gluconeogenesis, and detoxification through P450 activity.

The first biologically based liver assist device to be tested in FDA-approved phase II/III trial was HepatAssist™ by Arbios (formerly Circe, Waltham, MA). The device employed a hollow fiber extracorporeal bioreactor loaded with cryopreserved primary porcine hepatocytes. A randomized, controlled, multicenter phase II/III clinical trial was conducted in patients with fulminant/subfulminant liver failure and primary graft nonfunction (Demetriou et al. 2004) The study demonstrated favorable safety, but failed to demonstrate improved 30-day survival in the overall study population. Although sub-groups of the study population showed significant survival benefits, HepatAssist is not yet approved by the FDA. The Extracorporeal Liver Assist Device (ELAD™) by Vital Therapies (San Diego, CA) utilizes hollow fiber cartridges loaded with cells from the C3A human hepatoblastoma cell line. The most current model also contains a conventional hemodialysis unit. An early randomized controlled trial of 24 patients with acute alcoholic hepatitis demonstrated that therapy with ELAD produced reduced levels of ammonia and bilirubin along with improvement in hepatic encephalopathy when compared to controls (Ellis et al. 1999). However, a statistically significant survival advantage was not demonstrated. The Modular Extracorporeal Liver Support System (MELS™; Charité, Berlin, Germany) is a hepatocyte based liver support therapy composed of four independently functioning hollow fiber capillary cell compartments. A phase I study in 2003 including eight patients with ALF demonstrated safety, with all eight patients being successfully bridged to transplantation (Sauer et al. 2003). Clinical experience with MELS has been limited by the infrequent and unpredictable supply of human hepatocytes and concerns of xenozoonosis involving pig hepatocytes, which are prevalent in Europe. The Bioartificial Liver Support System (BLSSTM) by Excorp Medical (Minneapolis, MN) is a system that utilizes ~100 g of primary porcine hepatocytes in a single hollow fiber cartridge. Venovenous bypass is used to circulate the patient's blood through the system. A phase I trial, in which four patients were treated with BLSS demonstrated safety (Mazariegos et al. 2001). Currently, a phase II/III study is underway, and results will further define the role of this device. The Amsterdam Medical Center bioartificial liver (AMC-BALTM; AMC, Amsterdam, The Netherlands) uses 100 g of primary porcine hepatocytes bound to a spiral-shaped polyester fabric with integrated hollow fibers. During treatment, the bioreactor is perfused with the patient's plasma. A phase I study of the system examined seven patients with ALF who underwent multiple treatments with AMC-BAL (Van De Kerkhove et al. 2002). Six patients were successfully bridged to transplantation, and one patient recovered liver function without transplantation. Improvements were observed in both clinical and biochemical parameters including a decrease in both bilirubin and ammonia. No adverse events were associated with treatment. While preliminary results were encouraging, larger randomized, controlled trials are needed to determine the role of AMC-BAL.

5.6.5 *Hepatocyte and Stem Cell Transplantation*

In acute liver failure hepatocyte transplantation may act as a bridge to recovery and regeneration of the injured native liver or alternatively to orthotopic liver transplantation until an organ becomes available. The procedure may also be used in patients who are not candidates for organ transplantation due to co-morbidities like pre-existing malignancies or severe cardiovascular disorders. A major advantage of hepatocyte transplantation is the immediate availability of cryopreserved cells. Sufficient cell mass (approximately 10–15 % of liver cell mass) is needed to provide enough metabolic function (Asonuma et al. 1992). The mass of cells, which can be transplanted into the liver, is, however, limited by the effect on portal hypertension. Other options include intrasplenic or intraperitoneal transplantation, which allow a larger volume of cells. The spleen has been used successfully as injection site in animal (Kobayashi et al. 2000; Cai et al. 2002) and human transplantation (Bilir et al. 2000); however, in view of the number of immunologically active cells located in the spleen, rejection or destruction of the non-native cells needs consideration. Hepatocyte transplantation in patients with ALF has resulted in a reduction in ammonia and bilirubin with improvements in hepatic encephalopathy and cardiovascular instability (Bilir et al. 2000; Fisher and Strom 2006). In the absence of any randomized controlled trials, it is difficult to comment on the true efficacy of the intervention.

There are a few studies on liver cell therapy for treatment of acute liver failure in humans with the intention to bridge the patients to orthotopic liver transplantation or recovery (Bilir et al. 2000; Schneider et al. 2006; Strom et al. 1997). Main challenges for future applications are the appropriate timing of cell transplantation, the restricted uptake capacity of the recipient liver, the availability of cells and the need for immunosuppression to prevent the rejection of the transplanted cells. The latter point may become more important than considered previously, because the liver failure gives a high risk for septic complications itself, which will be aggravated by immunosuppressive drugs.

Extended liver resections have been associated with significant morbidity and mortality due to hepatic dysfunction or hepatic failure in the postoperative period. Autologous bone marrow stem cell therapies may offer the potential to enhance hepatic regeneration in this setting, perhaps increasing the safety of the procedure. Preclinical models and initial translational studies have suggested that autologous bone marrow stem cell administration can facilitate hepatic regeneration following both acute and chronic liver disease (Stutchfield et al. 2010; Dhawan er al 2006). Infusion of HSC in three patients after extended liver resection demonstrated the therapeutic potential, however, more and controlled clinical trial data are needed (am Esch et al. 2005).

5.6.6 Chronic Liver Disease and Liver Fibrosis

Chronic injury and inflammation triggers a gradual loss of liver function and deposition of extracellular matrix components, which leads to fibrosis and finally to cirrhosis of the liver. Although acute injury does activate mechanisms of fibrogenesis, more sustained signals associated with chronic liver diseases result in a fibrogenic response, which engages several different cell types. Cirrhosis of the liver as a clinical endpoint of the fibrogenic process is probably an irreversible condition and the only long-term therapeutic solution for end-stage chronic liver disease today is liver organ transplantation. However, experimental and clinical data indicate that earlier events of the perpetuated fibrogenic process in the liver could be stopped or even reversed.

New therapeutic targets interfering with fibrogenesis are emerging from translational research and have been recently addressed in clinical trials. Interferon-gamma1β (IFN-γ1β) is a pleiotropic cytokine that displays antifibrotic, antiviral, and antiproliferative activity. Initial studies conducted in patients with HCV-related liver diseases have shown a fibrosis reduction in some of the patients (Muir et al. 2006). In particular, patients with elevated interferon-inducible T cell-alpha chemoattractant (ITAC) levels in their blood and perhaps less advanced disease stage, may best be suited for IFN-gamma1β based therapy (Pockros et al. 2007).

Interleukin-10 (IL-10) was first described as a cytokine synthesis inhibitory factor for T-lymphocytes produced from T-helper-2 cell clones. In fact various cell populations produce IL-10 in the body, including T-cell subsets, monocytes, macrophages and also various other cell types present in organs such as the liver. IL-10 gene polymorphisms are possibly associated with liver disease susceptibility or severity. Recombinant human IL-10 is currently tested in clinical trials in patients not responding to standard Peg-IFN α therapy.

PDGF is the most potent mitogen for hepatic stellate cell-derived myofibroblasts and levels of the growth factor have been shown to increase in liver diseases. Autocrine signalling by PDGF was the first cytokine loop discovered in hepatic stellate cell activation and is amongst the most potent ones (Borkham-Kamphorst et al. 2008). Hepatic PDGF-α overexpression using the CRP-gene promoter was accompanied by a significant increase in hepatic procollagen III mRNA expression as well as TGF-β1 expression. Liver histology showed increased deposition of extracellular matrix in transgenic but not in wildtype mice. These results point to a mechanism of fibrosis induction by PDGF-α via the TGF-β1 signalling pathway (Thieringer et al. 2008). On the other hand, Dominant-negative soluble PDGF receptor beta is currently investigated as a possible new antifibrogenic target.

TGFβ1 remains, however, the classic fibrogenic cytokine. TGF β1 activates stellate cells via the SMAD proteins pathway and also stimulates collagen expression in stellate cells through a hydrogen peroxide and C/EBPβ -dependent mechanism. There is experimental evidence that hepatocyte-specific overexpression of TGFβ1 in transgenic mice increases fibrosis in vivo, and that soluble TGFβ receptor type II

treatment inhibits fibrosis in vivo. Also, it has been shown that the application of adenoviral vectors encoding antisense TGFβ mRNA inhibits fibrogenesis in vivo.

More experimental strategies aim to reduce extracellular matrix deposition by over-expression of MMP's. Siller-Lopez et al. have used an extrahepatic human neutrophil collagenase complementary MMP-8 DNA cloned in an adenovirus vector (AdMMP8) as a therapeutic agent in cirrhosis using CCl_4 and bile duct–ligated cirrhotic rats models. Liver fibrosis in bile duct–ligated cirrhotic animals was decreased by 45 % along with reduced hydroxyproline levels in AdMMP8 treated animals. Treatment in both models correlated with improvements in ascites, functional hepatic tests and gastric varices indicating diminished portal hypertension in animals injected with AdMMP8 (Siller-Lopez et al. 2004).

Alternative treatment concepts aim to protect existing hepatocytes and/or to increase the hepatocyte mass. Hepatocyte growth factor (HGF), originally identified and cloned as a potent mitogen for hepatocytes (Nakamura et al. 1984, 1989; Russell et al. 1984; Miyazawa et al. 1989) has mitogenic and morphogenic activities for a wide variety of cells (Boros and Miller 1995; Michalopoulos 1997) and also plays an essential role in the development and regeneration of the liver (Schmidt et al. 1995). It has also been shown to have antiapoptotic activity in hepatocytes (Bardelli et al. 1996). Transduction of the HGF gene has suppressed the increase of transforming growth factor-β1 (TGF-β1), which plays an essential part in the progression of liver cirrhosis and inhibited fibrogenesis and hepatocyte apoptosis leading to complete resolution of fibrosis in the cirrhotic liver in a rat model (Ueki et al. 1999).

5.6.7 Stem Cell Therapy of Chronic and Acute of Chronic Liver Disease

Although the concept of cell therapy for various diseases is principally accepted, the practical approach in humans remains difficult. Bone marrow derived mononucleated cells, hematopoietic stem and progenitor cells, mesenchymal stem (stromal) cells and sinusoidal endothelial cells are currently being investigated. There are several proposed mechanisms, by which stem and progenitor cells might support regeneration in targeted organs including the liver: intercellular signalling through cell-cell contacts, paracrine signalling (growth factors, cytokines, hormones) or cell fate change in the target organ (Aurich et al. 2007).

The concept of stem/progenitor cell infusions exerting a paracrine regenerative effect on the liver is gaining support and is backed up by both rodent and human studies, although the latter are small and uncontrolled. Endothelial precursor cells (EPC) have been shown in rodent models to promote angiogenesis and the degradation of liver scar tissue thereby contributing to liver regeneration (Taniguchi et al. 2006; Nakamura et al. 2007; Ueno et al. 2006; Wang et al. 2003). By participation in neovascularisation and by the expression of multiple growth factors, transplanted EPCs significantly accelerate liver regeneration. This is achieved by enhancing pro-

liferative activity of hepatocytes leading to improved survival after chemically induced liver injury (Taniguchi et al. 2006).

Sakaida et al. have demonstrated that transplanted bone marrow cells degrade extracellular matrix in carbon tetrachloride (CCl_4)-induced liver fibrosis, with a significantly improved survival rate in this animal model. Their findings suggest that transplanted bone marrow cells can degrade collagen fibers and reduce liver fibrosis by strong expression of MMPs, especially MMP-9 (Sakaida et al. 2004).

Other groups have raised concerns about the role of certain subtypes of bone marrow stem cells in liver fibrogenesis (Russo et al. 2006). It has been shown that bone marrow derived myofibroblasts significantly contributed to fibrogenesis in a chronic liver injury model in mice. They originated predominantly from bone marrow cells enriched for mesenchymal progenitor cells. These cells were located in the region of hepatic scarring and actively expressed collagen. The data suggest that an axis of recruitment from the bone marrow to the liver does exist in chronic injury and that the therapeutic application of certain subsets of bone marrow derived cells may contribute to, rather than resolve scarring of the liver tissue. The choice of the transplanted bone marrow cell type might thus be important with regard to supporting liver regeneration or fibrogenesis.

Taken together the infusion of stem cells might provide an array of factors supporting not only liver regeneration but also the remodelling of impaired liver architecture by interfering with fibrogenesis. Important experimental findings, however, suggest that infused bone marrow cells may also contribute to fibrogenesis (Takezawa et al. 1995; Kisseleva et al. 2006) giving some cautious notes for the uncritical use of stem cells for chronic liver disease outside of controlled clinical trials (Sakaida et al. 2004; Fang et al. 2004; Zhao et al. 2005; Oyagi et al. 2006).

Several clinical trials already investigated the effect of bone marrow (stem) cells in patients with liver disease. They were mainly uncontrolled, with only small numbers of patients enrolled and have provided variable results. The trials can be categorized in four groups according to the main endpoint and source of cells: (1) effects of granulocyte colony–stimulating factor (G-CSF) mobilized bone marrow cells in advanced chronic liver disease, (2) effects of infusion of autologous mononuclear cells collected from bone marrow in advanced chronic liver disease, (3) effects of collection (with or without ex vivo manipulation) and infusion of mobilized bone marrow cells in advanced chronic liver disease and (4) effects of bone marrow infusions on liver regeneration (after selective portal venous embolization) prior to extended hepatectomy for liver tumors (Houlihan and Newsome 2008; Gaia et al. 2006; Terai et al. 2006; Mohamadnejad et al. 2007a, b; Lyra et al. 2007a, b; Gordon et al. 2006; Levicar et al. 2008; Yannaki et al. 2006; am Esch et al. 2005; Pai et al. 2008).

The trials are quite heterogeneous with regard to the source of stem cells used and the number of patients included. The following stem cells sources have been used: bone marrow from iliac crest (50–400 ml), mobilization of endogenous stem cells by G-CSF only, G-CSF mobilization into peripheral blood, followed by leukapheresis and CD-34+ selection and subsequent reinfusion. All but one trial were non-randomized. The stem cells were administered by peripheral vein infusions

(three studies), by hepatic artery infusions (five studies) or portal vein infusions (two studies). The largest study conducted so far by Lyra et al. was also the only randomised 1 and included 30 patients. Eight out of 11 trials have shown a moderate improvement in liver function (albumin, INR, bilirubin, Child-Pugh score, MELD score) and the follow-up period has ranged from 2 to 12 months.

In one study safety and efficacy of hepatic artery administration of mobilized autologous and ex vivo expanded adult $CD34^+$ hematopoietic stem cells in patients with alcoholic cirrhosis (ALC) was assessed (Pai et al. 2008). This study reported one of the largest numbers of CD34 positive stem cells infused in cirrhotic patients so far. Nine patients with biopsy-proven ALC and abstinence from alcohol for at least 6 months were included in the study and all patients tolerated the procedure well, with no treatment-related side effects or toxicities observed. Significant improvement in liver function was shown by decrease in serum bilirubin levels, serum alanine transaminase and aspartate transaminase. The Child-Pugh score improved in seven out of nine patients and in five patients ascites production had declined.

Two studies so far aimed to ameliorate acute on chronic liver disease by administration of granylocyte – colony stimulating factor (G-CSF) treatment. In contrast to an earlier study by Campli et al. (2007) a more recent study from India showed profound effects on short term survival, which was associated with a marked increase of CD34 stem cells in the liver of recipients (Garg et al. 2012).

5.6.8 Hereditary Liver Disease

Liver organ transplantation can be viewed as a form of gene therapy for inherited liver diseases since the procedure substitutes a defective gene with a normal copy from a healthy donor. Animal studies have shown that for most monogenetic liver diseases partial substitution of a missing or defective protein is able to reverse the clinical phenotype and can result in complete remission of the disease. This redundancy opens the possibility to apply minimally invasive therapies such as cell and gene therapies to correct an existing gene defect. Although many hurdles still exist, feasibility has been proven unequivocally in animal models and therapeutic protocols are now emerging in the clinical arena.

5.6.8.1 Transplantation of Primary (Adult) Hepatocytes

In recent years the interest in liver cell therapy has been increasing continuously, since the demand for whole liver transplantations in human beings far outweighs the supply (Nussler et al. 2006). From the clinical point of view, transplantation of hepatocytes or hepatocyte-like cells may represent an alternative to orthotopic liver transplants for the correction of genetic disorders resulting in metabolically deficient states. The aim of hepatocyte transplantation in metabolic disease is to partially

replace the missing function without the need to replace the whole organ. More than 50 children and adults who received liver cell therapy for metabolic liver disease are reported in literature (Fisher and Strom 2006; Fitzpatrick et al. 2009). Clinical therapies up to now have been performed by infusing fresh or cryopreserved primary hepatocyte suspensions isolated from donated organs. The availability of high quality liver tissue for cell isolation, however, has slowed the widespread application of this therapy. Furthermore, the clinical situation of target patients is rarely immediately life threatening and often acceptable conventional therapies are available. Therefore, the potential benefit must be carefully weighed against any possible complications, such as side effect from immunosuppression, hepatocyte embolisation of the pulmonary vascular system, sepsis or hemodynamic instability.

Objective parameters such as laboratory data (i.e. bile acid, clotting factors, etc.) can be determined to unequivocally assess the efficacy of the treatment. The results of hepatocyte transplantation for many metabolic liver diseases have been encouraging with demonstrable, although short-term correction of metabolic deficiency in the majority of cases. Therapeutic benefit has been reported in a girl with Crigler–Najjar Syndrome Type I, which is a recessively inherited metabolic disorder characterized by severe unconjugated hyperbilirubinaemia (Fox et al. 1998). Isolated hepatocytes were infused through the portal vein and partially corrected plasma bilirubin levels for more than 11 months. Similarly, a 9-year-old boy received 7.5×10^9 hepatocytes, infused via the portal vein, which resulted in a decrease in bilirubin level from 530 ± 38 μmol/L (mean ± SD) before to 359 ± 46 μmol/L (Ambrosino et al. 2005). Hughes et al. also reported a 40 % reduction in bilirubin levels in a Crigler–Najjar Syndrome Type I patient following transplantation of hepatocytes (Hughes et al. 2005). Although these data demonstrate efficacy and safety, a single course of cell application seems not sufficient to correct Crigler–Najjar Syndrome Type I completely.

Sustained response was reported in a patient with argininosuccinate lyase deficiency after repeated hepatocyte transplantation. Engraftment of the transplanted cells was analyzed in repeated liver biopsies for more than 12 month by fluorescence in situ hybridization for the Y-chromosome and by measurement of tissue enzyme activity (Stephenne et al. 2006). Promising results have also been obtained in a 47-year-old woman suffering from glycogen storage disease type 1a, an inherited disorder of glucose metabolism resulting from mutations in the gene encoding the hepatic enzyme glucose-6-phosphatase (Muraca et al. 2002). 2×10^9 ABO-compatible hepatocytes were infused into the portal vein. Nine months after cell transplantation, her metabolic situation had clearly improved. Successful hepatocyte transplantation has also been achieved in a 4-year-old girl with infantile Refsum disease, an inborn error of peroxysome metabolism, leading to increased levels of serum bile acids and the formation of abnormal bile acids (Sokal et al. 2003). A total of 2×10^9 hepatocytes from a male donor were given during eight separate intraportal infusions. Abnormal bile acid production (for instance pipecholic acid) had decreased by 40 % after 18 months. Recently, hepatocyte transplantation has been used successfully to treat inherited factor VII deficiency (Dhawan et al. 2004). Two brothers (aged 3 months and 3 years) received infusions of 1.1 and 2.2×10^9 ABO-

matched hepatocytes into the inferior mesenteric vein. Transplantation clearly improved the coagulation defect and decreased the necessity for exogenous factor VII to approximately 20 % of that prior to cell therapy. As with the other metabolic liver diseases, hepatocyte transplantation has been shown to provide a partial correction of urea cycle defects. Patients showed clinical improvement, reduced ammonia levels and increased production of urea (Horslen et al. 2003; Mitry et al. 2004; Stephenne et al. 2005; Meyburg et al. 2009).

The investigational medicinal product "Human Heterologous Liver Cells" (HHLivC, Cytonet®) is being developed as an advanced therapy medicinal product (ATMP) for the treatment of urea cycle disorders. HHLivC consists of a cryopreserved dispersion of liver cells prepared for intraportal administration. HHLivCs are isolated from non-transplantable donor organs and refined in a manufacturing process under Good Manufacturing Practices (GMP) conditions (Alexandrova et al. 2005). The study medication is infused via the portal vein through branches of the inferior or superior mesenteric vein. 3×10^8 viable liver cells/kg body weight are infused in equal fractions over period of 6 days. After initial applications of HHLivCs in 4 patients with urea cycle disorders with promising results a total of 21 patients with Ornithine Transcarbamylase (OTC), Carbamoylphosphat-Synthetase I (CPS1) and Argininosuccinate Synthase Deficiencies (ASSD) at the age of 0–5 years have subsequently been recruited for two pivotal studies (CCD02 and CCD05) in Germany, USA and Canada (Meyburg et al. 2009). Primary efficacy endpoints as defined by the incidence of severe (>500 μM) and moderate (>250 μM) hyperammonemic events are compared with matched historical controls (n = 63 patients). Although efficacy data have not yet been published the market approval application was submitted to the European Medicines Agency (EMA) in December 2014.

5.6.8.2 Transplantation of Stem Cells

In the last few years, many reports have suggested that extrahepatic stem cells participate in liver regeneration and may be useful for treating many diseases (Alison et al. 2000; Herzog et al. 2003; Lagasse et al. 2000; Petersen et al. 1999; Theise et al. 2000). However, subsequent work by several independent groups has clearly shown that hepatocyte replacement levels after injection of extrahepatic stem cells or by bone marrow transplantation are low (<0.01 %), unless those bone-marrow-derived hepatocytes have a selective growth advantage (Cantz et al. 2004; Kanazawa and Verma 2003; Wagers et al. 2002). Furthermore, in most of the cases, fusion with host hepatocytes rather than transdifferentiation of extrahepatic cells, has been described as the underlying mechanism (Alvarez-Dolado et al. 2003; Quintana-Bustamante et al. 2006; Vassilopoulos et al. 2003; Willenbring et al. 2004). So far no convincing evidence has yet been provided in animal models that stem cells including HSC, MSC, iPS or cells derived from cord blood or the amnion can generate therapeutically significant numbers of hepatocytes for the correction of hereditary metabolic liver diseases.

A clinical study with stem cells derived from human liver has recently been launched by Promethera Biosciences, a Belgian biotech company. HepaStem® consists of a "Heterologous Human Adult Liver Progenitor Cells" (HHALPC) suspension, which is generated from normal adult human liver tissue. The cells are described as fibroblastic in morphology expressing mesenchymal as well as hepatocytic markers and can be expanded from cultured primary hepatocytes (Najimi et al. 2007; Berardis et al. 2014). Preclinical studies in animals have shown safety and engraftment in recipient livers. The first transplantation of HepaStem® in a 3-year-old girl suffering from ornithine carbamoyltransferase (OTC) deficiency showed 3 % engraftment after 100 days as determined by the Y chromosome FISH technique. The clinical outcome of this patient was not reported (Sokal et al. 2014) in the publication. At the annual meeting of the Society for the Study of Inborn Errors of Metabolism (SSIEM 2014), oral presentation safety of the treatment was demonstrated in 14 patients with urea cycle defects and in 6 patients with Crigler-Najjar Syndrome. Preliminary efficacy data in this Phase I study showed variable results (Dobbelaere et al. 2014). Approval for enrollment of patients with urea cycle defects has been granted by Belgian authorities for a Phase IIb/III trial in October, 2014.

5.6.8.3 Liver-Directed Gene Therapy in Humans

Gene therapy has the potential to offer a definitive cure for monogenic diseases by achieving a long-term correction of the underlying pathology. Monogenic diseases in the liver are divided into two groups depending on whether cell damage in the liver is involved or not. For example, hemophilia, familiar hypercholesterolemia, and phenylketonuria show systemic manifestations without significant liver cell damage, and have the least risk for hepatotoxicity in orthotopic gene delivery. In fact, phase I/II clinical trials for hemophilia B were completed with promising results (Manno et al. 2006). Unfortunately, however, the development of inhibitory antibodies against the exogenous factor IX and/or components of viral vectors diminished a persistent phenotypic correction (Manno et al. 2006; Mingozzi and High 2007). Possible solutions to avoid antibody development are gene delivery into the fetal liver to induce tolerance to the exogenous proteins (Seppen et al. 2003; Waddington et al. 2004; Sabatino et al. 2007), alternative injection routes (Tominaga et al. 2004) or screening of patients for pre-existing antibodies against viral proteins. It is important to point out that significant difference exists between animal studies and human clinical trials with respect to immunological reactions (Ye et al. 2004; Gao et al. 2006). In a recent study Nathwani and coworkers have treated adult patients with haemophilia B with AAV serotype 8 and demonstrate blood factor IX concentrations up to 5 % of normal over a period of 3 years. Neo-expression of factor IX in these patients resulted in reduction of bleeding episodes by 90 % (Nathwani et al. 2011, 2014).

In case of the monogenic liver diseases with substantial hepatocellular damage, gene therapy should not be a primary indication unless gene delivery can be completed in all hepatocytes in the liver. Successful delivery of human fumarylacetoac-

etate hydrolase gene into hepatocytes protected $FAH^{(-/-)}$ – mice mimicking hereditary tyrosinemia type 1 disease from fulminant liver failure by restoring the enzyme activity (Overturf 1997; Grompe et al. 1998). However, hepatocellular damage continued in the remaining non-transduced hepatocytes and resulted in development of hepatocellular carcinoma. Liver transplantation should thus be a primary option for the diseases in this category at this moment.

5.7 Advanced Therapy Medicinal Products Regulation

For many of the regenerative therapies discussed in this chapter a new legal framework on advanced therapy medicinal products (ATMPs), which was implemented by the European Medicine Agency (EMA) in December, 2008, is now applicable. ATMPs are defined as "innovative, regenerative therapies, which combine aspects of medicine, cell biology, science and engineering for the purpose of regenerating, repairing or replacing damaged tissues or cells" and fall into three categories: (a) gene therapy 'medicinal products' (b) somatic cell therapy 'medicinal products' or (c) tissue engineered products. As a result of this European Union legislation, the EMA has introduced the Committee for Advancced Therapies (CAT). The CAT will play a central role in safety and efficacy assessment of new ATMP's prior to formal marketing approval. This central European legislation will surely facilitate the development and wide spread application of regenerative therapies in hepatology and other medical disciplines.

5.8 Future Directions

Regenerative therapies involving various types of cells as well as gene transfer technologies are currently being investigated in research laboratories around the world and more and more find the way into therapeutic algorithms in the clinic. Bioartificial liver support systems and cell therapies are currently limited by the availability of good quality hepatocytes. A renewable source of highly metabolically competent hepatocytes will be essential for any successful bioartificial liver system. To date porcine hepatocytes are most commonly used in bioratificial support systems, although with limited acceptance due to ongoing concerns of xenozoonosis. Immortalized human hepatocytes have not shown expression of the required hepatocyte functions including ammonia detoxification. Other limitations of first-generation bioartificial liver systems, which need to be solved, include excess device complexity, insufficient number of hepatocytes to support a failing liver, early hepatocyte death, and absence or loss of differentiated function.

The application of stem cells in liver cell therapies seems to be a promising approach for the treatment of liver diseases. However, several issues still have to be addressed to fulfil this promise. We need to identify, both inside and outside of the

liver, the stem cell candidates that are able to form mature hepatocytes in vitro and functional liver tissue after transplantation in vivo. The fundamental molecular pathways involved in the differentiation of hepatocytes and cholangiocytes from stem/progenitor cells, the factors that are responsible for in vitro differentiation of various stem cells into hepatocytes, the mechanisms involved in the fusion of stem cells and hepatocytes and the aspects that can potentially enhance these mechanisms need to be studied in more detail. With future progress in stem cell research, the various stem cell sources including hepatic stem/progenitor cells, embryonic and adult extrahepatic stem cells should provide great opportunities for the treatment of liver disorders.

Additional work is also needed in the development of an ideal gene delivery system. The efficacy of delivery and the level of transgene expression achieved by the current methods have resulted in phenotypic correction of various hereditary liver diseases in animal models. The most efficient vehicles for gene delivery to the liver developed so far are viral vectors. Adeno-associated viral vectors have become a promising tool for liver directed gene transfer and have now been successfully applied in humans with factor IX deficiency. Techniques to further increase the transduction efficacy and to control of immune rejection will result in improved therapeutic results.

In summary, advanced approaches in regenerative hepatology will cover strategies to improve endogenous liver regeneration, to correct monogenetic liver diseases by gene therapy, and to support organ function with hepatic cells, either in extracorporal devices or as cell transplants. For the latter aspect, improved cell isolation and propagation techniques to utilize cells from donor organs or advanced stem cell-differentiation protocols become of utmost importance to ensure the supply of functional hepatic cells.

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Chapter 6
Gastrointestinal Tract and Endocrine System

Carsten Keil, Elmar Jäckel, Michael P. Manns, and Oliver Bachmann

Abstract The absorption of nutrients in the small intestine and the control of blood glucose levels are crucial to ensure energy homeostasis of the organism. These functions can be severely impaired by diseases like type I diabetes, short bowel syndrome, and inflammatory bowel disease, for which the standard treatment involves either life-long replacement, immunosuppression, or transplantation, and is often not satisfactory. After outlining the diseases and established as well as experimental approaches, this chapter summarizes recent preclinical and clinical studies on novel therapeutic options relating to regenerative medicine, including growth factors and stem cells.

Keywords Diabetes mellitus • Insulin • Short bowel syndrome • Crohn's disease • Intestinal stem cells • GLP-2

6.1 Introduction

6.1.1 Type I Diabetes Mellitus

Human type 1 diabetes (T1D) is an autoimmune disease that arises in genetically predisposed individuals due to the destruction of insulin producing beta cells of the pancreatic islet of Langerhans, a process triggered by autoaggressive CD4+ and CD8+ T cells. This results in a lack of control of blood glucose levels culminating in hyperglycemia if more than 90 % of beta cells are destroyed. This in time leads to severe chronic complications such as widespread microvascular (retinopathy, nephropathy and neuropathy) and macrovascular damage (myocardial infarction, stroke, peripheral arterial vascular disease) (Atkinson and Maclaren 1994; Daneman 2006). The search for a cure for T1D or effective treatments that can prevent the complications associated with T1D is still ongoing.

T1D accounts for 5–10 % of all diabetes cases and commonly occurs in people of European descent with estimates of two million people affected in Europe and

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Table 6.1 Comparison of T1D in NOD mice and human patients

Characteristic	Humans	Mice
Genetic predisposition and polygenetic trait	Yes	Yes
MHC-loci contribution	Multiple	Multiple
Environmental influence	Yes	Yes
Defective peripheral immune regulation	Yes	Yes
Impaired dendritic-cell maturation	Possibly	Possibly
Autoantigens	GAD65, IA2, insulin and Hsp60	GAD65, IA2, insulin and Hsp60
Initiating auto antigen	Unknown	Unknown
Islet auto immunity linked to early gluten exposure	Yes	Yes

Adapted from Roep et al. (2004)

North America (Daneman 2006; Gillespie 2006). T1D is largely known as a childhood disease accounting for 90 % of childhood-onset diabetes (Daneman 2006; von Herrath et al. 2007). It has been reported that the incidence of T1D is on the rise with predictions suggesting a doubling of new cases in European children under the age of 5 years by the year 2020, with the prevalence of cases expected to rise by 70 % in individuals that are below 15 years of age (Gillespie 2006; Patterson et al. 2009).

6.1.1.1 Etiology of T1D

Much of our understanding of the etiology and pathogenesis of T1D stems from research conducted in the spontaneous non obese diabetic (NOD) mouse and biobreeding rat (BB rat) rodent models of T1D. The NOD mouse is the most widely used animal model of T1D that shares major disease characteristics with human disease as shown in Table 6.1 (Atkinson and Leiter 1999; Anderson and Bluestone 2005).

Studies conducted in the NOD mouse show that like in humans, T1D is a complex disease that is precipitated by a combination of factors such as genetic susceptibility, environmental triggers and immune dysregulation (Kishimoto and Sprent 2001; Anderson and Bluestone 2005; Daneman 2006).

Genetic susceptibility to T1D is heritable and lies predominantly within the major histocompatibility (MHC) or human leukocyte antigen (HLA) locus in the NOD mouse and human disease respectively (Anderson and Bluestone 2005). MHC/HLA molecules function in the initiation of immune responses to foreign antigens by presenting antigens to T cells bearing the respective T cell receptor (TCR) specificity. Furthermore these molecules are also involved in tolerance induction to self-antigens and account for both positive and negative selection of autoreactive T cells within the thymus. Therefore the occurrence of allelic variability within the MHC/HLA loci may lead to immune dysregulation. Indeed the MHC/HLA class II loci have been shown to determine susceptibility to T1D in the NOD

mouse and human disease. In human disease, strong associations have been made between T1D susceptibility and genes located within the HLA-DR and HLA-DQ loci (Cucca et al. 1993; Noble et al. 1996). In addition, the risk assessment of familial T1D can be conducted by screening for allelic variations within the HLA-DR and HLA-DQ loci (Nejentsev et al. 1999; Ilonen et al. 2002).

Variability within the insulin gene, in particular a variable number tandem repeat (insulin-VNTR) in the insulin promoter, has been shown to also contribute to disease susceptibility, albeit to a lesser extent than the MHC/HLA. The insulin-VNTR controls the expression of insulin in the thymus, therefore potentially regulating the autoimmune repertoire. Shorter forms of insulin–VNTR are reported to be associated with T1D development, whilst the longer forms are associated with greater protection of thymic insulin message correlating with T1D protection (Bluestone et al. 2010; Gillespie 2006).

Disease susceptibility has been further associated with the global immune dysregulation that occurs in both the NOD mouse and human disease. Indeed genetic variations in genes associated with immune homeostasis such as the regulatory cytotoxic T-lymphocyte antigen 4 (CTLA-4), the co-stimulatory molecule CD28, PTPN22 (which encodes the lymphoid protein tryrosine phosphate–LYP), IL-2RA (CD25), and PD-1 have been demonstrated to be associated with T1D development. Proteins encoded by these genes maintain homeostasis by either activating T and B cells or regulating their activity through regulatory cell populations which is key to regulating autoimmunity (Bluestone et al. 2010; van Belle et al. 2011). On the whole, genetic susceptibility to T1D results from genetic variability in genes that are associated with antigen presentation, central tolerance induction and immune regulation.

Environmental factors are believed to be involved in the causation of T1D. Indeed, the increase in disease incidence amongst young children has been reported to be occurring rather rapidly to result from genetic alterations alone, thereby implying that environmental factors may be responsible (Gillespie 2006). The question of the identity of the environmental triggers involved is still under debate, though it is widely viewed that viruses such as enteroviruses (particularly Coxsackie B viruses), rubella, and rotaviruses act as environmental triggers for T1D (Ginsberg-Fellner et al. 1985; Peltola et al. 2000). Furthermore, environmental toxins and foods such as cow milk proteins, cereals, or gluten have also been reported to be associated with the causation of T1D (Daneman 2006).

6.1.1.2 Immunopathogenesis of T1D

T1D results from a series of complex events and is believed to be initiated in genetically susceptible individuals by environmental triggers. How environmental factors trigger T1D is still under elucidation. However studies suggest that factors such as virus infection or environmental toxins prompt the up regulation of IFN-γ and MHC class I molecules by pancreatic beta cells. This in turn leads to a loss of tolerance culminating in the release of beta cell antigens such as insulin, glutamic acid

decarboxylase 65 (GAD65), the zinc transporter (ZnT8), protein tyrosine phosphatase (IA-2), islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), and heat shock protein 60 (Hsp60) (Jaeckel et al. 2008). These beta cell antigens are then taken up by antigen presenting cells and transported to the pancreatic lymph nodes where they are presented to potentially autoreactive T cells.

Studies in the NOD mouse show that dendritic cells and macrophages infiltrate the pancreas in the initial phase of T1D. Shortly thereafter, B cells and potentially autoreactive CD4+ and CD8+ T cells migrate from the pancreatic lymph node and infiltrate the pancreas without initially destroying the beta cells. This stage of the disease is known as insulitis (Gianani and Eisenbarth 2005). At this stage, B cells initiate a humoral response that leads to the production of beta cell autoantibodies. The role of beta cell specific autoantibodies in the pathophysiology of T1D is still controversial, yet they serve as a very useful biomarker for the development of autoimmunity (Waldron-Lynch and Herold 2011; Daneman 2006). Furthermore, insulin-specific autoantibodies can be detected months to years prior to the onset of clinical symptoms. This lag period between initiation of autoimmunity and development of overt diabetes also explains the difficulties in identifying a causative environmental agent for T1D.

After a gradual increase in cellular infiltrates, the progressive destruction of beta cells progresses. At this stage, beta cells are destroyed by perforin, interferon gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α), that are produced by the activated CD4+ and CD8+ autoreactive T cells leading to the release of new beta-cell antigens that are then taken up by antigen presenting cells including migrated B cells and presented in the pancreatic lymph nodes to new T and B cell specificities. As a result of epitope spreading, new antibody specificities can also be detected, such as antibodies against GAD65, ZnT8 and IA-2. The number of detectable autoantibodies has been used in T1D risk assessment, with patients positive for three or more specificities exhibiting a higher risk of developing diabetes in comparison to patients positive for a single specificity (Waldron-Lynch and Herold 2011; Daneman 2006). Subsequently, the beta cells undergo a more aggressive immune attack that leads to a severe depletion of the beta call mass. This leads to a complete loss of insulin production and dysregulation of glucose metabolism, a stage known as overt diabetes (van Belle et al. 2011; Gianani and Eisenbarth 2005). Hyperglycemia results as a consequence of insulin deficiency, and T1D is diagnosed when there is about 10–30 % of functional beta cells remaining (Gianani and Eisenbarth 2005).

6.1.1.3 Current Therapeutic Options for T1D

Insulin Replacement Therapy

Treatment for T1D currently involves insulin replacement therapy by subcutaneous injections of exogenous insulin. This treatment also requires daily blood glucose level measurements in order to determine the correct dosage of insulin required to

control hyperglycemia, and to prevent hypoglycemia. For the best outcome, a multidisciplinary health team is required that also assists with dietary planning and screening for diabetes related complications (Daneman 2006). Although insulin replacement therapy can control hyperglycemia, it does not induce immune tolerance and patients are still in danger of developing microvascular and macrovascular complications of diabetes due to suboptimal glucose control. Furthermore, there is the issue of compliance as patients have to treat themselves for decades beginning from childhood. Side effects such as hypoglycemia, weight gain, and excessive diurnal glucose fluctuations have also been associated with insulin replacement therapy (Waldron-Lynch and Herold 2009). Therefore there is a need for new and potentially curative therapies for T1D that can improve the quality of life for patients.

Pancreas Transplantation

As previously stated, hypoglycemia is a dangerous side effect associated with insulin replacement therapy. This is particularly problematic in a subgroup of patients with erratic glycemic control (i.e. labile diabetes). For these patients whole organ pancreas or allogeneic islet transplantation are an alternative therapeutic approach (Vardanyan et al. 2010).

Since the first pancreas transplant in 1966, more than 30.000 pancreas transplants have been performed worldwide. About 7 % of pancreas transplants were single organ transplants, with 72 % performed in patients undergoing simultaneous pancreas-kidney (SPK)-, 17 % undergoing pancreas after kidney (PAK)- and less than 4 % undergoing combined heart, liver or intestine procedures (Gruessner and Sutherland 2008). One year survival rates after transplantation were over 95 and 83 % after 5 years. Furthermore, SPK exhibited the best graft survival in comparison to the other procedures (Gruessner and Sutherland 2008). Despite its success, pancreas transplantation is limited by the scarcity of tissue pancreas donors and the side effects of immunosuppressive drugs taken to prevent allograft rejection. Due to the invasive nature of pancreas transplantation, it is limited to a subgroup of individuals with labile diabetes and those undergoing kidney transplantation at the same time. SPK improves survival compared to patients with T1D on dialysis, and in addition, SPK also improves survival of the kidney graft. Despite these facts, just 150–160 patients with T1D received a pancreas transplantation, although an estimated 3000–5000 patients with T1D are on hemodialysis.

Islet Cell Transplantation

Due to the limitations of whole organ transplantation, islet transplantation has been considered as an alternative approach to restore normoglycemia. Islet transplantation via percutenous transhepatic portal embolism combined with a corticoid-free

immunosuppressive regimen (Edmonton protocol) was demonstrated to result in a 1-year insulin independence in 50–80 % of treated patients (Shapiro et al. 2000). Patients exhibited better glycemic control and no hypoglycemic events. However, by year 5 90 % on patients were again dependent on exogenous insulin, although remaining C-peptide production of the graft could still be observed in many of those patients. The latter result was attributed to the side effects of the immunosuppressive regimen that involved administration of daclizumab (humanized anti-CD25 mAb), rapamycin (sirolimus), and FK-506 (tacrolimus). This regimen was reported to result in lymphopenia and led to the induction of homeostatic cytokines that expanded autoreactive T cells, thereby accounting for recurrent autoimmunity in transplanted patients. Furthermore, rapamycin was shown to impair engraftment, induce insulin resistance and inhibit beta cell replication (Zhang et al. 2006; Zahr et al. 2007; Fraenkel et al. 2008; Monti et al. 2008). Clinical trials for islet transplantation using different combinations of immunosuppressive drugs are currently underway (van Belle et al. 2011).

Although promising, islet transplantation is limited by the isolation of adequate islets from the little available donor tissue. In order to meet demand xenogeneic islets especially from pigs have been considered as an alternative source of islets for transplantation. Porcine islets are ideal as they are physiologically similar to human islets and are readily available due to rapid breeding of pigs. However, this procedure is not without its drawbacks such as the possibility of zoonotic infections and an aggressive xenogeneic immune response against the islet xenografts. The latter, however, could potentially be overcome by co-stimulation blockade or encapsulation of the islet xenografts (Cardona et al. 2007; Kobayashi et al. 2008). Initial xenogeneic islet transplantations performed in New Zealand and Moscow, however, proved to be safe as no transmission of porcine pathogens or viruses was observed. The effectivity of the transplant procedure still needs to be determined (Garkavenko et al. 2008).

6.1.1.4 Therapeutic Advances for T1D

Our increased knowledge of the etiology and pathogenesis of T1D has led to the identification of a number to potential targets for therapy. Preclinical and clinical observations strongly suggest that a successful therapeutic strategy for T1D should fulfill three requirements: Firstly, the therapy should be short term and able to re-establish immune tolerance by regulating the ongoing beta cell destruction. Secondly, the therapy must be able to maintain immune tolerance in order to facilitate beta-cell regeneration. And finally, the therapy should be acceptable to T1D patients and should have a similar or even better effect than the existing insulin replacement therapy (Waldron-Lynch and Herold 2009). In the section below we explore some of the therapeutic advances made thus far and their clinical applications.

Immunomodulatory Therapies

The goal of immunomodulatory therapies is to inhibit the destructive autoimmune response against the pancreatic beta cells in order to preserve and potentially restore beta cell function. Immunomodulatory therapies can be classified as either polyspecific (i.e. use of global or cell targeted immunosuppressants) or antigen specific (mediated by antigen specific tolerance induction).

Polyspecific Immunomodulatory Strategies: Immunosuppressive Drugs

The calcineurin inhibitor cyclosporine A and the corticosteroid prednisone were shown to deplete or inactivate T cells in solid organ transplantation. This led to their use in the initial clinical trials for T1D. Treatment of T1D patients with either cyclosporine A or prednisone in combination with the purine analogue azathioprin induced disease remission, with 50 % of patients requiring no exogenous insulin during treatment (Feutren et al. 1986; Stiller et al. 1987; Silverstein et al. 1988). However, treatment with immunosuppressive drugs did not restore tolerance to pancreatic beta cells as disease remission was largely limited to the duration of drug administration. Therefore, maintenance of normoglycemia would mean chronic treatment with these drugs. However, side effects associated with prolonged usage of cyclosporine A and prednisone, such as systemic immunosuppression, induction of nephrotoxicity and insulin resistance prevented their further clinical use (Bougneres et al. 1990; Parving et al. 1999).

Polyspecific Immunomodulatory Strategies: Targeted Cell Therapies

It was shown in the mid-1980s that short term treatment with depleting or non depleting isotypes or F(ab′)2 fragments of CD4 antibodies induces long-term tolerance to skin and islet allografts (Gutstein et al. 1986; Carteron et al. 1989). The results obtained using F(ab′)2 fragments and non-depleting isotypes of CD4 antibodies further demonstrated that non-depleting monoclonal antibodies can be used for tolerance induction in vivo. Since then, antibodies against CD40L, CD25, CD3 and CLTA4-Ig amongst others have been shown to facilitate tolerance induction in transplantation and autoimmunity (Waldmann and Cobbold 1998).

Anti-CD3ε Therapy (Teplizumab and Otelixizumab)

One of the first monoclonal antibodies (mAb) described to be effective in preventing organ allograft rejection and in treating acute rejections after transplantation was the anti-CD3 antibody (OKT3, a murine immunoglobulin). However the success of the OKT3 in solid organ transplantation was hampered by development of a severe cytokine release syndrome that resulted from activation of T cells enhanced by cross-linking the murine Fc portions by human Fc receptors (FcR) (Cosimi et al. 1981; Abramowicz et al. 1989). These findings led to the development of CD3

mAbs, which were humanized making them less immunogenic. In addition the Fc binding site was mutated to prevent cross-linking of Fc-receptors (Friend et al. 1999).

The immunosuppressive capacity of anti-CD3 therapy in T1D was initially tested in the NOD mouse using a brief course of low dose non-Fc binding Fab-fragments of anti-CD3 mAb (145-2C11). These studies showed disease reversal in both treated recent onset and overtly diabetic NOD mice (Chatenoud et al. 1994, 1997). The mechanism of disease reversal by anti-CD3 was shown to involve induction of peripheral tolerance via ignorance due to short term internalization of TCR complex after anti-CD3 binding, induction of anergy or Fas mediated apoptosis of activated Th1 cells and induction of TGF-β dependent adaptive CD4+CD25lowFoxp3+ Tregs from peripheral CD4+ CD25- T cells (Chatenoud et al. 1982, 1994; Belghith et al. 2003; You et al. 2007). Owing to the success of the preclinical studies, two non Fc binding anti-CD3 antibodies were used for T1D clinical trials, namely humanized OKT3γ1 (Ala Ala, named teplizumab), and chAglyCD3 (aglycoslated FcR non-binding, named otelixizumab). Administration of a single course of teplizumab or otelixizumab in recent onset patients halted disease progression, and these patients exhibited better preservation of stimulated C-peptide levels and lower insulin usage compared to control groups. This effect lasted up to 4 years after treatment. Furthermore, patients that had the highest endogenous insulin production at the commencement of the clinical trial exhibited the greatest effect. However, despite the promising clinical outcome, the effect of teplizumab and otelixizumab was short-lived. This implies either disease re-emergence or a limitation of drug efficacy to time of disease onset. Recently, phase III clinical trials with teplizumab and otelixizumab were declared a failure owning to their inability to meet their primary endpoints, that is reduced insulin usage and serum HbA1c levels. Furthermore, the phase III trial using otelixizumab failed because of 90 % dose reduction between phase II and III trials (Herold and Bluestone 2011; Waldron-Lynch and Herold 2011). However, it has to be emphasized that especially teplizumab could demonstrate its ability to stabilize stimulated C-peptide secretion repeatedly. Taken together both antibodies are still promising partners for combination therapies described below.

CTLA4-Ig (Abatacept and Belatacept)

The activation of T cells requires two signals: The first signal comes from antigen recognition of MHC-peptide complexes by the TCR while the second signal emanates from the recognition of co-stimulatory molecules (e.g. CD28, CD40L, ICOS) expressed on T cells and their receptors (e.g. B7.1/B7.2, CD40, ICOSL) expressed on activated antigen presenting cells. The best characterized costimulatory pathway is the CD28/B7 pathway. Conversely, the binding of the T cell surface molecule CTLA-4 to B7.1/B7.2 results in negative regulation of T cell activation.

CTLA4-Ig is a fusion protein consisting of a CTLA-4 extracellular domain and an IgG Fc domain. CTLA4-Ig has a higher affinity for B7 molecules than for CD28 and hence acts as a competitive inhibitor of the CD28/B7 pathway. CTLA4-Ig

(abatacept) in combination with methotrexate has been shown to be immunosuppressive in rheumatoid arthritis patients. However, abatacept was shown to be non-tolerogenic and patients require monthly infusions to maintain immunosuppression (Waldron-Lynch and Herold 2011; Kremer et al. 2008). On the other hand, administration of CTLA4-Ig in preclinical trials for T1D yielded conflicting results. CTLA4-Ig was shown to prevent diabetes development in an adoptive transfer model of diabetes. In these studies, tolerance induction mediated by expansion of Tregs was observed in treated mice (Rigby et al. 2008). However, in an islet transplantation model, anti-CD4 mAbs were shown to be more effective at preventing disease resurgence than CTLA4-Ig. This could be attributed to the fact that some studies have shown that the CD28/B7 pathway is important for Treg development and survival, and hence administration of CTLA4-Ig may interfere with Treg homeostasis (Guo et al. 2001; Salomon and Bluestone 2001). Recently, a phase II trial using abatacept did just initially (3–6 months) slow the loss of β-cells despite continued use for 24 months. Additionally, belatacept (a high affinity variant of CTLA4-Ig) is currently being tested in a phase I/II islet transplantation trial (van Belle et al. 2011). Taken together, CTAL4-Ig does not seem to be a promising partner for combination therapy in new onset T1D.

Anti-CD20 (Rituximab)

The role of B cells in the pathogenesis of T1D has been overshadowed by the predominant role of T cells. However, evidence of detectable autoantibodies prior to disease onset suggests that B cells may play a role in disease initiation. The first studies to demonstrate a role of B cells in disease pathogenesis were conducted in NOD mice engineered to express a humanized form of the B cell surface molecule CD20 (hCD20). These studies showed that depletion of B cells with an anti-hCD20 antibody resulted in a delay in disease onset and also managed to control already established diabetes. The efficacy of this treatment was attributed to the expansion of regulatory T and B cells (Hu et al. 2007).

Successful treatment of autoimmunity with the anti-CD20 drug (rituximab) was demonstrated in clinical trials for rheumatoid arthritis and systemic lupus. Conversely, disease remission was limited to drug administration bringing to question long-term tolerance induction by rituximab in human disease (Kazkaz and Isenberg 2004; Looney 2005). A phase II clinical trial for T1D using rituximab showed some preservation of C-peptide levels and reduced insulin usage between 3 and 6 months after treatment. However, the effect of rituximab on T1D was short lived and modest (Waldron-Lynch and Herold 2011).

Antigen Specific Strategies

The rationale behind antigen specific immunomodulatory strategies is based on the evidence that oral, intranasal, or subcutaneous administration of antigens can induce peripheral immune tolerance. The therapeutic capacity of major beta cell antigens such as insulin, GAD65 and Hsp60 was tested in the preclinical NOD mouse with

much success (Atkinson et al. 1990; Zhang et al. 1991; Muir et al. 1995; Daniel and Wegmann 1996; Tian et al. 1996b; Bockova et al. 1997; Elias et al. 1997; Ma et al. 1997). Owing to the success of these preclinical studies, antigen specific immunomodulation for T1D was translated to clinical trials. Furthermore, antigen specific therapies were favored as they can ensure a tissue specific response, which would circumvent the problem of systemic immunosuppression observed by the use of global immunosuppressants.

Insulin Trials

Insulin is considered to be a major autoantigen in both the NOD model and in human T1D. Ins B9-23, a peptide that is recognized by both CD4+ and CD8+ T cells, has been shown to be a prerequisite for T1D development in NOD mice and a target of autoreactive CD4+ T cells in T1D patients (Nakayama et al. 2005). Oral and intranasal administration of insulin prevented disease development in the prediabetic NOD mouse through the induction of Th2 (IL-4/IL-10), Th3-(TGF-β) secreting, CD8+-and IL-10 dependent Tr-1 regulatory T cells (Atkinson et al. 1990; Zhang et al. 1991; Daniel and Wegmann 1996; Harrison et al. 1996; Faria and Weiner 2006a, b). However, parenteral and intranasal administration of insulin in at risk patients did not have an effect on disease progression in prediabetic and recent onset patients (Pozzilli et al. 2000; Pozzilli 2002; Kupila et al. 2003; Harrison et al. 2004). A beneficial effect was however observed in a subgroup of patients that had high titers of insulin autoantibodies. The results were sustained over 8 years in patients on continued therapy (Vehik et al. 2011).

GAD65 Trials

Glutamic acid decarboxylase (GAD) is an enzyme that exists in two isoforms (GAD65 and GAD67) and is involved in the production of the neurotransmitter γ-aminobutyric acid (GABA). GAD is expressed exclusively in the brain and pancreas. GAD65 reactivity has been primarily associated with T1D with the presence of GAD65 reactive T cells and autoantibodies reported in T1D patients and at risk individuals (Fenalti and Rowley 2008). Intranasal and intravenous administration GAD65 in prediabetic or recent onset NOD mice resulted in disease prevention that was mediated by antigen specific CD4+ regulatory T cells with a Th2 phenotype (Tian et al. 1996a, b; Tisch et al. 1998, 1999; Chen et al. 2003). Administration of Diamyd (a recombinant human GAD65 formulated in alum in patients with latent autoimmune diabetes in adults (LADA) or recent onset T1D resulted in the preservation of insulin secretion. The effect of GAD alum treatment was attributed to the induction of TGF-β secreting FOXP3+ regulatory T cells (Hjorth et al. 2011; Agardh et al. 2005, 2009; Ludvigsson et al. 2008). Unfortunately, two recently conducted Phase III clinical trials in Europe and the USA did not reproduce these effects and failed (Wherrett et al. 2011). However, due to the low side effects of GAD65 administration it might be a potential candidate for combination therapy in GADA positive patients.

DiaPep277 Trials

p277 is a major T cell epitope of the heat shock protein60 (HSp60) and has been shown to be an immunodominant epitope in human and NOD type 1 diabetes (Horvath et al. 2002). Additionally murine and human p277 have been shown to differ in a single position and NOD T cells have been shown to respond to stimulation with the human peptide (Birk et al. 1996; Horvath et al. 2002). Furthermore, subcutaneous vaccination of the human p227 prevented diabetes in recent onset and overtly diabetic NOD mice by a Th2 mediated cytokine burst and induced Qa-1 CD8+ regulatory T cells (Elias and Cohen 1994; Bockova et al. 1997; Elias et al. 1997).

DiaPep227 is a modified version of the p277 in which cysteines were substituted with valine in order to improve stability. Administration of DiaPep277 in phase II trials resulted in the preservation of C-peptide for up to 18 months in adult recent onset diabetes patients. However the effect on C-peptide level was not accompanied by a reduction in insulin usage or lower HbA1c levels (Raz et al. 2001, 2007). A phase III study in adults is currently ongoing while no DiaPep277 effect has been reported in young children (Lazar et al. 2007).

6.1.2 Short Bowel Syndrome

Short bowel syndrome occurs after an extensive loss of small intestinal length, typically after surgery, which leads to a malabsorption of fluid and nutrients. In a timespan of about 24 months following surgical resection of small intestinal segments, the remaining intestine undergoes adaptation through several mechanisms which aim at increasing the absorptive capacity, a process which has been recognized first 100 years ago (Flint 1912). These include villous cell hyperplasia, increased crypt depth, intestinal dilatation, increased mucosal enzyme activity and reduction of intestinal transit (Nightingale and Lennard-Jones 1993). This was shown to involve growth factor and specific nutrients, such as growth hormone, insulin-like growth factor 1, glucagon-like peptide 2, glutamine, short chain fatty acids and pancreatic-biliary secretions (Tamada et al. 1993; Jacobs 1983; Seguy et al. 2003; Ellegard et al. 1997).

Nevertheless, a subset of patients will develop intestinal failure, meaning the inability to maintain an adequate balance of nutrients and water even after the postoperative adaptation phase, and suffer from dehydration and malnutrition without dietary support. In particular, a residual small bowel length of less than 100 cm leading to an end stoma or less than 50 cm connected to a functioning colon poses a risk factor for the need of long-term parenteral nutrition (PN) or, in a selected number of patients, small bowel transplantation. In the past, medical management was predominantly supportive and directed to reduce stool output to <2 l per day using agents that reduce secretion, such as proton pump inhibitors and octreotide, and motility, such as loperamide and opium, but also dietary advice to meet the requirements of the postoperative anatomy.

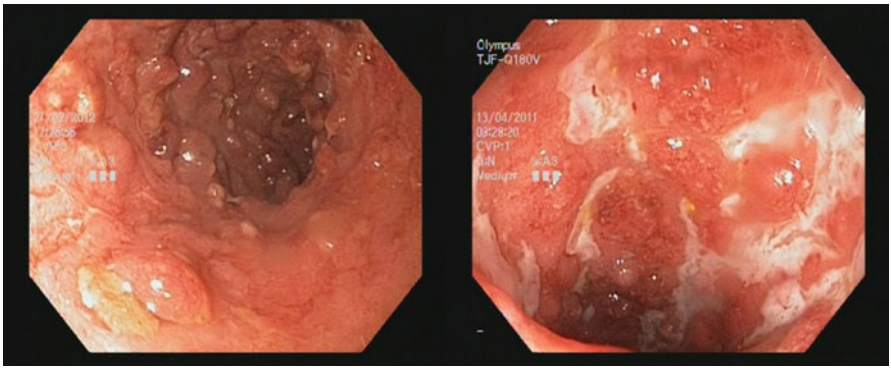

Fig. 6.1 Endoscopic aspect of Ulcerative colitis (*left panel*) with multiple pseudopolyps and Crohn's disease (*right panel*) with severe longitudinal ulceration

While patients with mild short bowel syndrome gain nutritional autonomy by dietary modification such as enlarged meals, increased frequency of oral intake and oral rehydration fluids, PN is the therapy of choice for intestinal failure for Patients with moderate to severe impairment, even though PN carries considerable risks such as hepatic failure, central vein thrombosis, recurrent infections, and a reduced life expectancy. However, it is still superior to small bowel transplantation, which is encumbered by high incidences of graft rejection and other postoperative complications (Pironi et al. 2008, 2011).

Teduglutide, a recombinant analog of glucagon-like-peptide-2, showed a significant reduction in parenteral nutritional support (20–100 %) by improving intestinal absorptive capacity with a to date adequate safety profile in three randomized controlled trials, and was approved as the first therapeutic drug for the treatment of short bowel syndrome with intestinal failure in June 2012 by the EMEA, and in December 2012 by the FDA (Vipperla and O'Keefe 2014).

6.1.3 Inflammatory Bowel Disease

Inflammatory bowel diseases (IBD) are chronic inflammatory disorders of the gastrointestinal tract, including Crohn's disease (CD) and Ulcerative colitis (UC). They are characterized by recurrent mucosal inflammation and ulceration (Fig. 6.1), leading to various intestinal and extra-intestinal manifestations. Crohn's disease mostly involves the distal ileum and/or the colon, whereas Colitis ulcerosa is restricted to the colon. The pathogenetic mechanisms that cause the two types of inflammatory bowel disease are still under investigation. It has originally been suggested that they develop in a genetically predisposed subject due to a dysregulated adaptive immune response to unknown antigens, resulting in continuous immune mediated inflammation (Ardizzone and Bianchi 2002; Fiocchi 1997). Currently,

there is broad agreement that luminal microbes are playing an important role in the development of IBD since both disease locations are characterized by high concentrations of intestinal bacteria and the adaptive immune response is directed against the microbiota. Increasing evidence has shown that defects in the innate immunity are at the centre of both types of IBD. In healthy mucosa, an adequate secretion of antimicrobial peptides and the mucus layer act as a barrier against the microbes. It was shown in the last years that the differentiation from the intestinal stem cell towards the Paneth cell in ileal CD and the goblet cell in UC might be impaired, which leads to a defective antimicrobial barrier and thus, microbes can invade the mucosa and cause inflammation (Gersemann et al. 2012).

Current treatment procedures for CD and UC variably affect the inflammatory events, and indeed no available drug is at present curative. Therapy is often implemented stepwise through aminosalicylates, antibiotics, corticosteroids, immunosuppressive medications including thioguanine compounds, methotrexate, ciclosporin, anti-TNF drugs and the recently approved humanized monoclonal IgG1 antibody vedolizumab, which is specific for α4β7-integrin that binds to MAdCAM-1 and not to VCAM-1, which presumably results in gut specificity. Many patients require surgery to combat complications. In the future, patients may benefit from new therapeutic approaches stimulating the protective innate immune system.

6.2 Medical Regenerative Therapies for Type 1 Diabetes and Intestinal Disease

6.2.1 Beta Cell Regenerative Strategies for Type 1 Diabetes

The results from islet cell transplantation demonstrated that diabetes can be cured by replenishing the beta-cell mass. As a result treatment strategies have been developed aimed at restoring beta cell mass and function such as stimulation of insulin secretion and islet neogenesis. However it must be emphasized that based on preclinical and clinical results a combination of both immunomodulatory and regenerative strategies could greatly improve clinical outcome.

6.2.1.1 Stimulation of Beta Cell Function

Incretin hormones, such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) function by regulating after meal blood glucose levels via mechanisms including enhancement of glucose-stimulated insulin release and reduction of postprandial glucagon levels (Drucker 2003). In murine models, GLP-1 and its analog exendin-4 were also reported to function by increasing beta-cell replication, decreasing beta-cell apoptosis, stimulating beta-cell neogenesis, and inducing beta-cell expansion (Xu et al. 1999; Farilla et al. 2003; Li et al. 2005). Combination therapy trials using either GPL-1 and anti-lymphocyte serum (ALS)

or exendin-4 and anti-CD3 resulted in disease reversal in overt diabetic and recent onset NOD mice, respectively. Treated mice showed an increase in insulin secretion with no effect on beta cell replication or beta cell apoptosis (Ogawa et al. 2004; Sherry et al. 2007). In type 2 diabetes patients, GLP-1 and exendin-4 have been shown to stimulate insulin secretion in remaining beta cells (Kolterman et al. 2003; Buse et al. 2004). However clinical trials for type 1 diabetes conducted with a combination of exenatide (a synthetic version of exendin-4) and daclizumab (an immunosuppressive anti-CD25 monoclonal antibody) yielded disappointing results: Exenatide resulted in delayed gastric emptying, suppressed endogenous incretin levels, but did not increase C-peptide secretion in most trials (Rother et al. 2009). Besides this, β-cell replication in humans is more difficult to stimulate compared to rodents.

6.2.1.2 Beta Cell Neogenesis

The beta cell mass is dynamic and undergoes expansion and contraction depending on metabolic needs (e.g. during normal growth, pregnancy, obesity) (Bonner-Weir 1994). Mechanisms such as replication of pre-existing beta-cells or the formation of new beta cells from progenitor cells (neogenesis) have been associated with beta cell mass expansion. The exact contribution of these two mechanisms to beta cell mass regeneration is still under debate. However, differences in the balance of these pathways have been shown to be species- and age- dependent. Murine beta cell expansion dynamics differ from human dynamics, with β-cell replication being predominant in murine models, whereas neogenesis was more evident in type 2 diabetes patients and non-diabetic individuals (Bonner-Weir et al. 2010; Bouwens and Pipeleers 1998; Butler et al. 2003a, b; Dor et al. 2004).

Murine models of injury such as partial pancreatectomy and partial duct ligation (PDL) have been used to study beta cell neogenesis (Bonner-Weir et al. 1993; Wang et al. 1995). Beta cell neogenesis occurs either via stem/progenitor cell activation and/or transdifferentiation. The identity of the stem/progenitor cell for neogenesis is however still elusive but the PDL model demonstrated that regeneration is limited to the portion distal to the site of ligation. Furthermore, neurogenin 3 (a transcription factor involved in development of pancreatic endocrine cells), was induced in cells in or adjacent to the pancreatic ducts after PDL. These neurogenin 3+ cells yielded islets including beta cells (Xu et al. 2008). These studies hence suggest that beta cells can be formed from progenitor cells within the pancreatic duct epithelium. Conversely, administration of the beta-cell toxin alloxan prior to PDL resulted in the generation of new beta-cells from adult alpha cells (Chung et al. 2010), thereby suggesting that alpha cells of the islets of Langerhans may be a source of beta cell progenitors.

Taken together, these studies show that beta-cell neogenesis is feasible in vivo; therefore, induction of beta cell neogenesis could be an excellent way to restore beta-cell mass for effective T1D treatment. Hormones and growth factors have been shown to induce beta-cell neogenesis (Wang et al. 1993; Rooman et al. 2002;

Rooman and Bouwens 2004). However, any form of beta cell replacement in T1D will also need induction of immune tolerance to prevent the destruction of cells by the autoimmune response.

Gastrin and Epidermal Growth Factor

Infusions of the peptide hormone gastrin in a rodent PDL model induced neogenesis and expansion of beta-mass from transdiffentiated exocrine pancreas. These studies showed an increase in beta cell mass in the ligated portion of the pancreas that was not associated with increased proliferation and hypertrophy or reduced beta-cell death (Rooman et al. 2002). Furthermore a combination of gastrin and epidermal growth factor (EGF) was shown to restore normoglycemia, increase beta-cell mass, density and pancreatic insulin content in alloxan treated mice. Monotherapy with either hormone was reported to have no effect on hyperglycemia (Rooman and Bouwens 2004). The effect of gastrin and EGF was further confirmed in NOD mice as treated recent onset diabetic NOD mice exhibited increased beta cell mass and reversal of hyperglycemia (Suarez-Pinzon et al. 2005). Furthermore combination therapy with gastrin and GLP-1 resulted in reversal of hyperglycemia, downregulation of autoimmune response and protection of beta cells from apoptosis in NOD mice (Suarez-Pinzon et al. 2008). Gastrin and EGF therapies have been translated into clinical trials with results from a phase II clinical trial conducted with an EGF analog E1-1NT showing a 35–75 % reduction in insulin usage and maintenance of blood glucose levels in some T1D patients. Currently, a phase I combination trial with EGF and gastrin is ongoing (van Belle et al. 2011). In parallel a phase II trial is being performed using gastric proton pump inhibition to increase endogenous gastrin in combination with a GLP-1 analogue.

Islet Neogenesis Associated Protein (INGAP)

Islet neogenesis associated protein (INGAP) is a member of the regenerating gene (Reg) family of proteins. The Reg family is part of the C-type lectin super family and is mainly involved in the proliferation or differentiation of liver, pancreas, gastric and intestinal cells (Zhang et al. 2003). INGAP is believed to be the initiator of neogenesis in particular the INGAP104-118 peptide. This peptide stimulates an increase in beta cell mass in mice, rats, hamsters and dogs (Lipsett et al. 2007). INGAP has been found to be overexpressed in islets from patients with recent onset-type1 diabetes, and administration of INGAP into streptozotocin induced diabetic mice resulted in reversal of disease and an increase in beta cell mass (Rosenberg et al. 2004). Phase I and II trials with INGAP showed an increase in C-peptide secretion, improved glycemic control but no decrease in HbA1c levels. A trial is ongoing to optimize dosing, exposure, formulations and possible combination therapies (Dungan et al. 2009).

6.2.2 *Growth Hormone and Glutamine in Short Bowel Syndrome*

Growth hormone exerts its trophic effects on the intestine via IGF-1, which originates from lamina propria mesenchymal stem cells. The resulting increase in DNA and protein production involves ornithine decarboxylase activity, for which glutamine is a substrate. Previous animal studies have shown that growth hormone and glutamine both have beneficial effects on intestinal adaptation in the early phase after surgery (Gouttebel et al. 1992) and act synergistically on intestinal function (Gu et al. 2001). Subsequently, further basic research could document the benefit of growth hormone and glutamine in human intestine (Scheppach et al. 1994; Inoue et al. 1994). Byrne and colleagues first demonstrated 15 years ago the efficacy of growth hormone and glutamine in promoting intestinal adaptation in an open-label clinical trial and a case series (Byrne et al. 1995a, b) with SBS patients. These results attracted much interest, and several randomized controlled trials were carried out in the following years, which were also the topic of a Cochrane review in 2010 (Wales et al. 2010).

The clinical studies examining the effects of growth hormone with or without glutamine demonstrated an increase in weight, lean body mass and absorptive capacities, but the benefit was short-lived after therapy cessation (Byrne et al. 1995a; Ellegard et al. 1997; Scolapio et al. 1997; Seguy et al. 2003; Jeppesen et al. 2001). Only one study was able to document a sustained effect on PN volume, calories and infusion number in growth hormone and glutamine-treated patients at the 3 months follow-up (Byrne et al. 1995a). Analysis of the fat- and energy absorption yielded heterogeneous results, which is likely due to the different outcome measures and differences in patient selection (e. g. with or without underlying mucosal disease, age, or nutritional status). Only two of the trials found a positive effect on fat absorption at the end of treatment (Jeppesen et al. 2001; Seguy et al. 2003). Furthermore, the dose and therapy duration may explain the differences between the clinical trials. While a lower dose of growth hormone led to an increase in absorption in one trial (Seguy et al. 2003), higher doses in two different trials did not. This may be partially explained by observations from animal studies, where an excess of growth hormone caused a reduced, possibly compensatory, responsiveness of crypt proliferation to growth factor signaling (Lund 1998; Dahly et al. 2004). The question whether glutamine addition to growth hormone treatment further enhances the clinical benefit is still controversial; In the studies using both compounds (Byrne et al. 1995a; Scolapio et al. 1997; Jeppesen et al. 2001), neither the crude results nor the subgroup analyses from the Cochrane review (Wales et al. 2010) detected significant differences.

Overall, the data is still insufficient to routinely recommend growth hormone treatment for SBS. There is some benefit in terms of weight gain and fat absorption, but the patient numbers are very small, and the effects short lived. Evidence regarding

long-term safety is non-existent. Furthermore, the question whether glutamine addition is beneficial remains unresolved. Growth hormone treatment may not be justified when considering benefit and costs.

6.2.3 *Intestinal Growth Factors in Inflammatory Bowel Disease*

IBD treatment has largely focused on decreasing inflammation. Given a component of dysfunctional epithelial repair, several studies have investigated the effect of intestinal growth factors to treat not only growth retardation in pediatric IBD, but also inflammation children and adults. In a preliminary study, 37 patients with moderately to severely active Crohn's disease were treated with subcutaneous growth hormone vs. placebo and were instructed to increase their protein intake. At 4 months, CDAI had decreased by a mean of 144 points in the treatment group, and by only 19 points in the placebo group, which represented a statistically significant difference. In terms of side effects, several patients experienced headache and edema. The authors concluded that growth hormone treatment can be beneficial in active CD (Slonim et al. 2000). In a pediatric study, 20 patients were treated with systemic corticosteroids plus either growth hormone or placebo. Remission rates after 12 weeks of treatment were 65 % in the combined treatment group vs. 20 % in the monotherapy group. While the addition of growth hormone produced a positive effect on growth failure, the intestinal mucosal inflammatory state as assessed by endoscopy was not different between the groups (Denson et al. 2010).

Epidermal growth factor can induce epithelial growth by activation of PI3-kinase, AKT and MAPK pathways. Based on concerns that systemic EGF may induce epithelial neoplasia, one study investigated the effect of recombinant EGF enema (5 μg) vs. placebo (n = 12 patients in each group) in mild-to-moderate Ulcerative colitis. Disease remission defined as a St. Mark's score <4 was achieved by ten patients in the EGF group and two patients in the placebo group. However, both groups received additional mesalamine, which may have biased the results towards a more positive outcome, considering that a EGF-only group was not included (Sinha et al. 2003).

Controlled clinical trials with keratinocyte growth factor (Sandborn et al. 2003), trefoil factor (Playford et al. 1996), GMCSF (Dieckgraefe and Korzenik 2002), and sargramostim (Korzenik et al. 2005) did not reveal a benefit over placebo. Although the approach to use intestinal growth factors to treat inflammatory bowel disease, possibly as an adjunct to anti-inflammatory therapy, is intriguing and may aid the restitution of the damaged mucosa, none of the above therapies can today be recommended for widespread use, and concerns regarding their potential to induce neoplastic growth remain.

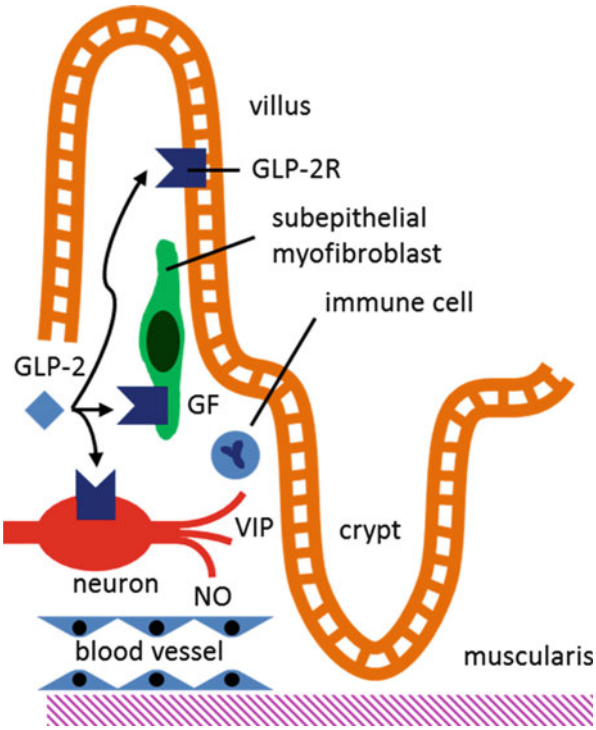

Fig. 6.2 GLP-2 action in the intestine. GLP-2 receptor (GLP-2R) is expressed on endocrine cells, subepithelial myofibroblasts and neurons. The effects of GLP-2 on epithelial proliferation, inflammation and blood flow are probably conferred to a large part in an indirect manner, involving nitric oxide (NO), vasoactive intestinal polypeptide (VIP), and growth factors (GF) such as IGF-1

6.2.4 Targeting GLP-2 Signaling to Treat Intestinal Diseases

The 33 amino acid peptide GLP-2 is a key mediator of intestinal adaptation (Scott et al. 1998). It is secreted from neuroendocrine cells and increases absorptive capacity by augmenting crypt cell proliferation and reducing villous cell apoptosis (Drucker et al. 1996). Additionally, it inhibits gastric emptying and acid secretion, reduces intestinal permeability, and modulates inflammatory responses and mesenteric blood flow (for review see Tee et al. 2011). Its half-life is very short, since it undergoes N-terminal truncation by the proteolytic enzyme dipeptidyl peptidase IV.

G-protein coupled GLP-2 receptors are expressed on enteroendocrine cells, enteric neurons, and subepithelial fibroblasts of the small intestine (Guan et al. 2006; Yusta et al. 2000; Orskov et al. 2005). A variety of effectors appear to be involved in mediating the downstream signal, including insulin-like growth factor-1 for epithelial proliferation, nitric oxide for the upregulation of intestinal blood flow, vascular endothelial growth factor and transforming growth factor-β for wound repair, and vasoactive intestinal peptide for anti-inflammatory effects (Rowland and Brubaker 2011) (Fig. 6.2).

Teduglutide is a DPPVI-resistant analog of GLP-2 lacking the N-terminal cleavage site to extend its half-life. Initially, an open label study of SBS patients demonstrated an increase in wet-weight absorption of up to 1 l daily without a significant effect on energy absorption, which was maintained for 24 months of treatment, but was quickly reversed upon treatment discontinuation (Jeppesen et al. 2005). Subsequently, a multinational, randomized, placebo-controlled study involving 83 patients suffering from SBS in need of PN at least three times a week for 1 year or more was carried out (Jeppesen et al. 2011). After a 4–8 week stabilization period to allow for optimization of PN, patients were randomized to receive either placebo, 0.05 mg/kg/day teduglutide, or 0.1 mg/kg/day teduglutide for 24 weeks. The primary endpoint was a graded response score accounting for the intensity and the duration of the response, which was defined as a reduction of the requirement for PN by 20 %, with urine production being used as a surrogate marker. While the graded response score as well as a 20 % reduction of PN were achieved by a significantly higher proportion of patients in the 0.05 mg/kg/day group than in the placebo group (16/35 [46 %] vs. 1/16 [6 %]), the difference of the 0.1 mg/kg/day arm vs. placebo was not significant. The latter result was explained by group differences and the fact that the high-dose group displayed a significant reduction in oral fluid intake. Additionally, there was a small increase in body weight despite reduced calorie provision via PN, and a positive effect on trophic markers of the intestine. In an open label extension study for additional 28 weeks, 52 patients continued their dose of teduglutide. At week 52, a progressive effect in both dose groups was observed. A reduction by more than 20 % of initial PN (i.e. end of initial RCT) was found in 68 % of the 0.05 mg/kg/day group and in 52 % of the 0.1 mg/kg/day group. Furthermore, a reduction of greater or equal to 1 day/week needing PN was noted in 68 % and 37 %. A total number of four patients became entirely independent from PN. Seven patients dropped out due to adverse events, most of them (four patients) because auf gastrointestinal symptoms. The safety profile was comparable to that of the initial RCT (O'Keefe et al. 2013).

In a confirmatory phase 3 study (STEPS – study of teduglutide in PN-dependent short bowel syndrome, NPS pharmaceuticals press release), 86 patients were treated with either 0.05 mg/kg/day active drug or placebo for 24 weeks to investigate whether a 20–100 % reduction in weekly PN volume at weeks 20 and 24 was feasible. 63 % of teduglutide-treated vs. 30 % of placebo-treated patients reached this endpoint. In addition, patients in the teduglutide group were able to reduce the weekly PN volume from 12.9 to 8.5 l, which was also significant vs. placebo. First results of a 2-year follow-up study (STEPS 2) with 88 patients enrolled and 74 % completing the 2 year period showed clinical response in 93 % of the patients. A mean PN volume reduction between 3,1 and 7,6 l/week depending on the treatment group with 58,5 % achieving one or greater than 1 day off of PN was observed. Thirteen patients achieved enteral autonomy and complete independence from PN.

Most studies with GLP-2 and teduglutide have not shown a significant increase of adverse events. Abdominal complaints, headache, and injection site reactions were commonly reported. In patients with congestive heart failure or a history of bowel obstruction, these therapies should be administered with caution. A remain-

ing concern arises from the proliferative properties of GLP-2 agonists and therefore their potential ability to induce malignancies. This is of particular importance, since treatment of SBS with teduglutide may be life-long. In azoxymethane-treated mice, GLP-2 indeed had pro-carcinogenic effects (Iakoubov et al. 2009). The most common adverse event in the ex-ante published STEPS-2 study were abdominal pain (34 %), blood stream infections (28 %), and decreased weight episodes (25 %). While treatment-emergent adverse events (AEs) occurred in 95 % patients, serious adverse events occured in 64 % of the patients of which 10 % were considered treatment related. Three cases of cancer and three deaths occurred with one case of cancer and one AE leading to death considered to be treatment related (Gabe et al. 2014). Until the safety profile with long-term treatment has been clearly established in clinical studies, thorough screening for premalignant lesions, such as colonic adenomas, is clearly advisable.

Based on the positive clinical data, teduglutide has been approved as the first therapeutic drug for the treatment of short bowel syndrome with intestinal failure in June 2012 by the EMEA and in December 2012 by the FDA.

In summary, trials with teduglutide in SBS have shown encouraging results, making this drug a new therapeutic option for SBS with the potential to reduce PN dependence and complications. Future studies will have to delineate the long-term outcomes, safety, and optimal therapeutic regimen.

Since it has been shown that GLP-2 can act anti-inflammatory and promote epithelial repair, studies have been carried out to investigate the effect of manipulating GLP-2 signaling in Crohn's disease. In an 8-week, controlled pilot study, 100 patients were treated with teduglutide, and their CDAI determined (Buchman et al. 2010). The higher teduglutide dose led to remission rates of 55.6 % vs. 33.3 % (placebo). This difference was not statistically significant, and secondary outcomes like surrogate markers of inflammation, were not reported.

6.3 Cell-Based Therapies

6.3.1 Stem Cells Approaches for Diabetes Therapy

Stems cells have been reported to hold great promise for T1D therapy due to their immunomodulatory role and regenerative capacities. Furthermore, drawbacks encountered in the optimization of islet cell transplantation have prompted researchers to search for other potential sources of glucose producing tissues including stem cells. Stem cells are defined by their ability to self-renew and to differentiate into many specialized cell types, tissues or organs. Stem cells can be classified as either pluripotent (with the ability to differentiate into all cell types) or multipotent (with a limited differentiation capacity) (Tuch et al. 2011).

6.3.1.1 Pluripotent Stems

Embryonic Stem Cells (ESC)

ESCs are derived from the inner cell mass of a blastocyst. These cells express the transcription factors Oct-4, Nanog-1 and Sox2, which are involved in self-renewal and act as markers of pluripotency (Friel et al. 2005). ESCs are able to differentiate into the ectoderm, mesoderm, and endoderm (from which the pancreas is derived). The rationale behind the use of ESCs for T1D therapy is that under certain conditions, these cells can be steered to differentiate into pancreatic islet cells that can in turn be transplanted into patients.

Various ESC differentiation protocols have been developed based on the development of the embryonic pancreas. Many of the earlier attempts to generate functional islets in vitro from ESCs were limited by final cell homogeneity, immaturity of differentiated cells, low numbers of insulin producing cells, and poor insulin responses to glucose exposure (McCall et al. 2009). However, a recent study from Baetge and colleagues showed that implantation of pancreatic endoderm derived from human ESCs (hESCs) into mice could efficiently generate glucose-responsive endocrine cells after implantation. These results imply the need for in vivo differentiation in order to generate glucose responsive cells. Furthermore, these cells could protect against streptozotocin-induced hyperglycemia (Kroon et al. 2008). Conversely, a separate study conducted by implanting hESCs derived pancreatic endoderm into athymic nude rats confirmed the development of islet-like structures but upon glucose challenge no increase in C-peptide or insulin was observed. These results led the researchers to conclude that though islet-like structures were formed from implanted hESC differentiated pancreatic endoderm, the extent of endocrine cell formation and secretory function is not yet sufficient to be clinically relevant (Matveyenko et al. 2010). Apart from the complexities of generating functional islets, ESCs use is also limited due to ethical concerns. Furthermore, since ESCs are developed from an allogeneic donor, strategies need to be developed to protect the ESCs from immune attack. Moreover, ESC cells have been shown to give rise to teratomas and teratocarcinomas in humans (Guleria et al. 2007).

Induced Pluripotent Stem Cells (iPs)

A major breakthrough in the pluripotent stem cell field was the development of induced pluripotent stem cells (iPS). iPS cells are derived from somatic cells in which pluripotency is restored by the induced expression of the transcription factors Oct-4, Sox2, Nanog, c-Myc, LIN28, and Klf4 (Stefanovic et al. 2009). With regards to morphology, self-renewal capacity, and differentiation iPS and ESC cells are very much alike; however, iPS cells have an added advantage over ESC cells in that they allow for the possibility of autologous cell therapy. Indeed, Maehr and

colleagues showed for the first time that it was possible to generate iPS cells from dermal fibroblasts of T1D patients. This process involved retroviral transduction of the fibroblast with Oct4, Sox2, and Klf4. Furthermore, these iPS cells showed a normal karyotype and beta-cells derived from these iPS cells were shown to be C-peptide positive and were capable of releasing insulin after in vitro stimulation with glucose (Maehr 2011). However, a lot of research still needs to be done before iPS cells can be considered for clinical trials. Furthermore, iPS cells are also limited by the formation of teratomas, and the use of retroviral vectors for delivery of reprogramming factors could further lead to malignant transformation.

6.3.1.2 Multipotent Stem Cells

Hematopoietic Stem Cells (HSCs)

Adult stem cells/multipotent stem cells can be classified into hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). HSCs are by far the best characterized and best studied stem population. HSCs are located in the bone marrow niche and can be readily harvested from bone marrow and umbilical cord blood (UCB). HSCs can also be collected from peripheral blood after mobilization from bone marrow with granulocyte colonystimulating factor (G-CSF) (Brignier and Gewirtz 2010; Flomenberg et al. 2005). Additionally HSCs can be isolated on the basis of their surface markers, that is, lineage specific antigen negative (lin-), CD34,+ CD38, −CD133,+ c-Kit/CD117,+ CD59,+ Thy1/CD90+, and CXCR4+ cells. HSCs preferentially differentiate into lymphoid and myeloid lineages.

The rationale behind the use of HSC transplantation (HSCT) for the treatment of autoimmunity is that transient lymphoablation followed by autologous HSCT will allow for immune regeneration and resetting of immune self-tolerance (Couzin-Frankel 2010). A clinical trial for T1D was conducted in Brazil using high dose cyclophosphamide plus rabbit polyclonal anti thymocyte globulin (ATG) for lymphoablation followed by an autologous HSCT. Analysis of this study showed an increase in C-peptide levels, good glycemic control, and insulin independence in a majority of the treated patients (20 out of 23 recent onset diabetics). Furthermore 12 of these patients were insulin dependent for 31 months whilst the other 8 patients had periods ranging from 6 to 47 months when they were insulin free after which they resumed insulin therapy albeit at a lower dose than pretransplant (Voltarelli et al. 2007; Couri et al. 2009). Additionally, using the same HSCT protocol in eight recent onset patients, Snarkski and colleagues achieved insulin independence in all the patients, except one who resumed low dosage insulin treatment 7 months after transplantation (Snarski et al. 2011). Although these results seem promising, this treatment was associated with side effects such as nausea, vomiting, fever and alopecia. Two patients presented with nosocomial pneumonia and cases of Grave's

disease, transient hypergonadotropic hypogonadism, and autoimmune hypothyroidism were reported. However, no mortality was observed so far (Couri and Voltarelli 2009). Long-term consequence of the conditioning regimen need to be monitored, and it remains questionable how much risk of immune interventions we dare to take for a disease which can safely be treated with insulin replacement for decades. Taken together, the studies involving HSCs transplantation have taught us what can be achieved with appropriate immune therapies.

Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are a heterogeneous population of multipotent stem cells that can differentiate into various mesodermal cell lineages including myocytes, osteoblasts, chondroblasts, fibroblasts and adipocytes. MSCs can be found in almost every organ, but for therapeutic use they are isolated from the UCB and bone marrow. Human MSCs can be identified by their lack of HSC markers, their expression of CD105, CD73 and CD90 and ability to adhere to plastic. MSCs possess anti-inflammatory and immunomodulatory properties mediated by the secretion of factors such as indolamine 2,3-dioxygenase, IL-6, TGF-β1, inducible nitric oxide synthetase and prostaglandin (Brignier and Gewirtz 2010) . Furthermore, MSCs have been shown to induce regulatory T cells and suppresses effector and cytotoxic T cells and B cells in vitro (Selmani et al. 2008). The immunomodulatory effects of MSCs are still under exploration for their application in T1D therapy. However, transfer of MSCs into prediabetic NOD mice was shown to result in disease prevention and induction of IL-10 secreting FOXP3+ regulatory T cells (Madec et al. 2009). Furthermore, transfer of human MSCs (hMSCs) into a streptozotocin induced diabetes model resulted in decreased hyperglycemia associated with an increase in beta cells mass and insulin production. In addition, it was observed that the hMSCs selectively homed to both pancreatic islets and renal glomeruli of the diabetic mice leading to tissue repair. This suggests that hMSCs may be useful in enhancing insulin secretion and perhaps improving the renal lesions that develop in patients with diabetes mellitus (Lee et al. 2006).

MSCs have also been reported to be a potential alternative source for β-cell neogenesis. Retroviral transduction of the MSCs with the β-cell development transcription factor pancreatic and duodenal homeobox 1 (Pdx1), followed by in vitro steering in the presence of islet hormones resulted in the generation of insulin producing cells. Transplantation of these cells under the kidney capsule resulted in decreased hyperglycemia in a streptozotocin induced diabetes model. Furthermore, insulin producing cells could be generated from diabetic patients without genetic manipulation thereby demonstrating that long-term hyperglycemia does not constitute a factor against iPS generation in T1D patients (Li et al. 2007; Sun et al. 2007). Phase I/II clinical trials aimed at assessing the efficacy and safety of allogeneic or autologous MSCs therapy for T1D are currently ongoing (Fiorina et al. 2011).

6.3.1.3 Combination Therapy: The Future of T1D Treatment

Most of the clinical trials discussed thus far have been largely based on monotherapies which were either aimed at tolerance induction and beta cell preservation, or restoration of beta cell mass and function. However, many researchers in the field of T1D argue that like in cancer, T1D treatment could benefit from combination therapy thereby resulting in tolerance induction and beta cell regeneration (Bresson and von Herrath 2007). They propose combinations of treatments with either a documented effect as a monotherapy in T1D or a combination treatment that has been efficacious in another autoimmune disease (van Belle et al. 2011). Combination therapies have been largely successful in preclinical rodent models. For example, studies in BB rats showed disease delay or prevention after administration of the purine biosynthesis inhibitor mycophenolate mofetil (MMF) and anti-CD25 mAb (Ugrasbul et al. 2008). Furthermore, intranasal insulin in combination with anti-CD3 mAb was shown to be more efficacious than the monotherapies at reversing recent onset diabetes in NOD mice and a RIP-LCMV diabetes model. This treatment resulted in the expansion of CD25+ Foxp3+ and IL-10, TGF-β and IL-4 insulin-specific Tregs, and regulatory T cells could confer dominant tolerance to immunocompetent recent onset diabetic (Bresson et al. 2006). These results suggest combination therapy with immune modulators and islet antigen specific vaccines could be effective. Additionally based on the success of anti-CD3 mAb in combination with exendin-4 treatment in recent onset diabetic NOD mice, combination treatments with immune modulators and compounds that enhance β-cell mass and function have also been proposed for future clinical trials (van Belle et al. 2011; Sherry et al. 2007). This approach is postulated to have a higher success rate than the gastrin and exenatide β-cell regeneration trials owing to the fact that anti-CD3 induces tolerance whilst exenatide increases insulin secretion from residual β-cells. Clinical trials so far have shown that intensive insulin therapy in combination with exenatide and anti-CD25 mAb showed no improved function of remaining β-cells in patients with long-standing diabetes (van Belle et al. 2011; Rother et al. 2009). However, these results do not rule out the efficacy of combination therapy in recent onset diabetes patients.

The aforementioned anti-CD3 and intranasal insulin preclinical study demonstrated that adoptive transfer of regulatory T cells results in reversal of disease in recent onset diabetic mice (Bresson et al. 2006). The efficacy of adoptive transfer of CD4+ CD25+ Foxp3+ natural Tregs (nTregs) in human trials has been demonstrated in the treatment of leukemia by hematopoietic stem cell transplantation (HSCT) and donor lymphocyte infusions (DLI). Adoptive transfer of ex vivo purified nTregs in this setting was shown to be safe and the transferred nTregs promoted lymphoid reconstitution, did not overtly weaken the graft vs. leukemia effect, and prevented graft versus host disease (Edinger and Hoffmann 2011). These findings open up the possibility of nTreg transfer as a potential immunomodulatory approach for T1D patients. The HSCT trials mentioned above utilized polyclonal nTregs however adoptive transfer of antigen specific nTregs was shown to be more effective at disease prevention and reversal in recent onset and overtly diabetic NOD mice (Katz

et al. 1993; Salomon et al. 2000; Wu et al. 2002; Tang et al. 2004; Tarbell et al. 2006; Masteller et al. 2005; Jaeckel et al. 2008). The frequency of antigen specific nTregs coming from a polyclonal repertoire is very low however the successful treatment of colitis in a murine model with nTregs redirected by antigen-specific chimeric receptor gives hope for the potential use of nTregs with redirected specificities for T1D treatment (Elinav et al. 2009). Furthermore, the fact that T cells with redirected specificities are currently in use for a plethora of clinical trials for cancer (Jena et al. 2011; Morgan et al. 2010) and nTreg transfer was shown to be safe in HSTC implies that adoptive transfer of redirected nTregs could also be an acceptable therapy in T1D patients. Furthermore, one could envision combination therapies of adoptive transfer of redirected Tregs with compounds that enhance beta cell mass and compounds that favor in vivo Treg expansion such as rapamycin (Battaglia et al. 2005).

6.3.2 Stem Cell Approaches in the Therapy of Intestinal Disease

Experimental and clinical research increasingly utilizes stem cell therapy for IBD. Initially, the excitement surrounding the stem cell field was based on the unique biological properties of these cells and their capacity to self-renew and regenerate tissue and organ systems. Later on, the immunomodulatory ability of stem cell therapy has become apparent. Conventionally, the definition of stem cells refers to the hematopoietic stem cells (HSC), with reference to myeloid and lymphoid lineages. However, a distinct lineage is now known to consist of mesenchymal stem (stromal) cells (MSC). Stem cell therapy for IBD is thought to both repair damaged intestinal tissue and the immune system. For active luminal disease three forms of stem cell therapy have been attempted. First, bone marrow derived donor (BMD) or autologous stem cells (bone marrow contains both hematopoietic as well as mesenchymal stem cells), second, peripheral blood donor or autologous stem cells and third, donor or autologous mesenchymal stem cells from adipose tissue. Autologous and donor stem cell transplantation has involved pre-transplantation bone marrow ablation, while mesenchymal stem cell studies have avoided bone marrow ablation (Table 6.2).

6.3.2.1 Hematopoietic Stem Cells

The possibility that HSCT might be an effective treatment in CD arose from a case report published in 1993, reporting a female patient with CD who remained symtom-free during a 6 month follow-up after HSCT for non-Hodgkin's lymphoma (Drakos et al. 1993). This report was followed by several other case reports and a case series in 1996. In this case series, remission was observed in four out a five patients with CD and leukemia after allogenic bone marrow transplantation (Lopez-Cubero et al.

Table 6.2 Types of stem cells for treatment in IBD

	Hematopoietic	Mesenchymal	Intestinal
Origin	Bone marrow, peripheral blood	Bone marrow, adipose, placenta	Intestinal crypt
Function	Autologous or allogeneic hematopoietic stem cell transplantation (HSCT)	Autologous or allogeneic transplantation	Keeps intestinal barrier functional
	Elimination of autoreactive lymphocytes (lymphoablative effect)	Antiproliferative for stimulated T-cells	Keeps crypt sterile through secretion of antimicrobial peptides and defensins
	Altered immune reconstitution after immuneablation	Inhibits inflammatory response of innate and adaptive immune system (immunomodulatory effect)	Genetic repair approach in future might supply defective genes through genetic transfer techniques
	Generation of naive cells to restore tolerance	Reparative effect on inflamed tissue	
		Adipose derived stem cells for treatment of fistulizing diseases	

1998). Five of these six patients had active CD, and four of the five patients had sustained remission of CD 54–183 months after transplantation. In another case series, remission of symptoms occurred in all patients (six with CD, four with UC) after myeloablative treatment and allogeneic bone marrow transplantation (Ditschkowski et al. 2003). In this study, one patient had a mild self-limiting recurrence and another died of infectious complications, all patients except two maintained immunosuppressive therapy at the end of follow-up (follow-up 3–117 months). Although these studies were not designed to investigate the role of HSCT on IBD, they supported the notion that lymphoablation and generation of new self-tolerant lymphocytes might induce remission in patients with IBD.

Based on these results, attempts with HSC as a primary treatment were initiated. The first case was reported in 2003 (Craig et al. 2003). These initial trials are integrated in the final report of a phase I study in which infusion of autologous HSCs from peripheral blood after mobilization, expansion and conditioning in 12 patients with refractory CD was performed (Oyama et al. 2005). Eleven patients had remission after 6 month, monitored by Crohn's Disease Activity Index (CDAI) <150, and after 18.5 month of follow-up only one patient has experienced recurrence. Another trial used peripheral HSC unselected for CD34 in four patients successfully (mean follow-up 16.5 months) (Cassinotti et al. 2008). In a long term follow-up of 24 patients (the largest cohort to date) by the group from Chicago, the percentage of clinical relapse-free survival, defined as the percentage of patients restarting medical therapy after transplantation, was 91 % in 1 year, 63 % at 2 years, 57 % at 3 years, 39 % at 4 years and 19 % at 5 years, showing that 81 % of these patients had to begin medical therapy again within 5 years after transplantation,

with an annual percentage of 70–80 % being in remission in the 5 years after transplantation (Burt et al. 2010).

Regarding safety, Snowden et al. showed results on HSCT in Patients with immun-mediated diseases from 1997 to 2009. The survival rates were 85 % in the first year and 78 % after 5 years after autologous cell transplantation, and 87 % and 65 %, respectively, after allogenic transplantation in 70 transplantations in 69 patients, including 55 autologous transplantations and 15 allogenic transplants. The disease free rates were 51 % in the first year, and 33 % after 5 years for autologous transplantations and 80 % and 65 %, respectively, for allogenic transplantation (Snowden et al. 2012).

The sustained clinical remission with hematopoietic stem cell therapy seems not only to be due to cyclophosphamide und G-CSF during mobilization (Kreisel et al. 2003). The mechanisms underlying the beneficial effects remain unclear, but are probably the result of initial eradication of T-cells and memory cells because of the lymphoablative effects of drugs used in the conditioning regimen. Later on, there may be an effect of altered immune reconstitution. In 2005, the international committee established for the development of guidelines on entry criteria and transplant protocols for IMIDs (immune mediated inflammatory diseases) recommended that autologous HSCT should be preferred to allogeneic HSCT because of a lower risk of severe toxicity (Gratwohl et al. 2005). It has to be kept in mind that stem cell collection and autologous transplantation are associated with morbidity and mortality, and that flares of the disease and lethal complications during mobilization have been reported (Kapoor et al. 2007). Therefore, and due to the low number of patients transplanted in each center with different regimens, the European society for blood and marrow transplantation Autoimmune Diseases and Immunobiology Working Parties have undertaken a joint initiative to develop and implement guidelines for immune reconstitution studies in patients with autoimmune disease before, during and after SCT (Alexander et al. 2015).

In a study based on genetic linkage analysis and candidate-gene sequencing on samples from two unrelated consanguineous families with children with early-onset inflammatory bowel disease, three distinct homozygous mutations in genes IL10RA and IL10RB were identified that segregated with disease phenotype (Glocker et al. 2009). One member of the family with IL10RB mutation suffered from a severe CD since his third month of life with proctitis, abscesses and multiple surgical interventions. This subject underwent allogeneic HSCT using an HLA matched sibling not carrying the mutation. Fistulas resolved shortly after transplantation, and the patient remained in continuous remission from ileocolitis during a follow-up period of 2 years after transplantation. This was the first study showing a curative approach to severe CD by means of allogeneic HSCT that was justified by a monogenic cause of the disease in this case.

Currently, there are two trials active but not recruiting on stem cell transplantation in patients with Crohn's disease (NCT02225795, NCT01288053). An interventional trial is now recruiting patients in the USA to measure safety and clinical benefit of lymphoablation followed by autologous HSCT as a rescue in therapy refractory Crohn's disease (NCT01932658).

6.3.2.2 Mesenchymal Stem Cells

When cells from a bone marrow aspirate are cultured in plastic flasks, hematopoietic cells and stem cells do not adhere to the plastic and are removed when changing media. The remaining plastic adherent cells are termed MSCs, an abbreviation for both mesenchymal stem cells and mesenchymal stromal cells: Mesenchymal stromal cells, because they can contribute to the structural matrix of bone marrow and support hematopoesis; mesenchymal stem cells, because they have the ability to differentiate under ex vivo conditions into different mesenchymal-derived cells (Pittenger et al. 1999). MSCs may be isolated from bone marrow, skeletal muscle, adipose tissue, synovial membranes and other connective tissue of human adults, as well as cord blood and placenta.

The cytokine secretion profile as well as the immunmodulating effect from MSCs from adipose tissue differ from those obtained from bone marrow with more potent immunmodulating effect in the MSCs acquired from adipose tissue (Melief et al. 2013). Mesenchymal stem cells have been shown to inhibit inflammatory responses of innate and adaptive immune cells as well as have reparative effects on inflamed tissues. Recent reports showed that most of their biological effect seems to be derived by paracrine mechanisms such as secretion of chemokines, growth factors and cytokines (Burdon et al. 2011; Parekkadan and Milwid 2010; Burchfield and Dimmeler 2008). Furthermore, there are observations of a reduction of inflammation, apoptosis and fibrosis as well as improved wound healing and regeneration (van Poll et al. 2008; Qi et al. 2014). The biological effects probably are also due to the induction and stimulation of the host progenitor cells for an optimization of the regenerative process (Semont et al. 2013).

Successful preclinical studies using MSCs in models of autoimmunity, inflammation and tissue damage have paved the way for clinical trials without the need of a conditioning phase of either autologous or allogenic origin because of their lack of immunogenicity (Voswinkel et al. 2013).

In one trial, expanded autologous bone marrow mesenchymal stem cells (BM-MSC) were applied intravenously and recorded an improvement in CDAI in four out of six patients (Duijvestein et al. 2010). In a phase II trial with expanded allogeneic bone marrow derived adult MSCs that were administered in two doses in patients with moderate-severe refractory Crohn's disease (CDAI >220), a clinical response defined as a reduction in CDAI of at least 100 points in three out of nine patients was observed (Onken et al. 2006). In a 12 month study with eight patients, seven adverse events were reported (Onken et al. 2008). No tumors or formations of ectopic tissue were found. In another completed phase I trial of intravenous autologous bone marrow derived MSCs that included nine patients confirmed that the treatment is feasible and safe, but without apparent benefit for patients with severe refractory luminal disease (Duijvestein et al. 2010).

In 2012, Liang et al. showed results on allogeneic mesenchymal stem cell transplantation in four patients with Crohn's disease and three patients with Ulcerative colitis. All the patients maintained on their medication and received one million cells/kg bone marrow or umbilical cord, healthy donor derived MSCs intravenously. Remission was

induced in five of the patients and was maintained over 24 months in two patients. Side effects were mild (flush, insomnia, transient fever) and resolved without any further therapy (Liang et al. 2012). In 2014, Forbes et al. published a phase 2 study of allogeneic mesenchymal stromal cells for luminal Crohn's disease refractory to biologic therapy in 16 patients of whom 15 completed the study. Improvement considering the CDAI was shown in all 15 patients (median 327 to median 129 at day 42), whereas clinical response was found in 12 patients reaching clinical remission in 8 of the patients. A serious adverse event occurred in one patient, but was probably not caused by MSCs (two dysplasia-associated lesions) (Forbes et al. 2014).

At the moment, four clinical studies are registered to recruit patients for systemic MSC therapies in Crohn's disease (NCT01874015, NCT01540292, NCT02000362, NCT01915927), and one considering treatment for Ulcerative colitis with adipose tissue derived MSCs. Furthermore, there are four ongoing, but not any longer recruiting trials registered at clinicaltrials.com to further investigate the safety and treatment outcome of intravenous human mesenchymal stem cells (previously Prochymal, now Remstemcel-L) as a therapy for Crohn's disease (NCT01510431, NCT01233960, NCT00482092). The most important work in this field is the phase III study (NCT00482092) that includes 330 patients who will be treated with MSCs at different doses with final results expected 2018.

Taken together, MSCs are a promising tool in the treatment of IBD. However, quite an amount of work remains to be done to solve the still not completely understood mechanisms how MSCs regulate homeostasis, tissue repair and the immune system. Furthermore, methodological concerns remain taking the small numbers of patients, the variation in cell products and the lack of published controlled studies into consideration. Until new tools as an effective MSCs-based treatment for IBD can be implemented as proposed further investigation is crucial (Flores et al. 2015).

Regarding fistulizing disease, there is an 11-year experience of the Spanish group with MSCs taken from fat tissue. First encouraging results from an initial phase I clinical trial using locally administered adipose-derived stem cells (ACS) to treat complex perianal fistula were published in 2005 (Garcia-Olmo et al. 2005). These results have been confirmed in a phase II multicenter randomized trial including patients with complex perianal fistulas (cryptoglandular origin n = 35, associated with Crohn's disease n = 14) observing fistula closure in 17 out of 24 patients who received ASCs in addition to fibrin glue, compared to 4 out of 25 who received fibrin glue alone (Garcia-Olmo et al. 2009). In 2013 a phase I/II trial was published using expanded adipose-derived allogenetic mesenchymal stem cells showing a safe and beneficial treatment option (de la Portilla et al. 2013).

Also, a single study using bone marrow cells in 12 patients with complete closure in seven cases and incomplete closure in three cases is available (Ciccocioppo et al. 2011). Recently, two Korean studies using autologous ASCs to evaluate the safety of the treatment (Cho et al. 2013) followed by a phase II study (Lee et al. 2013) have been published. A total of 43 patients were locally treated with ASCs. Of these patients, 33 were included in the modified per protocol analysis. A complete sealing of the fistula was achieved in 27 patients 8 weeks after the final injection of ASCs without any case of serious adverse effects.

Three studies are currently recruiting assessing the use of bone marrow mesenchymal or adipose derived stem cells in fistulizing Crohn's disease (NCT02403232, NCT01874015, NCT01915927, NCT02000362).

In summary, it is important to define the source and type of MSC (autologous or allogenic) in order to standardize cell expansion conditions and to adopt uniformal study protocols. Also, significant issues remain regarding the design and interpretation, such as patient selection, disease stage, disease activity, MSC source (bone marrow, adipose tissue, placenta) and long-term safety. Although the perspective of immune reeducation and regulation seems fascinating, it is unrealistic to believe that cell-based therapies can eradicate immune disease, because most processes have a genetic predisposition that remain unaltered by an autologous transplant. Especially for IBD, where the primary defect is probably a stem-cell differentiation problem in the intestine, new therapeutic strategies should be based on stimulation of the protective innate immune system.

6.3.2.3 Intestinal Stem Cells

Intestinal stem cells maintain the rapidly self-renewing intestinal tract tissue. Concerning the localisation of intestinal stem cells in the gut, there are two major models still under debate. The first is called the "+4 position model", which assumes that the stem cell is located above the Paneth cells at position +4 related to the crypt base (Haegebarth and Clevers 2009), the second is called the "stem cell zone model". This model proposes, that the crypt base columnar cells represent the intestinal stem cell (Haegebarth and Clevers 2009).

Intestinal stem cells differentiate into four epithelial cell types, namely absorptive columnar cells, goblet cells, neuroendocrine cells and Paneth cells. LGR5 (Leucine-rich-repeat-containing G-protein-coupled receptor 5) is a marker for intestinal stem cells since it could be shown that LGR5-positive cells are pluripotent, self-renewing and differentiate into all four epithelial cell types (Barker et al. 2007).

Paneth cells are located at the crypt of the cell and secrete defensins and other antimicrobial peptides to keep the crypt sterile. For ileal Crohn's disease it could be shown, that a defective differentiation from the intestinal stem cell toward the Paneth cell, because of a diminished expression of the Wnt signaling transcription factor TCF4 and the WNT coreceptor LRP6 (Koslowski et al. 2009, 2012), resulting in a defensin deficiency. This in turn leads to a dysfunctional mucosal barrier and an invasion of luminal microbes resulting in an inflamed mucosa. For Ulcerative colitis, a defective differentiation from intestinal stem cell to goblet cell, mediated by the transcription factors Hath1 and KLF4, might lead to a goblet cell depletion and impaired mucin induction (Gersemann et al. 2009). This causes a defective mucus barrier and again invasion of luminal microbes triggering inflammation. Despite these exciting new perspectives, interventions focusing intestinal stem cells are difficult. One approach might be the genetic repair approach, supplying the defective genes to local crypt cells using a variety of gene transfer techniques. In a way, the alternatives of hematopoietic or mesenchymal versus intestinal stem cell therapies

replays the current paradigm shift from adaptive to innate immunity centered on a barrier disease. Moreover, a promising alternative is the stem cell and mesenchyme dependent implantation of autologous organoid units to create a tissue-engineered small intestine which is also under investigation for SBS (Belchior et al. 2014).

6.4 Conclusions and Future Perspectives on Regenerative Therapies

Medical therapies aiming at beta-cell neogenesis like gastrin, EGF, and INGAP (islet neogenesis associated protein) are in early stages of clinical development, with insufficient data available to date. Similarly, treatment of short bowel syndrome with growth hormone with or without glutamine is ill-defined with regards to the optimal treatment regimen and safety. Intestinal growth factors are being tested for inflammatory bowel disease, and may be possibly of value as an adjunct therapy in the future. The GLP-2 analogon teduglutide, on the other hand, demonstrated promising results in the treatment of SBS and has approved by the EMEA and FDA for this indication. Regarding cell-based regenerative approaches for T1D, pluripotent stem cells are still in preclinical development, and the first studies involving multipotent stem cells are ongoing. In inflammatory bowel disease, trials using hematopoietic or mesenchymal stem cells have reported limited efficacy. Given the widespread damage in IBD and the different intestinal cell types cell-based therapies will probably need to be a synergic combination of the present investigated pathways for a sustained therapeutic response. New promising approaches involve intestinal stem cells, which seem to account better for new clues to the pathogenesis of IBD as a barrier defect and includes their regulating function concerning tissue engineered small intestine.

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Chapter 7
Kidney

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Abstract Regenerative medicine is an area of intense excitement and potential. Despite the increasing rate of end-stage renal disease, dialysis and transplantation remain the only treatment options to date. However, there is hope that stem cells and regenerative medicine may procure additional therapeutic options for renal disease. Such new treatment options may include induction of repair using endogenous or exogenous stem cells or the reprogramming of the kidney to reinitiate development. This chapter reviews the current state of understanding with respect to stem cell functions in the kidney, regenerative principles in kidney diseases, as well as clinical implications and implementation of regenerative medicine in renal disease.

Keywords Kidney development • Kidney disease • Kidney stem cells • Regenerative principles • Regenerative therapies

7.1 Introduction

The term *regenerative medicine* spans bioengineering, cell biology and matrix biology with the objective to repair or re-grow a damaged organ or tissue. It can be defined as the use of cells for the treatment of a disease and covers both organ repair and the *de novo* regeneration of an entire organ. Organ repair can be delivered in situ or ex vivo. In situ possibilities include the recruitment of stem cells to the kidney to trigger repair and the induction of self-repair of resident renal cells. Whereas some regard in situ approaches as more likely to be successful for an architecturally and anatomically constrained organ such as the kidney, the other approach is the ex vivo culture of stem cells for re-delivery to the damaged kidney. This might involve autologous or non-autologous stem cells from a variety of sources. Finally, a bioengineering approach that relies on cells, factors, and matrix may be achievable. Although seemingly the most difficult, it may be the more feasible approach for genetic conditions such as polycystic kidney disease.

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7.2 Kidney Development, Stem Cell Function

Regenerative biology draws on an understanding of normal developmental processes. Understanding the molecular basis of kidney development will be the key to the development of regenerative therapies for chronic renal disease (Little 2006). During mammalian development, three separate excretory organs develop: The pronephros, the mesonephros, and the metanephros. In mammals, it is the paired metanephroi that persist postnatally and constitute the permanent kidney (Horster et al. 1999). The permanent kidney arises via reciprocal interactions between two tissues, the ureteric bud and the metanephric mesenchyme, the latter arising from the intermediate mesoderm. The ureteric bud gives rise to the collecting ducts and the ureter. The metanephric mesenchyme, which shows much broader potential and gives rise to all other elements of the nephrons, the interstitium, and the vasculature, is regarded as the renal progenitor population (Herzlinger et al. 1992). As the ureteric bud reaches the metanephric mesenchyme, signals from the tips of the branching UB induce areas of adjacent metanephric mesenchyme to aggregate and undergo a mesenchyme-to-epithelial (MET) transition. Each MET event represents the birth of a new nephron with the first nephrons "born" in the center of the metanephric mesenchyme. The peripheral metanephric mesenchyme, which has not yet undergone induction, is referred to as the nephrogenic zone. Nephrogenesis in humans is complete by week 36 of gestation, whereas it continues for 1–2 weeks after birth in the mouse and the rat. At that time, it is assumed that the peripheral nephrogenic zone is exhausted.

Embryonic metanephroi, differentiating into the adult kidney, have come to be a generally accepted model system for organogenesis. Nephrogenesis implies a highly controlled series of morphogenetic and differentiation events that starts with reciprocal inductive interactions between two different primordial tissues and leads, in one of two mainstream processes, to the formation of mesenchymal condensations and aggregates. These go through the intricate process of mesenchyme-to-epithelium transition by which epithelial cell polarization is initiated, and they continue to differentiate into the highly specialized epithelial cell populations of the nephron. Each step along the developmental metanephrogenic pathway is initiated and organized by signaling molecules that are locally secreted polypeptides encoded by different gene families and regulated by transcription factors. Nephrogenesis proceeds from the deep to the outer cortex, and it is directed by a second, entirely different developmental process, the ductal branching of the ureteric bud-derived collecting tubule. Both systems, the nephrogenic (mesenchymal) and the ductogenic (ureteric), undergo a repeat series of inductive signaling that serves to organize the architecture and differentiated cell functions in a cascade of developmental gene programs.

The development of the metanephric (permanent) mammalian kidney begins at gestational week 4–5 in humans and at E11 in mouse. Organogenesis and its governing principles have been studied mostly in the mouse. Metanephros formation, i.e., organogenesis of the permanent kidney, is initiated by the ureteric bud, which

sprouts out of the posterior end of the Wolffian duct and invades the metanephrogenic mesenchyme. The subsequent interaction between the two primordia induces the ureteric bud to branch dichotomously, thus initiating the morphogenesis of the collecting duct system. Induced metanephric mesenchyme condenses at the tips of the ureteric buds, and mesenchymal cells form aggregates, thus beginning the mesenchyme-to-epithelium transition. Each aggregate epithelializes and proceeds in stages to the vesicle stage, comma stage, and S-stage, from where each S-shaped body, after fusion with the ureteric bud-derived collecting duct, differentiates into one of the ($\sim 2 \times 10^6$) nephrons of the human kidneys. The architectural pattern, therefore, as a result of the sequential ureteric bud arborization, is designed to proceed from the deep cortex to the periphery in a repeat series of induction, morphogenesis, and differentiation (Horster et al. 1999).

The bulk of the nephron, unlike the ureteric bud-derived collecting duct system, is created from mesenchymal cells by an intricate cascade of events. The early events result in the acquisition of an essentially epithelial character by the future nephron cells while these polarized cells form a sphere or vesicle. The process of modeling the subsequent stages of comma and S-shape is not yet fully understood, although plenty of morphoregulatory molecules and transcription factors are sequentially and differentially expressed. These stages of morphogenesis are the onset of nephron differentiation, i.e., epithelial segments begin to express their specific properties. These stages of nephrogenesis have an ancestry that begins at the blastula stage, which determines the mesoderm; it follows the induction of the pronephros and the directed migration of the pronephric duct to proceed through the stage of the Wolffian duct and to induce the metanephric mesenchyme, which in turn directs branching of the ureteric tree. Cells of the metanephrogenic mesenchyme are induced by ureteric bud cells to become multipotent progenitor cells after rescue from apoptosis; they go on to condense and, guided by regulatory circuits of gene expression and repression, to enter the mesenchyme-to-epithelium transition, and to polarize to apicobasal expression patterns (Horster et al. 1999). In parallel, a pool of distinct progenitor cells within the metanephrogenic mesenchyme gives rise to cortical and medullary interstitial cells, mesangial cells, and pericytes that accompany the developing vascular network.(Kobayashi et al. 2014) For the metanephric mesenchymal blastema to produce the ~15 epithelial cell types of the metanephric kidney, it must be induced to undergo a conversion to the epithelial phenotype and subsequently differentiate into the highly specialized cell types of the nephron. Hypothetically, this pathway could start from two different points. One starting point would be a homogeneous mesenchymal population consisting of one multipotent cell type from which all nephron epithelial cell types are derived. Alternatively, the primary inductive event is not the conversion to the epithelial phenotype but a commitment of the mesenchymal cell type to different developmental pathways, and the secondary inductive event of phenotypic conversion then destines already committed cells to be recruited for the early nephron (Koseki et al. 1991; Qiao et al. 1995). Studies on the temporospatial expression of two transcription factors: *BF- 2* (Hatini et al. 1996) and *Pax-2* (Dressler et al. 1990; 1997) have shed some light on this situation. It seems now justified to favor the hypothesis that

all peripheral mesenchymal blastema cell types are induced to become stem cells through the first signal interactions. This initial step rescues most of the nephrogenic stem cells now expressing *Pax-2* from apoptosis whereas the uninduced mesenchymal cells enter programmed cell death. Induction is a two-step event that had been postulated already from earlier tissue recombination work (Ekblom 1989) where it was found that a short-time (hours) exposure of uninduced mesenchyme to the ureteric inductor led to the stem cell phenotype but no further. Nevertheless, this first step to the stem cell phenotype rescues most of the mesenchyme from apoptosis. The second step, however, very likely differs in molecular nature from the first one (Barasch et al. 1996). Molecular and cell-fate studies clearly indicate the existence of at least two distinct key progenitor populations within the mesenchyme. Six2+ progenitor cells undergo an epithelial transformation and give rise to all components of the main nephron from podocytes to the connecting segment (Kobayashi et al. 2008) while stromogenic Foxd1+ (previously known as BF-2) progenitor cells, produce major stromal interstitial cell types, including pericytes and mesangial cells (Humphreys et al. 2010). Recent evidence highlights the heterogeneity of these cell populations within the metanephric mesenchyme supporting a crucial inductive and inhibitory crosstalk in which Foxd1+ stroma inhibits nephron progenitor cell expansion and promotes its differentiation (Das et al. 2013). It is interesting to note that hemangioblast precursors that give rise to renal endothelial cells, might transdifferentiate from additional populations and potentially also from a subset of Foxd1+ precursors within the nephrogenic mesenchyme. (Risau 1997; Sims-Lucas et al. 2013) Cell lineage analysis based on classic embryologic work clearly indicates that the definitive kidney is derived from two independent tissue compartments of the intermediate mesoderm, namely, the metanephrogenic mesenchyme and the Wolffian duct. This traditional view has been broadened by a set of data derived from embryonic kidney organ culture; (Sariola et al. 1983; Qiao et al. 1995) when uninduced mesenchyme was isolated and tagged so that cells could be followed to their final destination, and then cocultured with isolated ureteric bud, mesenchymal cells were found to be inserted into the collecting duct, although the majority of collecting duct cells were derived from the ureteric bud. Organogenesis of the kidney has long become a model system that represents principles in morphogenesis and cell differentiation. The continuous process of morphogenesis is guided by cascades of interactions between two different cell populations. Regulation involves diverse families of genes and their products, including protooncogene-encoded receptors and their polypeptide ligands, transcription factors and their target genes, and regulating extracellular matrix proteins and cell adhesion molecule-mediated signals. All of these diverse systems interact to initiate and guide embryonic renal morphogenesis and cell differentiation.

As distinct from other tissues, such as bone marrow, it has been difficult to isolate or confirm the existence of stem cells in the kidney, although several studies have suggested the existence of stem cells in the adult renal interstitium (Hishikawa and Fujita 2006; Cotsarelis et al. 1989).

7.2.1 Slow-Cycling Cells in the Papilla

Adult stem cells are considered to have a slow cycling time (Johansson et al. 1999; Lavker and Sun 2000) and thus Oliver et al. tried to distinguish the cells in the kidney by measuring the retention of the nucleotide label bromodeoxyuridine (BrdU), which is incorporated into the DNA of cells during DNA synthesis (Oliver et al. 2004). BrdU was administered to 3-day-old rat and mice pups. At this age, because nephrogenesis in rodents continues after birth, many cells in the kidney were probably dividing and thus could incorporate BrdU. After a chase period of at least 2 months, during which the multiple cell divisions required for kidney growth, would have diluted the BrdU content of most cells, incorporation of BrdU was analyzed in the kidney tissue. Oliver et al. found that only the interstitium of the renal papilla contained an abundant population of cells that retained a strong BrdU signal. They also found that the cells entered the cell cycle and the BrdU signal quickly disappeared from the papilla in transient renal ischemia models. Moreover, the isolated renal papilla cells were multi-potent and displayed other characteristics of adult stem cells, and when they were injected directly into the renal cortex, the cells incorporated into the kidney parenchyma (Horster et al. 1999). Despite these promising data, several approaches have not supported the notion that BrdU retaining cells in the papilla are renal stem cells. Humphreys et al. failed to confirm, the migration and proliferation of papillary BrdU retaining cells after renal ischemia (Humphreys et al. 2011). A report by Adams and Oxburgh (2009) suggested that the BrdU retention was simply explained by the postnatal localization of the cells. They demonstrated that the centripetal cell cycling gradient of postnatal mammalian nephrogenesis (decreasing from cortex to inner medulla) caused BrdU dilution in the bulk of the kidney while the label remained undiluted in cells of the papillary region. However, a novel cell type with epithelial clonogenic capacity and pan-mesodermal potential was recently identified in the renal papillary collecting duct extending the previous observations of a potential papillary stem cell niche (Li et al. 2015).

7.2.2 Side Population Cells

In 1996, Goodell et al. reported a new method of obtaining an enriched population of hematopoietic stem cells from adult bone marrow in a single step by Hoechst 33342 staining and FACS sorting (Goodell et al. 1996). The isolated cells were called side population (SP) cells, and the SP phenotype can be used to purify a stem cell–rich fraction. The SP phenotype is determined by the BCRP1/ABCG2 gene, and enforced expression of BCRP1/ABCG2 prevents hematopoietic differentiation (Zhou et al. 2001) To determine core genes comprising a stem cell genetic program, several comprehensive microarray studies have been performed (Ivanova et al. 2002; Ramalho-Santos et al. 2002). However, the number of overlapping genes

among the reports was limited, and BCRP1/ABCG2 was the only gene that was expressed in ES cells, hematopoietic stem cells and neurosphere cells (Easterday et al. 2003). These results suggest that SP cells may play a role as adult stem cells. In fact, skeletal muscle SP cells (Asakura et al. 2002) may differentiate into endothelial cells (Majka et al. 2003) and bone marrow–derived SP cells into cardiomyocytes, endothelial cells (Jackson et al. 2001) and osteoblast precursors (Olmsted-Davis et al. 2003). Recently, Hishikawa et al. found that kidney SP cells differentiated into multiple lineages in the presence of leukemia inhibitory factor via kidney-specific cadherin 16 (Hishikawa et al. 2005a). Moreover, the function of kidney SP cells was found to be regulated by basic helix–loop-helix transcription factor MyoR, and the cells resided in the interstitial spaces of the kidney (Hishikawa et al. 2005b).

7.2.3 CD133⁺ Cells in the Interstitium

CD133 is a surface marker of endothelial progenitor cells, hematopoietic progenitor cells and neural stem cells. Bussolati et al. reported that CD133-positive cells in the interstitium of the adult human kidney have characteristics of stem cells (Bussolati et al. 2005). The cells expressed the early nephron developmental marker Pax2, as well as several markers typical of bone marrow stromal cells, but were negative for hematopoietic cell markers such as CD34 or CD45. By using different culture conditions, the authors indicated that CD133+ renal cells might be pluripotent, having the capacity to differentiate into either type of tubular cells with the appropriate cues.

The Romagnani group reported the presence of CD133 + CD24 + CD106- cells in the urinary pole of human Bowman's capsule as well as in proximal and distal tubules (Angoletti 2012). These cells showed proliferation upon tubular injury in patients and they engrafted in murine experimental kidney injury generating novel tubular cells (Angelotti et al. 2012). A rodent study from another group came to a different conclusion, indicating that renal CD133 + CD24+ cells represent transiently dedifferentiated tubular cells that are expressing CD24 during the renal repair process but without progenitor potential (Smeets et al. 2013). Ongoing research is trying to dissolve these discrepancies.

7.2.4 rKS56 Cells

In the developing kidney, there are two major distinct areas of cell proliferation, the nephrogenic zone in the outer cortex below the renal capsule and the area in the corticomedullary junction corresponding to the primitive S3 segment of the proximal tubule (Cha et al. 2001). Kitamura et al. dissected individual nephrons from adult rat kidneys, then separated them into segments and cultured them (Kitamura et al. 2005). Outgrowing cells were replated after limiting dilution so that each well

contained a single cell. In this way, they were able to isolate the cell line showing the most potent growth, which they designated rKS56. rKS56 cells expressed immature cell makers relating to kidney development and mature tubular markers. The location of rKS56 cells in kidney tissue is unclear, but rKS56 cells possessed self-renewal and multi-plasticity, and differentiated into mature tubular cells defined by aquaporin1,2 expression under different culture conditions. Recent lineage studies indicated that the normal repair of the renal tubule is governed by terminally differentiated epithelia that re-express apparent stem-cell markers (e.g. CD24) (Kusaba et al. 2014). In the light of these data, it will be crucial to define whether progenitor-like cells, such as rKS56 cells, can be exploited for cell therapy even if their role in normal repair might be limited.

7.3 Kidney Diseases, Regenerative Principles

Most renal diseases can be envisioned as the consequence of a dysbalance between tissue damage and repair. Hypoxia, infection, immune reactions, and toxic substances can damage renal tissue (Rookmaaker et al. 2004; Toback 1992). On the other hand, regenerative mechanisms counteracting the damage inflicted on renal tissues have been reported as well, both in tubuli (Abouna et al. 1983) and the glomeruli (Imasawa et al. 2001). Insights into the nature of these regenerative mechanisms have evolved over the years. In tissues with a high cell turnover like the intestine or the hematopoietic system, organ- or tissue-restricted stem cells have been shown to replace cells that have completed their life cycle. It is becoming increasingly apparent that in organs with a relatively low rate of cellular turnover like the liver and kidney similar regenerative mechanisms are operational (Pabst and Sterzel 1983; Poulsom et al. 2001).

In the mammal, partial nephrectomy stimulates hypertrophy of remaining tissue, even in the contralateral kidney, but not the generation of new nephrons. However, whereas the resection of an adult kidney does not lead to the regeneration achieved in the liver, the mammalian kidney shares with the majority of organs the ability to repopulate and repair structures that have sustained some degree of injury. This process, termed *cellular repair*, can be achieved by reentry into mitosis and proliferation of neighboring cells. As a result, the kidney can undergo significant remodeling in response to acute damage. For example, obstruction of the ureter can result in the near destruction of the kidney medulla, but once the obstruction is removed, there is a rapid process of reconstruction and repair that will regenerate the tubules of the medulla without forming new nephrons. It has been proposed that the cells that elicit such repair come from tubular cell dedifferentiation and migration into the areas odamage before redifferentiation (Humphreys et al. 2011; Kusaba et al. 2014), However, the mammalian kidney seems to have a very limited potential for *structural repair* or true regeneration. While nephrogenesis is occurring in the fetus, there is evidence that a systemic humoral response to nephrectomy allows the enhanced nephrogenesis of the remaining organ. However, nephrogenesis in

mammals ceases just before or shortly after birth, and the birth of new nephrons has never been reported after this point in time. Chronic injury of the kidney, which is responsible for the majority of cases of end-stage renal failure, results in irreversible glomerular and tubular damage and resultant loss of renal function. Hence, mammalian kidneys respond to chronic damage by fibrosis, scarring, and irreversible functional loss.

7.3.1 Regeneration of Renal Endothelium

A number of studies have addressed maintenance and regeneration of the specialized renal glomerular capillaries. In normal rats, the rate of total glomerular cell renewal is about 1 % per day with the endothelial fraction being the most predominant cell type. However, in response to injury, the rate of vascular regeneration could well be increased. An established model to study glomerular injury and repair in rats is experimental anti-Thy1.1 glomerulonephritis. Injection of a complement-fixing antibody to the mesangial cell antigen Thy1.1 causes acute mesangiolysis and matrix dissolution, leading to ballooning of glomerular capillaries, formation of aneurysms, and loss of endothelial cells. In the subsequent repair phase increased proliferation and migration of endothelial and mesangial cells is observed resulting in (partial) restoration of glomerular structure and function. Using this model it was shown that glomerular capillary repair is associated with a marked increase in endothelial cell proliferation (Iruela-Arispe et al. 1995). Several studies have provided evidence that circulating endothelial progenitor cells (EPC) may contribute to glomerular capillary repair. Experiments by Rookmaaker et al. with rat hematopoietic chimeras demonstrated low levels of bone marrow–derived cells staining for the rat endothelial cell antigen RECA-1 (Rookmaaker et al. 2003). The number of these cells gradually increased over time suggesting that EPC contribute to normal physiologic glomerular endothelial cell turnover. Following anti-Thy- 1.1–induced glomerular injury they observed a 4x increase in bone marrow-derived endothelial cells in the glomeruli. These data indicate that glomerular repair cannot only be attributed to migration and proliferation of resident endothelial cells but also involves bone marrow-derived cells. Participation of circulating EPC to renal regeneration has also been demonstrated in human adults. As early as 1969 the presence of acceptor endothelial cells in kidney allografts was first reported (Williams and Alvarez 1969). It has been reported that in human renal transplants the extent of replacement of donor endothelial cells lining the peritubular capillaries by those of the acceptor was related to the severity of vascular injury (Lagaaij et al. 2001). It was suggested that this endothelial replacement could be explained by the involvement of acceptor-derived EPC. Rookmaaker et al. demonstrated male, donor-derived endothelial cells in the renal macrovasculature of a female patient who developed thrombotic microangiopathy after gender-mismatched bone marrow transplantation (Rookmaaker et al. 2002). Taken together these observations confirm a novel role for bone marrow–derived endothelial cells in maintenance and repair of renal endothelium.

7.3.2 Regeneration of the Renal Mesangium

Glomerular mesangial cells provide structural capillary support to the glomerulus and display a smooth muscle cell–like phenotype. They play a central role in the pathogenesis of a number of human and experimental glomerular inflammatory diseases. In particular, mesangial hyperplasia is a prominent histopathologic feature associated with impaired glomerular function. Although transient hyperplasia is thought to reflect a physiologic response required for successful glomerular reconstitution and renal tissue repair, tight regulation of mesangial proliferation, function, and apoptosis is needed for recovery without fibrosis. Initially, mesangial maintenance and repair after injury was thought to depend solely on proliferation of viable resident intraglomerular mesangial cells. These mature mesangial cells dedifferentiate before they proliferate (El-Nahas 2003). Like the glomerular endothelial cells, in normal rats, mesangial cell turnover amounts to less than 1 % per day. Hugo et al. first demonstrated that during recovery of anti-Thy1.1 glomerulonephritis, proliferating immature mesangial cells migrated from the juxtaglomerular apparatus and hilar region into the glomerulus (Hugo et al. 1997). Recently, the same group demonstrated that the responsible cells are renin-positive which represent a major precursor cell source in the adult kidney (Starke et al. 2015). Reminiscent to mesangial cell recruitment during embryonic glomerulogenesis, the involvement of extraglomerular mesangial progenitor cells in glomerular repair was reported by several investigators. The involvement of bone marrow–derived cells in normal mesangial cell turnover was also demoenstrated. Lethally irradiated mice given transplants of T-cell-depleted bone marrow cells from syngeneic donor transgenic for green fluorescent protein (GFP) manifested a time-dependent increase in GFP-positive cells in their glomeruli. When isolated and cultured, these cells stained positive for the mesangial cell marker desmin and the cells contracted in response to angiotensin II, confirming that bone marrow–derived cells have the potential to differentiate into glomerular mesangial cells. Similar experiments with mice transplanted with purified clonally expanded hematopoietic progenitor cells were carried out to confirm the hematopoietic origin of bone marrow–derived mesangial cells (Masuya et al. 2003). In similar experiments, using a rat allogenic bone marrow transplant model and antibodies to the mesangial cell–specific antigen Thy-1 (ox7), this time-dependent increase of bone marrow–derived mesangial cells in the glomerulus was confirmed. Also, a major increase of bone marrow– derived mesangial cells during recovery from anti-Thy- 1.1–induced mesangiolysis in bone marrow transplantation models in rats was observed (Ito et al. 2001). Cornacchia et al. demonstrated that glomerulosclerosis can be transmitted by bone marrow transplantation in mice (Cornacchia et al. 2001). Transplantation of bone marrow cells from sclerosis-prone mice in normal background mice invoked glomerulosclerosis in the recipients. These data not only point to the contribution of bone marrow–derived cells to glomerular maintenance and repair but also show that dysfunctional or diseased mesangial progenitor cells can have a negative influence on the kidney.

7.3.3 Regeneration of the Renal Tubules

The renal tubule is known for its high capacity for regeneration. Acute tubular necrosis, as a result of ischemia or toxic substances, can be followed by active migration and proliferation to restore normal tissue architecture and function. Different sources of these proliferating progenitor cells have been reported. Isolated resident proliferative epithelial cells from the tubuli of mature rabbit kindeys displayed a high capacity for self renewal and differentiated into complete three-dimensional tubular structures in vitro Similar experiments were later performed with human epithelial cells (Humes et al. 1996). Bone marrow-derived extrarenal tubular progenitor cells were reported by Poulsom et al.: In the tubules of renal biopsy specimens from eight male patients transplanted with female kidneys, they found Y-chromosome–positive tubular cells within the tubules that co-expressed epithelial markers (Poulsom). However, the proportion of Y-chromosome–positive tubular cells ranged from 1.8 to 20 %. Gupta et al. reported finding Ychromosome–positive tubular cells in renal biopsies taken from two men transplanted with female kidneys, but the positive cells made up less than 1 % of the tubular cells examined (Gupta et al. 2002). In female mouse recipients of male bone marrow grafts co-localization was observed of Y-chromosomes and tubular epithelial cell markers, suggesting participation of bone marrow-derived cells in normal tubular cell turnover. Moreover, bone marrow ablation diminished functional recovery after tubular ischemia, while infusion of a progenitor cell reversed this effect, suggesting an important functional role for the hematopoietic stem cell in tubular repair. When male kidney transplant patients who received a female kidney and who recovered from acute tubular necrosis were studied, a Y chromosome could be demonstrated in few (less than 1 %) of the tubular cells. This is consistent with the observation that surviving tubular cells are the major contributor to repopulating the injured kidney epithelium in murine lineage dilution studies (Humphreys et al. 2011). However, the data from human transplantation do provide us with a proof-of-principle observation of bone marrow derived tubular repair. The functional importance of this phenomenon in the clinical situation is still uncertain but opens the possibility for potential therapeutic exploitation.

7.4 Clinical Implications

Most therapies in nephrology focus on reducing renal damage. However, insight in renal repair and maintenance may offer new therapeutic strategies. Progenitor cells appear to participate in renal repair and turnover of the major renal cell types. Therefore, renal progenitor cells may encompass a new target for therapeutic strategies aimed at the reduction or even prevention of renal disease. Such strategies could be directed toward different populations of progenitor cells. The advantage of circulating progenitor cells may be that they are more accessible for isolation in

comparison to the resident progenitor cells. Autologous progenitor cells from the patient are preferable to allogenic progenitor cells because of possible rejection. Obviously, in case of inherited progenitor cell disease, allogenic cells should be considered. One approach to harness progenitor cells for therapeutic purposes is to increase the available pool of progenitor cells. Such expansion may be achieved by growth factor therapy both in vivo and ex vivo. VEGF and erythropoietin are probably good candidates to stimulate progenitor cell–mediated endothelial repair. Both have EPC mobilizing and proangiogenetic activities (Kale et al. 2003; Asahara et al. 1999; Bahlmann et al. 2003; Heeschen et al. 2003).

Another approach to enhance cellular repair and maintenance is reinforcement of progenitor cell function. EPC dysfunction has been shown in diabetic patients Sorrentino et al. 2007). The decreased re-endothelialization capacity of EPCs from diabetic patients was restored after oral therapy with the PPAR-γ agonist rosiglitazone.

Mesangial and mesangial progenitor cell dysfunction has been described, too (He et al. 1996). Replacement of these prosclerotic cells by healthy allogenic mesangial progenitor cells may potentially reduce or even prevent progressive renal disease. The relatively low turnover rate of mesangial cells of 1 % per day might however hamper this strategy. Controlled mesangial injury by pharmacologic agents combined with healthy allogenic or transfected autologous mesangial precursor cell infusion might increase mesangial turnover and improve cell replacement. Finally, progenitor cells can be used as so-called “magic bullets.” Progenitor cells are able to home and participate in their target tissue. This ability can be used to deliver certain gene products very locally. Gene therapy has already successfully been used. Transfection of skeletal muscles with the gene of a transforming growth factor-b1 (TGFb1) inhibitor was able to reduce glomerulosclerosis in a rat nephritis model (Isaka et al. 1996).

7.4.1 Stem Cell Aging Leads to Regenerative Loss

The renal capacity for normal regeneration is reduced with advancing age (Schmitt and Cantley 2008). This deficit contributes to an increased risk for acute and chronic kidney disease in the elderly. Advanced age is known to affect repair processes in all organs and was linked to an exhaustion of endogenous stem cells in terms of functionality and numbers (Behrens et al. 2014). Although it is currently not clear whether age-dependent changes in stem cell biology have direct effects on kidney aging deleterious effects have been clearly demonstrated for other organ systems in which stem cells loose paracrine activity, proliferation potential and efficiency of transplantation with advanced age (Ju et al. 2007). In the light of our aging society with an increasing longevity major efforts to improve organ repair using stem cell rejuvenation strategies are undertaken. An important potential target has been identified in the aged cellular environment which might disrupt stem cell functionality. In parabiotic pairing experiments it was found that the regenerative potential of old

stem cells markedly improved when exposed to a young systemic environment (Brack et al. 2007). Recent advances in deciphering distinct systemic factors that are responsible for the underlying rejuvenation effect (Sinha et al. 2014) might help to develop pro-regenerative approaches that could also be used to improve kidney regeneration in aging.

7.5 Clinical Studies, Experience, Outcome/Side Effects of Kidney Regenerative Therapies

Preclinical studies suggest that the administration of exogenous stem cells may ameliorate acute kidney injury and accelerate regeneration (Table 7.1). In consideration of the role of endogenous bone marrow-derived stem cells, a possible approach could also be stem cell mobilization. However, the possible effects of bone-marrow-recruited cells and of inflammatory cells in this experimental setting require further investigation. Currently, the most promising approach may be the administration of in vitro expanded mesenchymal stem cells (also referred to as mesenchymal stromal cells) applied to acute tubular and glomerular injury (Bussolati et al. 2009; Fleig and Humphreys 2014). Injected mesenchymal stem cells were shown to home to the injured kidney and to accelerate morphological and functional regeneration, possibly by a paracrine or even endocrine mechanisms. A meta-analysis of 21 experimental studies demonstrated a consistent improvement in renal function after acute administration of mesenchymal stem cells (Wang et al. 2013). A major role in the effect of mesenchymal stem cells has been attributed to the production of growth factors and cytokines with immunosuppressive, antiinflammatory, anti-apoptotic and proliferative effects. Further research is needed to characterize the underlying soluble factors that are produced by mesenchymal stem cells. Several clinical phase 1 and 2 trials are in progress to evaluate the effect of mesenchymal stem cells administration in renal transplantation, acute renal injury or chronic allograft nephropathy (Table 7.2, www.clinicaltrial.gov). So far, reported data showed that intravenous treatment with mesenchymal stem cells seems to be safe and well tolerated in renal transplant recipients and some initial results suggested effective immune suppression. Trials that will evaluate the effect of mesenchymal stem cells administration in native kidneys with chronic renal damage are currently in the recruiting phase (Table 7.2).

7.6 Conclusions and Future Perspectives on Kidney Regenerative Therapies

Acute and chronic kidney diseases have a complex pathophysiology that may involve both ischemic and inflammatory as well as immunological injury. In contrast to most current pharmacological agents that target only a single

Table 7.1 Therapeutic administration of stem cells in experimental animal models of renal damage

References	Model	Stem cells	Outcome
Morigi et al. (2004)	Cisplatin ARF	MSC	Improved urea; decreased tubular damage
Togel et al. (2005)	I/R ARF	MSC	Improved creatinine; lower renal injury score
Lange et al. (2005)	I/R ARF	MSC	Improved creatinine; lower renal injury score
Duffield and Bonventre (2005)	I/R ARF	MSC	Improved creatinine
Broekema et al. (2005)	I/R ARF	MSC	Morphological / functional recovery
Bi et al. (2007)	I/R ARF	MSC	Morphological / functional recovery
Kale et al. (2003)	I/R ARF	MSC	Morphological / functional improvement
Herrera et al. (2004, 2007)	Glycerol ARF	MSC	Improved creatinine; decreased tubular damage
Ninichuk et al. (2006)	$^{5}/_{6}$ nephrectomy	MSC	Decreased interstitial fibrosis
Kunter et al. (2006)	MPGN (Anti-Thy1.1)	MSC	Decreased mesangiolysis, improved creatinine and decreased proteinuria
Uchimura et al. (2005)	MPGN (Anti-Thy1.1)	MSC	Decreased glomerular injury score
Wong et al. (2008)	MPGN (Anti-Thy1.1)	MSC	Decreased glomerular injury score
Rookmaaker et al. (2002, 2007)	MPGN (Anti-Thy1.1)	MSC, EPC	Recovery and vascularization
Prodromidi et al. (2006)	Alport	MSC	Improved renal function, decreased glomerular scarring and interstitial fibrosis
Sugimoto et al. (2006)	Alport	MSC	Partial restoration of expression of type IV collagen α3 chain with concomitant α4 and α5 chain expression, improved glomerular architecture, reduction of proteinuria
Chade et al. (2009)	Renal artery stenosis	EPC	Decreased microvascular remodeling, preserved microvascular architecture

ARF acute renal failure, *MSC* mesenchymal stem cells, *I/R* ischemia/reperfusion, *MPGN* mesangioproliferative glomerulonephritis, *EPC* endothelial progenitor cells

pathophysiological pathway, cell-based therapies such as mesenchymal stem cells act through multiple mechanisms and have the potential to target immunological, vascular and inflammatory pathways. In addition, mesenchymal stem cells have the capacity to engraft and survive long-term in a specific target tissue and are both non-immunogenic and immunosuppressive. This has important implications for the therapeutic application of mesenchymal stem cells in tissue repair and regeneration, in that mesenchymal stem cells derived from healthy unrelated volunteer donors can be cryopreserved, thus making them available in a timely manner for patients in

Table 7.2 Current clinical trials using stem cells to treat kidney disease

Title	Disease	Cells	Trial	Time	Clinical trials.gov identifier
Allogenic multipotent stromal cell treatment for acute kidney injury following cardiac surgery	Acute renal failure	MSC	Phase I, non-randomized, open label, single group assignment	8/2008 – 7/2009	NCT00733876
Effect of mesenchymal stem cell transplantation for lupus nephritis	Lupus nephritis	MSC	Phase I + II, open label, active control, single group assignment	5/2008 – 5/2010	NCT00659217
Mesenchymal stem cell transplantation in recipients of living kidney allografts	Kidney Transplantation	MSC	Randomized, open label, active control, parallel assignment	3/2008 – 3/2009	NCT00658073
Mesenchymal stem cell transplantation in the treatment of chronic allograft nephropathy	Chronic allograft nephropathy	MSC	Phase I + II, open label, historical control, single group assignment	5/2008 – 5/2010	NCT00659620

a variety of acute and chronic clinical settings. The clinical application of mesenchymal stem cells is broad and has generated significant interest in clinicians from diverse fields, with preclinical and clinical data in a wide variety of conditions, including osteogenesis imperfecta, osteoarthritis and cardiac regeneration. However, many questions remain about the basic biology and long-term safety of mesenchymal stem cells. More research is needed to understand the physiological role of these cells, their stimuli for migration and the pathways that mediate their apparent beneficial effects in regeneration and repair. Protocols that limit the differentiation potential of the cells into a specific lineage when used for treatment of a specific disease are needed, along with studies that determine the correct dose, schedule and administration route. Despite the lack of apparent adverse effects seen in trials to date, longer-term follow up is required given the possibility of malignant transformation (McTaggart and Atkinson 2007) And although there is excitement about the application of many of these novel regenerative approach. es, many hurdles remain. The unique architecture of the kidney creates substantial obstacles to the functional integration of a stem cell–derived nephron. Indeed, despite major recent advances the functional capacity of a bioengineered organ to provide anything like the filtering and resorptive capacity of the endogenous kidney is doubtful.

The final major obstacle is the degree of damage that is present in a patient with chronic renal disease. It is unlikely than any organ-based repair process will over-

come the extent of damage that is seen in a patient who has reached end-stage renal failure. This has major implications for the adoption of any autologous therapy. Even if an adult stem cell population does exist in the adult kidney, would it remain in an end-stage kidney? Indeed, the adoption of any organ-based cellular therapy is likely to succeed only if chronic renal disease can be diagnosed early and if such therapies are implemented well before end-stage renal failure is reached. As we move closer to that point in time, the ethical debate about whether trials can proceed before end-stage renal disease will become critical. A lack of surrogate end points with which to assess the success of a cellular therapy in renal disease will make clinical trials long and expensive, eroding the will of the developers to continue to support the trials.

However, the imperative to continue to forge such novel approaches is clear from the rate at which the incidence of chronic renal failure is rising in both the developed and the developing world. In the end, it is unlikely that any such therapies will produce a physiologic outcome that is equivalent to that of a healthy kidney, but as patient numbers inevitably increase the use of dialysis for treatment, a novel therapy that creates an improvement over dialysis will become not only a major achievement but also a necessity.

7.7 Recent Developments

During the last 2 years several areas of renal regeneration have been further developed. A major achievement has been the demonstration of regeneration in the fish. Mammals can partly repair their nephrons, but cannot form new ones. By contrast, fish add nephrons throughout their lifespan and regenerate nephrons *de novo* after injury providing a model for understanding how mammalian renal regeneration may be therapeutically activated. We have shown in the shark kidney that specific stem cells are the main source for regenerating renal tissue (Elger et al. 2003). . In fact, fish exhibit a multifactorial regenerativeresponse to renal injury that distinguishes them from mammalian species; they restore nephron epithelia and make new nephrons. Davidson et al. traced the source of new nephrons in the adult zebrafish to small cellular aggregates containing nephron progenitors (Diep et al. 2011). Transplantation of single aggregates comprising 10–30 cells is sufficient to engraft adults and generate multiple nephrons. Serial transplantation experiments to test self-renewal revealed that nephron progenitors are long-lived and possess significant replicative potential, consistent with stem-cell activity. Transplantation of mixed nephron progenitors tagged with either green or red fluorescent proteins yielded some mosaic nephrons, indicating that multiple nephron progenitors contribute to a single nephron. Consistent with this, live imaging of nephron formation in transparent larvae showed that nephrogenic aggregates form by the coalescence of multiple cells and then differentiate into nephrons. Taken together, these data demonstrate that the zebrafish kidney probably contains self-renewing nephron stem/progenitor cells. The identification of these cells paves the way to isolating or

engineering the equivalent cells in mammals and developing novel renal regenerative therapies.

Another area is the identification of novel molecules which regulate renal repair and regeneration. Molecules associated with the transforming growth factor β (TGF-β) superfamily, such as bone morphogenic proteins (BMPs) and TGF-β, are key regulators of inflammation, apoptosis and cellular transitions. Kalluri and his colleagues have shown that the BMP receptor activin-like kinase 3 (Alk3) is elevated early in diseased kidneys after injury. They also found that its deletion in the tubular epithelium leads to enhanced TGF-β1-Smad family member 3 (Smad3) signaling, epithelial damage and fibrosis, suggesting a protective role for Alk3-mediated signaling in the kidney Sugimoto et al. 2012). A structure-function analysis of the BMP-Alk3-BMP receptor, type 2 (BMPR2) ligand-receptor complex, along with synthetic organic chemistry, led them to construct a library of small peptide agonists of BMP signaling that function through the Alk3 receptor. One such peptide agonist, THR-123, suppressed inflammation, apoptosis and the epithelial-to-mesenchymal transition program and reversed established fibrosis in five mouse models of acute and chronic renal injury. THR-123 acts specifically through Alk3 signaling, as mice with a targeted deletion for Alk3 in their tubular epithelium did not respond to therapy with THR-123. Combining THR-123 and the angiotensin-converting enzyme inhibitor captopril had an additive therapeutic benefit in controlling renal fibrosis. Their studies show that BMP signaling agonists constitute a new line of therapeutic agents with potential utility in the clinic to induce regeneration, repair and reverse established fibrosis.

A fascinating area of kidney regeneration is the use of progenitor cells for the repair and regeneration of damaged renal tissue. There is a pressing need for improved strategies to arrest or reverse intra-renal injury in kidneys with chronically impaired blood flow. Endogenous endothelial progenitor cells (EPC) are often mobilized to mediate neovascularization and endothelial replacement that contribute to healing ischemic tissues. The mobilization from bone marrow and subsequent homing of progenitor cells can be regulated by a variety of mediators such as stromal cell-derived factor (SDF)-1, stem cell factor (SCF), erythropoietin (EPO), or angiopoietins, which are released by injured tissue to attract the cells and ensure their adherence. In turn, the cells express corresponding cognate receptors such as CXCR4, cKit, EPO-receptors (EPO-R), and Tie, respectively, which allow them to be recognized, recruited, and retained at the injured tissues Ebrahimi et al. 2012; Chade et al. 2010 Jun).

However, the endogenous system may be overwhelmed or dysfunctional, and hence fail to repair the tissues. Therefore, exogenous delivery of EPC collected and expanded in-vitro offers the potential for targeted treatment of conditions such as chronicallydamaged kidneys. Lerman and co-workers have recently shown the beneficial effects of intra-renal administration of autologous EPC in a porcine model of chronic non-atherosclerotic RAS. Conceivably, a decrease in tissue damage may resolve the injury signals and homing cues that it releases.

Specific signals that portend chronic ischemic injury and regulate the homing and adherence of endogenous circulating cells into the ischemic kidney, or the abil-

ity of successful renal repair to alleviate these signals, have not been elucidated. Lerman et al. tested in an experimental study the hypotheses that, firstly, renovascular disease activates homing signals detectable in both the ischemic kidney and EPC, and secondly, that these signals are attenuated upon renal repair using selective intra-renal cell-based therapy. For this purpose they utilized a pig model of experimental atherosclerotic RAS (ARAS), which recapitulates many characteristics of early human atherosclerotic renovascular diseass. Pigs were treated with intra-renal autologous EPC after 6 weeks of ARAS. Four weeks later, expression of homing-related signals in EPC and kidney, single-kidney function, microvascular density, and morphology were compared to untreated ARAS and normal control pigs. Compared to normal EPC, EPC from ARAS pigs showed increased stromal cell-derived factor (SDF)-1, angiopoietin-1, Tie-2, and ckit expression, but downregulation of erythropoietin and its receptor. The ARAS kidney released the ckit-ligand stem-cell factor (SCF), uric acid, and erythropoietin, and upregulated integrin β2, suggesting activation of corresponding homing signaling. However, angiopoietin-1 and SDF-1/CXCR4 were not elevated. Administration of EPC into the stenotic kidney restored angiogenic activity, improved microvascular density, renal hemodynamics and function, decreased fibrosis and oxidative stress, and attenuated endogenous injury signals.

With the advent of induced pluripotent stem cell (PSC) technology, it is now possible to derive patient-specific cell lines that can be used for both basic research and the development of new therapeutic options in human disease. The importance of PSC technology is underlined by the fact that there were only 6 years between the discovery of induced PSC reprogramming [Takahashi] and the award of the Nobel Prize in Medicine to Shinya Yamanaka in 2012. Despite major advances in PSC use for regeneration in several organs it is still challenging to generate functional kidney structures from these cells. Recently Taguchi et al. introduced a model, which recapitulates the in vivo stepwise nephrogenesis with induction of metanephric nephron progenitors from both mouse and human PSC (Taguchi et al. 2014). Transplantation of the metanephric nephron progenitors under the kidney capsule of immunodeficient mice induced massive tubulogenesis and vascularized glomeruli. Although the PSC derived structures did not produce urine, these results are a major first step towards obtaining functional kidney tissue using PSC technology.

7.8 Conclusion

The ARAS kidney releases specific homing signals corresponding to cognate receptors expressed by EPC. EPC show plasticity for organ-specific recruitment strategies, which are upregulated in early atherosclerosis. EPC are renoprotective as they attenuated renal dysfunction and damage in chronic ARAS, and consequently decreased the injury signals. Importantly, manipulation of homing signals may potentially allow therapeutic opportunities to increase endogenous EPC recruitment.

These studies may allow novel clinical studies whereby EPC are used in patients after opening an atherosclerotic renal artery to enhance the regeneration of the damaged and destroyed tissue. A similar approach using mesenchymal stem cells forms the basis for a clinical study which is currently in the recruitment phase (Table 7.2). The identification of intra-renal stem cells, novel molecules which regulate tissue repair and the use of progenitor cells may be the areas where clinically relevant progress for the repair and regeneration of renal tissue will be made.

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Chapter 8
Urogenital Tract

B. Amend, W.K. Aicher, and Arnulf Stenzl

Abstract Urogenital tract diseases with potential relevance for regenerative therapies affect different parts of the urogenital system. Domains of urogenital medicine are the upper and lower urinary tract, urinary incontinence, erectile function and pelvic organ prolapse. Thereby congenital, traumatic, oncologic, inflammatory, or iatrogenic causes may be responsible for physical and psychological impairment of affected humans. Regenerative medicine in urology consists of cell-based regenerative therapy, the application of different scaffolds and increasingly a combination of both regenerative principles. A widespread collection of preclinical studies in all fields of interest resulted in a considerably smaller quantity of clinical studies. However, only few established applications have been introduced into clinical routine. Particularly potentially poor clinical study arrangement and lacking or deficient cross-national counselling may be responsible for the imbalance of excellent fundamental research and legally approved regenerative therapies. Currently increasing cooperation and growing insight into stem cell mechanisms will offer the possibility of extended success in the future.

Keywords Tissue engineering • Stem cell therapy • Urinary tract • Scaffolds • Urinary incontinence • Erectile dysfunction • Reconstructive urology

8.1 Introduction

Urogenital cancer, urinary incontinence, erectile dysfunction, ureteral and urethral stricture, congenital malformations or pelvic organ prolapse – all of these exemplarily listed urogenital diseases impair lifetime and quality of life in their own way and intensity. Current guideline-based therapeutic approaches of academic medicine facilitate treatment of the diseases, but frequently complete rehabilitation namely "restitutio ad integrum" could not be promised or reached for the respective individual. Sometimes only symptomatic improvement can be offered. This situation poses the access for regenerative medicine. Atala, certainly one of the most well-known stem cell researcher in urology, stated in 2005: "In regenerative medicine,

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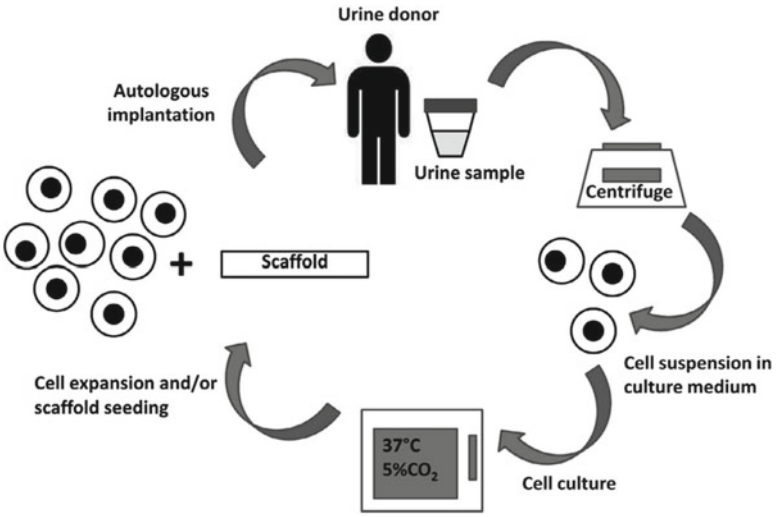

Fig. 8.1 Autologous implants generate from urine samples (Reprint from Kloskowski et al. (2015) with permission of Elsevier)

efforts are currently underway to develop virtually every type of tissue and organ within the human body" (Atala 2005). Ten years later this sentence still encourages young scientists in regenerative medicine but actually we are still several steps away of a comprehensive clinical use of regenerative principles (Atala 2012).

Unchanged critical questions of stem cell research include effective cell resources, practical scaffolds for cell application and especially a wise handling of ethical considerations. The optimal combination of these factors needs to be identified to reach the goal of target-oriented and long-term active application of regenerative therapeutic strategies (Kollhoff et al. 2011).

Scaffolds for application in the genitourinary tract either consist of synthetically arranged biodegradable substances or have been extracted as acellular matrices from different tissues or organ. For the latter possibility the acellular bladder matrix became a challenge for intensive research during the last decade. In contrast to the limited numbers and kinds of scaffolds employed in different studies, a large variety of cells has been used with different outcome in urogenital tissue regeneration. Embryonic stem cells and induced pluripotent stem cells are characterized by a wide differentiation potential but o ethical and safety concerns limit their therapeutic applications in some countries. Reasonable alternatives are tissue-derived progenitor cells with predetermined differentiation potential or adult multipotent stem cells, both of them naturally available in necessary amounts (Smaldone et al. 2009; Lin 2010). Urological specificity may be reached with the development of inherent available urine-derived stem cells. Kloskowski et al. gave a nice illustration of the re-use of a putative "waste product" in Fig. 8.1 (Kloskowski et al. 2015).

The present chapter introduces relevant urological indications for regenerative medicine with special attention to current treatment options, diagnostics, limitations and possibilities of cell-based therapy as well as fundamental and clinical studies.

8.2 Urogenital Tract Development, Stem Cell Function

In human embryonic development, the urinary system arises from the intermediate mesenchyme, which is mainly responsible for the development of the kidney (see Chap. 7), internal genitalia and their ducts. Between about days 18 and 26 of growth the urogenital ridge becomes detectable. In the fifth week, development of permanent kidneys is observed and in the ninth week they become functional. Now the ureteric bud and its stalk generate the ureter. Parallel – between week 5 and 12 – the bladder develops by a separation of the cloaca by the urorectal septum to generate the urogenital sinus and the rectum. The epithelia of the bladder are derived from the endoderm of the urogenital sinus, the other tissues of the bladder wall develop from the adjacent mesenchyme. Again, the epithelium of the female urethra and most parts of the male urethra are derived from the endoderm of the urogenital sinus, and only the distal part of the epithelia of the penis is derived from ectodermal cells. The connective tissue of bladder and urethra including the smooth muscles are derived from the splanchnic mesenchyme (Schiebler et al. 1999). Detailed information regarding the structure and function of the ureter, bladder and urethra has been delineated from studies in embryology, molecular analyses of congenital abnormalities in humans, and in combination with suitable models. The later include studies using recombinant animals.

Malfunctions of the renal and urogenital organs are relatively common. They represent about a third of all abnormalities diagnosed by fetal ultrasound analyses (Woolf 2000) and they occur in about 0.5 % of all live births (Yosypiv 2012). Since hemodialysis and exchange of carbon dioxide and oxigen are performed through the placenta efficiently, intrauterine production of urine is not considered to be essential for a healthy development of a fetus. Therefore functional defects often become detrimental only after birth and contribute to chronic renal failure in children (Drozdz et al. 1998). Advances in diagnostic and surgical technologies paved way to better treatment of such infants and children. Nowadays babies can be treated by dialysis and toddlers may receive organ transplants when they are about 12 months of age.

Analyses of pedigrees facilitated a detailed mapping of congenital syndromes and a search at the Online Mendelian Inheritance in Man (OMIM) at www.omim.org in September 2015 yielded more than 7000 entries for the key words "urinary system congenital" and roughly 5000 entries each for the key words "*bladder abnormalities*" or "*urethral abnormalities*", respectively. As this list is updated on a regular basis, the reader is referred to investigate by the OMIM tools.

The advent of recombinant gene technology enabling researchers to generate animal models of development or diseases by general or conditional overexpression or knock-out of genes complemented the studies of familiar cases in many instances. Functional mutations or knock-down of the genes encoding the Zinc-finger transcription factor Wilms Tumor-1 (WT1) caused renal agenesis (Kreidberg et al. 1993) and WT1 is also involved in renal tumorigenesis (Lee et al. 1999). However, data delineated from animal models have to be interpreted with outmost care as the genetic background of the animal model may influence the effects of a mutation, transgene or knock-out, and

sometimes several factors involved a given pathway have to be modulated in order to give the full phenotype of corresponding malfunction or disease observed in humans (Vogelstein and Kinzler 2004; Frese and Tuveson 2007). In addition, chemical manipulations ("poisoning") of developing tissues and organs helped to detect mechanisms of development and of cancer (Hill et al. 2005; Poirier 2012).

Among important transcription factors and proto-oncogenes involved in urogenital development is the paired box 2 (PAX2) gene. Mutations of PAX2 yield in nerval defects, renal abnormalities and vesicourethral reflux, and similar abnormalities were reported Krd mice (Sanyanusin et al. 1995). Lack of functional PAX2 causes different perturbances in urinary tract growth, kidney development, renal hypoplasia and other defects (Woolf 2000). Experimental overexpression of PAX2 in mice yields renal cysts (Dressler et al. 1993). Congenital abnormalities of the kindey and urinary tract have been associated with other genes as well, but in most cases evidence for single gene malfunctions are missing (Yosypiv 2012). The situation is complex as healthy, symptom-free individuals have been identified which carry a mutation in a given gene, which was associated with urinary tract symptoms in others. This finding suggested that both, congenital defects arising early after births as well as the development of symptoms at later stages of live may be caused by a multifactorial etiology. To solve this issue, studies in bioinformatics and systems biology are well on their way to explore the regulatory networks resulting in malfunction of individual cells, tissues and complete organs.

Stem and progenitor cells resident in a given tissue are important for its permanent rejuvenation and in case of tissue damage or degeneration for the regeneration of the defect. Stem and progenitor cells therefore hold great promise when a part of the healing process is exported temporarily to a laboratory in the course of tissue engineering or when such cells are employed as drug to act in situ in patients. For regenerative medicine and tissue engineering of bladder, sphincter and urethra different types of progenitor cells stick out. (A) The urothelial progenitors give rise to the urothelial cells that cover the bladder and urethra. (B) Mesenchymal stromal cells (MSC) and pericytes give rise to smooth muscle cells (to target e.g. the lissosphincter), (C) Myoblasts which generate multinuclear myotubes (for regeneration of the rhabdosphincter), and (D) the epithelial progenitors which cover in males the distal part of the urethral tubing. These progenitor cells reside in the corresponding tissues, sometimes even in geographically defined stem cell niches (Mitsiadis et al. 2007). Normally true stem cells and progenitor cells are not expected to proliferate at high rates in situ. This enables them to provide a life-long basis for cellular rejuvenation and at the same time prevents premature replicative senescence in the stem cell pool (Ballew and Lundblad 2013). When stem cells divide, proliferation and differentiation competent progenitors arise which then represent the cellular source for the actual tissue rejuvenation or regeneration while the true stem cells returns to a non-proliferative stage of the cell cycle. Due to the toxic stress urine causes, the epithelia of bladder and urethra require a constant regeneration. Therefore numbers of proliferating progenitor cells may be more frequent in this tissue compared to types of tissues. In addition, during proliferation

cells have to detach at least in part from the extra cellular matrix and/or neighbouring cells to grant space for the daughter cells. This partial detachment of proliferating progenitor cells together with the natural stretch of bladder and urethra and the shear-forces of fluids in bladder and urethra facilitates a complete detachment of a few cells. But this is sufficient to isolate different proliferation competent progenitor cells from urine samples, including urothelial precursors (Zhang and Atala 2013) and urine-derived mesenchymal stromal cells Fig. 8.1 (Bharadwaj et al. 2011; Zhang et al. 2014).

While the urothelial precursor cells, myoblasts and epithelial progenitors seem to be mostly limited to differentiate in to one type of effector cells (= unipotent progenitor cells), the mesenchymal stromal cells (MSC) and the closely related pericytes are multipotent progenitor cells. Based on recommendations of consensus conferences human bone marrow-derived MSC were defined as fibroblast-like cells, expressing a set of mesenchymal cell surface antigens, and differentiating in vitro at least in three different types of cells (Dominici et al. 2006; Horwitz et al. 2005). MCS and pericytes were described many different tissues and were investigated in detail (Caplan 2008). MSC and pericytes share many features, but show quite distinct characteristics as well (Crisan et al. 2008; Mitchell et al. 2006; Pilz et al. 2010; Aicher et al. 2011). Depending on the environment MSC may differentiate along the smooth muscular, adipogenic, chondrogenic, osteogenic and other lineages lineages (Crisan et al. 2008; Pittenger et al. 1999; Zuk et al. 2001; In ‘t Anker et al. 2004; Bu et al. 2014). Therefore they are well-studied cells in the field of regenerative medicine and tissue engineering.

However, at least in vitro the efficacy of differentiation seems to be not very high. This finding is no problem in translational research but may become a difficulty in a clinical context when all components applied to human patients in the course of a study have to meet the quality criteria of the health authorities (e.g. EMA, London). The efficacy of differentiation also depends on the source of the cells (Sacchetti et al. 2007) and cell culture conditions (Muraglia et al. 2000). In most studies published, MSC were prepared as bulk cultures. They therefore represent a blend of cells. This may contribute to the overall low efficacy of in vitro differentiation (Aicher et al. 2011). In contrast, by using monoclonal antibodies to enrich defined MSC subsets ex vivo, an improved adipogenic and chondrogenic differentiation of MSC was achieved (Battula et al. 2009). Comparably, another antibody allowed the separation of more osteogenic from less osteogenic MSC (Ulrich et al. 2013; Amend et al. 2015). To the best of our knowledge, monoclonal antibodies for enrichment of MSC with improved differentiation efficacy towards the smooth muscular lineage have not been disclosed. But differentiation to yield mature cell or long-term survival and functional integration seems not to be required for the therapeutic success of MSC applications (Passipieri et al. 2014). The regenerative activity of MSC has also been associated with their paracrine activities (Caplan and Correa 2011).

In situ MSC are an important source for paracrine release of growth factors and cytokines modulating not only inflammation and immune reactions (Uccelli et al. 2007; Adamowicz et al. 2013), but also wound healing (Caplan and Correa 2011;

Table 8.1 Pathophysiology of urogenital diseases

Pathophysiology	Urogenital example
Oncologic	Bladder cancer with radical cystectomy
	Retroperitoneal metastasis with ureteric stricture
Traumatic	Urethral stricture after urethral trauma
	Neurogenic bladder after spinal cord injury
Inflammatory	Retroperitoneal fibrosis with ureteric stricture
	Urethral stricture after bacterial infection
Hereditary	Bladder exstrophy
	Hypospadia
Iatrogenic	Stress urinary incontinence after radical prostatectomy or radiotherapy
Neurogenic	Detrusor underactivity with urinary retention
	Erectile dysfunction
Vascular	Erectile dysfunction
Aging/Atrophy	Pelvic organ prolapse

Bhargava et al. 2008). Human MSC express among other cytokines transforming growth factor β1 (TGFβ1), hepatocyte growth factor (HGF), insulin-like growth factor-1 and -2 (IGF-1, -2), basic fibroblast growth factor (bFGF) (Madrigal et al. 2014) (and unpublished observations). These factors are known to facilitate the regeneration of striated muscle and smooth muscle tissues.

Moreover, the health of the cell donor has a significant influence on the regenerative potential of MSC, and cells from healthy donors yielded a significantly better renal regeneration in an animal model of renal malfunction (= *Lpr* mice) and immuno modulatory activity compared to MSC from lupus patients (Collins et al. 2014). Such differences in the regenerative potential of MSC were not observed by in vitro experiments. This reinforces the importance of suitable animal models to study regenerative agents in a relevant in vivo context. It also suggests that autologous treatment with MSC may not grant optimal results when the biological condition of the patient is compromised in the first place. In such cases stromal cell donations by healthy volunteers may become a clinical need.

8.3 Urogenital Tract Diseases, Regenerative Principles and Current Therapies

Different parts of the urinary tract can be affected by diverse underlying pathophysiological mechanisms. Table 8.1 summarizes possible causes and gives a review about urological examples. Thereby organs can be impaired directly or through a restriction of a superordinate system (e.g. neuronal/vascular diseases).

8.3.1 Ureter

Although malformations of the ureter are common, the need of additional tissue to treat these patients is rare compared to the large group of patients with ureteric stricture. Strictures arise either from traumatic injury, extrinsic compression due to retroperitoneal fibrosis or metastasis or intrinsic causes like carcinoma of the upper urinary tract, benign reasons (e.g. endometriosis) or endoscopic treatment of different ureteric diseases (iatrogenic). To date endoscopic or percutaneous stenting is the initial treatment option and in many patients the long-term treatment as a result of age or ineffective treatment alternatives. Endoscopic treatment of strictures either by balloon dilatation or incision by cold-knife or laser surgery often results in recurrent occlusion. If patients are willing and feasible for open or laparoscopic surgery, reconstruction of the ureteric can be performed with different techniques: ureteroureterostomy, simple ureteroneocystostomy, psoas hitch and boari technique for impairment of the middle or lower ureter (success rates >80 %) and ileal ureter replacement for the upper third or long distance strictures of the ureter (anastomotic stricture rate 6 %). Autotransplantation of the involved kidney is reserved for selected cases. Especially ileal ureter interposition comprises short and long-term risks for the patient: beside the surgical extent bowel segments may influence kidney function, vitamin B12 metabolism and acid-base equilibrium (Campbell et al. 2007; Guidelines on Urological Trauma 2015; Guidelines on Urothelial Carcinomas of the Upper Urinary Tract 2015).

8.3.2 Urinary Bladder: Physical and Functional

Malfunction of the urinary bladder can be subdivided into diseases affecting the structural integrity of the urinary bladder and diseases, which disturb the physical functioning of the bladder. Bladder function includes the compliance of the bladder wall to ensure filling volumes between 300 and 500 ml, day- and night-time continence and controlled micturition (Abrams et al. 2013).

8.3.2.1 Bladder Cancer

Bladder cancer this is the second most common genitourinary cancer. Worldwide about 380,000 cases of bladder cancer are newly diagnosed and 150,000 deaths per year are recorded. Main risk factors for bladder cancer in Western countries are smoking and exposure to chemicals. Non-muscle-invasive bladder cancer can be treated endoscopically with or without additional installation therapy in most cases, whereas muscular invasion leads to a more extensive treatment: radical cystectomy.

After cystectomy patients need surgical urinary diversion. The kind of diversion depends on oncological, physical and patient-depending factors. To date the orthotopic ileal neobladder and the incontinent ileal conduit are the most common surgical options. Again, similar to ureteral reconstruction, the use of bowel segments, which are typically between 40 and 100 cm long, are accompanied by multiple short and long term side effects for the patient. In addition the body image is negatively influenced in patients with an incontinent urinary diversion. Continent cutaneous reservoirs formed out of bowel segments with the need for intermittent catheterization and an ureteroileosigmoidostomy are treatment options or selected patients, which are informed about the increased rate of side-effects such as functional stone formation, infection and malignant transformation (Campbell et al. 2007; Guidelines on Chronic Pelvic Pain 2015; Guidelines on Muscle-invasive and Metastatic Bladder Cancer 2015).

8.3.2.2 Functional Urinary Bladder Complications

Urge urinary incontinence and detrusor underactivity with residual urine are contrary diseases, but in both cases either neuronal problems with detrusor innervation or intrinsic factors of the urinary bladder wall (muscular or intrinsic nerve system) can be responsible for the patient's symptoms. Current disease management can only focus on symptomatic treatment – causal solutions have not been identified yet.

Urge urinary incontinence can be addressed by behavioural therapy, medical treatment including anti-muscarinic drugs and newly designed beta-3-agonists, sacral neuromodulation and the injection of onabotulinumtoxin. In refractory disease again the use of bowel segments for bladder augmentation is the last resort.

In the field of neurourology, patients with neurogenic detrusor overactivity do not only suffer from urinary incontinence, but these patients are confronted with an impairment of renal function. Treatment algorithms are similar to patients with idiopathic overactivity but, in consideration of renal function, the control of treatment effects by urodynamics is necessary. Voiding of the bladder is handled by catheterization independent of detrusor function.

Detrusor underactivity is mainly treated by intermittent catheterisation because of the reduced effect of drugs. Sacral neuromodulation is able to reduce residual urine and frequency of catheterization with a high need of patient compliance. Latissimus Dorsi Detrusor Myoplasty (LDDM) includes to transplantation off the latissimus dorsi muscle over the urinary bladder with the success rate of up to 70 % in a very selected group, but patients have to be informed about the increased surgical extent with the relevant risks (Guidelines on Neuro-Urology 2015; Guidelines on the Management of Non-Neurogenic Male Lower Urinary Tract Symptoms (LUTS) and incl. Benign Prostatic Obstruction (BPO) 2015; Guidelines on Urinary Incontinence 2015).

In the end the symptom complex of chronic bladder or urogenital pain syndrome is not well understood, but morphological abnormalities could not be found often and

therefore functional problems are assumed. Complaints range from mild controllable pain to massive reduction of the quality of life in patients with formerly called interstitial cystitis. Although the multimodal treatment is recommended the outcome is poor in many affected patients (Guidelines on Chronic Pelvic Pain 2015).

8.3.2.3 Bladder Exstrophy/Epispadias Complex

Bladder exstrophy is a very rare congenital malformation of the development of the urinary bladder, urethra and the anterior abdominal wall. An incidence of 1:10,000–1:50,000 is reported. Affected neonates should be referred immediately after birth to a specialist urologic centre. Treatment includes either primary repair or staged repair depending on bladder capacity with closure of the bladder, pelvic bone dehiscence and urethra. Bladder augmentation and treatment of urinary tract strictures or incontinence as far as urinary diversions are typical risks in the future of concerned patients (Inouye et al. 2014).

8.3.3 Stress Urinary Incontinence: Urinary Sphincter Mechanism

Stress urinary incontinence is characterised by the involuntary leakage during coughing physical effort or even lying in pronounced disease. Incidence and prevalence of urinary incontinence are high worldwide; for example about 17 million people in the United States and more than 200 people worldwide suffer from urinary incontinence (Abrams et al. 2013; Guidelines on Urinary Incontinence 2015; Wang et al. 2011). Age dependency of urinary incontinence will lead to an increased problem within upcoming decades, which leads more and more to social isolation of the individual (Bae and Yoo 2010).

The pathophysiology of female stress urinary incontinence can be subdivided into two major categories: urethral hypermobility and intrinsic sphincter deficiency (Smaldone and Chancellor 2008). First line treatment of choice consists of pelvic floor exercises, which can be combined with electrotherapy or biofeedback. Oral medication with duloxetine can only lead to symptom release but will not result in a causal treatment. In case of insufficient conservative treatment surgery is indicated. So-called "Burch-colposuspensionon" represents the surgical golden standard for a long time with success rates of up to 85 % after 1 year. Over the time minimally invasive approaches with tension free vaginal tapes became more and more popular with first similar therapeutic outcome especially in patience with hypermobile urethra (Furuta et al. 2007). Sling placement is possible through the retropubic or the transobturator approach as far as mini-slings, which only need one anterior vaginal wall incision. Treatment failure can be handled with an additional sling, colposuspension or an artificial sphincter (Guidelines on Urinary Incontinence 2015).

Male stress urinary incontinence is treated accordingly to female patients. After a conservative management surgical options include: the artificial sphincter (gold standard), retrourethral functional slings in urethral hypermobility after radical prostatectomy and upcoming adjustable slings (Guidelines on Urinary Incontinence 2015).

8.3.4 Urethra

8.3.4.1 Urethral Stricture

Urethral strictures can be mainly a result of trauma and urethral infection (especially chlamydia trachomatis and neisseria gonorrhoe) or iatrogenic sequela after catheterization or transurethral instrumentation. Stricture disease does not only involve the urethral epithelium, moreover spongiofibrosis typically extends the length of the epithelial stricture and should be treated in addition. Endoscopic treatment either by cold knife or laser incision is characterised by recurrence of rates of 30 % and more, which makes open reconstructive surgery of the urethra necessary. Stricture excision with primary anastomosis and reconstruction with buccal mucosa are the most favourable approaches. Success rates between 80 and 90 % are possible but the availability of autologous tissue especially long strictures limits this technique in case of future recurrences (Campbell et al. 2007; Austoni 2010).

8.3.4.2 Hypopadia

Hypospadias are classified according to the position of the meatus of the urethra: glandular, coronar, penile, scrotal, perineal. Whereas glandular and coronar hypospadias facilitate physiological micturition and surgery is discussible in consideration of primarily cosmetic aspects, dorsal hypospadias require surgery to avoid social stigma of the patient. Multiple surgical approaches, for example preputial island flaps, urethral plate tabularization or skin grafts, with good results have been described, but there is still a risk of urethral stricture and urethral fistula. Especially in young patients with scrotal or perineal hypospadias autologous tissue with disadvantages has to be used: e.g. dermal grafts with hair growth (Kollhoff et al. 2011; Campbell et al. 2007; Austoni 2010).

8.3.5 Erectile Function: Vascular and Innervation

Erectile dysfunction is often caused by multiple factors and demonstrates an age-related increase of incidence and prevalence starting in the fourth decade. Teaching leads to endothelial dysfunction with deactivation of the NO-releasing system and

increased penile vascular tone. Diabetes mellitus (vascular and neuronal damage), hypertension and hyperlipidaemia disturb the structural and functional integrity of the cavernosal system. In addition iatrogenic damage of the pelvic nerves after surgery (prostatectomy, rectal surgery) and radiotherapy may result in a certain degree of erectile dysfunction (Alwaal et al. 2015; Guidelines on Male Sexual Dysfunction: Erectile dysfunction and premature ejaculation 2015). Peyronie's disease with deformation of the tunica albuginea typically presents with a various mix of penile deviation, penile pain and erectile dysfunction (Guidelines on Penile Curvature 2015).

Treatment of erectile dysfunction is still only symptomatic. Beside behavioural therapy oral phosphodiesterase type 5 inhibitors show a therapeutic response in up to 75 % of patients. Secondline options include transurethral or intracavernosal application of prostaglandin E1 and vacuum devices. Finally semi rigid or hydraulic penile prosthesis can be offered to suitable patients (Guidelines on Male Sexual Dysfunction: Erectile dysfunction and premature ejaculation 2015; Guidelines on Penile Curvature 2015).

8.3.6 Pelvic Organ Prolapse

The specific field of pelvic organ prolapse can only be touched upon in this chapter. In accordance with female urinary incontinence pelvic organ prolapse affects predominantly women between 60 and 70 years of age; the prevalence is reported 3–6 % for symptomatic prolapse and up to 50 % depending on vaginal examination. Pelvic organ prolapse is subdivided related to the location (anterior/middle(central)/posterior compartment) and to the grade of extend (following the mostly used pelvic organ prolapse quantification system POP-Q grad 0–4) (Barber and Maher 2013; Hofmann and Wagner 2014). Indications for surgical intervention of pelvic organ prolapse include a symptomatic disease (vice versa: incidental findings do not need treatment), utilization of conservative treatment (e.g. treatment of constipation in case of rectocele) and information about side effects, especially with regard to potentially used implants (e.g. alloplastic materials, xenogeneic implants). Different treatment options according to the localization of the defect are summarized in Table 8.2. Many patients suffer from combined defects of more than one compartment, which has to be addressed during individual surgical planning.

8.4 Clinical Principles, Diagnostics, Indications of Urogenital Tract Regenerative Therapies

The following paragraphs address the question of specific diagnostic aspects with special regard to the indication and need of regenerative therapies of the urogenital tract. Additionally physiological and regulatory limitations of the current

Table 8.2 Examples of the treatment of different pelvic organ prolapse pathologies (Hofmann and Wagner 2014)

Compartment	Treatment options
Anterior	Cystocele (central defect): anterior colporrhaphy (+/− implant)
	Cystocele (lateral defect): paravaginal repair (+/− implant)
Middle/Central	Sacrocolpopexy (open/laparoscopic/robotic)
	Hysterectomy (increasingly strict indication; (open/laparoscopic/robotic)
	Colpopexy (Amreich and Richter, abdominal fascial sling colpopexy)
Posterior	Rectocele: posterior colporrhaphy (+/− implant)

therapeutic strategies (based on Sect. 8.3) will be outlined as well as limitations of regenerative therapies itself.

8.4.1 Ureter

Diagnostics in ureteric strictures need to clarify the length of the stricture, urodynamic influence of the stricture with an increased risk of renal function impairment and finally the current clearance of the affected renal unit. In case of extrinsic compression of metastases the oncological prognosis is important to decide about the extent of ureteral reconstruction.

Retrograde urography facilitates both morphological identification of the ureteric stricture and drainage of the renal pelvis and calices. Intravenous urography, CT- or MRI-urography can be used as alternatives without the advantage of repeated examination at one time point. However only cross sectional imaging includes information of retroperitoneal abnormalities, especially in oncologic patients.

Radioisotope renography with mercaptoacetyltriglycine (MAG-3) is recommended prior to extensive reconstructive surgery to evaluate renal unit clearance (nephrectomy in case of reduced function (< 10–15 %)) and if contrast medium-enhanced imaging does not certainly clarify the drainage capacity of the ureter (Campbell et al. 2007).

As reported above, minimal invasive therapy with dilatation or incision is associated with poor long-term outcome. Therefore patients with recurrent stricture either need lifelong stenting of the renal unit or reconstructive surgery. Treatment of distal strictures can be managed with reconstructive principles (ureterocystoneostomy, bladder remodelling (psoas-hitch plasty, boari flap)) efficiently with good results. Mid-urethral and proximal stricture without the chance of primary anastomosis and in particular long-term strictures need extensive surgery with principally bowel segments. Especially unfit patients and patients with recurrent strictures might benefit from ureteral substitutes, which avoid the use of bowel segments with increased risks in the postoperative clinical course.

Any kind of regenerative ureteral substitute needs sufficient nutrition and oxygen supply. The anatomy of ureteral vascularization with multiple segmental pedicles (renal artery, aorta, common and internal iliac artery) (Schiebler et al. 1999; Benninghoff and Drenckhahn 2003), which is disjoined by the disease or even removal of the naïve ureter, results in a presumably insufficient nutrition through the anastomosis. Therefore un-vascularized tubular segments need surrounding tissue for an adequate supply and a base for neovascularization. Omental flaps surrounding tubular grafts might be one idea to ensure an optimal microenvironment in comparison to peritonealisation of ureters in patients with retroperitoneal fibrosis (Campbell et al. 2007).

8.4.2 Urinary Bladder: Physical and Functional

Key facts of an adequate urinary bladder function include: urinary bladder volume between at least 250 and 500 ml with physiological bladder sensation and controlled micturition resulting in urinary continence (day- and night-time) (Campbell et al. 2007; Guidelines on the Management of Non-Neurogenic Male Lower Urinary Tract Symptoms (LUTS) and incl. Benign Prostatic Obstruction (BPO) 2015; Guidelines on Urinary Incontinence 2015).

8.4.2.1 Bladder Cancer

Regenerative therapies will be applied in the future in case of radical cystectomy, diminished bladder capacity after multiple resections or multimodal treatment (radio-chemotherapy) or as a part of partial cystectomy in selected patients.

Oncological prognosis is necessary to know for an optimal patient selection – especially patients with a presumably curative treatment attempt will benefit in the long term from regenerative therapeutic principles. Diagnostics include transurethral resection of the tumour to determine the extent of the disease as well as the tumour grading. CT- or MRI-scan will discriminate a localized or locally advanced from a metastatic disease and in addition information about the urothelium of the upper urinary tract (exclusion of upper urinary tract carcinoma) can be obtained.

Renal function testing helps to decide about the possibility of neoadjuvant chemotherapy in locally advanced disease and confirms possibility of a continent reservoir (orthotopic or with catheterization) in case of an at least satisfactory renal clearance (>50–60 ml/min). Furthermore, due to the risk of urinary retention, patients suitable for an orthotopic solution have to be informed and should be able to practice intermittent catheterization.

Patients, which are not candidates for a continent diversion, have to accept an incontinent urostomy. The low-pressure situation carries a reduced risk of renal impairment but the body image is compromised and patients need to handle the collecting bag.

Postoperative care includes renal function evaluation, oncological follow up with cross sectional imaging, control of the acid-base equilibrium and vitamin B12 evaluation in the long-term.

Critical factors in the decision of the application of regenerative therapies may be the following:

- Definition of the oncological prognosis depends also on postoperative histopathology in many patients (node positive disease).
- Adjuvant chemotherapy in patients with extensive or node positive disease interferes with the necessary growth and neovascularization of a tissue engineered partial or complete reservoir.
- The healing properties, which are necessary for regenerative therapies, may be negatively influence by the surgical extend and risks of radical cystectomy.
- Autologous urine derived stem cells will be critical to use due to the fact of a generally increased oncologic degradation of even healthy bladder urothelium.

As a result cell source for regenerate therapy needs to be selected carefully to avoid oncologic dedifferentiation and the time point of the application needs to be determined referring to the assumed oncologic outcome. There will be a certain group of patient in which reconstructive principles are applicable in a staged procedure (Kollhoff et al. 2011; Campbell et al. 2007; Guidelines on Muscle-invasive and Metastatic Bladder Cancer 2015).

8.4.2.2 Functional Urinary Bladder Complications

Diagnostics to evaluate storage and emptying function with adequate bladder compliance (elasticity of the stratified bladder wall) and capacity as well as continence includes bladder diaries, ultrasound (residual urine and evaluation of the upper urinary tract), cystoscopy to rule out morphological pathologies, physical examination to exclude genital prolapse and especially (video-)urodynamics. Urodynamics offers the possibility to identify detrusor underactivity versus obstruction and detrusor overactivity versus hypersensitivity versus low compliance and low capacity urinary bladder, respectively (Guidelines on Neuro-Urology 2015; Guidelines on the Management of Non-Neurogenic Male Lower Urinary Tract Symptoms (LUTS) and incl. Benign Prostatic Obstruction (BPO) 2015).

In contrast to bladder cancer functional urinary bladder deceases facilitate a long-term planning for the application of tissue engineering and cell-based therapy. Thus functional problems provide a wide range of capabilities:

- Bladder augmentation in idiopathic and especially neurogenic overactive bladder
- Treatment of detrusor underactivity with special regard to patients with myogenic problems with preserved innervation of the urinary bladder
- Urinary bladder substitution in patients with an almost non-functional urinary bladder (fixed low bladder capacity, severe forms of bladder pain syndrome)

- Targeted influence of intrinsic urinary bladder nerve system dysfunctions

Regenerative therapy can be limited by the commonly restricted or completely interrupted innervation (spinal injured patients, severe autonomous neuropathy in patients with metabolic syndrome). In general the regeneration of a complete bladder wall does not consist only of a multilayer urethral scaffold, in fact also innervation and vascular ingrowth to the muscular structures need to be established. Even though substitution with growth factors (e.g. nerve/vascural epithelial growth factor) might enhance the regeneration of acellular matrices, these structures need to be tethered to the genuine vascular and neural system, respectively (Sievert et al. 2007a).

8.4.2.3 Bladder Exstrophy/Epispadias Complex

With regard to congenital dysplasia increased prenatal diagnostics nowadays leads to an early diagnosis of these challenging diseases, usually prior to childbirth. Although the time frame between birth and necessary primary surgery is too short to apply regenerative therapy immediately, early diagnosis creates the potential to preserve precious autologous stem cell resources like umbilical cord blood, placenta or amnionic fluid cells. Although cryopreservation of these sources is already possible, the clinical implementation in urogenital regenerative therapy is still beyond reality.

8.4.3 Stress Urinary Incontinence: Urinary Sphincter Mechanism

Stress urinary incontinence results from intrinsic sphincter deficiency or urethral hypermobility in most cases as reported above. Interestingly our knowledge of sphincteric anatomy has been changed recently. In contrast to the formerly reported circular striated external sphincter in the pelvic floor (Schiebler et al. 1999; Benninghoff and Drenckhahn 2003), current literature illustrates a clear anatomic view for males and females with a basically horse-shoe shaped striated muscle (rhabdosphincter) with an inner smooth muscle component (lissosphincter) ranging from the bladder neck to the pelvic floor (including the prostate in male patients) as described by Wallner et al. (2009)). Specific anatomy is especially addressed for surgical dissection of the urethra and bladder neck in radical prostatectomy and cystectomy in both genders.

Stress urinary incontinence is basically a clinical diagnosis during examination with cough stress test. Diagnostics as listed in Sect. 8.4.2.2 is first and foremost to exclude other forms of urinary incontinence and concomitant urogenital disease, which need treatment first. The pad test in different varieties offers the possibility of urine loss quantification to stratify the therapeutic concept. Valsalva leak point

pressure and urethral profilometry as part of urodynamics may add interesting information but the use of these examination is debateable (Campbell et al. 2007; Abrams et al. 2013; Guidelines on Neuro-Urology 2015; Guidelines on Urinary Incontinence 2015).

The main question for regenerative therapy is: what should we treat: muscular strength (intrinsic sphincter deficiency), location of the sphincter complex (urethral hypermobility) or even innervation? Current treatments target either on urethral repositioning (slings) or intrinsic sphincter deficiency (bulking agents, artificial sphincter), but possible injuries of the pudendal nerve, as described for vaginal delivery (Abrams et al. 2013), are out of focus probably due to the current treatment limitations of the neural system (Gill et al. 2013).

Regenerative therapy should address the following needs and limitations:

- Optimized diagnostics to identify the target(s) of regenerative medicine
- Determination of the time point of application: possibility of prevention (e.g. prostatectomy associated incontinence, nerve regeneration after delivery), treatment of long-lasting incontinence
- Identification of the cutting side off regenerative principles: muscular regeneration and/or paracrine support of feeling mechanisms
- Application of regenerative therapy: treatment of small delicate structures, navigation during application, measurement of efficacy

8.4.4 Urethra: Urethral Stricture and Hypopadia

Location and length of the urethral stricture can be determined either by retrograde urethrography, cystoscopy or voiding cystourethrography, if a suprapubic tube is placed. Almost no information is gathered about the involvement of the corpus spongiosum as one of the major disadvantages of the mentioned examinations. This can be overcome by the use of ultrasound in combination with are gel-filled urethra, which facilitates an excellent quantification of spongiofibrosis. Increasingly open reconstructive surgery becomes the therapy of choice as the primary surgery especially in young patients due to the reduced outcome of endoscopic treatment. To date most urological surgeons use buccal or labial mucosa as an ideal urethral graft. Advantages are the easy harvest of the graft, the absence of hair follicles and the similarities of the epithelial stratum. Regenerated therapy might solve the problem of buccal mucosa availability but tissue engineered scaffolds or cell layers have to meet criteria for an optimal implementation: feasibility to handle the implants by the surgeon (membrane stability), integrity of the implant adjacent to transurethral catheter, (cell) stability to contact with urine and optimal conditions for neovascularization (Campbell et al. 2007; Austoni 2010; Ribeiro-Filho and Sievert 2015).

8.4.5 *Erectile Function: Vascular and Innervation*

In face of the insight into the pathophysiology of erectile function, particularly with regard to the identification and development of phosphodiesterase type 5 inhibitors, a causal treatment is still not in sight. Diagnostic workup with medical history, examination, validated questionnaires, laboratory test (hormonal and metabolic assessment) and artificial erection of Doppler ultrasound distinguishes psychological, hormonal, vascular and neurogenic reasons (Guidelines on Male Sexual Dysfunction: Erectile dysfunction and premature ejaculation 2015; Guidelines on Penile Curvature 2015).

The "construction" of the cavernosal bodies mimics a blood sponge, which is supplied by deep and superficial penile arteries. Hereby administration of cell based therapies to regenerate vascular and/or neuronal defects can be performed easily and directly by intracavernosal injection. Again the question about timing is raised bearing in mind that patients with postoperative erectile dysfunction might benefit from an early/preventive therapy (Schiebler et al. 1999; Austoni 2010; Alwaal et al. 2015; Benninghoff and Drenckhahn 2003).

8.4.6 *Pelvic Organ Prolapse*

Physical examination and medical history with attention to specific complaints are still the key to correctly identify patients seeking for prolapse treatment. Dynamic MRI imaging is helpful for surgical planning for patients with critical anatomical conditions, but it has to be taken in account, that the grade of urogenital prolapse is frequently over-interpreted (Barber and Maher 2013; Hofmann and Wagner 2014).

After a period of tremendous use of different synthetic meshes and biological components either as implant systems or to support colporrhaphy, surgeons went back to classic techniques as a result of increased complication rates associated with certain implants. These problems have been addressed by the US Food and Drug Administration already in 2008 with a FDA safety communication (FDA 2011) due to side-effects and possible non-superiority of surgery with implants versus surgery alone. Therefore main aspects for the use of regenerative therapy in pelvic organ prolapse in the future are biocompatibility with avoidance of side effects (mesh erosion, pain, inflammation, bleeding) and improvement of current therapeutic strategies.

8.5 Standardized Treatment Technologies

Applied regenerative therapy in urogenital medicine: fact or fiction? Despite extensive fundamental and clinical research, including sophisticating animal and also human studies, still only a few regenerative products have been introduced into

current clinical practice. The comparison of the section lengths of "standardized treatment technologies" and "clinical studies" mirrors this situation as expected. This section focuses on regenerative products, which are available and approved for clinical use. Thereby scaffolds represents for the most part.

8.5.1 Scaffolds: General Aspects for Human Use

In vivo most cells attach to the extracellular matrix or to neighbouring cell forming the highly organized structures of functional tissues and organs. The contact of cells to the environment is essential for their viability and function, as receptor-mediated contact signals facilitate cellular survival. When detached, such cells undergo cell death (called anoikis) (Frisch and Screaton 2001). Only a few types of cells, such as T- and B-lymphocytes, monocytes and some others are optimized for survival while floating. This has interesting consequences when for instance the progenitor cells mentioned above or mature effector cells are explored for their therapeutic potential. For short periods of time MSC, urothelial cells, myoblasts, smooth muscle cells, endothelial cells, fibroblasts or other sessile cells can of course survive when detached and resuspended in suitable media. However, after only a few hours the viability of sessile cells in suspensions drops significantly. This depends of course of the cell type involved and several technical aspects including the method for cell detachment, composition of the medium, and temperature. In contrast, when sessile cells are mixed with or seeded on biomaterials, higher viability can be achieved. In addition, biomaterials allow the generation of shaped cell-augmented implants, implants with different types of cells placed on defined (sc., predetermined) locations, or cells in pattern. Therefore, for regeneration of bladder tissue, urethra and sphincter, a brief overview on scaffolds or biomaterials merits discussion.

Synthetic polymers such as the biodegradable polymers poly(glycolic acid) (PGA) and poly(lactic acid-co-glycolic acid) (PLGA) are biomaterials made of macromolecules assembled with covalent links. The main advantage of synthetic polymers is the capacity to manufacture any form of tissue or organ in three dimensions, in a quantitative and reproducible way, and at relatively low cost. Modern 3D-printers produce custom-made scaffolds adapted to an individual's anatomical or functional needs. To this end clinical imaging data of the patient (CT, MRT) are computed to control the 3D-printer. There are however some biological difficulties arising from the chemistry of normal polymer-based scaffolds. Cells attach to the extracellular matrix mainly (ECM) by integrins. The integrins are heterodimeric receptors, which bind defined peptide motifs. The archetype integrin ligand is the -arginine – glycine – aspartic acid tripeptide (RGD-peptide) originally defined in fibronectin. When incubated on normal polymeric scaffolds, the integrins can't attach to the materials surface. When the cells under investigation are seeded on the scaffold in media containing proteins (e.g. bovine serum), the latter can be adsorbed to the polymer surfaces and facilitate cell binding to the polymer. Alternatively

fibroblasts, smooth muscle cells or other cells may express for instance by collagens and thereby produce their own coating to cover the polymers. Next generation polymers take this into account and either co-polymerize monomers containing a linker with a RGD peptide or use nano-enabelled coating technology to cover the polymer by thin layer of gelatine, fibronectin or another component. Such modifications can also be used to control the proliferation and differentiation of the cells attached.

A second type of scaffold is routinely produced from natural sources. These natural scaffolds include polysaccharides such as agar, isolated from algae or proteins, such as various collagens and gelatine isolated from cadaveric organs and tissues, fibrin isolated from blood of animals for slaughter. Isolation of recombinant proteins from the milk of transgenic cattle is a modern variant to obtain a specific protein, albeit at high costs. To avoid transfer of pathogens and to have a defined starting material, proteins are produced in bioreactors by cell cultures under well-defined conditions as well and the (recombinant) protein is isolated from the supernatants or cell extracts. Scaffolds generated from natural components under mild conditions may preserve the structure and composition of even complex materials. Thereby the cellular responses may mimic a rather physiological behaviour when compared to a cell in a petri dish.

An even more advanced type of scaffolds are chemically and mechanically decellularized tissues (such as small intestinal submucosa (SIS), bladder acellular matrix (BAM), see below). They consist of collagen and other ECM components and have the advantage of providing an organotypic structure, inherent bioactivity and mechanical similarity to an organ and its native ECM. Moreover, decellularized tissues may contain growth factors that are released when cells start to partially degrade the tissue and replace the scaffold with a new ECM (Orabi et al. 2013a). Decellularized tissue can be produced from organs of slaughter animals of one species and seeded with autologous cells from second species. Using this strategy complex implants with vascularization and defined structural composition may be possible (Rosario et al. 2008).

8.5.2 Xenogeneic Collagen Matrices with Human Haemostatic Sealant

Tachosil® and Hemopatch® (the latter with additional pentaerythritol-polyethylenglycol-ether-tetra-succinimidyl-glutarat coating) are the main products of this kind of scaffold produced from equine collagen matrix and modified by haemostatic substances (human thrombin and fibrinogen), which are activated physiologically by intracorporal contact with blood components. Main field of application is haemostatic sealing during complex surgical procedures. Nephron-sparing surgery is one specific indication in urology for the application of these products, whereas a recent study could not identify benefits in regard of renal function in contrast to classic renorrhaphy (Lewis et al. 2014).

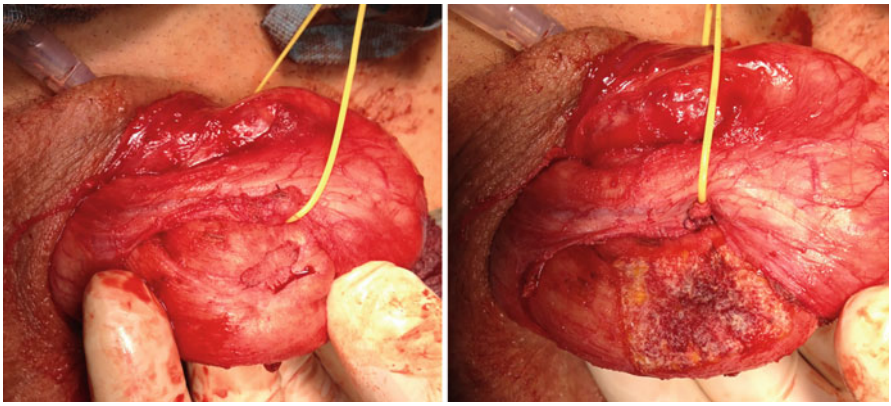

Fig. 8.2 Surgical treatment of Peyronie's disease with excised plaque (*left*) and grafting with Tachosil® patch (*right side*) (University Hospital of Tuebingen, Department of Urology)

Furthermore literature about Peyronie's disease (induration penis plastic) reported feasibility and sustainable therapeutic effects. Disadvantages are the functional outcome in concern of erectile function. Overall reported patient numbers are low and additional clinical studies with increased follow up are needed (Horstmann et al. 2011; Hatzichristodoulou et al. 2013) (Fig. 8.2).

8.5.3 (Porcine) Small Intestinal Submucosa (SIS)

Porcine small intestinal submucosa is a result of extensive mechanical treatment and additional decellularization protocols of porcine small bowel segments. However data exists that there is still a risk of residual porcine DNA within the implant, which might be a source of different kinds of "foreign body reaction" by the immune system (Zheng et al. 2005). Clinical use of SIS is described for different indications: hernia repair, dural reconstruction, abdominal wall restoration and fistula repair. Urogenital indications are Peyronie's disease and prolapse surgery. The last years the use of SIS and other xenogeneic implants decreased due to reported postoperative complications especially for transvaginal placement as reported above (FDA 2011). Consequently manufacturers withdrew their products from this indication.

SIS is still available for Peyronie's disease treatment. In contrast to collagen matrices with human haemostatic sealant SIS enables a watertight suture of the corporal defect after incision. Begemann et al. found no significant difference between dermis, fascia, or SIS for repair of Peyronie's disease (Sievert et al. 2007a). EAU guidelines for Peyronie's disease recommend "lengthening" procedures with tunical incision in patients with a deviation of more than 60°, normal erectile function an

inadequate penile length. SIS is characterized by tissue-specific regeneration and neovascularization by the support of endothelial cells, but reported clinical data is still sparse (Guidelines on Penile Curvature 2015).

8.5.4 *Porcine Dermal Graft, Bovine Pericardium and Cadaveric Allogeneic Grafts (Pericardium, Fascia Lata)*

In contrast to the previous scaffolds/grafts porcine dermal graft, bovine pericardium and cadaveric allogeneic grafts are less often used for urological indication and existing data is not sufficient to draw specific recommendations. Porcine dermal grafts have been tried for fistula repair, pelvic organ prolaps and sacrocolpopexy, but results did not show outcomes encouraging an increased use (Hviid et al. 2010; Dahlgren et al. 2011).

Pericardium patches could be applied for Peyronie's disease in the style of SIS placement. Especially the combination of tensile strength and multi-directional elasticity is described as an advantage of this material. Although 45 % of the patients reported recurrence of deviation, intercourse was possible and thus associated with sufficient patient satisfaction (Guidelines on Penile Curvature 2015).

8.5.5 *Tissue Engineered Buccal Mucosa (TEBM) Urethroplasty (Mukocell®)*

Tissue engineered urethral grafts with oral keratinocytes and fibroblasts seeded on different scaffolds is under investigation since several years (Osman et al. 2015). In 2008 Bhargava et al. reported the clinical course of five patients with de-epidermized cadaveric dermis served as scaffold for TEBM. Within a follow up of 33.6 months two patients needed extensive revision due to fibrosis; the remaining three patients received sufficient urethral diameters with the need of minor instrumentation (Bhargava et al. 2008).

To our knowledge Mukocell®, a commercially available form of TEBM, is the first legally approved cell based therapy in urology (UroTiss). A small buccal mucosa biopsy (0.4–0.8 cm^2) has to bee retrieved as the cell source for TEBM (Fig. 8.3). Although published original data is expected, Osman et al. reported in their review based on scientific two stricture recurrences out of ten treated patients after mean follow up of 18 months (Osman et al. 2015). A recently published review with participation of the manufacturer reported about 22 patients with a mean stricture length of 5.5 cm with a success rate of 80.9 % (17 patients). At the time of this review 103 patients are under prospective observation (Ram-Liebig et al. 2015).

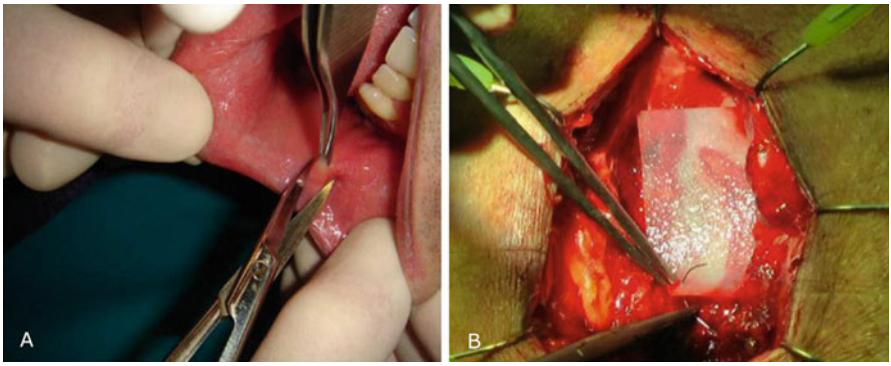

Fig. 8.3 Excision of small piece of buccal mucosa (**a**). Ventral only grafting of the urethra with TEBM (**b**) (Reprint from Ram-Liebig et al. (2015) with permission of Elsevier)

Beside the advantage of nearby unlimited autologous grafting material, original data of the TEBM treated patient cohort is needed to clarify potential fibrosis in the long-term and definite outcome (Osman et al. 2015).

8.6 Clinical Studies, Experience, Outcome/Side Effects of "Urogenital Tract" Regenerative Therapies

8.6.1 General Aspects: Cells and Scaffolds

Cells and scaffold should fulfil several requirements: capacious source, simple retrieval, reproducible preparation and results, reduced immunogenicity and avoidance of ethical concerns, to mention just a few important aspects among many others. Inevitably that raises the question of the optimal cell and scaffold for the specific urologic indication. The answer poses a challenge and the following sections try to focus on these individual urogenital aspects of regenerative medicine.

Kollhoff et al. provided a clearly arranged summary of (stem) cell resources and possible scaffolds with special attention to urological application in 2011, which is reflected in Table 8.3 (adapted).

In line with special needs for urogenital applicable scaffolds (see Sect. 8.5.1 and Table 8.3) different cell sources and cell lines are suitable for regenerative therapy in urology.

Different progenitor cells provide characteristics to implement epithelial regeneration. Autologous urothelial cells can be obtained from the urinary bladder wall by biopsy. Special techniques and protocols (bladder washing, extraction from voided urine) have been reported to avoid invasive procedures. Autologous epidermal cells (e.g. from foreskin) are easily available but reduced specificity may restrict possible applications. The clinical use of buccal mucosa for urethral reconstruction

Table 8.3 Table adapted from Kollhoff et al. (2011) with permission from regenerative medicine as agreed by future medicine Ltd

Biologic/synthetic polymerics and stem cells	Applications in urology
Acellular ECM grafts	
BAM	Bladder augmentation, urinary incontinence, urethral strictures
SIS	Bladder augmentation, hernia repair, urinary incontinence, urethral strictures, urethral replacement
ADM	Hernia repair, abdominal wall reconstruction, vaginal reconstruction, bladder augmentation
UBM	Bladder augmentation, urinary incontinence
Biodegradable synthetic polymers	
PGA/PLGA	Urethral grafts, bladder augmentation, urological stents
Polydiolcitrate	Bladder augmentation, urethral reconstruction, urological stents
PEUU	Ureter replacement, bladder augmentation
Nanomolecules	Delivery of growth factors and protein epitopes
Stem cells	
MSC	Smooth muscle regeneration, anti-inflammatory effects
AFSC	Neuronal regeneration, muscle regeneration
USC	Urethral reconstruction
MDSC	Stress urinary incontinence
ESC	Potential to regenerate different genitourinary tissues
iPS cell	Potential to regenerate different genitourinary tissues
EPC	Vascular regeneration

BAM bladder acellular matrix, *SIS* small intestinal submucosa, *ADM* acellular dermal matrix, *UBM* urinary bladder matrix, *PGA* polyglycolic acid, *PLGA* polylactic-co-glycolic acid, *PEUU* polyester urethane urea, *MSC* mesenchymal stem cell, *AFSC* amniotic fluid stem cell, *USC* urine stem cell, *MDSC* muscle derived stem cell, *ESC* embryonic stem cell, *iPS* induced pluripotent stem, *EPC* endothelial progenitor cell

implicates the use of oral keratinocytes and fibroblasts for tissue regeneration as summarized by Osman et al. (2015). Smooth muscle cells, retrieved from bladder wall excisions or deep biopsies, are necessary to achieve both functional contractility and tissue elasticity for reservoir compliance (Orabi et al. 2013a).

In contrast to progenitor cells with limited differentiation potential due to advanced cell development stem cells facilitate the design of various cell lines as well as the utilization of the secretoma with special regard to paracrine effects (Damaser and Sievert 2015). Starting in the 1960s as reported by Kollhoff et al. mesenchymal stem cells became more and more in the centre of interest for cell based therapy (Kollhoff et al. 2011). Pluripotent embryonic stem cells (ESC) typically bear the risks of malignant transformation and ethical boundaries. Currently bone marrow-derived stem cells (BMSC), urine-derived stem cells (UDSC) and especially adipose-derived stem cells (ADSC) avoid these concerns and are of utmost interest to investigate cell-based therapy in urology. The latter cell

source demonstrates an almost unlimited availability of stem cells and cell differentiation of ADSC to cells with smooth muscle characteristics has been demonstrated (Orabi et al. 2013a). Myogenic differentiation including muscular contraction of UDSC has been reported recently as well (Bharadwaj et al. 2011).

8.6.2 Ureter

Regeneration of ureteral replacement grafts is dependent on a tube-like scaffold. Shi et al. used human ADSC seeded on poly-lactic/collagen scaffolds in a co-culture system with urothelial cells to construct a ureteric graft. After subcutaneous implantation in mice urothelial differentiation could be demonstrated, which was explained mainly by the co-culture arrangement (Shi et al. 2012). Different scaffolds (acellular aortic arch and poly(L-lactide-co-caprolactone) have been investigated in a rat modell to restore ureter defects or an urinary conduit. The study concluded that the biodegradable synthetic scaffold showed superior outcome with regard to urothelial and smooth muscle cell integration and integritiy of the implants after 4 weeks (Kloskowski et al. 2014). To evaluate the influence of tissue specific extracellular matrix others compared human urothelial cell proliferation on synthetic and composite (synthetic plus SIS versus synthetic plus human ureteric extracellular matrix) scaffolds. Scaffolds with organ-specific extracellular matrix were favourable, which was explained by the hypothesis of an optimized cell-specific micro-environment (Xu et al. 2015). The effective use of a bioreactor to engineer tubular three-dimensional scaffolds was explored recently. Again the possibility of co-cultures with urothelial and smooth muscle realized with two fluid streams was evaluated with promising results (Seifarth et al. 2015).

8.6.3 Urinary Bladder

Urothelial progenitor and urothelial stem cells for specific cell regeneration are located in the niche of the upper and lower urinary tract for an easy harvest as required for benign (Bharadwaj et al. 2013). But despite of the reported widespread differentiation potential there are also several concerns in the use of urine-derived cells for regenerative application. Cytotoxic effects of urine (varying pH-value, high concentrations of excreted substances (urea, electrolytes, …)) on cell expansion were investigated. BMSC demonstrated a significantly reduced proliferation rate in pure urine or a mix of urine and cell medium compared to medium alone. If urine derived cells behave more robust in such extreme conditions remains an open question (Adamowicz et al. 2012). These aspects need to be considered not only for cell resources in the urinary tract (UDSCs), all the more urine contact may negatively influence the growth of tissue-engineered implants in vivo.

Patients with urinary tract malignancy may need an autologous source for UDCs. The possibility of post-mortem retrieval of urothelium for regenerative therapy was persued. The immediate post-mortem changes to the bladder and especially the urothelium were described, which makes urine-derived cells of healthy non-heart beating donors bootless (Garthwaite et al. 2014).

Consequently non-urothelial cell lines have been studied within different urinary bladder pathologies. Others reported the potential of presumptive BMSCs to restore bladder contractility in a rat model with outlet obstruction; hereby it remained unclear if BMSC or other (contaminated) cells differentiated into smooth muscle components (Vaegler et al. 2012). The capability of ADSCs to improve bladder function in overactive bladder syndrome has been described in hyperlipidaemic rats (Vaegler et al. 2012). The injection of these cells into the tail vein seems to be sufficient to detect improvement, which implicates a "homing process" of these cells into defected tissues.

Particularly with regard to the treatment of female patients the use of endometrial stem cells was addressed recently and successful differentiation of human endometrial stem cells into smooth muscle cells on hydrogels was reported. The recommends bioabsorbable nanocomposite scaffolds seeded with endometrial stem cells for bladder wall regeneration (Shoae-Hassani et al. 2013).

Yang et al. compared the in vitro effect of different recombinant growth factors on the behaviour of human bladder smooth muscle and urothelial cells. Combination of PDGF-BB, EGF, and VEGF was identified as an optimal factor for regeneration and angiogenesis (Yang et al. 2014).

Induced pluripotent stems cells might be one possibility to overcome the limitations of cell source (oncologic patients), cell availability or development possibility of the mentioned cell types, but may encounter ethical concerns. Li et al. gave a comprehensive illustrated overview (Fig. 8.4) (Li et al. 2015).

Different scientific groups successfully reported feasibility and protocols to achieve cell components of the urogenital tract by induced (trans-) differentiation (Osborn et al. 2014; Eberli et al. 2012; Moad et al. 2013).

Several different scaffolds have been examined with the aim to optimize bladder wall regeneration. The use of composite scaffold of collagen and polylactic acid esters permitted cell compatibility, mechanical strength and reduced inflammation compared to solely synthetic meshes (Engelhardt et al. 2011). The use of biodegradeable polymers in a canine model gained physiologic functional and morphologic results (Oberpenning et al. 1999). SIS for bladder regeneration was used as a basic principle in several animal studies with promising outcome. In 1996 SIS was reported as part of partial cystectomy in 19 male dogs with histologically physiologically layered bladder wall (Kropp et al. 1996). In contrast others reported in 2006 increased adhesion, graft shrinkage and stone formation with no difference between seeded and unseeded SIS in a partial cystectomy dog model (Zhang et al. 2006). Bladder acellular matrix has been also evaluated in a partial cystectomy dog model. Superior functional outcomes of grafted versus ungrafted subjects were reported and morphologic regeneration was confirmed by histology (Probst et al. 2000). In accordance several other publications also support and summarize the advantages

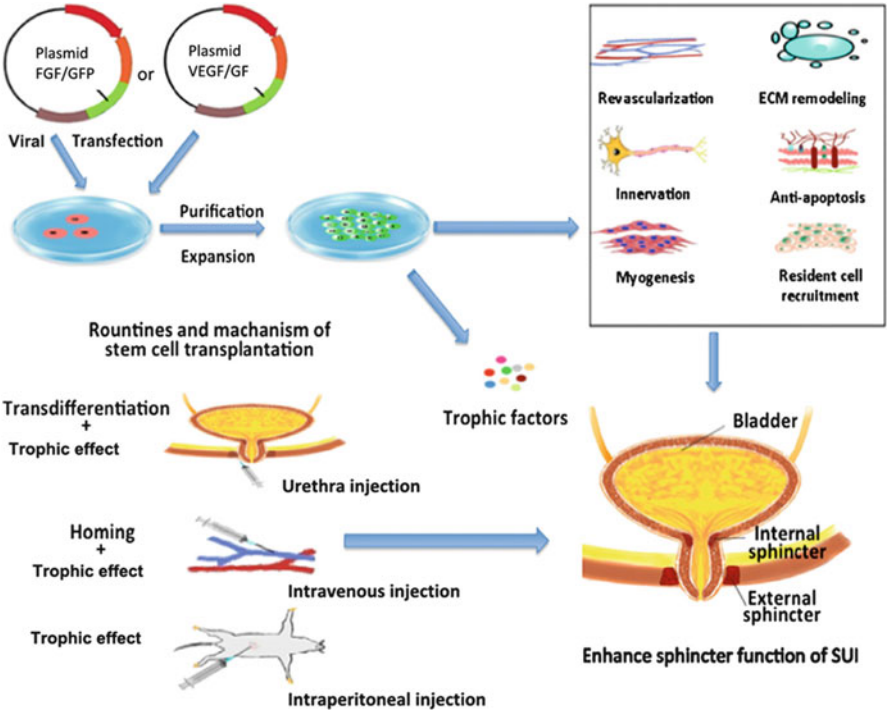

Fig. 8.4 Use of induced pluripotent stem cells for urogential regenerative medicine (Reprint from Li et al. (2015) with permission of Elsevier)

of bladder extracellular matrix in urinary bladder regeneration (Song et al. 2014; Pokrywczynska et al. 2015). To maximize benefits the composition of a mixed scaffold of biodegradable polymers and acellular bladder matrix was evaluated in vitro with upper urinary tract cells with positive characteristics (Jang et al. 2014).

Although the ideal combination of scaffold and cell source has not been identified, sophisticated clinical studies have been conducted. Certainly impressive is a study published in Lancet in 2006. Seven patients with neurogenic bladder received tissue-engineered cystoplsaty based on collagen +/−polyglycolic acid scaffolds and urothelial and muscle cells obtained by autologous biopsy. Figures 8.4 and 8.5 exemplifies the outstanding human procedure (Atala et al. 2006).

Despite the reported excellent clinical outcome of the study, further investigations in a larger cohort of patients are still missing.

Clinical application of cell-based therapy has been reported also for detrusor underactivity, although only one patient received treatment with endoscopic autologous muscle-derived stem cells injection. Beside the absence of relevant side-effects only minor effects with reduction of cystometric capacity and micturition of small amounts with continuous intermittent catheterization have been reported (Levanovich et al. 2015).

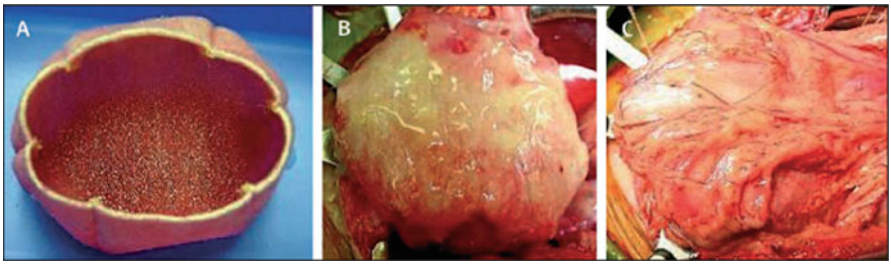

Fig. 8.5 Scaffold for bladder augmentation seeded with cells (**a**). Bladder augmentation with seeded scaffold in situ (**b**). Omental flap covers augmentation for nutrition (**c**) (Reprint from the Lancet (Atala et al. 2006) with permission of Elsevier)

Anti-inflammatory nanofibres were employed to reduce inflammatory effects during bladder regenerative therapy (Bury et al. 2014).

Overall regeneration of the urinary bladder is characterized by multiple encouraging in vivo animal studies and first clinical steps have been done, but larger studies are needed to prove the therapeutic principal in the long-term follow-up and to identify relevant cells and scaffolds.

8.6.4 Urethra

Critical aspects of urethral reconstructions are the size and length of the graft and the dimensional shape envisioned(flat versus tubular graft). To date only unseeded acellular scaffolds are used sporadically in clinical routine, therefore natural or artificial scaffolds are available (Fu and Cao 2012). SIS as one possible scaffold for urethral reconstruction is reported with partly contrary results (success rates between 20 % and 85 % in ventral urethral onlay technique) (Sievert et al. 2007a). Existing data is too sparse to decide about the ideal scaffold, especially regarding seeded implants, which have been reported in less than 20 patients (Ribeiro-Filho and Sievert 2015).

Encouraged by the lack of data, we investigated the fabrication of a multi-layered urothelial sheet, in which bladder washings provided the necessary autologous cell material (Fig. 8.6) (Nagele et al. 2008; Sievert et al. 2007b).

The same technique has been applied in a successful evaluation of autologous urethral cells in a porcine urethral stricture model (Seibold et al. 2012). Using cells from bladder washings we were able to produce a multi-layered urothelial transplant from human cells under GMP-conditions and obtained approval from the local authorities. Stabilization of the tissue with fibrin seems to facilitate surgical appliance in animal models (unpublished data), but the clinical application of such constructs still awaits legal approval. Currently our experiments focus on a combination of urine-derived progenitor cells and a collagen scaffold for urethral reconstruction in a porcine model.

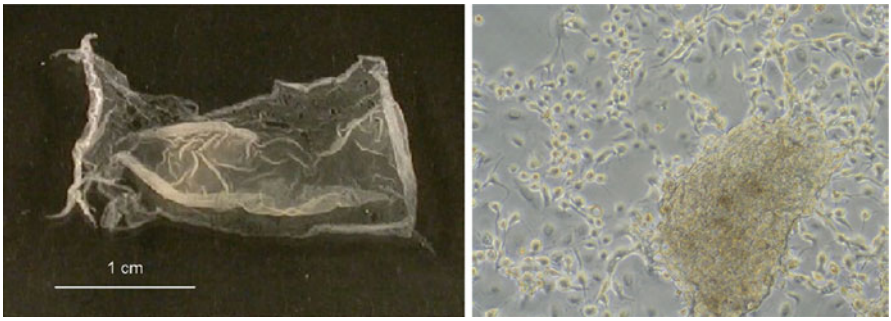

Fig. 8.6 Urothelial cell sheet tissue engineered from bladder irrigation (*left*). Cell growth in the petridish (*right*) (Reprint from Nagele et al. (2008) with permission from Elsevier)

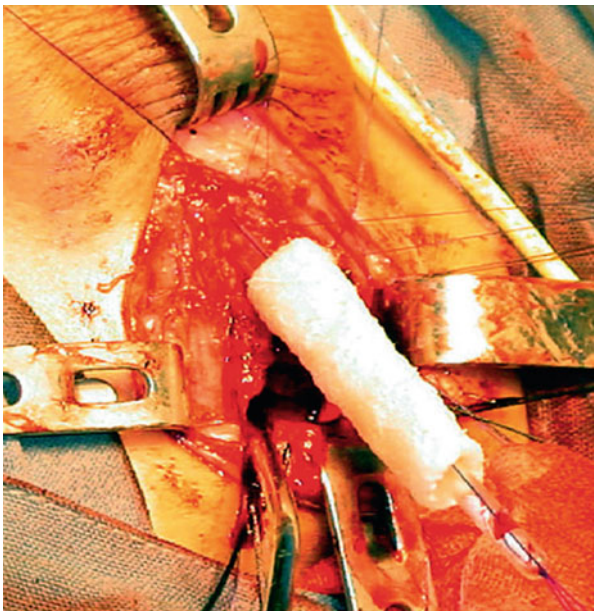

Fig. 8.7 Tissue engineered, cell seeded tubular graft sutured to the naive urethral margin (Reprint from Raya-Rivera et al. (2011) with permission of Elsevier)

Treatment with tubular grafts consisting of synthetic absorbable scaffolds with muscular and epithelial seeding was studied in five boys recently (Fig. 8.7). Within a follow-up period of 6 years the functional and histological urethral character could be confirmed (Raya-Rivera et al. 2011).

Chronically after the individual treatment attempts in humans the results were confirmed in a dog model with collagen tubularized scaffolds seeded with epithelial and smooth muscle cells. Additionally seeded implants indicated superior outcomes due to collapsed unseeded repairs (Orabi et al. 2013b). With regard to bladder acellular matrix favourable outcomes were for peracetic acid treated scaffolds in the

reconstruction of rabbit penile urethra (Huang et al. 2014). Comparable results have been obtained with a combination of bladder acellular matrix and autologous urethral tissue (Chun et al. 2015).

A clinical study randomly compared buccal mucosa onlay versus acellular bladder matrix in complex urethral stricture. Equivalent results could be obtained in healthy urethras, whereas spongiofibrosis and prior therapy negatively influenced the tissue-engineered outcome (el-Kassaby et al. 2008).

Urethral reconstruction, in contrast to bladder regeneration, noticeably seems to benefit of an approach with seeded scaffolds to reach the aim of a full functional urethra. On the over hand data is too limited to draw specific recommendations for clinical use with the available scientific data (Damaser and Sievert 2015; Vaegler et al. 2015).

8.6.5 Stress Urinary Incontinence: Urinary Sphincter Mechanism

Different animal models have been introduced to study cell-based therapies for urinary incontinence: vaginal distension, pudendal nerve crush or transection to imitate neural induced stress urinary incontinence (SUI); urethrolysis, electrocauterization, sphincterotomy and repeatedly vaginal distension mimic urethral hypermobility and/or intrinsic sphincter deficiency (Vaegler et al. 2012; Herrera-Imbroda et al. 2015). The basic question of regenerative principles in SUI is the aim of the applied treatment: smooth muscle, striated muscle or pudendal nerve. Most scientists would point to the striated muscular component of the sphincter complex. Regeneration of striated muscle tissue requires component muscular progenitor cells such as myoblasts or muscle-derived (satellite) stem cells (Amend et al. 2015). To restore continence, techniques target on either cell-based therapy for intrinsic sphincter deficiency and scaffold for urethral hypermobility.

Muscle precursor cells have been reported to regain 80 % of contraction with histologically proved integration of cells in a surgically induced dog incontinence model (Eberli et al. 2012). Another group applied the same model and highlighted the safety of cell injection independent of the injected cell numbers (Williams et al. 2015). Chun et al. investigated human amniotic fluid-derived cells in mice. Combinations of three different lineages (muscle/neuron/endothelial progenitor cells) cells showed better results than individual use alone (Chun et al. 2014). Additionally pre-clinical safety was reported in terms of side effects and oncogenicity in (Choi et al. 2015).

Clinical implementation in human studies started early in the field of stress urinary incontinence. Elmi described the injection auf autologous myoblast in seven patients with SUI after treatment of bladder exstrophy. Overall each patient demonstrated improvement with a dry rate of 71 % after 4 years with minimal morbidity (Elmi et al. 2011). Gras and colleagues used the same cell source in 20 female SUI patients. Thereby uncomplicated SUI has been cured in 25 % of the cases and

reduction has been reported in 63 % of patients treated; again with only minor side effects (Gras et al. 2014). Comparable results have been reproduced by Stangel-Wojcikiewicz in 16 female patients (Stangel-Wojcikiewicz et al. 2014).

Application of ADSCs in humans has been published only in small cohorts. Injections of ADSC with bovine collagen gel in five patients yielded a negative cough test after 12 months in three patients. The authors concluded feasibility and safety but outcome needs further improvement (Kuismanen et al. 2014). It is an open question if bulking effects of collagen or a "real" influence of the transplanted cells provokes the incontinence reduction. Similar results and limitations (with regard to bulking) can be extracted from a study of Yamamoto et al. in three male SUI patients (Yamamoto et al. 2012). In 2014 r an encouraging study using ADSC in 11 patients for treatment of SUI were reported (one dry patient, seven improved) (Gotoh et al. 2014), but it has to be kept in view that an injection of 20 ml of adipose cell solution was used.

Certainly a break in the progress of regenerative medicine for SUI or even urologic indications marks a recent study with the injection of autologous myoblasts and fibroblasts in female SUI patients (Strasser et al. 2007). Different critical circumstances lead to the retraction of the manuscript (Kleinert and Horton 2008) with the consequence of a sceptical public perception of regenerative medicine in urology.

The group of Chancellor recently published data of patients with autologous muscle derived cell treatment based on initial animal models (Chancellor et al. 2000).

Yet another study reporting on 80 patients reported at least 50 % reduction of pad weight and leak episodes. No relevant adverse events were noted and, in addition, a correlation between the amount of injected cells and the treatment outcome has been observed (Peters et al. 2014).

In view of critical studies and the unknown effects of different cells used for sphincter regeneration, undifferentiated MSCs were injected in the urethral sphincter of minipigs to develop a model for cell-based SUI therapies (Hart et al. 2015; Vaegler et al. 2013). The cells could be localized in this animal model by histology in the tissue targeted (unpublished data). Beside endoscopic tracking of cell injection Blaganje and Lukanovic reported positive results of ultrasound guidance for injections of cells (Blaganje and Lukanovic 2013).

The use of pre-differentiated stem cells raises the question of differentiation protocols. Thereby mechanical stimuli may represent a new strategy to promote muscular differentiation necessary for sphincter regeneration (Bu et al. 2014; Zhao et al. 2013). In addition microbeads with inclusion of different growth factors has been reported as a chance for specific and continuous in vivo stimulation after cell injection (Shi et al. 2014; Liu et al. 2013a).

Contrary to cell-based treatment of SUI only few studies focused urethral stabilization, which is currently performing by urethral slings. With regard to FDA statements as reported above (FDA 2011), main aspect is the increase of biocompatibility to reduce side effects. Coating of synthetic non-absorbable meshes of SUI patients with autologous plasma with a residence time of 30 min preoperatively was

evaluated. Outcomes with respect to feasibility, side effects an efficacy were positive and the authors plan a randomized study (Barski et al. 2014). In rodents composite slings made of polylactic acid scaffolds and ADSCs were investigated and good acute integration into the host tissues was observed (Roman Regueros et al. 2014; Roman et al. 2014).

In summary current available studies for SUI regenerative therapies provide sufficient evidence to proceed in this field of research with a hopefully effective and especially biocompatible approach (Hakim et al. 2015).

In our opinion the following issues and questions need to be addressed purposefully:

- Congruence of therapeutic indication and the specific pathophysiology of SUI
- Smooth muscle or striated muscle regeneration? Neuronal regeneration?
- Cell source: Stem cells with the potential to differentiate variably or progenitor cells to reach striated muscle regeneration?
- Mode of action: Secretome with paracrine effects? Cell differentiation and ingrowth? Both?

8.6.6 Erectile Function and Peyronie's Disease

Restoration of erectile function by regenerative therapies implies repair mechanisms for both neuronal and vascular structures. Preclinical studies favour the intracavernosal injection of cells for administration of regenerative therapy. Moreover ADSC currently represent the typically used cell population for erectile function recovery (Alwaal et al. 2015; Lin et al. 2008), additionally BMSC and muscle-derived progenitor cells were discussed as well (Zhang et al. 2012). ADSCs seem to play a key role for erectile dysfunction recovery) (Liu et al. 2013b). Recently the value of putative paracrine effects of injected urine-derived stem cells for recruitment of resident cells and tissue regeneration was discussed (Ouyang et al. 2014).

Only a few clinical trials used stem cells to treat erectile dysfunction. Positive effects of umbilical cord stem cells on erectile function were observed in seven diabetic patients, but results were far away from complete recovery (Vaegler et al. 2012; Bahk et al. 2010).

Two interesting studies targeted on penile tissue regeneration. Orabi et al. demonstrated the capability of ADSC to differentiate into smooth muscle and endothelial cells. Implantation of tissue-engineered cavernosal tissue without scaffolds in a rat model resulted in suitable integration after 1 and 2 months (Orabi et al. 2012). Others published on the engineering of an entire corporal body based on a cavernosal collagen matrix with autologous smooth muscle and endothelial cells. Structural and functional aspects similar to naïve tissue could be demonstrated (Chen et al. 2010).

Cavernous nerve regeneration to treat neurogenic erectile dysfunction was addressed as well. In the style of former sural nerve interpositions acellular grafts were used in 80 male rats after nerve transection. Significant recovery after 1 and 3

months has been seen (Connolly et al. 2008). Angeloni et al. introduced the sonic hedgehog protein applied with amphiphile nanofibers in a rat model to successfully improve erectile dysfunction after cavernosal nerve crush (Angeloni et al. 2011).

We have already described different commercially available scaffolds to treat Peyronie's disease. An interesting study evaluated the use of acellular bladder matrix for corporal tunica albuginea substitute in rabbits. Replacement of about 50 % of the dorsal tunica with acellular bladder matrix resulted in positive biomechanical, histological and biocompatible performance (Eberli et al. 2007).

8.6.7 Pelvic Organ Prolapse

In contrast to other urologic indications for regenerative medicine, the use of cell-based tissue engineering strategies to restore pelvic organ prolapse are only rarely studied (Boennelycke et al. 2013). The aspects of mesh biocompatibility has been described in Sect. 8.6.5. Commonly studies for POP base on a combination of a scaffold and promising cells. Different kinds (oder types) of scaffolds were seeded with oral fibroblasts and polylactic acid scaffolds and SIS were identified as potential candidates for POP repair (Mangera et al. 2013). Human vaginal fibroblast in combination with a scaffold produced from collagen gel was evaluated with regard to POP treatment. Tissue-engineered fascia with characteristics to facilitate POP surgery were developed (Hung et al. 2010). Different studies reported endometrial MSCs as an obvious cell source for POP correction. Human endometrial MSCs seeded on biodegradable scaffolds reduced immunogenicity and increased biocompatible properties (Ulrich et al. 2014; Su et al. 2014). This, however, needs to be discussed with care as the experiments have been conducted in immune-deficient nude rats and properties of both, cells and scaffolds may be very different in immune-competent hosts.

A milestone in tissue engineering with a vaginal substitute was applied in patients with Mayer-Rokitansky-Küster-Hauser syndrome. Four patients received vaginal extensions reconstructed from autologous vulvar biopsies and biodegradable scaffolds. Within a follow up of up to 8 years patients were sexually active as well as MRI-scans and histology showed similarity to naïve vaginal formation an tissue, respectively (Raya-Rivera et al. 2014).

8.7 Conclusions and Future Perspectives on Urogenital Tract Regenerative Therapies

How far is regenerative medicine in urology? Taken together, preclinical studies and preliminary clinical results suggest an import role of cell-based therapy and tissue engineering in urogenital diseases (Aboushwareb et al. 2011). But despite encouraging data clinical studies have not yet elucidated mechanisms of action and

Table 8.4 Summary of regenerative therapies for urogenital tract dysfunction

Current status of regenerative therapy for urogenital tract dysfunction			
Tissue	Disease	Present approach to therapy	Current status of regenerative therapy
Urethral sphincter	Stress urinary incontinence	Conservative treatment	Autologous cell injection therapy (MDSC, ADSC, etc.) => *Clinical trials*
		Urethral slings	
		Artificial sphincter	Cytokine therapy => *Animal experiments*
Bladder	Neurogenic bladder	Urinary diversion or orthotopic bladder substitution	Acellular ECM graft or multipotent progenitor cell-seeded graft => *Clinical trials* (*in part*)
	Bladder cancer		
	Radiation cystitis		
	Interstitial cystitis/ bladder pain syndrome	Conservative treatment	Composite cystoplasty => *Animal experiments*
Urethra	Hypospadias	Urethral dilation	Acellular ECM graft or multipotent progenitor cell-seeded graft => *Animal experiments*
	Urethral stricture	Urethral incision	
	Urethral trauma	Urethroplasty (end-to-end; buccal mucosa graft)	
Penis	Erectile dysfunction	PDE-5 inhibitor	Acellular ECM graft or multipotent progenitor cell-seeded graft => *Animal experiments* (*ED*) => *Clinical trials* (*Reconstruction*)*s*
	Peyronie's disease	Vacuum device	
	Trauma	Prostaglandin E1	
	Penile cancer	Penile prosthesis	
		Penile reconstruction	
		Penile straightening (with/without graft)	

Adapted reprint from Sumino and Mimata (2013) with permission from John Wiley and Sons

data from animal models is conflicting (Vaegler et al. 2012). Neither the "ideal" scaffold nor the "perfect" cell has been yet identified for the different urogenital indications. Recently the status of regenerative therapy for urogenital tract dysfunction was summarized. This is reflected in an adapted fashion in Table 8.4.

Bladder acellular matrix, SIS and biodegradable synthetic scaffolds are the consistently used scaffolds for tissue engineering. Cell-type specific progenitor cells seem to be promising if cell growth and integration is the main aim of application, but they are limited in differentiation capabilities if different cell lines want to be achieved. Induced pluripotent stem cells may be a solution for this issue but they are inevitably accompanied by ethical considerations and the fear to receive genetically manipulated biologic material. Adult mesenchymal stem cells could be a reasonable option between differentiation potential and both biological and ethical safety, but the mode of action of these cells in tissue regeneration is not clearly understood yet (Garriboli et al. 2014). More and more data supports and favours a paracrine support of undifferentiated MSCs than a direct tissue- or cell lineage-specific differentiation (Damaser and Sievert 2015).

After careful consideration of outstanding clinical results with entire urogenital organ replacement (Atala et al. 2006; Raya-Rivera et al. 2011, 2014; Chen et al. 2010) the question of successional clinical studies to prove the concepts in larger cohorts with long-term follow-up care will automatically be raised. Nevertheless these positive efforts are encouraging and a scientific stimulus to promote more intense engagement. Especially the wide range of indications, cell resources and scaffolds necessitate an intensive communication among scientific leaders of regenerative medicine, which is for instance currently facilitated by cross-national platforms (e.g. European Cooperation in Science and Technology (COST)).

An important future aspect, which has not been focused in this chapter, may be the application of human spermatogonial stem cells, which might offer new possibilities for scientists and patients. From special interest might be the definitely discussable question of the generation of germ cells in patients with different causes of unfulfilled desire to have a child (Hwang and Lamb 2010). Similarly the importance of legal requirements (namely good manufacturing practice and good laboratory) should not be underestimated for the future – ultimately this will be chokepoint to bring scientifically proven concepts "from bench to bedside" (Feil et al. 2011).

Wezel et al. pronounced in a clear and unequivocal way the main need of regenerative medicine to be present in daily routine: "To make the step into clinical practice, tissue-engineered or cell-based approaches have to be shown to be better than existing 'gold standard' approaches in long-term follow-up studies and supported by transparent and objective evaluation" (Wezel et al. 2011).

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Chapter 9
Bone

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Abstract Commonly applied therapies to achieve bone reconstruction or function are restricted to the transplantation of autografts and allografts, or the implantation of metal devices or ceramic-based implants. Bone grafts generally possess osteoconductive and osteoinductive properties. They are, however, limited in access and availability, and harvest is associated with donor site morbidity, hemorrhage, risk of infection, insufficient transplant integration, and graft devitalisation. Research therefore focuses on alternative therapeutic concepts such as tissue engineering to aid bone regeneration. However, bench to bedside translations are infrequent as the process towards approval by regulatory bodies is protracted and costly. Approval requires both comprehensive in vitro and in vivo studies necessitating the utilization of well-standardized large preclinical animal models, fixation devices, surgical procedures and methods of taking measurements. Only then reliable data pools can be generated which consecutively serve as a base for further research directions.

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The following chapter gives insight into bone morphology and physiology, and describes the clinical background necessitating research for alternative treatments. Furthermore, basic principles of bone tissue engineering are introduced as well as key points to consider when discussing preclinical animal models. Finally, successfully translated bone regeneration concepts are summarized.

Keywords Bone regeneration • Tissue engineering • Bone defect • Stem cell • Scaffold • Growth factor • Large animal model

9.1 Bone Morphology and Physiology

Bone is a complex, constantly altering tissue and consists of cancellous and cortical bone. The specific architecture of bone allows the skeleton to fulfil its mechanical functions. In adults, cortical bone accounts for 80 % of the total bone mass. It responds slowly to changes in loads and aids to protect organs, provides levers for movement, and (together with cancellous bone) stores minerals. Cancellous bone is found interiorly and comprises of a trabecular network that reduces organ weight and provides space for blood vessels and marrow. Cancellous bone has a large surface area per unit volume and a greate rate of metabolic activity. The external surface of bone is covered by periosteum (Hutmacher and Sittinger 2003).

Specific collagen fibres (Sharpey) connect periosteum and bone. These fibres penetrate the cortex at sites exposed to high tensile forces. The periosteum comprises of an external, fibrous layer (collagenous and reticular fibres) and an inner, proliferative layer (cambium). The cambium layer hosts osteoblasts and osteoprogenitor cells. It is capable of lamellar bone apposition and of forming primary, woven bone after a fracture. The outer fibrous layer provides elasticity and flexibility facilitating the insertion of tendons, ligaments and muscles.

Bone has a rich vascular supply receiving 10–20 % of the cardiac output. In long bones, one or two principal diaphyseal nutrient arteries represent the most important supply of arterial blood. These arteries pass obliquely through the cortical bone and divide into ascending and descending branches to supply the inner two thirds of the cortex and medullary cavity. Numerous arteries supply metaphysis and epiphysis. These blood vessels mainly arise from arteries that supply the adjacent joint, anastomose with the diaphyseal capillaries, and terminate in bone marrow, cortical bone, trabecular bone, and articular cartilage. In growing bones, these arteries are separated by the epiphyseal cartilaginous plates. Periosteal arterioles supply the outer layers of cortical bone and the periosteum.

The majority of bone mass is made of extracellular bone matrix. It consists of an organic component, primarily composed of type I collagen, which provides tensile strength and an inorganic component, primarily hydroxyapatite, providing compressive stiffness. Specialized populations of bone cells form, maintain and remodel this matrix. Four types of bone cells are distinguished based on their location, morphology and function: Osteoprogenitor cells, osteoblasts, osteocytes and osteoclasts. Osteoblasts develop from undifferentiated cells while osteocytes form from osteo-

blasts. Osteoclasts arise from hematopoetic stem cells and develop from blood-borne monocytes. Monocytes are attracted to the bone matrix by chemotaxis triggered by a range of stimuli such as cytokines released by resident cells. These cytokines then stimulate monocyte differentiation into osteoclasts. The processes of bone modelling and remodelling require osteoclastic resorption of bone matrix and deposition of a new matrix by osteoblasts. Modelling shapes and reshapes bones during growth and stops at skeletal maturity. Physiologic remodelling does not change bone shape and consists of bone resorption and subsequent bone deposition in approximately the same location. Since it continues throughout life, it appears to be important for the maintenance of the skeleton. Its exact function, however, remains unclear. Adaptive remodelling is the bone's response to altered mechanical conditions and may result in changes of strength, density and shape. In recent years, the understanding of the processes associated with the control of bone cell function has increased significantly.

9.2 Clinical Background

In general, bone displays a high intrinsic regenerative capacity following insult or disease. Consequently, the majority of bone defects and fractures heal spontaneously. Improved surgical techniques, advanced implant designs and adjustments of postoperative management have contributed to improve outcomes after complex injuries caused by high energy trauma, disease, developmental deformity, revision surgery, and tumour resection (Perka et al. 2000; Gugala and Gogolewski 2002; den Boer et al. 2003; Komaki et al. 2006; Laurencin et al. 2006; Wildemann et al. 2007). Extensive soft tissue injury, infections, and mechanical instability can, however, compromise the intrinsic regenerative potential and result in formation of large defects (Perry 1999). Their surgical treatment is challenging and associated with high socio-economical costs. To increase our understanding of factors and microenvironmental cues that favour the incidence of slow or non-healing defects therefore poses a major research challenge (DeCoster et al. 2004; Clements et al. 2008).

Fractures of cancellous bone are often impacted and result in defect formation after reduction (den Boer et al. 2003). Segmental cortical defects most commonly occur tibial diaphyseal as soft tissue coverage is marginal, which increases the risk of bone loss (DeCoster et al. 2004).

To date, the transplantation of autologous bone grafts still remains the gold standard treatment to augment or accelerate bone regeneration (Einhorn et al. 1984; Perka et al. 2000; Komaki et al. 2006) (Fig. 9.1). Nevertheless, considerable shortcomings are associated with bone grafting. Graft harvest requires prolonged anaesthesia and personnel for graft collection (Bucholz et al. 1989; Gao et al. 1996; Liu et al. 2008). Harvested graft amounts are often insufficient while donor site accessibility is limited (Stevenson 1998; Blokhuis et al. 2000; Oest et al. 2007; Liu et al. 2008). Persistent pain at donor sites or haemorrhage can occur, and the risk of infection is significantly increased. Once transplanted, grafted bone is associated with high failure rates (Sciadini et al. 1997; Blokhuis et al. 2000; den Boer et al. 2002;

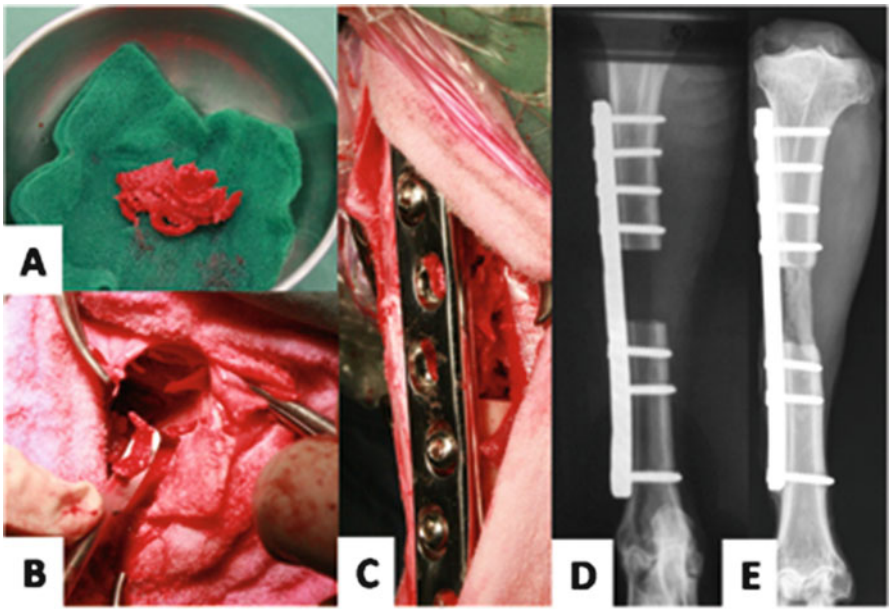

Fig. 9.1 Autologous, cancellous bone graft (**a**) harvested from the iliac crest (**b**) was used to reconstruct a 3 cm critical sized defect in an ovine tibia (**c**, **d**) Defects were stabilized with a 4.5 mm broad dynamic compression plate (Synthes). 12 weeks after surgery, new bone formation had resulted in solid bony union (**e**)

Liu et al. 2008) due to incomplete transplant integration. Graft devitalisation and subsequent resorption can compromise mechanical stability (Younger and Chapman 1989) and healing. Alternatively applied vascularised autografts are technically challenging whereas allografts and xenografts are prone to immune-mediated rejection, graft sequestration and transmission of infectious disease (Taylor et al. 1975; Dell et al. 1985; Gazdag et al. 1995; Puelacher et al. 1996; Chapman et al. 1997; Lindsey et al. 2006; Muscolo et al. 2006; Clements et al. 2008).

The high density of cortical bone allografts hinders both sufficient revascularization and cellular invasion from the surrounding host tissue (Oest et al. 2007). The limited revascularization and remodelling ability of allografts accounts for graft failure rates of 25 % and complication rates of 30–60 % (Cacchioli et al. 2006; Oest et al. 2007).

Callus distraction aims to circumvent these graft and integration related issues. It is successfully applied to treat large bone defects, infected non-unions and limb length discrepancy (Cierny and Zorn 1994). The procedure is, however, long-lasting, inconvenient for the patient (Goldstrohm et al. 1984; Ilizarov 1989) and recurrent pin track infections and pin loosening are common complications (Lindsey et al. 2006; Gugala et al. 2007).

To avoid the limitations related to current standard treatments, research interest has focused on bone graft substitutes, and the concept of tissue engineering has

emerged as an important alternative to regenerate compromised bone. Despite advances in the field and promising results achieved by the application of novel concepts in small and large animal models, translation of research results into clinical treatments remains rare.

9.3 Bone Tissue Engineering

Tissue engineering approaches can generally be classified into strategies that primarily aim at tissue conduction, induction or cell transplantation.

Tissue conduction utilizes graft materials such as hydrogels, microspheres/beads or scaffolds to mediate cell attachment. These materials usually provide an interconnected structure to support cell migration and blood vessel formation.

Tissue induction refers to the ability of a graft or substance (growth factors, lyophilized cell fractions, peptides, etc.) to induce progenitor cells migration, proliferation and differentiation into functionally mature cell types.

Cell transplantation aims at the replacement of limited structural and/or biochemical functions of the target tissue. Examples include the transplantation of chondrocytes in combination with a periosteal flap, the injection of myocardiocytes into heart muscle, haematopoetic bone marrow cell transplantation, and epidermal cell sheets for skin regeneration (Hutmacher et al. 2004).

Other approaches combine these principles with 3D cell culture to mimic natural extracellular matrices as closely as possible. Such 3D environments can be realized in form of sandwich cultures, by cell encapsulation into hydrogels, and by seeding solids or scaffolds with cells (Tibbitt and Anseth 2009).

In the past a multitude of matrices has for example been used as carriers to deliver MSCs. These include ceramics, collagen sponges and gels, and biodegradable polymers. A comprehensive report on scaffold design and fabrication for bone engineering is beyond the scope of this chapter and has been reviewed elsewhere (Hutmacher 2000; Hutmacher and Cool 2007). There is ample evidence that the nature and properties of a scaffold play an important role in bone engineering. It is, however, unclear what defines an ideal scaffold-cell or scaffold-neo-tissue construct as human tissues perform multiple functional roles. Consequently, it is unlikely that a single scaffold can serve as a universal foundation for the regeneration of a tissue.

At present bone tissue engineering efforts mainly concentrate on adult stem cells, which were found to undergo subsequent differentiation after in vivo transplantation in combination with scaffolds. These approaches rely on scaffold guided host cell and tissue in growth as well as transplanted cells as a part of an engineered device as the main factors regulating cell behaviour and performance in vivo are local cells and non-soluble factors within the extracellular matrix (ECM) (collagens, glycosaminoglycans (GAGs), cytokines, hormones, nutrients, minerals and waste products) (Table 9.1).

Table 9.1 Scaffold requirements

Scaffold requirements	
Biocompatibility	The material should not elicit an immunological or chemically detectable primary or secondary foreign body reaction
Biodegradability	Degradation at a controlled rate into non-toxic and easily excreted products
Mechanical strength	Maintenance of structural integrity during culture; support and transfer of loads after implantation
Ease of fabrication	
Porosity	Controlled and adequate porous architecture facilitating cell attachment, growth, tissue regeneration, vascularization and clearance of waste products
Osteoconductivity	Facilitation of vascular invasion, cell infiltration and attachment as well as appositional bone formation
Drug delivery	Allow release or attachment of active compounds
Ability to integrate	Integration with the host tissue following in vivo implantation
Availability	General availability; adequate shelf-life and handling properties
Sterilization	Ease of sterilisation without loss of characteristic properties

9.4 Pre-clinical Evaluation in Large Animal Models

To simulate human in vivo conditions and to assess the effects of bone grafts and tissue engineered constructs various large animal models have been developed. Most models, however, are not well described, defined, or standardized, and provide only rudimentary information on the process of model establishment.

Experimentally defects to study bone repair are postulated to be of dimensions to preclude spontaneous healing (Einhorn 1999). Such critical- sized defects can be defined as “the smallest size intraosseous wound in a particular bone and species of animal that will not heal spontaneously during the lifetime of the animal” (Gugala and Gogolewski 1999; Rimondini et al. 2005; Cacchioli et al. 2006) or as a defect which shows less than ten percent bony regeneration during the lifetime of an animal (Gugala and Gogolewski 1999).

The minimum size that defines a defect as “critical” is not well understood. Nevertheless, it has been described as a segmental bone deficiency exceeding 2–2.5 times the diameter of the affected bone (Lindsey et al. 2006; Gugala et al. 2007). However, defect healing also depends on the species’ phylogenetic scale, anatomic defect location, associated soft tissue, and biomechanical conditions in the affected limb as well as age, metabolic and systemic conditions, and related co-morbidities (Lindsey et al. 2006; Rimondini et al. 2005).

The selection of a specific animal species as a model system requires consideration of multiple factors. The chosen animal model should clearly demonstrate close physiological and pathophysiological analogies with humans regarding the scientific question under investigation. Moreover, it must be manageable to operate and observe a multiplicity of study objects over a relatively short period of time

(Schimandle and Boden 1994; Liebschner 2004; Egermann et al. 2005). Further selection criteria include costs for acquisition and care, animal availability, acceptability to society, tolerance to captivity and ease of housing (Pearce et al. 2007).

Our own research group has established and applied a 3 cm measuring critical sized tibial defect model in sheep to compare the gold standard autograft with biodegradable composite scaffolds consisting of medical-grade polycaprolactone and tricalcium phosphate combined with autologous bone marrow–derived mesenchymal stem cells (MSCs) or recombinant human bone morphogenetic protein 7 (rhBMP-7). Critical-sized defects were treated with autograft, rhBMP-7, or MSCs.

Bridging was observed within 3 months for both the autograft and the rhBMP-7 treatment. After 12 months, biomechanical analysis and microcomputed tomography imaging showed significantly greater bone formation and superior strength for the biomaterial scaffolds loaded with rhBMP-7 compared to the autograft. Axial bone distribution was greater at the interfaces. With rhBMP-7, at 3 months, the radial bone distribution within the scaffolds was homogeneous. At 12 months, however, significantly more bone was found in the scaffold architecture, indicating bone remodeling. Scaffolds alone or with MSC inclusion did not induce levels of bone formation comparable to those of the autograft and rhBMP-7 groups (Fig. 9.2).

9.4.1 Clinical Applications in Orthopaedic Surgery

Vacanti et al. (2001) reported the replacement of an avulsed phalanx with tissue engineered bone. Treatment resulted in the functional restoration of a biomechanically stable thumb of normal length. Periosteal osteoblastic progenitor cells were obtained from sections of the distal radius and were seeded onto a coral based scaffold. A calcium alginate hydrogel encapsulating the cells was used to saturate the coral implant. During follow-up, MRI examination showed evidences of vascular perfusion and biopsy revealed new bone formation with a lamellar architecture. In a first clinical study, Quarto et al. have reported the use of cell based tissue engineering approaches to treat large bone defects in three patients suffering from various segmental defects (4 cm bone segment loss in the right tibia, 4 cm in the right ulna, and 7 cm in the right humerus) (Quarto et al. 2001). Prior to transplantation, bone marrow derived osteoprogenitor were isolated and expanded. The cells were then seeded onto a macroporous scaffolds designed to fit the missing bone fragment. Defects were stabilized with an external fixator. The radiographs on follow up showed abundant callus formation along the implants and good integration at the host bone interface. In all patients recovery was reported. However, conclusions were drawn solely based on radiographic evaluation; no confirming biopsies were taken. Due to the high radiopacity of the ceramic material the assessment of bone formation within the ceramics might have been difficult as the gain in radiopacity due to new bone formation would have been overshadowed by scattering. It is furthermore unclear if the callus formation was induced by the implanted human MSCs or by bone-forming cells of the periosteum.

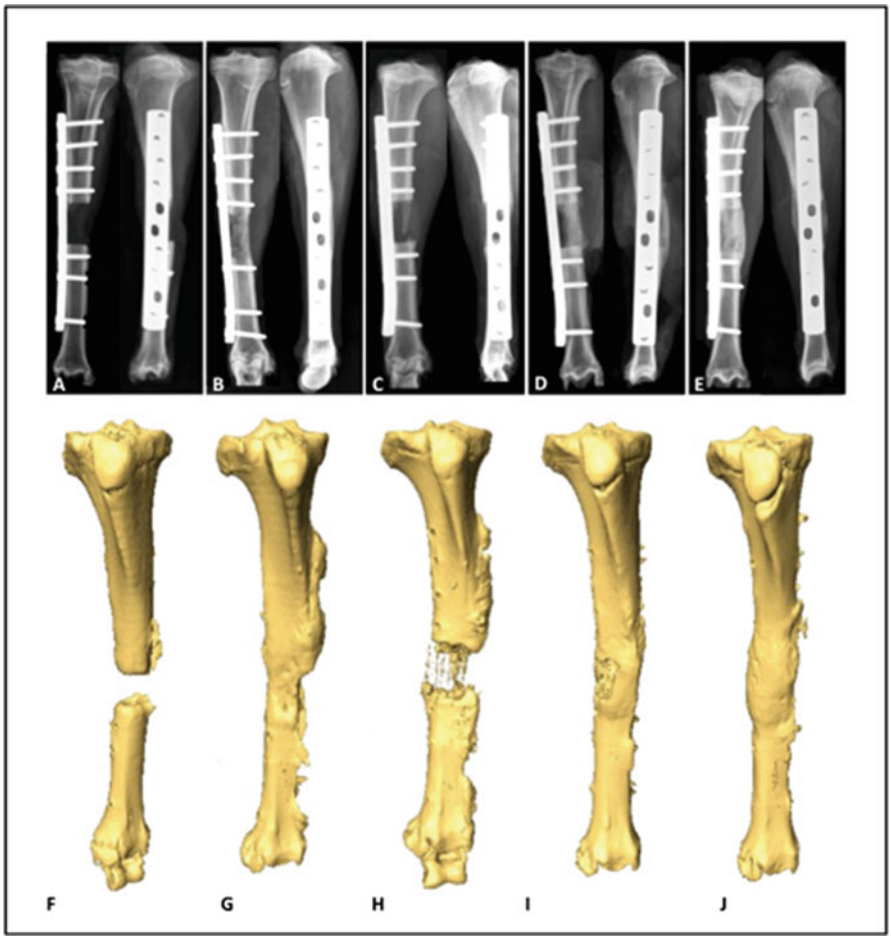

Fig. 9.2 X-ray images and CT 3D reconstructions after 3 months of an empty control defect (**a**, **f**), a defect reconstructed with cancellous bone graft from the iliac crest (**b**, **g**), a defect treated with a mPCL-TCP scaffold (**c**, **h**), and a defect augmented with mPCL-TCP + rhBMP-7 (1.75 mg D, I; 3.5 mg **e**, **j**). After 3 months, the images show clear radiographic signs of defect bridging for the autograft and rhBMP-7 group, with external callus formation in the rhBMP-7 groups. No bone formation was observed within the empty control defect and only little bone formation for the scaffold only group

The second published clinical study describes the augmentation of the posterior maxilla in 27 patients, using matrix derived from mandibular periosteum cells on a polymer fleece (Ethisorb; Ethicon,) (Schimming and Schmelzeisen 2004). In 12 patients, only radiographic and clinical assessments were performed. Limited conclusions can be drawn from the radiographic findings. The other 15 patients were treated in a biphasic approach. First, reconstruction of the host area was performed.

After a healing period of 3 months, prior to dental implant placement, a biopsy was taken. In 8 of these 15 patients a non-satisfying outcome was observed; the tissue engineered bone had been resorbed and replaced with connective tissue. In cases of positive biopsies (seven patients), the authors were unable to distinguish between bone formation induced by the implanted cells (osteoinduction) or by resident osteoblasts from the pre-existing bone (osteoconduction).

Bajada et al. (2007) reported on the successful healing of a 9 year old's left tibial mid-shaft non-union following a high-speed road traffic accident. The non-union had been resistant to various surgical procedures including the application of a monolateral external fixation, functional bracing, and two programmes of ring circular external fixation with autologous bone grafting. Using autologous bone marrow stromal cells (BMSCs) expanded in vitro to 5×10^6 cells within a period of 3 weeks combined with calcium sulphate ($CaSO_4$) in pellet form, the defect was reconstructed observing clinical and radiological convalescence 2 months after implantation.

Kim et al. investigated the effect of autologous osteoblast transplantation on healing of long bone fractures in patients (Kim et al. 2009). Autologous bone marrow derived osteogenic cells (1.2×10^7) were transplanted in combination with fibrin into fracture sites 2 weeks after internal fixation. After 8 weeks, the authors observe a significant acceleration of fracture healing compared with controls.

Due to their easy accessibility, peripheral blood progenitor cells represent a promising alternative cell source to marrow derived cells.

Kuroda et al. initiated a phase I/IIa clinical trial investigating the application of G-CSF-mobilized CD34+ peripheral blood cells for patients suffering from tibial or femoral nonunion (n = 7) (Kuroda et al. 2014). Five days following G-CSF injection, cells were magnet sorted to separate the CD34+ fraction. A number of 5.0×10^5 cells/kg body weight were incorporated into in atherocollagen gels (3 ml) and transplanted to the fracture site. Radiological fracture healing at 12 weeks was achieved in five of seven (71.4 %) patients, which exceeded the threshold (18.1 %) predefined by historical outcomes of standard care.

Giannotti et al. examined the long-term efficacy and safety of ex vivo expanded bone marrow MSCs, embedded in autologous fibrin clots, for the healing of atrophic pseudarthrosis of the upper limb (Giannotti et al. 2013). Bone marrow MSCs isolated from eight patients were expanded ex vivo and short-term osteodifferentiated. MSCs embedded in autologous fibrin clots were locally implanted in combination with bone grafts, calibrating their number on the extension of bone damage. Radiographic healing was evaluated with short- and long-term follow-ups (range averages: 6.7 and 76.0 months, respectively). All patients recovered limb function, with no evidence of tissue overgrowth or tumor formation.

The aim when treating femoral head necrosis is to preserve the femoral head and therefore avoid total hip replacement surgery. Core decompression has been shown to decrease intraosseous pressure, while additionally providing the opportunity to deliver bioactive materials and/or progenitor cells to enhance healing. We have [Nöth et al. 2007 #78] presented a therapeutic approach for patients suffering from femoral head necrosis stage ARCO II using bone marrow derived MSCs in

combination with a β-Tricalciumphosphate matrix. Kawate et al. (2006) reported on three cases of steroid-induced femoral head osteonecrosis stage Steinberg 4A (one patient) and C (two patients) treated with MSCs cultured with beta-TCP ceramics and with a free vascularized fibula. All hips showed preoperative collapse and radiographic progression was observed in two hips postoperatively, although osteonecrosis did not progress any further within the time frame reported. Moreover, Hernigou and Beaujean (2002) demonstrated that autologous bone marrow transplantations combined with core decompression before collapse of the femoral head made hip replacement surgery necessary in only 9 of 145 cases, compared to 25 of 44 cases when operated after manifest collapse. As well, Gangji et al. (2004) reported on successful treatment of 18 patients treated with bone marrow cells harvested from the iliac crest suggesting that the application of cell-based treatment concepts in case of femoral head necrosis might play a decisive role in future therapeutics.

Recently, Horch et al. reported on two cases of large bone defect reconstruction after debridement of an osteomyelitis. One of the defects was localized in the radius and one in the tibia (Horch et al. 2014). For osseus reconstruction, arteriovenous loops were created to serve as a vascular axis, and placed in the bony defects. In case 1, cancellous bone from the iliac crest in combination with fibrin glue was used to generate bone, in case 2 a clinically approved β-tricalciumphosphate/hydroxyapatite (HA), fibrin glue and bone marrow aspirated from the iliac crest. At final follow up after 36 and 72 months computed tomography (CT), magnetic resonance imaging (MRI) and doppler ultrasound revealed patent arterio-venous (AV) loops in the bone grafts as well as completely healed bone defects. The patients were pain-free and presented normal ranges of motion.

We report on a 50 year old, female patient. The patient was initially diagnosed with beginning osteoarthritis and underwent arthroscopic right knee chondroplasty at the age of 42. Despite anti-thrombotic prophylaxis, she developed a deep vein thrombosis postoperatively and ultimately an acute compartment syndrome of both flexor lodges of the lower leg. The compartment syndrome was subsequently treated by fasciotomy. Over the following 6 months she developed a progressive supination contracture of the right foot, which was corrected by subtalar arthrodesis. Consequently, the surgery related muscle imbalance in the operated foot resulted in the formation of claw toes DII-V, which were treated by tenotomy of the long and short flexor tendons, respectively. Furthermore, as another long-term consequence of the compartment syndrome, the patient increasingly suffered from developing knee stiffness with an overall extension deficit of 40° greatly affecting her mobility and quality of life. The ability to fully extend was restored by a combined arthroscopic ventral and open dorsal arthrolysis. Within 6 months after discharge a supination malposition of the right forefoot occurred accompanied by a contraction of the toes DIII-V. Together with the patient, the decision was made to perform an arthrodesis of the tibio-tarsal joint with one medial and two lateral cortical screws, open arthrolysis DII-V and tenotomy of the short flexor tendons plantar. Three months after corrective surgery, the patient suffered a traumatic fracture of the arthrodesis as one of her crutches broke while walking stairs. The fracture was stabilized with a retrograde intramedullary (IM) locking nail (12 mm diameter, 150 mm

length). As no progress towards bony consolidation was observed radiologically after a period of 5 months, revision surgery was scheduled to perform a thorough debridement. The pseudarthrosis was re-stabilized with an IM nail and cortico-spongious autograft from the right iliac crest was transplanted into the defect for bone augmentation. Six months later the two proximal locking bolts were removed, after another 6 months, the distal two. However, the desired effect of telescope-like sintering of the bone was not achieved and a persisting non-union had developed. Again, the non-vital bone in the pseudarthrotic region was resected and the resulting defect was reconstructed with a cortico-spongious autograft transplant taken from the iliac crest following thorough debridement of the non-union. Fifteen months later, still no bony union was achieved. As an *ultima ratio*, it was decided to pursue a newly developed, cell-based, experimental treatment concept. Bone marrow was aspirated from the posterior iliac crest and processed by Aastrom Biosciences Inc. (Stuttgart, Germany).

The bone marrow sample taken from the patient in the first step of the process contains a range of cells including hematopoietic and mesenchymal cells. These cells are known to play important roles in the natural healing mechanisms of the human body (Bruder et al. 1997; Cui et al. 2007). Aastrom's patented single-pass perfusion technology controls gas and cell culture media exchange to enable the replication of these naturally occurring cells. The cellular therapy resulting from this process contains expanded populations of mixed stem and progenitor cells to support the regeneration of tissue (Tissue Repair Cells, TRC). This approach reduces the risk of rejection and increases the likelihood of integration with the surrounding tissues, eliminating the need for immunosuppressive drugs.

The patient's bone marrow derived cells were expanded to a total number of 104.81×10^6. During final revision surgery, the IM nail was removed, and the TRC were applied at a concentration of 7.65×10^6/ml (total volume 13,7 ml) to the pseudarthrotic area and the cavity caused by the IM fixation device in combination with 20 cc of a synthetic cancellous bone void filler (Vitoss, Orthovita, Malvern, USA).

Vitoss is composed of β-tri-calcium-phosphate (β-TCP) and is stable at physiologic pH. It resorbs during the natural remodeling process of bone. Evidence suggests that β-TCP resorbs in the most relevant time frame in comparison to other bone substitutes such as hydroxyapatite (HA) and calcium sulfate (CaS) (Anker et al. 2005). Vitoss has an open-interconnected structure that facilitates 3-D bone regeneration and is composed of nano-particle construction, allowing for physiologically relevant cell mediated resorption. Vitoss is highly porous with an overall porosity of 90 % (Hinz et al. 2002).

Postoperatively, the patient was supplied with a dorsal lower leg splint for a period of 2 weeks, afterwards with a circular cast. The patient was asked not to weight-bear for a period of 4 weeks after stem cell transplantation. Gradually, she was allowed to partially weight bear with 20 kg (4 weeks), subsequently with half of her body weight (4 weeks) to then fully weight bear after all. The patient was regularly followed up in our outpatient department, lastly, 4 years after stem cell transplantation surgery. After a period of 6 months solid bony consolidation of the pseudarthrosis was observed radiologically, half a year later, signs of cortical remodeling as an indicator of successful, biomechanically stable bone healing

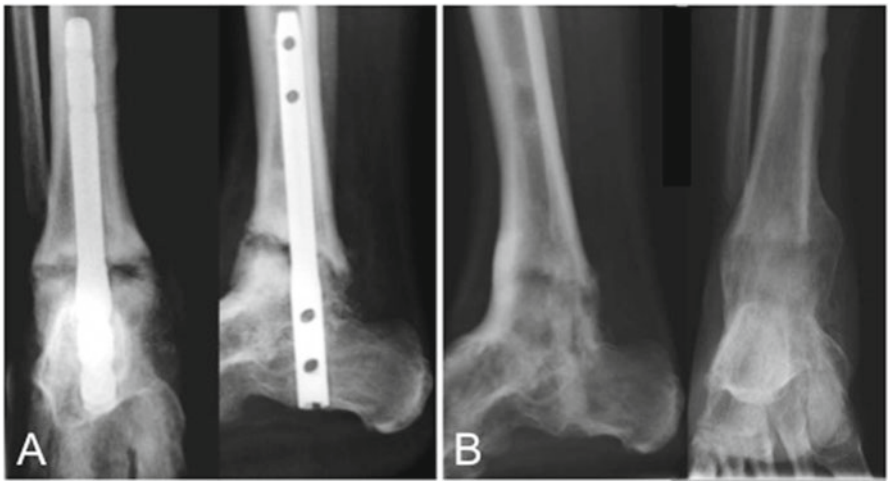

Fig. 9.3 Conventional x-ray images showing the pseudarthrotic tibial non-union in two standard planes (anterior-posterior view *left*, lateral view *right*) prior to autologous stem cell transplantation (**a**) and increasing bony consolidation with signs of cortical remodeling 1 year post mesenchymal stem cell application (**b**) forming a mechanically stable arthrodesis

(Fig. 9.3). The patient was additionally supplied with orthopaedic footware and henceforward free of complaints.

Bone repair and regeneration with bone morphogenetic proteins (BMPs) have advanced as an alternative treatment option in orthopaedic and trauma surgery. A number of animal studies and subsequent clinical trials have demonstrated the osteogenic potential of BMPs. The result has been the commercialisation of two of the early BMPs, BMP-2 and BMP-7 (also called osteogenic protein-1 or OP-1).

OP-1 is an attractive adjunct in the treatment of fractures and atrophic long bone non-unions.

The use of OP-1 in the treatment of open tibial shaft fractures was evaluated by the Canadian Orthopaedic Trauma Society. One hundred and twenty-four open tibial fractures (62 controls and 62 OP-1) were included in the study. After irrigation, debridement and intramedullary nailing, at the time of definitive wound closure, patients were randomised to standard wound closure or standard wound closure with the addition of OP-1 to the fracture site. Patients were followed up radiographicly, clinically and serologically until union. Outcomes showed that the number of secondary interventions for delayed union and non-union was significantly lower in the OP-1 group than in the control group (8 vs. 17; $p=0.02$). A significantly greater number of patients in the OP-1 group was able to fully weight bear without pain at 12 months compared to the control group. No OP-1 related adverse effects were clinically evident. The study investigators suggested that the use of OP-1 is safe for use in open tibial shaft fractures, and its use decreased the number of secondary procedures for delayed or non-union.

More recently, Ristiniemi et al. have evaluated OP-1 in the treatment of distal tibial fractures (Ristiniemi et al. 2007). Twenty patients with distal tibial fractures were treated with external hybrid fixators and OP-1 and compared to 20 matched patients treated without BMP. Outcome measures included time to radiographic union, duration of application of the external fixator, the number of secondary interventions due to delayed healing and length of absence from work. The mean time to union as well as to fixator removal was significantly shorter in the BMP group (15.7 weeks vs. 23.5 weeks, $p=0.002$; 15 weeks vs. 21.4 weeks, $p=0.037$). Revisions for delayed union were required in two patients in the BMP group and seven patients in the control group. Average time off work was significantly lower in the BMP group than in the controls.

The use of OP-1 in the treatment of tibial non-union was studied by Friedlaender et al. in a randomised controlled, prospective clinical trial (Friedlaender et al. 2001). Clinical and radiographic results were compared to assess the efficacy of OP-1 versus autograft in the treatment of tibial non-unions that had persisted for at least 9 months. One hundred and twenty-four tibial non-unions in 122 patients were randomised to either intramedullary nail and autograft or intramedullary nail and implantation of OP-1 at the non-union site. Nine months after surgery, 81 % of the OP-1 group and 85 % of the autograft group had achieved clinical union. Radiographic analysis indicated that 75 % of the fractures treated with OP-1 and 84 % of the autograft-treated group had healed. There was no statistically significant difference between groups clinically or radiographicly. More than 20 % of the bone graft group complained of persistent donor site pain. The authors concluded that OP-1 was a safe and effective alternative to bone grafting in the treatment of tibial non-unions. This study led to multiple regulatory approvals worldwide. Numerous studies in the literature suggest that OP-1 is a safe and effective treatment option for fractures and atrophic non-unions not only of the lower but also of the upper extremities.

9.5 Summary

The reconstruction of complicated fractures and large segmental bone defects remains a significant clinical problem. Large bone defects may occur as a result of extensive bone loss resulting from pathological events such as trauma, inflammation, and tumour resection. Present therapeutic approaches include the application of bone graft transplants (autologous, allogenic, xenografts), fixation devices consisting of different synthetic and natural biomaterials, and segmental bone transport. However, to date, no existing therapy has been fully satisfactory. A number of research groups therefore work on the development of new bone grafting materials, carriers, growth factors, and tissue engineered constructs for bone regeneration. To optimize cell-scaffold combinations and the application of locally or systemically active stimuli remains a complex process. It is characterized by a highly interdependent set of

variables with a large range of possible variations. Consequently, the evaluation of new developments in the field of bone tissue engineering must base on clinical experience, knowledge of basic biological principles, medical necessity, and commercial practicality. The area of bone tissue engineering relies on animal models to evaluate both experimental and clinical hypotheses. To overcome current limitations associated with bone tissue engineering, researchers must rely on the functional assessment of biological and biomechanical parameters of generated constructs. For comparison of different studies and their outcomes, the standardization of animal models, fixation devices, surgical procedures and methods of taking measurements is essential to accumulate a reliable data pool guiding further directions to orthopaedic and tissue engineering developments. Only then we can overcome the lack of translation in the field of bone engineering which is closely related to difficulties in integrating individual technical discoveries in model tissue engineering systems, in manufacturing scale up, in funding, and in regulatory approval.

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Chapter 10
Articular Cartilage Regeneration

Georg N. Duda, Michael Sittinger, Joshua O. Eniwumide, and Evi Lippens

Abstract The glassy translucent material found at the ends of bones, within synovial joints, is termed articular cartilage. While healthy, it provides a low-friction bearing surface, preventing bone-to-bone contact, and to an extent, absorb shock during vigorous activities. However, when damaged could lead to pain, deformity and reduced mobility; the social impact of which, entails high costs in terms of therapeutic treatments and loss of income. The present chapter reviews the common knowledge of the constraints to articular cartilage regeneration; namely cartilage structure, composition and major diseases. The first of the three sections detail the major constituents of the tissue and their structural organization; the tissues mechanical properties, and ends with a brief description of how these features change in an unhealthy cartilage; be it mechanical or disease. In the second section, both clinical and academic approaches are pooled together, to review the current strategies in restoring health to joints with diseased or damaged cartilage. The final section highlights the fact that progression of cartilage disease affects not only the cartilage, but its underlying bone. The implications of the subchondral bone in the propagation of cartilage degeneration are discussed, and finally, their considerations in cartilage defect healing.

Keywords Tissue composition • Structural organisation • Mechanical properties • Repair and regeneration strategies • Subchondral tissue

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10.1 In Health and Sickness

10.1.1 Structure and Function

Articular cartilage is found at the end of bones as a thin, white tissue. This tissue consists mainly of extracellular matrix (ECM), and has a relatively low density of cells. The adult tissue is known to be devoid of blood vessels, lymph vessels, and nerves (Poole 1997; Stockwell and Meachim 1979). The thickness of adult human cartilage generally varies between 2 and 7 mm (Meachim and Stockwell 1979). This variation is evident both between joints and also within different regions of the same joint.

The efficient functioning of the synovial joints is made possible by the presence of articular cartilage. Lining the surfaces of diarthrodial joints, articular cartilage provides a low-friction bearing surface and prevents bone-to-bone contact (Guilak and Mow 2000; Mow et al. 1980). Major load bearing joints, such as the hip, knee and ankle are subjected to peak stresses up to three times body weight during normal walking or higher during stumbling (Guilak and Mow 2000; Mow et al. 1992; Weightman and Kempson 1979). Under these high stresses, articular cartilage deforms, effectively reducing both the contact stresses and the pressures transmitted to the underlying bone (Ateshian and Wang 1997; Kim et al. 1994; McCutchen n.d.; Weightman and Kempson 1979). Cartilage also exhibits impact resistance, which permits a degree of shock absorbance during vigorous activities such as running and jumping.

10.1.2 Composition

Articular cartilage consists of Water, Collagen, and non-collagenous proteins, and cells. These are approximated to be 70–80 %, 10–15 %, and 5–10 % of the tissues wet weight, respectively, while the cells are approximately 5 % of the tissues volume. Notably these features vary amongst species, from joint to joint and within different locations of the same joint (Buckwalter and Mankin 1997; Stockwell and Meachim 1979).

10.1.2.1 Collagen

Collagen is a large protein family with at least 27 members (Boot-Handford et al. 2003; Eyre 2004). They can be distinguished by their distinct amino acid composition and hence polypeptide chains (Meisenberg and Simmons 1998). The collagen types present in articular cartilage are types II, VI, IX, X and XI (Eyre et al. 1992). Type II collagen makes up 95 % of the solid composition of the mature human articular cartilage (Eyre et al. 1992). It is derived from procollagen molecules

containing amino (NH_2)-and carboxyl (COOH)-terminal extension peptides that are cleaved, extracellular, prior to fibrilcollagens (Ryan and Sandell 1990).

Due to their abundance in articular cartilage, type II and type X collagen are often used as indicators for extracellular matrix (ECM) formation by cultured chondrocytes and to ascertain a chondrogenic phenotype of differentiating stem cells.

Type XI collagen contributes less than 5 % to the total collagen content of the articular cartilage. In cartilaginous tissues, collagen XI forms heterotypic narrow fibrils with collagens type II and type IX (Grant et al. 1988). Type VI collagen is a short-helical heterotrimer. Its monomers are arranged intracellular into anti-parallel staggered dimers and then into tetramers by lateral aggregation of two dimmers, then secreted into the ECM. In the pericellular environment, collagen VI has been implicated in both the maintenance of chondron integrity and cell-matrix signaling. Type IX collagen accounts for 10 % of human fetal cartilage. This proportion decreases with age, reaching 1–2 % in mature human articular cartilage. This molecule can be considered a protoglycan, due to its possession of a chondroitin sulphate chain (Huber et al. 1988). Type X collagen is a short-chain collagen, which forms a mat-like network in the hypertrophic cartilage matrix and around differentiating chondrocytes (Kwan et al. 1986). It is found in either the cartilaginous tissues undergoing endochondral ossification, such as the hypertrophic zones of the growth plate, or the calcified zone in mature articular cartilage (Ayad et al. 1987).

10.1.2.2 Proteoglycans

Proteoglycans (PGs) are the most abundant non-collagenous macromolecular components in mammalian cartilage, making up approximately 10–15 % of the mature mammalian articular cartilage. These are defined as having a protein core to which one or more glycosaminoglycan (GAG) chains are covalently attached. GAGs present in articular cartilage include chondroitin sulphate, keratan sulphate and hyaluronan. The GAG chains are often crucial to the functional properties of the PG (Hardingham and Fosang 1992). However, for PGs such as decorin and biglycan, which bind to growth factors and modulate their activities, evidence exist, suggesting that it is their protein core and not their GAG chains that mediates the binding function (Cheifetz et al. 1988; Ruoslahti and Yamaguchi 1991). The most abundant PG, accounting for up to 90 % of mature articular cartilage PG is aggrecan. As GAG builds up in the cells ECM, aggrecan molecules bind non-covalently along a hyaluronan chain to form an aggrecan-hyaluronan complex. The aggregate, having molecular weight approximately 50,000 kDa is associated with load distribution in articular cartilage (Hardingham and Fosang 1992). Smaller PG molecules present in cartilage include biglycan, decorin and fibromodulin (Knudson and Knudson 2001). These leucine-rich PGs bind to collagen type II and play important roles in ECM organization (Pulkkinen et al. 1990; Vogel et al. 1984).

PGs have important roles in collagen fibrillogenesis, organization of collagen networks and in providing rigidity to the ECM. Chondrocytes express cell surface PGs (Knudson and Knudson 2001), which interact with growth factors such as,

basic Fibroblastic Growth Factor (bFGF) in order to regulate cell activities. Similar to types II and X collagen, PG provide a reliable indicator of biosynthetic activities of chondrocytes in culture (Knight et al. 1998; Lee et al. 2003). Other common examples of such proteins are Anchorin CII. These bind to the surface of the chondrocytes and anchor it to the collagen fibrils in the ECM and cartilage oligomeric matrix proteins, which also bind to chondrocytes (Mollenhauer et al. 1984). These proteins maintain the chondrocytes phenotype, and are used as markers of cartilage turnover and can control the progression of cartilage degradation in osteoarthritic cartilage (Salter 1993). Other proteins, such as fibronectin and tenascin have roles in matrix organization, cell-matrix interaction and the tissue response to inflammatory conditions such as osteoarthritis (Buckwalter and Mankin 1997; Nishida et al. 1995).

10.1.2.3 Extracellular Matrix Fluid

Water is the largest component of articular cartilage. It makes up to 80 % of cartilage wet weight in the surface zone, and decreases to approximately 65 % within the deep zone. The high affinity of articular cartilage for water is due to the charge density of the hydrophilic PG molecules. The PGs encapsulate the water within their matrices, forming a gel-like substance, with pore size of approximately 6.9 nm (Meachim and Stockwell 1979). These pores contribute to the diffusivity of the small molecules and water through articular cartilage (Lusse et al. 2000; Maroudas 1979). Matrix water contains small gasses, proteins and dissolved electrolytes (Na^+, Cl^-, Ca^{2+}, SO_3^-, COO^- etc.). The cations balance the negatively charged PGs, thereby influencing the mechanical properties of the tissue (Guilak et al. 1999). A large proportion of the ECM fluid can move freely in and out of the tissue under applied load (Buckwalter and Mankin 1997). Therefore during normal joint loading, the cartilage is compressed and the water is squeezed out of the loaded region. As the region is unloaded, the water is re-imbibed and the original volume is restored with time. This movement of cartilage fluid is crucial in joint lubrication, transport of macromolecules within articular cartilage and nutrition of the cells therein.

10.1.2.4 Chondrocytes

The cartilage ECM and its associated proteins are synthesized, assembled and organized into a highly ordered framework by its cellular component, the articular chondrocytes (Buckwalter and Mankin 1997; Muir 1995). The chondrocytes attempt to maintain the ECM and their associated protein by their continual replacement in health, disease and following trauma. However, this depends on the cells ability to detect changes in the matrix composition, which may be due to macromolecular degradation, or the mechanical demands placed upon the tissue. Although relatively sparse in density, the chondrocytes are the only living units available to adapt cartilage to changes in its surroundings. The chondrocyte is surrounded by a thin layer of matrix, called the pericellular matrix (PCM) which together with the enclosed cell is called the chondron. The composition, structure and function of the PCM is

different from the surrounding ECM and plays an important role as transducer of both mechanical and biochemical signals to the chondrocytes (Wilusz et al. 2014). Not only the elastic modulus of the PCM is different from its surrounding ECM, also a higher proteoglycan content is found in the PCM compared to the ECM. Therefore, tissue deformation and the associated changes in interstitial water content that occur during loading will result in dynamic changes in the physicochemical and osmotic environment of the cell and may provide critical signals for regulating the cell's response to loading (O'Conor et al. 2014; Wilusz et al. 2014).

Individually, chondrocytes have been found to be metabolically active, with a glycolytic rate per cell similar to that of cells found in vascularized tissues. However, the adult tissue as a whole, has a comparatively low metabolic activity due to its low cell density of approximately one cell per 100 μm^3 (Buckwalter et al. 2005). Both cell proliferation and ECM synthesis decline following skeletal maturity in normal tissue.

10.1.3 Organization

The structure and composition of articular cartilage changes with depth from the joint surface (Buckwalter et al. 1987; Clarke 1971; Lane and Weiss 1975; Lipshitz et al. 1976; Muir et al. 1970; Ratcliffe and Mow 1976). Although these changes are continuous, articular cartilage has been divided into four distinct zones/layers (Fig. 10.1). These are termed the superficial zone (I), transitional zone (II), the deep or radial zone (III), and the zone of calcified cartilage (IV). PGs occupy the interfibrillar space and their concentrations increase from the surface to a maximum in the transitional zone, and then diminishes toward the deep zone (Comper 1996; Muir 1980; Poole et al. 1982). The volumetric concentration of collagen fibers in human articular cartilage increases from the superficial (16–31 %) to the deep zone (14–42 %), while that of the cell decrease by a factor of about three from the surface to the deep zone. The superficial zone is closest to the articular surface. It is approximately 250 μm thick for human articular cartilage, and is the thinnest of the four zones. Chondrocytes in this zone are flattened and oriented parallel to the articular surface (Meachim and Stockwell 1979). Collagen fibers in this region are very fine and, are arranged tangentially to the surface of the cartilage, thus deriving its alternative name; the tangential zone (Buckwalter et al. 1987). The most superficial part of this region is termed the lamina splendens and is devoid of cells, but consists of fine fibers and polysaccharides.

In the transitional zone, chondrocytes are spherical in form and fairly uniform in distribution. The collagen fibrils in this zone are generally larger and more randomly organized. There is a higher concentration of PGs and lower water content when compared with the superficial zone (Buckwalter and Mankin 1997). The deep zone has a thickness greater than 500 μm, making it the thickest of the four zones. Chondrocytes of the deep zone are spherical, and are arranged in columns of four to nine cells, oriented perpendicularly to the joint surface (Meachim and Stockwell 1979). Collagen fibers within the deep zone are arranged perpendicularly to the articular surface. The zone of calcified cartilage separates the radial zone and

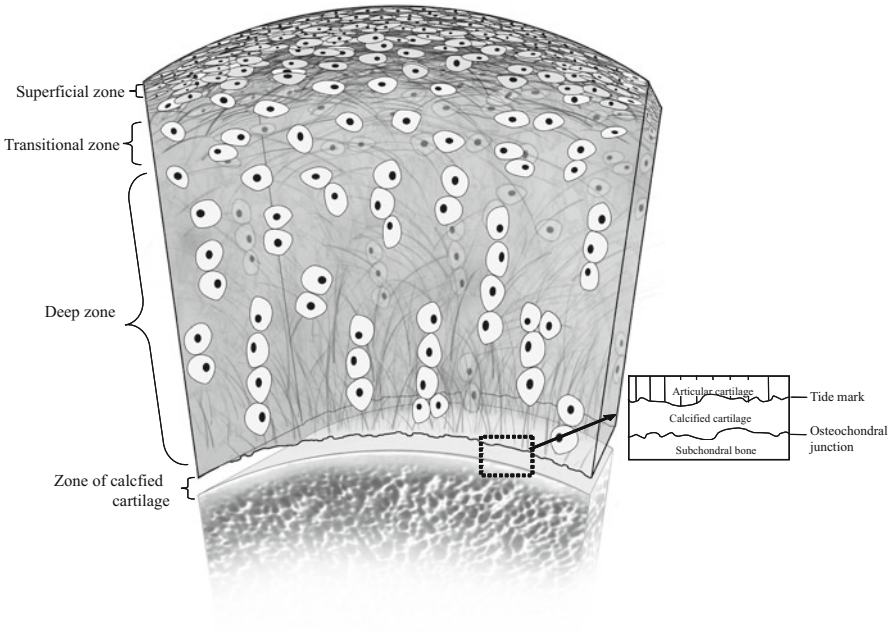

Fig. 10.1 Structure and composition of cartilage at different depths from the articular surface

subchondral bone. The deep and the calcified zones are separated by the *tide mark*. It is widely believed that the calcified zone of articular cartilage and the *tide mark* present barriers for solute diffusion via the subchondral bone and therefore nutrition is solely from the synovial fluid, via the articular surface (Honner and Thompson 1971; McKibbin and Holdsworth 1966). Evidence does exist, however to suggest the contrary, in that molecules can travel across to the articular cartilage. Notably, most of these studies have used immature synovial joint, and it is generally accepted that the route for nutrient delivery to the articular cartilage is affected by skeletal maturity (Honner and Thompson 1971). Compared to the deep zones, cells in the calcified zone have a smaller volume, and are associated with fewer Golgi membranes and endoplasmic reticula in the cytoplasm, thus suggesting a reduced metabolic activity (Buckwalter and Mankin 1997).

10.1.4 Cartilage Biomechanics

Mechanically, a healthy articular cartilage acts to limit the contact stresses acting on the underlying bone and provide an extremely efficient low wear bearing surface for smooth movement (Kempson et al. 1970; Mow et al. 1992). For these reasons, cartilage is more deformable than bone, and thus when loaded, can provide a considerable area of contact to support joint loads (Weightman and Kempson 1979).

Interactions between the two main solid components of the ECM have crucial roles in the tissues ability to sustain an applied load (Maroudas 1976; Wong et al. 2000). For example, PG exhibits a swelling capacity, resulting from the negatively charged GAG molecules repelling each other while attracting water and mobile cations. Therefore PG molecules impede the loss of water in the matrix by reducing the tissue's permeability (Quinn et al. 2001). When not loaded, the associated osmotic pressure is balanced by the hydrostatic pressure resulting from the tensile stresses within the network of collagen fibers.

10.1.4.1 Cartilage Loading

When cartilage is loaded, there is a short-lived response that alters the balance between the osmotic and hydrostatic pressures. The internal pressures increase within the joints, producing pressure gradients. Consequently, fluid flows away from the tissue, resulting in an increased in PG concentration (Weightman and Kempson 1979). To minimize this displacement, tensile stresses builds up in the collagen network. As the load is removed from the joint, fluid flows back into the cartilage and it regains its original shape. This response varies with the magnitude and type of load applied (Herzog et al. 1998).

During prolonged loading of cartilage, fluid flows out from the tissue and over time, there is a loss of volume, resulting in a time-dependent creep response. During static loading such as squatting and maintaining a 90° bend, the load transmission is confined to a relatively smaller area of the joint, the contact stress and deformation in this region is considerably greater than anywhere else the joint. Despite the loading rate being lower than that in knee bending exercises, the cartilage is loaded continuously at a local site over a long period, and this can lead to creep-behavior (Eckstein et al. 2000).

10.1.4.2 Creep

The mechanical properties of cartilage can be measured either in creep or stress relaxation. Creep is the slow time-dependent deformation process which occurs after the immediately, elastic deformation of cartilage during load application (Fig. 10.2a). This is then followed by a slow, time dependent increased deformation. The initial deformation is brought about by the tissues' matrix, thus there is no net change to its volume. Moreover, the tissues resistance to deformation is due to the network of collagen fibers. However, the time-dependent deformation, which occurs as the load is maintained, results directly from the imbibition of water from the tissues matrix. As the fluid leaves cartilage, the applied load is transferred from the matrix fluid to the solid components. An equilibrium is established when the load is totally transferred unto the matrix fibers. For creep measurement of cartilage, the tissue is compressed with a constant load and the resulting deformation is recorded.

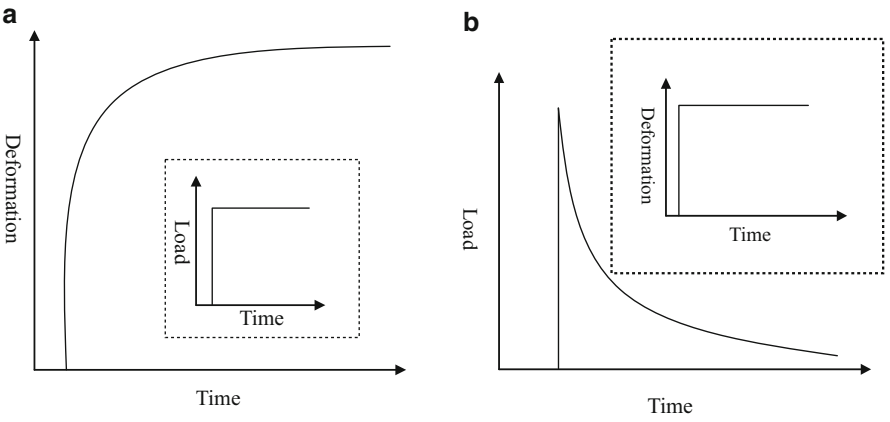

Fig. 10.2 (**a**) Creep and (**b**) load relaxation measurements of cartilage

10.1.4.3 Load Relaxation

In load relaxation, cartilage is compressed to a constant deformation and the load is measured (Fig. 10.2b). Initially, the load required to maintain a constant deformation is high. This is necessary in order to pressurize the matrix fluid. As the fluid begins to leave the tissue, the load required to maintain the deformation decreases and tend towards a plateau. At this stage, the matrix is compacted and the interfibrillar pore space is reduced. Equilibrium is then established when fluid is no longer exiting the cartilage, and the remaining fluid is redistributed within the tissue. Both the time-dependent creep and load relaxation of cartilage is largely due to the fluid flow, whereas the equilibrium is controlled by the solid matrix. In fact, the equilibrium stiffness of articular cartilage has been tied to the tissues PG content (Jurvelin et al. 1988; Mow et al. 1980).

10.1.4.4 *In Vitro* Mechanical Testing

Three common methods used for determining the mechanical properties of cartilage are confined compression, unconfined compression and indentation testing. In confined compression (Fig. 10.3a), cartilage is placed a non-porous chamber and compressed with a porous platen, so fluid is forced out only via the porous platen. During unconfined compression (Fig. 10.3b) however, cartilage is compressed between two non-porous platens and fluid exits laterally. For indentation tests (Fig. 10.3c), cartilage is compressed with an indenter that is either porous or non-porous. In cases where a porous indenter is used, fluid expelled from the cartilage may flow laterally or axially. However, non-porous indenters impede axial fluid flow.

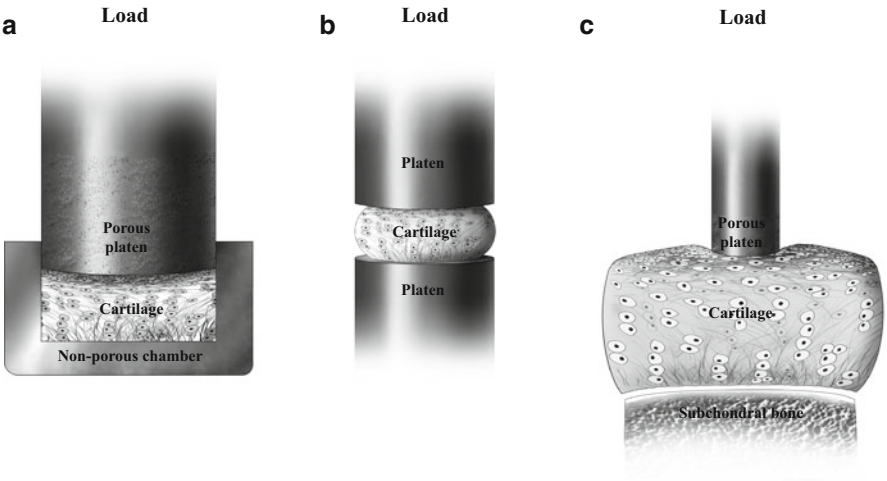

Fig. 10.3 Schematic representation of experimental setup used for the (**a**) confined, (**b**) unconfined compressive and (**c**) indentation testing of cartilage explants

Although common mechanical parameters may be obtained using any of the three strategies, it has been recognized that the values of mechanical properties are dependent on the measurement technique employed. For example, Hurtig and co-workers (Korhonen et al. 2002) reported that values of compressive stiffness and poisson's ratio of bovine cartilage derived from confined compression were slightly higher than values derived from unconfined compression tests, and values derived from indentation testing were significantly higher than both the confined and unconfined values. This technique dependence of mechanical properties is due to the inhomogeneous structure and anisotropic mechanical properties of cartilage.

10.1.4.5 In Vivo Mechanical Testing

The ability to monitor the health status of an intact cartilage, predict or diagnose osteoarthritis, and monitor the healing process of the tissue after a treatment had necessitated in vivo strategies, be it simple observation, or qualitative or quantitative measurements. Earlier techniques were based on magnetic resonance imaging (MRI). To this extent, Eckstein and co-workers (1999) analyzed the deformation and recovery, as indicated by interstitial fluid flow rate in seven healthy patellae joints. Similarly, O'Byrne et al. (2003) assessed the biochemical composition of cartilage in goat knees, in response to papain injection. The authors were able to demonstrate a compromise of the tissues collagen integrity with the magnetic resonance technique. This dose-dependent degradation was confirmed by post-mortem biochemistry and histology.

High-frequency ultrasound and mechanical indentation in now commonly combined to measure both structural and mechanical parameters, such as stiffness, and thickness, respectively. In an example, Kiviranta et al. (2008) compared the dynamic stiffness of healthy and degenerated patella cartilage, thereby diagnosing early stages of OA. In a similar study, Nishitani et al. (2008) were able to arthroscopically determine the thickness and surface roughness of ten male athletes while undergoing mosaicplasty for osteochondritis dissecans.

10.1.5 Modelling Theories for Articular Cartilage

During indentation tests, after a sudden application of constant load on cartilage, a rapid compression takes place, which is then followed by a slow creep process towards equilibrium at a rate which is governed by the applied load and test conditions (Mow et al. 1984). Early explanations for this viscoelastic behavior did not take into account the interstitial fluid flow and internal redistribution of the organic matrix and the compaction within the cartilage specimen. Although the possible influence of the multiphasic nature of cartilage on its deformational characteristics was realized by Hirsch (1944) as early as 1944, the role of fluid flow on the dynamic deformational behavior of articular cartilage was not recognized until later. One of these studies (Elmore et al. 1963) showed that the creep response observed in indentation testing of cartilage was largely due to the efflux of interstitial fluid from the tissue. They also observed that upon removal of the load, complete recovery of the tissue occurred only if sufficient fluid was available to re-imbibe into the tissue. Indeed, Linn and Sokoloff (1965) recorded a positive correlation between creep response and the amount of fluid exuded from cartilage tissue. Such studies stimulated a range of models describing both physicochemical and mechanical properties of cartilage.

10.1.5.1 Biphasic Theory

The biphasic model depicts cartilage as a soft, porous and permeable material comprising 20 % (wt/vol) of elastic solid, and filled with an incompressible fluid (Mow et al. 1980; Torzilli and Mow 1976). The model accounts for the effect of the drag forces arising from the relative motion between the fluid and solid phases. It incorporates all existing known mechanical properties of cartilage, namely, inhomogeneity, anisotropy, stress-strain non-linearity, interstitial fluid flow and finite deformation. However, several assumptions are associated with the theory (Mow et al. 1992), namely,

- The solid matrix is porous, permeable, and elastic.
- The solid matrix and interstitial fluid are intrinsically incompressible; i.e. volume change of the tissue as a whole is possible only if there is fluid exudation or imbibition.

- Frictional drag is directly proportional to the relative velocity between the interstitial fluid and the porous-permeable solid matrix – the proportionality coefficient is the drag coefficient [*K*], which may be strain-dependent.
- The frictional drag of the interstitial fluid flow is the dominant mechanism controlling tissue viscoelasticity in compression.

A modified, general form is the Kuei, Lai and Mow biphasic theory for cartilage. This form differs to the previous by the addition of more constitutive assumptions, such as an infinitesimal strain, linear, isotropy, constant elastic coefficients, and constant or strain-dependent permeability. However, some authors have associated the biphasic theory with inherent flaws (Brown and Singerman 1986). In particular; the theory relies on the ability to define the distinct phases, which is problematic as there are no distinctive barriers between the matrix and the fluid components. When applied to the prediction of creep behavior of an isotropic, homogeneous and linearly elastic material undergoing small strain deformation the biphasic theory was found to be inadequate. For example, it was incapable of modelling the substantial portion of the transient phase of cartilage response when load under a slow rate in unconfirmed compression.

10.1.5.2 Triphasic Theory

It has been observed that when unloaded cartilage specimens are soaked in a sodium chloride (NaCl) solution at constant temperature, the tissue dimensions decrease exponentially with increasing NaCl concentration. The influence of ionic movements in cartilage on its swelling and deformational behavior has long been recognized (Maroudas 1979). This has led to the development of the triphasic theory. The theory couples both the physicochemical aspects of cartilage swelling and the biphasic view of solid matrix deformation and interstitial fluid flow. The theory describes the equilibrium free swelling and confined compression behavior of cartilage and other soft hydrated tissues. In this theory, cartilage is considered as a mixture of three phases: an *incompressible solid phase*, which is the matrix, consisting of collagen and PG, *an incompressible fluid phase*, which is the interstitial water, and an *ionic phase* of two species of a single salt, the cations and the anions. The theory can be applied to equilibrium as well as transient problems, and has been found capable of predicting the stress-strain fields in the solid matrix, the interstitial fluid flow along with the distribution of the ions, and fluid pressure (Gu et al. 1997; Lai et al. 1991).

10.1.5.3 Poroviscoelastic and Poroelastic Theories

Both the biphasic and triphasic theories fail to incorporate the anisotropy and viscoelasticity of cartilage, which are of great importance when determining cartilage mechanical properties. To this extent, several models exist, whose details are

beyond the scope of the present review. More relevant is the consideration of the interfibrillar pores in cartilage, which control the transport of soluble nutrients and the flow-independent viscoelasticity of cartilage mechanical and physicochemical properties. These are described by the poroelastic and the poroviscoelastic theories.

The poroviscoelastic model describes the viscoelasticity exhibited by cartilage with a combination of a fluid flow-dependent, fluid flow-independent mechanisms and the intrinsic viscoelasticity of the solid matrix (Mak 1986). In the poroelastic model of cartilage, the tissue is modelled as an isotropic solid matrix containing fluid-saturated pores, entrapped by a fibrillar network. Both the solid and the fluid phases are assumed to be incompressible. The structure is defined by the Young's modulus, Poisson's ratio of the matrix and the hydraulic permeability. The model assumes that the hydraulic permeability depends on the dilatation of the bulk material. The fibrils are evenly distributed in the radial, circumferential and axial directions forming an elastic constituent attached to the porous matrix and that the stiffness of the fibrillar network depends on the longitudinal strain of the fibrils. These fibrils have no resistance to compression and the effect of lateral deformation of every single fibril is neglected (Li et al. 1999).

10.1.6 Pathologies

10.1.6.1 Mechanical

Single and multiple blunt impacts on cartilage yielding 20 % strains at strain rate of 6.7 %.s^{-1} have been found to cause destruction of bovine metacarpal cartilage. Moreover, strains of 40 % and above have evidently caused surface defects, correlating to collagen network failure and cell death. However, cartilage has been shown to survive impacts yielding less than 10 % strain, with no injury to chondrocytes on their ECM (Radin et al. 1970; Repo and Finlay 1977). On the other hand, low impact may lead to cell death despite structural integrity being maintained (Duda et al. 2001).

Defects of articular cartilage may or may not reach the surface of the underlying bone. In care of the latter, these are termed chondral, or partial thickness defects. Some of these superficial lesions result from surgical procedures (Rosenberg 1971; Thompson 1975). Full thickness lesions, also termed osteochondral defects (Fig. 10.4), cross the tidemark of articular cartilage and violate the underlying subchondral bone. In doing so, they have access to cells in the bone marrow cavities.

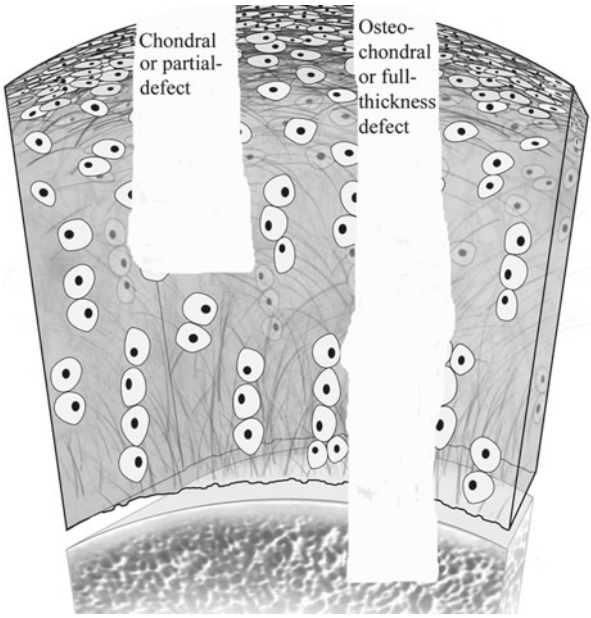

Fig. 10.4 Schematic representation of full and partial thickness defects in articular cartilage

10.1.6.2 Degenerative and Non-Degenerative Diseases

Common diseases, which affect the health and functionality of the joint, are osteoarthritis (OA), rheumatoid arthritis (RA), chondromalacia and disuse atrophy. OA is a slowly progressive disorder of unknown cause (Mankin 1974a), which generally occurs later in life, principally affecting major weight-bearing joints. It is characterized clinically, by pain, deformity and reduced mobility, and pathologically, by features including focal erosive lesion, cartilage destruction, subchondral sclerosis, cyst formation, and large osteophytes at the margins of the joints. With the progression of OA, cartilage exhibits histological, biochemical and metabolic changes, although their precise nature frequently depends on the underlying abnormality and the duration of the disease progression. At the early stages of the disease, the tissue erodes, disappearing completely from the focal areas of the surface, leaving a denuded, sclerotic and eburnated bone. Type II collagen degrades beneath the articular surface, and their organization is disrupted. Consequently, the tissue depletes in stiffness and strength and fibrillation follows (Mow et al. 1992).

RA typically affects many different joints and can be chronic in nature. This systemic disease affects the entire body and is one of the most common forms of arthritis. It is characterized by the inflammation of the membrane lining the joint, causing pain, stiffness, warmth, redness, and swelling. In a similar manner to OA, there is degradation of type II collagen, particularly around chondrocytes in the deep zones, rather than directly beneath the articular surfaces (Mow et al. 1992). PGs are also degraded, but can be partly replaced. Eventual cartilage thickness is reduced due to its exposure of migrating cytokines, produced in the adjacent subchondral bone, resulting in the erosion of underlying calcified cartilage and bone.

Pathological diseases of the articular cartilage are not necessarily confined to the elderly. Indeed, any form of joint immobilization, for example following an injury or surgery, will lead to tissue atrophy and joint stiffness. These conditions can be reversed with joint remobilization, starting with gentle exercise, which gradually increase in intensity. Another non-degenerative disease is chondromalacia patella, which is often caused by trauma, overuse, part misalignment or muscle weakness. Instead of gliding smoothly, the patella translates across the femur, thereby roughening the cartilage underneath the patella. The damage may range from a slight abnormality to a complete wear of the associated cartilage surface. Traumatic chondromalacia occurs when a blow to the patella bone tears off either a small piece of articular cartilage or a large fragment containing a piece of bone. The latter is termed an osteochondral fracture. Clinically, the process results in mild to moderate pain and stiffness. The resulting changes resemble those of mild OA, with fibrillation, surface irregularities and cartilage erosion (Mankin 1974b).

10.2 Repair and Regeneration Strategies

10.2.1 Response to Injuries

The effects of mechanical injuries to articular cartilage vary considerably, depending on its nature and severity. Its response to superficial defects, which violate neither its calcified layers nor the underlying bone typically lack inflammation of the cartilage, and has limited potential for self-repair (Buckwalter 1998; Mankin 1982). Characteristically, there is minimal attempt on the part of the cartilage to elicit cellular or matrix repair (Calandruccio and Gilmer 1962; Campbell 1969; DePalma et al. 1966). However, cartilage responds to lacerative injuries with an enhanced mitotic activity adjacent to the defect margins. This is associated with increased synthesis of the matrix components (Mankin 1962).

When a cartilage defect affects the vasculature of the subchondral bone, an enhanced biological response is elicited. This repair response is equivalent to that of other vascularized tissues in the body (Mankin 1982), filling the whole defect cavity with blood. In the proceeding events, a blood clot is formed, which contains both red and white blood cells, undifferentiated cells and marrow elements (DePalma et al. 1966). However, only the bone defect is filled with the new bone, and is fused

with the cartilage defect, with its edges united by the vascular fibrous tissue (Calandruccio and Gilmer 1962; DePalma et al. 1966). As observed with superficial defects, brief synthetic activities take place in the remaining cartilage, during which a small amount of cells and matrix is produced, replacing some of that lost to the initial damage (Mankin 1962, 1982).

The quality of the repaired cartilage is dependent on the initial defect size. For example, defects less than 3 mm in diameter often repair completely after 3 months and are difficult to locate after 9 months (Convery et al. 1972). However, defects 9 mm or larger may not completely repair. It has also been demonstrated that the site of an old osteochondral laceration may clearly be visible years after injury as a slightly discolored, roughened pit, or linear grooves on the otherwise smooth surface adjacent to the defect site (Bennett and Bauer 1935; Campbell 1969; Key 1931).

10.2.2 Non-invasive Therapies

Different interventions exist for the management of cartilage damage. The most topical of these are lifestyle changes, pharmacological and surgical methods. The emphasis on lifestyle becomes highly relevant due to the high contributions of obesity, and abnormal loading on the development and progression of osteoarthritis. Acute joint injuries, fractures of articular surface, along with tears of the meniscus and ligaments are all linked with osteoarthritis. Occupation and nutrition have also been deemed strong factors in degenerative cartilage diseases (Cooper et al. 1992; Felson and Zhang 1998; Lievense et al. 2001). For example, strong evidence exists, which suggests that the risk of OA doubles after 10 years of farming (Jensen 2008). Additionally, occupations which involve kneeling, squatting or heavy lifting also accelerates cartilage degeneration. Therefore, strategies such as weight control, recreational exercise, and injury prevention are all common interventions adopted as least-invasive therapies. More specific, exercises such as quadriceps strengthening; stretching and aerobic exercises are commonly prescribed for treatment of hip and knee OA (Bukowski et al. 2006; Hochberg et al. 2012; Jansen et al. 2011; Roddy et al. 2005). Other examples are ultrasound (Soren 1965; Welch et al. 2001) and acupuncture (Brinkhaus et al. 2007; Lin and Chen 2009; Reinhold et al. 2008).

Pharmacological interventions play a vital role in pain relief to OA patients. For mild or moderate pain, acetaminophen (paracetamol) is a common choice recommended by physicians (Towheed et al. 2006; Wegman et al. 2004). In the 2014 Osteoarthritis Research Society International (OARSI) guidelines for the non-surgical management of knee osteoarthritis, acetaminophen treatment is deemed appropriate as a short-term analgesic for knee OA pain, however conservative dosing and treatment duration consistent with approved prescribing limits is recommended (McAlindon et al. 2014). Other non-steroidal anti-inflammatory drugs (NSAID) such as naproxen and ibuprofen used for patients with either hip or knee OA have been found to be superior to paracetamol, in bringing pain relief, albeit,

at a higher health risks. However, both analgesics have been associated with discomfort, perforation, and bleeding of the gastrointestinal tracts (Chou et al. 2011; Zhang et al. 2004). Both paracetamol and NSAIDs work by inhibiting the actions of cyclo.oxygenase-1 and -2, thereby, relieving the patients of pain. As the disease progresses and the pain persists, intra-articular injection of corticosteroids can lead to a significant short-term decrease in pain (Bannuru et al. 2009; Bellamy et al. 2006). Other pharmacological interventions include chondroitin and glucosamine (Dahmer and Schiller 2008; Matsuno et al. 2009; Owens et al. 2004; Sawitzke et al. 2008). These glycosaminoglycans are taken as food supplements either alone or in combination with each other. However, it's beneficial effect both in terms as pain relief drug and disease-modifying osteoarthritis drug (DMOAD) is controversial and under constant debate (Henrotin et al. 2014) and they are labelled as having an uncertain appropriateness as analgesia for knee OA and not recommended as disease modifying OA treatment by the OARSI (McAlindon et al. 2014) nor by the American College of Rheumatology (ACR) (Hochberg et al. 2012). Although the benefits towards pain and physical function in knee OA of intra-articular injection of hyaluronic acid is controversial and currently its use for knee OA treatment is considered uncertain by the OARSI (McAlindon et al. 2014).

10.2.3 Surgical Interventions

In cases where non-invasive therapies are not possible, due to substantial cartilage damage, or detachment of cartilage fragments, surgical procedures are undertaken. These include joint lavage, subchondral stimulation, autogenic and allogenic transplantation and cell transplantation, performed either arthroscopically or with an open joint surgical approach.

10.2.3.1 Arthroscopic Cleaning

Arthroscopic procedures, such as lavage, involve thorough rinsing of the joint cavity with Ringer solution, lactate and sodium chloride solutions. Such a palliative approach is designed to reduce the degree of pain experienced by the patient. In the main, this strategy is successful, although the biological reasoning for pain relief is not established (Anderson et al. 1993; Chang et al. 1993; Gillespie and O'Connell 1992; Livesley et al. 1991). However, there are limits to its application, particularly with OA patients whose pain relief is generally considered to be a result of a placebo effect (Gibson et al. 1992; Moseley et al. 1996).

Other arthroscopic strategies include chondral shaving and debridement also called chondroplasty. The aim is to remove loose and unstable remains of damaged and fibrillated articular cartilage to a smoother surface while avoiding any damage to the healthy surrounding cartilage. As with lavage, the biological rationale behind each of these two procedures remains unclear. Indeed, cell loss along the lesion

borders of the remaining cartilage has been reported to follow chondral shaving, which is counter-productive for cartilage repair (Kim et al. 1991; Mitchell and Shepard 1987; Tew et al. 2000). In addition, debridement has been reported to be associated with skeletal misalignment and shown clinically to exacerbate the osteoarthritic condition (Messner et al. 2000). Both procedures can be carried out using a gentle cutting instrument incorporating laser light at a specific wavelength. Although the laser is useful for welding and fusing the tissue, performing a chondral shaving or a debridement using laser offers little advantages over mechanical cutting method (Vangsness and Smith 1995).

10.2.3.2 Subchondral Stimulation

A common repair strategy recommended for superficial defects, involves surgically accessing the adjacent bone-marrow spaces along with the bone and the vascular spaces so that residing bone marrow mesenchymal stromal cells can migrate into the cartilage defect. Examples of such procedures include Pridie drilling, abrasion arthroplasty and microfracture techniques. The three procedures are very similar, and start with a chondroplasty followed by a procedure to access the bone marrow. Pridie pioneered the drilling procedure by drilling with a Kirschner wire (K-wire) in the subchondral plate at the cartilage lesion (Insall 1967). Abrasion arthroplasty is similar to Pridie drilling, but instead of using a drill or wire, a high speed burr is used to reach the subchondral bone marrow space. The microfracture technique introduced by Steadman and his team (Steadman et al. 2003, 1999), is a refinement of the Pridie drilling. The approach starts with the debridement to a stable cartilage margin followed by a careful removal of the calcified cartilage layer while taking care of not damaging the underlying subchondral bone. Finally, using a sharp instrument, called an awl, subchondral bone perforations are made over the entire cartilage lesion approximately 3 mm apart to a depth of 4 mm. This should result in a defect filling with a well-anchored mesenchymal clot. The rehabilitation protocol, which includes quick mobilization of the joint after surgery by continuous passive motion, is an important part of the microfracture procedure (Steadman et al. 2003). Moreover, the holes are relatively small (1.0–2.0 mm diameter) when compared to Pridie drilling who recommended using wires of a quarter inch (6.35 mm) in diameter (Insall 1967).

By penetrating the subchondral bone beneath the defect, the void is immediately filled with a fibrin clot which, within 2 days adheres to the bony compartments of the wound, as opposed to the cartilaginous tissue. By the fifth day, mesenchymal stem cells have penetrated and completely resorbed the fibrin clot, filling the void. Thereafter, between days 10 and 14, the cells differentiate into chondrocytes and lay down a PG-rich matrix. By 8 weeks, the repair tissue begins to resemble normal cartilage and forms a continuous surface with the surrounding native tissue by approximately 6 months. However, by 12 months, there is generally evidence of degradation of repair tissue (Hunziker 1999; Wyre and Downes 2000).

Arthroscopic microfracture has increasingly replaced subchondral drilling as marrow stimulation technique in the clinical situation. However, it has to be noted that microfracture does not restore normal hyaline cartilage but primarily results in fibrous or hybrid repair cartilage tissue with variable repair tissue volume (Mithoefer et al. 2009). A long-term follow-up study by Steadman's team also indicates, that the success rate of the technique is dependent on the age of the patients and improved functional outcome and less pain is mainly seen in patients under the age of 35 years treated for isolated full-thickness chondral defects (Steadman et al. 2003). Two different routes are currently under investigation to improve the outcome of the bone marrow stimulation technique. On the one hand, research is directed to optimize the technique of drilling. Especially the drill hole size and drill depth are being addressed. The aim is to have ample access to the bone marrow, while drill hole diameters better mimic the normal physiological trabecular distance (Benthien and Behrens 2013; Eldracher et al. 2014; Min et al. 2013). On the other hand, the procedure of subchondral stimulation in combination with defect filling with a scaffold, to offer the infiltrating cells a substrate from where to repopulate the defect area, is another hot research topic (Sharma et al. 2013; Zantop and Petersen 2009).

There is an inherent discontinuity between the repair tissue and the surrounding cartilage, as the collagen fibrils within the two compartments fail to integrate. Additionally, some PGs in the cartilage matrix exhibit anti-adhesive properties, and hinder the bonding between the repair and the native cartilage tissues. The employment of abrasion chondroplasty for cartilage defects in rabbits and dogs have resulted in the formation of cartilage-like tissue, which originated from the subchondral bone (Altman et al. 1992; Kim et al. 1991), though other studies report the presence of significant quantities of fibrous cartilage (Furukawa et al. 1980). It has also been reported that the, repaired full-thickness cartilage lesion in rabbits are more durable following the Pridie drilling when compared to the abrasion chondroplasty strategy (Menche et al. 1996). Indeed the Pridie approach has been reported to be of great benefit to patients with conditions such as osteochondritis dissecans and gonathrosis (Pedersen et al. 1995), yielding both pain relief and restored joint function (Beiser and Kanat 1990; Goldman et al. 1997). The microfracture technique, being a minimally invasive arthroscopic procedure is relatively less disruptive to the subchondral bone. Nonetheless, its employment in treating young athletes and horses has resulted in an improved joint function and pain relief (Frisbie et al. 1999; Sledge 2001).

10.2.3.3 Tissue Grafting

The transplantation of cartilage into defect sites has been a viable strategy for several decades (Cohen and Lacroix 1955). The transplanted cartilage may be sourced autologously or extracted from human cadavers. In autologous cartilage transplantation, plugs of cartilage biopsy are extracted either from adjacent to the defect (periosteal) or from the rib (perichondral). These are either sutured or glued to the defect floor, such that the defect may be stimulated to form repair cartilage that

binds to the transplant, forming a continuous neo-tissue over the entire defect (Ohlsen 1976). Another more common autologous grafting procedure is the osteochondral autograft transplantation (OATS). When multiple small cylindrical autogenous osteochondral plugs are fitted together in the defect, the term autologous osteochondral mosaicplasty is used. These autologous biopsies are retrieved from low-weight-bearing areas and transferred to the defect. In contrast to marrow stimulation procedures, which result in fibrocartilaginous tissue, OATS aims to restore functional hyaline cartilage. With this technique, joint function and pain relieve have been reported to reach 80 % of cases (Bouwmeester et al. 1997; Homminga et al. 1990; Korkala and Kuokkanen 1991; Moran et al. 1992). Moreover, these strategies are advantageous because they minimize disease transfer and immunological rejection, which are commonplace with allografts from another donor. For these reasons, autografts have produced treatments with survival rates up to 70 % at 2–5 years (Temenoff and Mikos 2000). To minimize donor side morbidities, these explants are taken from non-load-bearing region of cartilage. When implanted in the defect the cartilage is often unable to withstand forces imparted at joint surfaces. Due to its lack of mechanical integrity, the matrix of the implant and cartilage in the vicinity of the defect breaks down to the extent that the implant and associated regions later exhibit signs of osteoarthritis. Additionally, this breakdown also occurs at the donor site (Kim et al. 1991; Mitchell and Shepard 1987), often necessitating undesirable and expensive second operation. The amount of autograft that can be harvested is limited, thus the technique is often unsuitable for clinical-sized defects.

Alternately, allogenic osteochondral grafts obtained from human cadavers have been used to fill cartilage defects. Unlike autologous grafts, no biological interaction is predicted between the transplant and its surrounding cartilage, such that the primary role of the transplant is to fill the defect site and replace the lost tissue volume. This approach has benefited patients with large osteochondral defects, particularly caused by trauma and osteo-necrosis. The benefits of osteochondral resurfacing in the human knee joint has been observed to last for many years (Bakay et al. 1998; Bell et al. 1994), with reported success rates around 80–85 % after 10 years (Levy et al. 2013; Mahomed et al. 1992; Meyers et al. 1989). Fresh osteochondral allografting is associated with inherent challenges related to availability of suitable donor tissue and donor tissue retrieval and storage. Although, adverse immunological reactions are associated with this procedure, the survival of allogenic transplants may be prolonged with the use of immunosuppression and histocompatibility techniques (Hickey et al. 1994; Stevenson 1987; Stevenson et al. 1989). General potential limitations related to both autologous and allogenic osteochondral grafting include differences in orientation, thickness, and mechanical properties between donor and recipient cartilage. In addition, absence of fill and the potential dead space between cylindrical grafts may limit the quality and integrity of the repair (Bedi et al. 2010).

The perichondrium, which is a dense membrane composed of fibrous connective tissue that closely wraps cartilage (except for the articular cartilage, which is covered by the synovial membrane) has been implanted unto cartilage defects in the human joints (Homminga et al. 1990; Kwan et al. 1989). These autologous

scaffolds were advantageous because they naturally contain autogeneous cells that are useful for cartilage repair. However, in addition to the limited availability of harvest sites, the neo-cartilage formation resulting from these grafts have been deemed unsatisfactory in patients over the age of 40 (Seradge et al. 1984). Moreover, regenerates formed from these scaffolds do not completely fill the defect and tend to detach and ossify (Hendrickson et al. 1994; Homminga et al. 1990).

10.2.3.4 Cell Transplantation

Isolated chondrocytes have been transplanted into articular cartilage defects for its repair. However, such an approach typically has a success rate of less than 40 %, as the cells are not retained within the defect site for sufficient period to produce neo-ECM (Temenoff and Mikos 2000). On the other hand, mesenchymal stem cells from the skeletal muscle of adult rabbits, seeded onto porous polyglycolic acid (PGA) mats have also been implanted into non-weight bearing defects in the rabbits femoropatella groove. The PGA matrix biodegrades and the stem cells remain in situ, producing a cartilage-like tissue containing type II collagen and subchondral bone that is morphologically similar to native tissue (Grande et al. 1997; Martin et al. 1999). However, in a similar approach using mesenchymal stem cells and collagen gel for cartilage defects in osteoarthritic human knees limited clinical improvement was observed after 42 weeks (Wakitani et al. 2002).

10.2.3.5 Autologous Chondrocyte Implantation (ACI)

Since its first clinical application for treating deep articular cartilage defects in the knee (Brittberg et al. 1994), ACI has been a fairly successful approach for treating cartilage defects (Peterson et al. 2010). The technique involves harvesting a healthy portion of cartilage, usually from a non-load bearing region of the patient, and enzymatically degrading the tissue to isolate the cell population. These cells are expanded in vitro to a sufficient density for implantation. In the first generation ACI, a periosteal patch is sutured over the debrided cartilage defect and the cells are injected in the defect. The injection site is closed with a suture and covered with fibrin glue (Peterson et al. 2010). Presently a collagen membrane is used as a cover, minimizing peri-operative morbidity and eliminating hypertrophy of the periosteal flap (Benthien and Behrens 2011). The cells used in the repair may either originate from the chondrocytes in the host-extracted cartilage, its precursor cells from the periosteum or possibly mesenchymal stem cells of the subchondral bone (of the defect site) had this been injured. The use of ACI has resulted in an improved joint function for at least 72 % of patients at 1 year post-operatively (Bentley et al. 2003; Minas 1998) and 84 % of patients at 3 years post-operatively (Micheli et al. 2001). In a 10 years follow-up study of cartilage knee lesions treated with ACI, 26 % of the patients experienced graft failure at a mean of 5,7 years after ACI. Of the 73 patients that did not fail, 88 % had an excellent to good outcome 10-years post-implantation.

It is known that the clinical results of ACI as the primary articular cartilage repair technique are superior to the results obtained when ACI is used as a salvage option after failure of marrow-stimulating techniques. This could account for the relative high failure rate in this 10-year follow-up study (Biant et al. 2014). ACI has been used for treating defects in other joints such as hips and ankles (Giannini et al. 2001; Romeo et al. 2002). The use of ACI as an alternative treatment to surgical excursion, allogenic grafting and autografting (Peterson et al. 2003) has been discussed to provide long-term joint restoration and pain relief for patients with osteochondritis dissecans.

Although quite successful, the ACI technique has been constantly optimized to overcome some of the surgical and biological short-comings of the procedure. Since the late 1990s matrix-assisted autologous chondrocyte transplantation (MACT) was introduced in the clinics (Behrens et al. 2006; Schneider et al. 2011). In this procedure, the cells are no longer injected as a suspension in the defect, but seeded onto a biomaterial that fills the defect. The surgeon no longer needs to worry about handling and retaining the chondrocyte cell suspension under the leakage preventing periosteal seal. The cells will colonize the biomaterial in vitro and will start to produce their own matrix prior to implantation. Some used biomaterials even promote cartilage regeneration (Filardo et al. 2011; Zeifang et al. 2010). ACI and MACT will not result in true native tissue regeneration, but in pain relief and formation of fibrocartilage and/or articular cartilage and therefore delay OA and its adverse effects (Ringe et al. 2012).

10.2.4 Tissue Engineering

Despite the numerous strategies available for treating cartilage defects, there is yet to be a standardized solution for restoring long term function, especially due to the large variability of defects to be treated. This limitation has encouraged a more sophisticated tissue-engineered approach (Ringe and Sittinger 2009). Tissue engineering combines the principles of cell and molecular biology with material technology, to create a new tissue, which has the potential to physically and biologically mimic its predecessor and restore function to the damaged tissue. Key activities in this approach are the attainment and expansion of cells and the development of scaffolds that act as carriers for the cells.

10.2.4.1 Chondrocytes

An important step in tissue engineering is the isolation and expansion of cells that are to be transplanted. The cells must be both appropriate for the intended tissue and of sufficient quantity to treat clinical-sized defects (LeBaron and Athanasiou 2000), whilst being free of pathogens and contamination. Cells sources can be either autologous, allogenic or xenogenic, the latter being derived from a different animal species.

Each approach has specific benefits and shortcomings (Breinan et al. 2001; Ma et al. 2005; Masuoka et al. 2005; Ostrander et al. 2001; Pavesio et al. 2003). For example, although autologous cells are free from immuno-related problems, they are relatively few in numbers and cell harvesting could lead to morbidity at the donor site. Also the age of the chondrocyte needs to be considered, since chondrocytes from older patients are metabolically less active in vitro (Dehne et al. 2009). Thus the autologous approach does not effectively lead to a readily available off-the-shelf solution.

Allogenic and xenogenic cells may be extractible in large numbers and are available off-the-shelf, but these are associated with immunological problems and in the case of xenogenic cells, there is often the possibility of animal virus transmission (Sirlin et al. 2001). Tissue-engineered constructs derived from these cells require additional steps to incorporate immune acceptance. In general one of the major drawbacks is that chondrocyte expansion in a monolayer is characterized by dedifferentiation of the cells to a fibroblast-like phenotype, causing a decreased proteoglycan synthesis and collagen type II expression and an increase in type I collagen (Goessler et al. 2005; Minegishi et al. 2013).

10.2.4.2 Stem and Progenitor Cells

A chondroprogenitor population resides in articular cartilage and has been shown to possess superior migration abilities (Schminke and Miosge 2014; Seol et al. 2012). Upon in vitro chondrogenic differentiation in 3D pellet culture, there is no expression of hypertrophic markers nor of calcification (Williams et al. 2010). The surface marker CD166 has been identified as a marker for this chondrogenic progenitor population that predominantly resides in the superficial and middle zone of articular cartilage (Pretzel et al. 2011).

Multipotent stem cells have been proposed to be a vital source of cells for tissue engineering applications. In a similar manner to differentiated cells, stem cells may either be autologous, allogenic or xenogenic in nature. Since mesenchymal stromal cells (MSC) show immunosuppressive properties they are being applied allogeneically in clinical settings today without the need of immunosuppression treatment. Stem cells offer the benefits of being able to be multiplied extensively, yielding high cell number; from which, the desired number may be extracted and differentiated into chondrocytes. The remaining stem cells may be further multiplied for future use. The intervention is simple, but cell preparation is expensive. As an example, mesenchymal stem cells derived either from the bone marrow and other adult connective tissues (Friedenstein et al. 1976) may differentiate to a selected range of cells including chondrocytes, osteoblasts, tenocytes or myocytes, irrespective of their origin (Jones et al. 2002; Minguell et al. 2001; Pittenger et al. 1999; Yoo et al. 1998). The most obvious stem cell source for articular cartilage regeneration are the MSC population that reside in the bone marrow. Moreover clinical trials are also underway to look at the use of umbilical cord derived MSCs and adipose

tissue MSCs. Currently 26 clinical studies are registered in the ClinicalTrial.gov database with a focus on articular cartilage and mesenchymal stem cells. One of them, has reported on a 2 year follow-up of intra-articular injections of bone marrow-derived MSCs for the treatment of knee osteoarthritis. Based on a small patient cohort (12 patients) their preliminary results reaffirm that autologous MSC may be a valid alternative for AO treatment because it attains effective and durable pain relief and objective cartilage improvement (Orozco et al. 2014). The same group also looked at the efficacy of using allogenic MSCs in 15 knee OA patients and compared the treatment against intra-articular hyaluronic acid injections. Although the procedure resulted in significant relief of pain and disability, and quantitative MRI evidence indicated partial articular cartilage healing, the effects appeared to be somewhat smaller than those reported for treatment with autologous MSCs (Vega et al. 2015). Besides the bone marrow, also other tissues in the joint harbor mesenchymal stem cells that have the potential to differentiate into chondrocytes and could contribute to the healing of articular cartilage lesions, including the synovium, the synovial fluid and the infrapatellar fat pad (Chang et al. 2013; Felimban et al. 2014; Suzuki et al. 2012).

Pluripotent stem cells such as embryonic stem cells (ESC), derived from the inner cell mass of the embryonic blastocyst and induced-pluripotent stem cells (iPSC) offer great potentials for tissue engineering. Directing human ESC through a step wise differentiation protocol over intermediate developmental stages, allows for a successful end-differentiation into chondrocytes. Aggregates of these ESC–derived chondrocytes produced a collagen type II and sulfated GAG rich matrix without the evidence of hypertrophy (Oldershaw et al. 2010). However, there are ethical and legal concerns with using human embryonic cells. For this reason, much of the research has been conducted on animals (Fuchs et al. 2005; Kramer et al. 2006). iPSC are generated by transducing adult somatic cells with reprogramming factors (c-MYC, KLF4,OCT3/4 and SOX2) to make them pluripotent and as such rejuvenate somatic cells (Takahashi and Yamanaka 2006). It was shown that after 21 days of in vitro chondrogenic differentiation of human iPSC cells a significantly higher GAG content and gene expression of collagen type II and aggrecan was detected compared to human bone marrow-derived MSCs and the expression of hypertrophic and osteogenic markers was very low. Implantation of these 21 day differentiated iPCS cells in a cartilage lesion in immuno-deficient rats resulted after 12 weeks in good restoration of the articular surface, albeit with a reduced amount of proteoglycans compared to the adjacent healthy tissue (Ko et al. 2014). The use of viral vectors to incorporate these reprogramming factors causes a major concern towards their clinical application. Optimization of the transfection step is ongoing to generate transient expression of the reprogramming factors (Tsumaki et al. 2015). Although possessing a tremendous potential as cell sources for tissue regeneration, iPSC and hESC applications currently are not a realistic clinical option in the foreseeable future because of ethical and regulatory issues and the potential of teratoma formation (Ringe et al. 2012).

10.2.4.3 Stimulatory Biochemical Factors

Several cytokines, hormones and growth factors are known to influence chondrocyte behavior and chondrogenesis and have been incorporated in various cartilage tissue engineering strategies. Especially the members of the transforming growth factor (TGF) superfamily such as TGFβ1, TGFβ2 and TGFβ3 induce chondrocyte proliferation and promote ECM production and TGFβ1 and TGFβ3 promote chondrogenesis of MSCs. Also certain bone morphogenic proteins (BMP), especially BMP-2 and BMP-7, promote chondrogenesis of MSCs and increase matrix production by chondrocytes and MSCs. Insuline growth factor-1 (IGF-1) enhances proteoglycan and type II collagen production, while fibroblast growth factor-2 (FGF-2) is added to the culture medium to stimulate the expansion of chondrocytes (Barry et al. 2001; Fukumoto et al. 2003; Hicks et al. 2007; Kock et al. 2012). However, the dose and administration timing does play an important role in governing chondrogenesis.

Recent developments in the tissue engineering domain focus on the delivery of chemokines at the side of injury to mobilize endogenous stem cells and as such stimulate tissue repair. The body possesses an inherent mechanism to guide stem cells to sites of tissue injury, however this process is often insufficient to achieve full tissue repair. The chemokine CXCL12 also referred to as stromal cell-derived factor-1 alpha (SDF-1α) is a prominent stem cell homing factor and is transiently up-regulated upon injury in the bone to promote subsequent recruitment of MSCs (Andreas et al. 2014). A collagen scaffold releasing CXCL12 has been employed in vivo to recruit endogenous MSCs in partial-thickness cartilage defects in rabbit (Zhang et al. 2013). Cell-free chemoattractant-based therapies are likely to become more important in the future, since they could offer a convenient off-the-shelf product as a therapeutic tool for regenerative therapies. However additional research is need to be sure that no detrimental cells such as immune cells or fibroblasts are recruited to the defect side.

Furthermore, both for the growth factors and chemokines application there is a need to present these factors at the defect side in an appropriate delivery vehicle. These biochemical signaling molecules possess a short half-live and are prone to protease cleavage and will diffuse rapidly upon bolus injection at the defect side, so in order to keep them at the side of tissue repair an appropriate scaffold delivery vehicle is required (Andreas et al. 2014).

10.2.4.4 Scaffold Technology

Cells and growth factors are commonly transplanted into the body with the support of a carrier scaffold. These carriers function to retain the cells at the defect site, allow them to multiply and synthesize their own ECM. Therefore, such scaffolds must provide a number of design properties including: (1) Biocompatibility: To prevent undesirable immune or biological responses. (2) Permeability: to demonstrate sufficient porosity to enable good nutrient supply to cells at all regions of the

construct, allow transport of signaling molecules between cells, permit removal of waste products and allow ingrowth of host tissue. (3) Biodegradability, where appropriate, to enable the scaffold to degrade in a controlled temporal manner, into non-toxic by-products as a neo-tissue is developed. In addition, this property may enable the controlled release of morphogens and/or pharmacological agents to encourage cellular activity. To date, a wide range of natural and synthetic materials are available for use as scaffolds for tissue engineered cartilage constructs (Barnewitz et al. 2006; Ossendorf et al. 2007; Perka et al. 2000; Risbud and Sittinger 2002; Sittinger et al. 1994, 2004).

Natural polymers can be subdivided into protein-based such as collagen, fibrin and silk or carbohydrate-based such as alginate, agarose, hyaluronan, chondroitin sulfate and chitosan. Many of these are hydrogels, which makes them appropriate for cartilage regeneration, since their highly hydrated polymer networks mimic a similar high water content as in the native cartilage ECM (Kock et al. 2012).

Encapsulation of chondrocytes within agarose and alginate hydrogels is a well-established protocol for in vitro cartilage models (Benya et al. 1988; Freeman et al. 1994; Lee and Bader 1995; Naqvi and Buckley 2015) and has been used to deliver autologous chondrocytes articular cartilage lesions in human patients (Selmi et al. 2008). These in vitro systems have demonstrated their value in studying the response of chondrocytes to many external stimuli while excluding the coupled influences of other factors that are also implicated within the native cartilage. Examples of such studies include the effects of dynamic mechanical stimulation on chondrocyte metabolism (Chowdhury et al. 2001, 2003; Lee and Bader 1997) and chondrocyte deformation (Buschmann et al. 1995; Knight et al. 1998). In a similar manner to many hydrogels, however, cell-seeded agarose or alginate constructs are limited by their poor resorption rate and their inferior mechanical and biochemical properties, making them unsuitable for load-bearing applications. The potential of fibrin based scaffolds as carriers of cells and growth factors for cartilage regeneration was investigated (Hendrickson et al. 1994). Although this natural clot-forming polymer produces a neo-tissue that is histologically similar to natural cartilage, it has poor mechanical properties and often evokes an immune response (Kawabe and Yoshinao 1991). The use of collagen-based scaffolds for delivering cell and growth factors to defect sites is extensive. As collagen naturally occurs in skeletal tissues, it promotes attachment of cells unto its surface. Accordingly, it has been used either cell-free, seeded with chondrocytes or MSC, in many animal studies (Russlies et al. 2002; Sams et al. 1995; Samuel et al. 2002). Chondrocytes seeded onto dense collagen scaffolds, implanted into rabbit femoral trochlea for up to 24 weeks has demonstrated to have produced a hyaline-like cartilage that was biochemically and mechanically similar to its surrounding cartilage (Frenkel et al. 1997). By contrast, other in vivo studies reported that although the repair appears adequate at earlier time point, subsequent thinning of the repair tissue occurs with time (Wakitani et al. 1994). Hyaluronan is a non-sulphated GAG that is essential for the aggregation of large proteoglycans such as aggrecans in articular cartilage. It has been used to

deliver mesenchymal stem cells to caprine chondral defects (Butnariu-Ephrat et al. 1996), and stabilize chondrocytes and osteochondral progenitor cells for cartilage defects in rabbits (Grigolo et al. 2001; Solchaga et al. 2002). Although the newly developed tissues exhibit good integration with the host cartilage, they are typically thinner and often induce the breakdown of cartilage matrix. Chitosan has been used to deliver cells and growth factors to the defect site. Chitosan, is derived from the exoskeleton from arthropods and is structurally similar to the glycosaminoglycans found in cartilage (Berger et al. 2004). Chitosan can form into thermo reversible hydrogels, offers the combined advantages of an implant with a uniform distribution of cells and direct injectable into the defect (Chenite et al. 2000). Autologous chondrocytes encapsulated in injectable chitosan hydrogels repaired non-weight bearing defects in adolescent sheep, with good integration with the surrounding tissue (Hao et al. 2010).

Synthetic polymers used in cartilage tissue engineering are mainly poly-α-hydroxyesters (especially polylactic acid (PLA), polyglycolic acid (PGA), Poly(lactic-co-glycolic acid) (PLGA) and polycaprolactone (PCL)) (Dahlin et al. 2014; Mooney et al. 1996) but also poly(vinyl alcohol) (PVA), poly(ethylene glycol) (PEG), poly(ethylene oxide) (PEO). Compared to natural scaffolds, the mechanical and biochemical properties of synthetic scaffolds are readily modified to suit specific applications. PLA and PGA have been demonstrated to support chondrogenesis (Haisch et al. 2005). Moreover, their degradation products are naturally occurring metabolites that can be cleared from the body through the metabolic mechanism and because of their biodegradability there have been approved for certain clinical applications by the FDA and EMA. However, PGA was found to be weaker than most synthetic scaffolds, and degrades very fast, often releasing acidic byproducts of degradation into its immediate environment, which may prove cytotoxic (Grande et al. 2003). The uses of PLA/PGA copolymers as scaffolds allow improved control of the degradation rate. Indeed success was demonstrated by Cohen et al., who observed good histological and biochemical response, after 12-week implantation of the co-polymer into rabbit-chondral-defects (Cohen et al. 2003). An exhaustive range of copolymers have been proposed for cartilage repair. These include PLA/PEG (Tamai et al. 2005), and nanofibrous forms of PLA/PCL (Li et al. 2005), the latter has been shown to elicit differentiation of human mesenchymal stem cells into chondrocytes exhibiting similar zonal morphology to that of native cartilage. Other synthetic copolymers include Poly(ethylene oxide)-terephthalate/poly(butylene-terephthalate) (PEOT/PBT) which have been used as filler of the donor side during mosaicplasty in humans (Bartha et al. 2013; Rey-Rico et al. 2015).

Hybrid composite scaffolds combing both synthetic and natural polymers are also tested in the cartilage engineering field. This allows to combine the tailorable mechanical and degradation properties of synthetic polymers with delivering tissue-specific clues of naturally occurring polymers. A macroporous PCL scaffold with desired mechanical properties was coated with hyaluronic acid to provide a

hydrophilic surface and chondroactive properties and showed 24 weeks after implantation in a cartilage full-thickness defect in rabbits a well-organized hyaline cartilage in the defect lesions (Lebourg et al. 2014). Hybrid composite scaffolds allow for the construction of scaffolds with a biomimetic microarchitecture. A study combining type-II collagen and Chitosan-PCL as main components enabled to create a layered scaffold with an altered average pore-size, porosity, swelling index and compressive modulus from one layer to layer in a gradient manner and a decreased collagen content from the top layer to the bottom layer (Zhu et al. 2014).

Multiphase implants consisting of PGA, Bioglass and calcium phosphates have been examined in osteochondral defects in goats (Niederauer et al. 2000). The PGA fibers were seeded with autologous chondrocytes and the calcium phosphates were used to modulate the construct stiffness at specific regions of the implants.

10.3 The Limiting Factor: What Lies Beneath

10.3.1 Subchondral Considerations in Cartilage Disease

Although little is known about the relationship between bone and cartilage in the etiology of osteoarthritis, an abnormal growth of the subchondral bone resulting in thickened subchondral bone plate, increased stiffness, and bone mineral density have been tagged with the progression of the disease (Fazzalari and Parkinson 1997; Grynpas et al. 1991; Li and Aspden 1997). Having observed these, and a decreased energy absorbing capacity Radin and co-workers proposed that stiffening of the subchondral plate was an initiating factor in osteoarthritis (Radin et al. 1970). They later hypothesized that trabecular microfracture due to impulsive loading initiates bone remodeling in the subchondral plate. This leads to localized stiffening that in turn produces increased shear stress in the cartilage, culminating in cartilage breakdown (Pugh et al. 1974).

It had been an ongoing debate that the calcified cartilage layer and the tide mark was an impenetrable structure, separating the articular cartilage from its underlying subchondral bone. However, microcracks and micro channels between the subchondral region and the uncalcified cartilage have been demonstrated (Clark and Huber 1990; Holmdahl and Ingelmark 1950). It is therefore conceivable that these microcracks, and the vascularization in the subchondral bone plate, could facilitate molecular transport from the subchondral region to the basal layer of cartilage. Evidence to support this transportation comes from the discovery of hepatocyte growth factor (HGF) within the deep zone of normal cartilage, and an elevated level in osteoarthritic cartilage (Pfander et al. 1999); despite it not being produced by chondrocytes, but by the osteoblasts in the subchondral region (Guevremont et al. 2003). It has therefore been proposed that, following its synthesis by subchondral osteoblasts, HGF can reach the deep layers of articular cartilage via these microcracks, and/or the vascularized subchondral plate, and promote cartilage breakdown

and/or enhances matrix remodeling. The association of HGF with Osteoarthritis comes from its incitement of MMP-13 production (Reboul et al. 2001); an enzyme present in the lower intermediate and deep layers of osteoarthritic cartilage. Other evidence for the role of subchondral bone in cartilage degradation are TGF-ß, Cathepsin K, and PGE2/LTB4, which are all produced by osteoarthritic subchondral bone cells, and yet found at the deep layers of osteoarthritic cartilage (Konttinen et al. 2002; Moldovan et al. 1997; Nakase et al. 2000).

10.3.2 Subchondral Considerations in Cartilage Healing

Although clinical studies have reported favorable results with osteochondral autografts at short and mid-term follow-up, animal studies 3 months after grafting found signs of degeneration, evidenced by chondrocyte clustering and hyper-cellularity in cartilage (Tibesku et al. 2004). Answering the question of whether the observed degradation may be detected histologically, Kleemann et al. (2007) characterized the mechanical competence and morphology of cartilage in osteochondral autografts. The ensuing study demonstrated that grafted tissues seem to undergo in a short period of time, where both substantial degenerative and regenerative processes occur. The compressive stiffness of the grafted cartilage was about 58 % of that of healthy tissue at 3 months, and rose to 82 % at 6 months. Fibrillation, hypercellularity, and cell clustering were observed at the edges of the grafts. Both the cartilage and the underlying bone were observed to be degrading, raising doubts as to their long term repair. Events such as disrupted nutrition (Malinin and Ouellette 2000), physical damage during the extraction, and transplantation of the graft (Buckwalter and Mankin 1998; Duda et al. 2001; Huntley et al. 2005; Redman et al. 2004; Whiteside et al. 2005) were thought to be contributing factors. Despite this, the intensity of type II collagen staining of the healthy and the grafted cartilage tissue were identical.

A bottom-upwards approach by Schell et al., had aimed to first support the reconstruction of the subchondral bone plate in an osteochondral defect, and thereby improving the mechanical and histological quality of the repaired cartilage (Schell et al. 2007). The authors transplanted crushed bone graft together with a collagen membrane into osteochondral defects, 8.3 mm in diameter and 10 mm in depth. Comparing its healing with unfilled control groups, they observed no difference in healing outcome between the two groups after 6 months. All defects, whether filled or not, showed an irregular, more or less advanced cartilage repair. However, the articular surface was not restored in any case.

A similar endeavor had attempted to encourage osteochondral healing through mechanical straining (Duda et al. 2005). Bone resorption and formation were observed at the base, and at the circumference of the defects, respectively. Defect filling, cartilage formation, and trabecular structures were observed for up to 12 weeks. Although their defects were completely filled, the neo-tissue mainly comprised of fibrous cartilage, and only partially with hyaline-like cartilage.

The importance of the subchondral bone in cartilage healing is undisputed. When a cartilage lesion is deep enough, the penetrated subchondral bone is prompted into action. Often, in cases such as abrasion chondroplasty and microfracture, it is strategically penetrated surgically for its input into the healing process to be realized. It is now a topical discussion that the state of the underlying bone itself, be it mechanical, physiological, or otherwise, is actually important for the quality of cartilage regenerated.

10.3.3 Subchondral Considerations in Cartilage Tissue Engineering

The functioning of articular cartilage is believed to be dependent on the mechanical support by the subchondral bone. In fact, the steep stiffness gradient in the subchondral bone is suggested to be responsible for the initiation and progression of cartilage damage (Radin and Rose 1986). Moreover, the stiffened subchondral bone associated with osteoarthritis (Radin et al. 1970) is said to cause transverse stresses at the base of the articular cartilage, potentially resulting in deep horizontal splits therein. Given their apparent differences, tissue-engineered solutions either favors cartilage repair, or bone regeneration, and seldom satisfy both tissues. Osteochondral repair strategies now aim to concurrently mimic the physiological properties and structure of the cartilage and bone using cell-seeded constructs. The resulting hybrid is an engineered scaffold consisting of both a cartilage-optimized phase and a subchondral bone-optimized phase (Temenoff and Mikos 2000). Principally, the two phases are produced separately, under their appropriate conditions, and with the appropriate cellular disseminations, and are later united prior to implantation. On this front, two kinds of hybrid scaffolds have been developed by Hutmacher and co-workers, using a combination of fibrin, polycaprolactone (PCL), and a PCL-TCP combination. In one instance, the fibrin served as the cartilage phase while the PCL scaffold substitutes for the subchondral phase. On another occasion, their hybrid consisted of PCL and PCL-TCP. The top PCL region promotes cartilage regeneration while the underlying PCL-TCP serves as the subchondral bone phase. Having been seeded with pre-cultured MSCs, the biphasic constructs were implanted into New Zealand white rabbits for up to 6 months. The researchers found that in terms of cartilage regeneration, PCL bettered the fibrin constructs; stipulating that the mechanical support provided by the fibrin was insufficient for cellular development, and its subsequent secretion of the essential ECM products. In fact, the fibrin degrades rapidly, while the porous PCL scaffold degraded slowly, providing an effective mechanical support (Swieszkowski et al. 2007).

Along a similar line, Schlichting et al. evaluated the healing of osteochondral defects using polylactide-co-glycolide scaffolds of differing stiffness, hypothesizing that a stiff scaffold creates sufficiently stable conditions necessary for subchondral bone formation and consequently cartilage regeneration compared with a softer

scaffold or to untreated controls (Schlichting et al. 2008). The stiff scaffold was found to improve the regeneration of subchondral bone, while the soft scaffolds provided less support, and consequently the surrounding subchondral bone became more sclerotic. Indeed, the regenerated cartilage that was formed over, the stiff scaffold exhibited higher elastic and dynamic moduli at 3 months than did the soft scaffold group. However these mechanical properties were not dissimilar for both groups at 6 months. Moreover, these values were inferior to that of native articular cartilage. These findings led to the conclusions that Materials used to fill subchondral defects should have a comparable stiffness to that of healthy subchondral bone rather than being too flexible. When this is not the case, degradation or resorption of filling materials will lead to loss of stiffness, and may compromise the defect healing.

10.4 Summary

The present review has looked at the current challenges faced when trying to regenerate cartilage. Most of these issues have been related to the structure, composition, and mechanical features of the tissue. In light of the topics discussed above, the following statements may summarize the current challenges associated with cartilage regeneration:

Cartilage is a complex tissue, with at least three phases. Moreover, cartilage illness may be systemic, local, acute or chronic. Current treatment options aim to reduce pain. By large, there is as of yet no solution that is all-encompassing, and can regenerate all the different types of cartilage defects. Despite the ongoing debate over the separation of articular cartilage from its subchondral bone by the tide mark, there exists an overwhelming amount of evidence to link the two regions, particularly at the onset of OA. Therefore, a good strategy for cartilage regeneration ought not to neglect the underlying subchondral tissue. Principally, a successful clinical outcome will have re-established both the damaged cartilage and its underlying subchondral bone.

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Chapter 11
Muscle, Ligament and Tendon Regeneration

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Abstract Muscle injury and degenerative muscle diseases are disabling conditions that are currently challenging orthopedic surgeons, neurologist and specialists in rehabilitative medicine. Upon traumatic or degenerative changes in the structure of the muscle, regeneration befalls mainly by increased proliferation of satellite cells. If the injury is extensive fibrosis and scar tissue formation occurs. Till now various alternative therapeutic ways have been proposed to boost muscle regeneration. These methods include the use of growth factors, antioxidative therapeutic approaches, cell based therapy and cell transplantation as well as the use of scaffolds. Growth factors, antioxidative substances and endogenous polypeptides can not only influence but also control the natural repair processes by acting on different intracellular pathways. Cell orientated therapies have been popular in muscle regeneration mainly because small quantities of cells are needed to achieve therapeutic effects. Transplantation of stem cells, myoblasts or genetically modified cells, have been used after injury to restore muscle structure and function. Furthermore, scaffolds have been used to repair muscle defects and to generate new muscle fibers.

Similar approaches have been made for regeneration of tendon and ligament. There are a number of cell sources that are potentially helpful for cell mediated tissue regeneration. Scaffolds provide temporary mechanical support and can carry cells that promote the tendon and ligament regeneration. Furthermore, growth factors can be used to stimulate tissue healing and accelerate regeneration mainly by modulating the proliferation.

Keywords Injury • Repair • Satellite cells • Fibrosis • Tenocytes

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11.1 Muscle Regeneration

11.1.1 The Skeletal Muscle: Injury and Repair

Muscle is defined as the anatomical construct of animals with the ability to contract. The skeletal muscle tissue derives from paraxial mesoderm. During development, myoblasts migrate to their destinations, where they fuse to form elongated skeletal muscle cells. Muscle cells contain contractile filaments that move past each other and change the size of the cell. The human body consists of more than 600 different muscles, which allow us to breath, to go and to perform daily tasks. About 40 % of the total body mass in males are muscles whereas 25 % in females, only.

Over a short period of time after muscle injury ischemic events as well as inflammatory damage of myocytes and interstitial muscle tissue cells occurs. Immediately after, the nutritive microvascular perfusion of the muscle gradually surceases and the inflammatory response with leukocyte endothelial cell interaction enlarges. Subsequently, normal function of the endothelial cell barrier is disrupted and interstitial muscle edema establishes (Oestern and Tscherne 1983). At later time points after muscle injury tissue regeneration is characterized by three phases (Järvinen et al. 2005): (1) Destruction phase: rupture and necrosis of muscle cells, hematoma formation and leukocyte cell infiltration; (2) Regeneration phase: phagocytosis of the necrotic tissue, regeneration of myofibers and formation of fibrotic tissue, capillary incorporation as well as angiogenesis into the traumatic tissue; (3) Remodeling phase: reconstruction and regeneration of myofibers, increase of the breaking strength of the traumatized tissue, reorganization of the scar tissue and functional remodeling of the muscle.

Similar pathological changes occur not only after muscle injury but also in dystrophic muscle disease. Muscular dystrophies are a heterogeneous group of hereditary diseases affecting both children and adults, and are characterized by muscle wasting and weakness. Degenerative muscle diseases, like muscular dystrophies, involve cycles of segmental necrosis and regeneration. The muscle tissue is thereby characterized by fiber size variability, necrosis, regeneration, inflammation and connective tissues deposition (Ciciliot and Schiaffino 2009).

11.1.2 Regenerative Capacity of Skeletal Muscle

The skeletal muscle is an irreversibly post-mitotic tissue that has under normal conditions a very low mitotic activity. Under certain conditions and in response to various stimuli like very mild trauma, regenerative cascades in the muscle become activated in order to restore the injured tissue. Under normal conditions muscle regeneration is initiated subsequently to muscle injury.

The most important cells during muscular regeneration are satellite cells. Satellite cells are undifferentiated reserve cells, which are located in the gap between basal lamina and plasma membrane of each individual myofiber (Mauro 1961). In

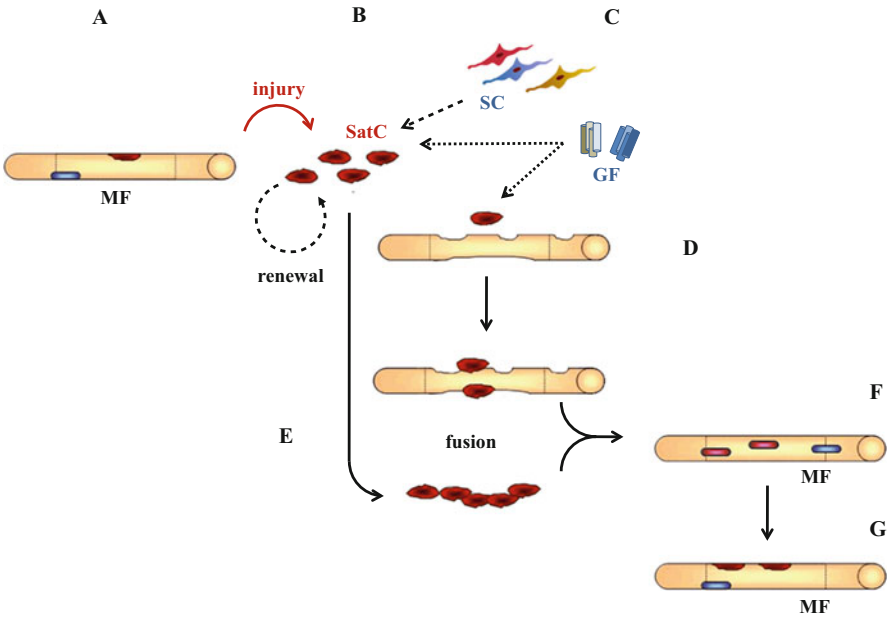

Fig. 11.1 The muscle regeneration: (**a**) If a normal myofiber (MF) is injured (**b**) satellite cells (SatC) became activated and start to proliferate. Circulating stem cells as well as local stem cells from the skeletal muscle (SC) differentiate to satellite cells (**c**) and participate during the repair process in the muscle regeneration. The satellite cells fuse with the injured myofibers (**d**) or together (**e**) and form new myofibers with central nuclei (**f**). Growth factors (GF) control the procedure and enhance the regeneration. At a later time point the newly formed nuclei move away from the center of the cell and reside beneath the cell membrane (**g**)

response to muscle injuries satellite cells proliferate, differentiate into myoblasts and finally fuse together to form a multinucleated myotube. The newly formed myotubes fuse with the injured myofiber that has survived the initial trauma.

In the mature muscle tissue two major populations of satellite cells reside. The first population of satellite cells directly repairs the injured tissue by proliferation and differentiation into myoblasts immediately after muscle injury. The second population of satellite cells proliferates after muscle injury and replenishes the existing satellite cell pool by undergoing cell divisions before differentiation. Furthermore, it is known that post trauma, stem cells from the bone marrow but also muscle residual stem cells are migrating to the injured skeletal muscle and refill the satellite cell pool. It is assumed that some satellite cells are capable to differentiate not only into myogenic cell lineages but almost any cells lineage of mesenchymal origin (Fig. 11.1).

However, this regenerative capacity is not infinite, as fatigue of the satellite cell population is an important factor during the regenerative process especially in patients with congenital myopathies such as Duchenne muscular dystrophy. Although the endogenous regenerative capacity of skeletal muscle can convention-

ally be supported by physical means (like rest, ice, cooling and elevation of the injured limb) the recovery is not always ample. Novel therapeutic strategies can be applied to restore, improve and maintain the function of the muscle tissue healing during injury, disease, age and congenital defects.

11.1.3 Growth Factors and Muscle Boosters

Muscular regeneration is a crucial biological process, which occurs during the natural repair cycle. The recognition of biological active proteins, which can enhance the repair process, is still under investigation. The ideal muscle booster would be a molecule that does not have side effects and can specifically and successfully be delivered into the injured muscle. Further goals of the generative therapies include the maintenance of pre-traumatic muscle mass, reduction of the post-traumatic muscle loss and up-regulation of muscle regeneration. Up to now, many factors have been recognized to control natural repair processes by acting on different intracellular pathways.

IGF-I and IGF-II (Insulin like Growth Factor I and II): The IGF-l and IGF-II increase the satellite cell proliferation, restores muscle mass after injury, mobilize non-muscle stem cells and improve regeneration in aged myopathy related skeletal muscle (Husmann et al. 1996; Singleton and Feldman 2001; Barton et al. 2002; McKay et al. 2008). Furthermore, IGF-I seems to utilize pathways in regulating the satellite cell pool.

TGF (Transforming growth factor): The TGF plays a crucial role in regulating the repair and remodeling following tissue injury. Further, TGF mediates many biological actions on extracellular matrix components (Husmann et al. 1996).

FGF (Fibroblast growth factor): The fibroblast growth factor family participates in the muscle regeneration and has been suggested as a potent activator of myocytes satellite cells (Charge and Rudnicki 2004). Especially FGF-2 and FGF-6 accelerate muscle regeneration (Israeli et al. 2004; Li et al. 2010).

HGF (Hepatocyte growth factor): The HGF plays a key role during the early stage of muscle regeneration. It promotes the satellite cell proliferation and migration into the site of injury as well as the stimulation of quiescence satellite cells (Charge and Rudnicki 2004).

MSTN (Myostatin): Myostatin is a potent negative regulator of skeletal muscle growth and member of the tumor growth factor-beta family. Disruption of the myostatin gene causes a combination of hypertrophy and hyperplasia which induces a remarkable increase in muscle mass. The depletion or inactivation of myostatin leads to a significant improvement in muscle regeneration processes, especially in degenerative diseases, mainly through stimulation of satellite cell proliferation and differentiation (Wagner 2005).

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells): The NF-κB is a protein complex that controls the transcription of DNA. NF-κB promotes

proliferation, inhibits differentiation and modulates muscle development (Peterson and Guttridge 2008). Further, it modulates immune response, inflammation and cell survival in skeletal muscle disease (Mourkioti and Rosenthal 2008).

EPO (Erythropoietin), G-CSF (Granulocyte-Colony Stimulating Factor) and PDGF (Platelet Derived Growth Factor): are endogenic polypeptides with pleotropic actions on a variety of hematopoietic and non-hematopoietic cells. In relation to muscle regeneration EPO has been recognized as anti-apoptotic and tissue-protective protein which increases the proliferation of local cells and improves muscle function (Rotter et al. 2008) after muscle injury as well as after muscle ischemia (Kim and Hong 2007). G-CSF has been found to increase satellite cell proliferation and reduce cell apoptosis after muscle injury resulting in faster and better muscle restoration (Stratos et al. 2007; Naito et al. 2009). The PDGF seems to increase myoblast proliferation, acts chemotactic for satellite cells and stimulates the angiogenesis (Husmann et al. 1996).

LIF (Leukemia Inhibitory Factor): The LIF is an interleukin 6 class cytokine that affects satellite cell growth and development as well as proliferation and differentiation after injury (Charge and Rudnicki 2004; Karalaki et al. 2009).

ß2 adrenergic agonists: ß2 adrenergic agonists stimulate the muscle growth and impede the muscle loss. They further suppress protein degradation (Yimlamai et al. 2005), provoke neuronal growth and activity as well as enhance the replacement of muscle specific proteins (Arai et al. 2006).

Calcineurin: Calcineurin is a calcium and calmodulin-dependent serine/threonine protein phosphatase, which mediates myotube differentiation, enhances myoblast recruitment and ameliorates injury to the dystrophic muscle (Mitchell and Pavlath 2002).

Melatonin: Melatonin is an indolamine that enhances muscle force, reduces apoptosis and impedes inflammation after muscle injury (Stratos et al. 2012). Additionally it has been shown that Melatonin protects against ischemia/reperfusion injury in skeletal muscle (Erkanli et al. 2005) by acting as a potent antioxidative substance (Halici et al. 2004).

Furthermore various other substances have been described to promote muscle regeneration like caplains (calcium dependent proteases) as well as diverse steroids and hormones that seem to promote myoblast recruitment, enhance proliferation and induce muscle growth.

11.1.4 Antioxidative and Antiapoptotic Therapy

Antioxidative substances have been used with intent to prevent oxidative stress and the experimental results showed mixed success. Antioxidants may potentially reduce certain types of muscle damage but supplementation probably cannot totally prevent muscle injury and degeneration. Ubiquinone seems to stabilize the cell membrane after injury (Kon et al. 2007) and to stop the ischemia reperfusion injury

(Bolcal et al. 2007). Vitamin C is a major antioxidant and has important functions in connective tissue and immune function. Vitamin C application before muscle injury preserves muscle function and reduced the local infiltration and edema (Kearns et al. 2004). Furthermore, Vitamin C prevents microvascular dysfunction in the skeletal muscle of the septic rats (Armour et al. 2001) and decreases the exercise-induced increase in the rate of lipid peroxidation (Evans 2000). Alpha-Lipoic acid (an essential cofactor for many enzyme complexes) and Isoflavonoids (a group of plant chemicals) have beneficial effects upon oxidative-induced damage of muscle tissue. Supplementation with vitamin E increases muscle force after injury (Warren et al. 1992) reduces the levels of protein oxidation in the skeletal muscle and lessens the exercise-induced lipid damage (Reznick et al. 1992). Several groups have tested combined supplementation treatments in relation to exercise-induced muscle damage. Combinations have included mainly vitamins E and C as well as other antioxidants (Baskin et al. 2000; You et al. 2005).

Antiapoptotic strategies have also shown enhanced muscle regeneration after injury. Additionally it is postulated that inhibition of caspase activation could be a potential therapeutic target during katabolic events, trauma and disease (Jackman and Kandarian 2004). Inhibition of caspase-mediated apoptosis supported the functional restoration of the injured muscle by amplifying muscle force and furthermore promoted the survival of the injured myofibers by decreased muscle atrophy (Stratos et al. 2011b). Interestingly, Hu et al. reported that transgenic mice, which selectively express the endogenous caspase inhibitor X-chromosome-linked inhibitor of apoptosis protein (XIAP) in skeletal muscles, have hypertrophic peripheral muscles compared to wild-type animals (Hu et al. 2010).

11.1.5 Cell Therapy and Muscle Regeneration

Till now, cell orientated therapies have been introduced which endorse muscular regeneration. Stem cell therapy is an attractive method to treat both muscle injury and dystrophic muscle tissue, because a small quantity of cells is required to achieve therapeutic effects (Price et al. 2007). A current classification of non muscle stem cells involved in muscle regeneration includes (1) non muscle stem cells from the ectoderm and endoderm e.g. neural stem cells (Galli et al. 2000) (2) non muscle stem cells from the hematopoietic system and (3) non muscle stem cells from the solid mesoderm.

Non muscle stem cells from the hematopoietic system: possible ways to induce muscular regeneration by non muscle stem cells from the hematopoietic system include the usage of cells from the bone marrow (Ferrari et al. 1998) or transplantation of bone marrow side population cells (Motohashi et al. 2008). Furthermore non muscle stem cells from the hematopoietic system expressing AC133+ are able to undergo myogenesis when cocultured with myogenic cells or when transplanted in-vivo (Torrente et al. 2004). Using genetically marked bone marrow derived cells in a mouse model Ferrari and colleagues could show that this cell population could

migrate into areas of induced muscle degeneration. Furthermore, this bone marrow derived cells could participate in the regeneration of damaged muscle fibers by undergoing myogenic differentiation (Ferrari et al. 1998). Differentiated myotubes can be formed in-vitro as well as in-vivo (Jiang et al. 2002; Muguruma et al. 2003) by using multipotent adult progenitor progenitor cells. Furthermore, it is known that in response to injury bone marrow-derived cells are not only capable to differentiate into satellite cells but also to fuse with the existing damaged myofibers and to regenerate the muscle fibers (LaBarge and Blau 2002; Corbel et al. 2003). For further support of his statements a parabiotic animal model was used. During these experiments, the vascular systems of observed mice were surgically joined. The authors could demonstrate that bone marrow-derived cells formed skeletal myofibers in injured and physiologically stressed muscle (Sherwood et al. 2004).

Non muscle stem cells from the solid mesoderm: Non muscle stem cells from solid mesoderm which participate in the muscular regeneration include mesenchymal stem cells (MSC). Isolated MSC from the bone marrow can be expanded in a cell culture, and differentiate into a variety of cells of mesenchymal origin including skeletal muscle. MSC have been used to restore the function and anatomy of degenerated peripheral skeletal muscle in cases of myopathy and other congenital muscle diseases (Dezawa et al. 2005). The injection of MSC into the muscle of mdx mice, led to the formation of functional myofibers and satellite cells (De Bari et al. 2003). Furthermore, it has been described that MSC are being mobilized into the peripheral blood in response to injury and to further migrate from the blood across endothelial cells into the injured tissue. Transplantation of MSC into the muscle after injury causes a site-specific differentiation of the MSC into myocytes. Multipotent adult progenitor cells can be differentiated in-vitro in mesenchymal lineages and participate in the muscular regeneration. Muscle derived stem cells reside in adult skeletal muscle, express CD34 as well as Sca-l and improve skeletal regeneration (Qu et al. 1998). Mesoangioblasts have a myogenic potential in culture and are capable of ameliorating the symptoms of a number of differing skeletal muscle pathologies (Otto et al. 2009). Further cells with myogenic capacity include endothelial progenitor cells (Takahashi et al. 1999), stem cells from adipose tissue (Rodriguez et al. 2005) as well as stem cells from synovium (De Bari et al. 2003).

Myoblasts: Up to now, many therapeutic attempts have been proposed to cure degenerative muscle disease using myoblasts. Based upon early experimental findings on mdx mice, dystrophin expression was restored after intramuscular transplantation of myoblasts. Irintchev et al. (Irintchev et al. 1997) applied in 1997 a muscle injury of the soleus muscle and induced an increased muscle force and functional improvement after transplantation of 106 myoblasts into the site of injury. Furthermore, muscular regeneration was preceded after muscle injury and immediate application of myoblasts into the site of injury (Irintchev et al. 1997; Wernig et al. 2000). These promising results were quickly followed by clinical trials. In the early 1990s intramuscular injection of allogenic myoblasts was performed on humans with Duchenne Muscular Dystrophy (Gussoni et al. 1992, 1997; Mendell et al. 1995). Unfortunately, the clinical benefit obtained from these studies was minimal, and research programs attempted to recognize the failures and pitfalls of these

clinical trials. Major limitations of previously mentioned attempts included insufficient cell distribution, immune rejection, and poor cell survival after cell transplantation. According to knowledge based on preliminary experimental results, only a small number of cells participated in muscle regeneration whereas the vast majority of the injected cells did not survive the transplantation (Farini et al. 2009). In virtue of current data, satisfactory myoblast transplantation requires an ample delivery system of cells to the injured or degenerated tissue as well as a sufficient immunosuppressive therapy.

Genetically modified cells for gene delivery into muscle: It has been suggested that overproduction of pleiotropic cytokines into the injured tissue via genetically modified cells may represent an attractive alternative to conventional therapeutic strategies. An interesting method, which has been already used, for tissues repair of muscle, skin, liver and cartilage is to transfect specific cells with genome which enhances repair processes. For this purpose transfection of myoblasts may be an interesting option since these cells are used to repair and regenerate damaged skeletal muscle by acting as vectors for gene therapy. Genetically modified myoblasts have been used for replacing degenerating muscle fibers in mdx mice. As a gene delivery vehicle, myoblasts were used to deliver growth hormones, VEGF, Factor IX, EPO, FGF and others. Recent findings predict that targeted delivery of mRNA or DNA into the site of injury or injured cells will specifically manipulate genes and enhance muscle regeneration (Järvinen et al. 2005; Caplan 2007; Krampera et al. 2007).

11.1.6 Scaffolds and Muscle Regeneration

Transplantation of cells or the use of growth factors is a suitable procedure to treat minor defects after muscle injury. The application of cell-containing-scaffolds into the site of injury shows advances compared to the previously mentioned methods particularly in the treatment of larger muscle defects. Detailed in-vitro studies have enabled the development of scaffolds with the ability to generate muscle tissue. Aim of experimental approaches with scaffolds is to restore the structure and function of the injured muscle without scar tissue formation (Grefte et al. 2007). Seeding of the matrix with autologous satellite cells reduces not only the inflammation but also decreases the fibrosis at the edge of the implant (Grefte et al. 2007). Moreover growth factors like FGF-2 or HGF inside the matrix have a positive effect on local myoblasts and improve myogenesis (Hill et al. 2006a, b). Further studies have shown that the matrix in which the cells are embedded plays a major role in the regenerative process. The use of myoblasts in scaffolds containing polyglycolic acid meshes, alginate or hyaluronic acid constructs promotes the vascularization and muscle neoformation (Saxena et al. 1999, 2001; Kamelger et al. 2004; Stratos et al. 2011a, b). Myoblast can furthermore fuse in-vitro and can develop physiological function like force production. For this purpose a three-dimensional matrix can be used, were myoblasts are seeded and differentiated on top of a fibrin gel (Huang et al. 2005).

11.1.7 Future Perspectives

Although major scientific efforts have been made to understand the principles of myogenesis and muscular regeneration, no definitive treatment for muscle injury and degenerative muscle disease exists. The primary focus of current studies has been to identify molecules and cascades that can regulate the proliferation of satellite cells, influence the tissue inflammation, control the angiogenesis and affect the apoptosis of skeletal muscle. Further studies are needed to define the specific role of stem cells, scaffolds and other growth factors in the regeneration after injury, degeneration and dystrophy. Further goal should be to identify molecules that can modulate muscle cell homeostasis as well as to be able to boost proliferation and cell survival. These results will enhance our understanding of cell biology and cell regeneration serving as a platform for patient oriented based therapies.

11.2 Regeneration of Ligaments

Ligaments are non-stretchable strings of the skeleton, they contain mainly collagen and connect bones together. Ligamental tissue consists of an extracellular matrix with its embedded fibroblasts. The fibroblasts are responsible for biologic adaption to the mechanical environment, remodeling and healing of the injured ligament. Immediately after ligament injury (phase I) bleeding of the injured tissue occurs. This phase is followed by an inflammatory response (phase II) as various cytokines and growth factors are released by the inflammatory cells. This results in neovascularization and initiation of granulation tissue formation. Later fibroblasts proliferate (phase III) and collagen is being formed. Remodeling (phase IV) occurs 6 or more weeks after injury as wound gap is being filled with unorganized granulation tissue. This phase of healing can extend up to many years and is responsible for restoration of tensile stiffness and strength (Woo et al. 2004).

11.2.1 Ligamentisation and Mechanical Load

A common surgical procedure in humans for ligament replacement is to use autologous tendon grafts. The biological and morphological changes which take place after tendon transplantation are defined as ‘ligamentisation’ (Amiel et al. 1986). During the first 2 months a fibroblast proliferation occurs followed by graft remodeling, angiogenesis, vascularization and necrosis. Throughout the steady maturation of the graft and within 3 years after transplantation the tissue undergoes a complete metaplasia to a ligamentous structure.

Application of mechanical loading has been reported to positively influence the cellular proliferation as well as to effect cellular morphology and alignment in the

regenerating ligament. Additionally mechanical load influences the ligamental regeneration by modulating the healing of the graft-bone interface. According to recent studies both the timing as well as the magnitude of mechanical stimulation after ligament injury are important for the optimal healing during the regeneration period (Rodeo et al. 2010).

11.2.2 Cell Therapy and Scaffolds

In-vivo studies have shown ligament regeneration by implanting mesenchymal stem cells and silk scaffold (Fan et al. 2008, 2009) resulting in a histological and functional improvement. Current in-vitro and in-vivo experiments suggest that subsequently to transplantation of autologous or allogenic mesenchymal stem cells, the transplanted cells display phenotypic characteristics of the endogenous surrounding tissue. These studies suggested that administration of mesenchymal stem cells at the site of injury reduces the injury size and enhances the regeneration (Arthur et al. 2009).

Clinical trials as well animal studies have pointed out the efficacy of platelet-rich plasma treatment for ligament and tendon injuries (Rodeo et al. 2010; Paoloni et al. 2011). Platelet-rich plasma is produced after centrifugation of whole blood. That centrifugate holds not only higher platelet concentration than that of the whole blood but contains also numerous growth factors that can participate into regeneration after injury. However, the efficacy of such treatment remains controversially discussed especially when comparing platelet-rich plasma treated subjects with corresponding sham groups (Paoloni et al. 2011).

Further scientific efforts have been made to generate adequate ligament scaffolds. The scaffolds should be biodegradable, porous, biocompatible, exhibit sufficient mechanical strength, and promote the formation of ligamentous tissue (Cooper et al. 2005). In summary collagen fiber scaffolds as well as hybrid biomaterial-biological ensembles have been proposed to enhance ligament regeneration after injury (Kew et al. 2011). Current literature distinguishes between biologic and synthetic scaffolds, however both of them seem to exhibit inadequate tensile strength as well as to have fatique properties (Ignatius and Durselen 2009).

11.2.2.1 Matrix Metalloproteinases, Ultrasound Application and Nitric Oxide (NO)

Recently, extracellular proteins were identified to modulate ligament regeneration. The metalloproteinases are proteins, which function, in both extracellular environment through transmembrane and intracytoplasmic domains. Matrix metalloproteinases induce further production of pro-inflammatory cytokines and are counterbalanced by the Tissue Inhibitors of Metalloproteinases (TIMPs). The major role of the matrix metalloproteinases is to contribute to normal tissue remodeling mainly through breaking down any extracellular matrix component. Furthermore

low-intensity ultrasound has been shown to have angiogenic actions on the ligaments as well as gene expression and proteoglycan synthesis. The NO is a free radical agent which acts in intracellular and extracellular environment. NO is enhanced during ligament healing (Deehan and Cawston 2005).

11.2.3 Growth Factors and Gene Therapy for Ligament Regeneration

A variety of growth factors have been identified to modulate the regenerative capacity of ligaments and to improve tissue function. Researchers have tried to develop strategies that improve ligament regeneration and repair mainly by modifying the extent of scar tissue formation due to the fact that ligament regeneration is similar to the healing process of the skin. Platelet-derived growth factor (Hildebrand et al. 1998), epidermal growth factor and transforming growth factor as well as the fibroblast growth factor (DesRosiers et al. 1996; Deehan and Cawston 2005), promotes the angiogenesis and scar tissue formation of the ligament. Growth factors can be applied to modulate many cellular activities, including cell proliferation, cell migration, and extracellular matrix synthesis and production.

Gene therapy introduces foreign nucleic acids into cells in order to alter their endogenous protein expression. Direct transfer involves the use of naked DNA from mammalian tissue and a one-step delivery of genes into host cells in vivo. Indirect transfer involves transfection of desired genes into cells followed by implantation of the cellular tissue into the host. By means of tissue engineering it is possible to transfect cells with beneficial factors and to inject them into the injured ligament (Menetrey et al. 1999). This results in an improved ligament healing and a faster maturation. One of the major drawbacks of this technique is the mutagenesis, which is clinically unacceptable especially in elective cases.

It is clear from the current literature, that regeneration of ligaments is possible and that growth factors, cell transplantation as well as scaffolds (Fig. 11.2) do have a capacity to repair. Unfortunately none of the previously mentioned methods has been analyzed clinically. Considering the limited number of clinical and experimental studies as well as our poor knowledge regarding ligament regeneration, we conclude that at present tissue engineering of ligaments only partially fulfills scientist's expectations due to the challenge of achieving a sufficient primary tensile strength and adequate tissue angiogenesis.

11.3 Regeneration of Tendons

Tendon is the anatomic construct that connects muscle to bone and is is capable of enduring tension and transmitting forces. However, as a result of its viscoelastic nature, tendon also absorbs energy, and as a result tendon lesions occur.

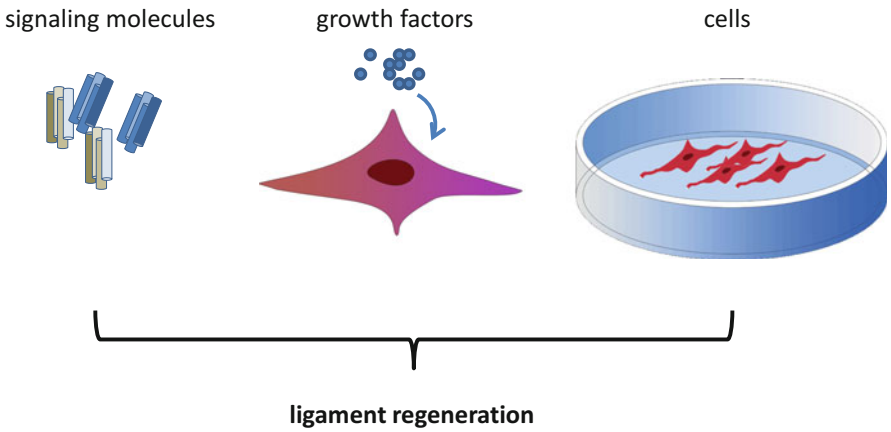

Fig. 11.2 A proposed approach achieving successful ligament regeneration combining signaling molecules, growth factors and cell transplantation

During tendon repair process the healing passes through three overlapping phases: inflammation, repair and remodeling. The initial inflammatory phase begins immediately after the injury and continuous for a few days. The reparative phase begins 48 h after injury and stops 4 weeks later. During this period proliferation occurs in the tissue. The repair process ends with the remodeling phase approximately 4 months after injury. Despite intensive remodelling over time, total regeneration of the tendon will not be achieved and the initial defect will be replaced by a hypercellular tissue. After the regenerative process the collagen fibrils are thinner and are responsible for the reduced biomechanical strength of the tendon (Maffulli et al. 2002).

The regenerative strategies upon tendon injury or primary disorders of tendons include the use of growth factors, cells with or without scaffolds and gene therapy.

11.3.1 Growth Factors

The roles of growth factors are complex hence growth factors interact with the tendon during the repair process. A variety of studies have been published and have analyzed the effects of growth factors during tendon regeneration. The vast majority of the growth factors were applied by local or systemic injection or by implanting scaffolds containing growth factors. There is evidence that interaction of several different growth factors is important for tendon healing. A short list of some well known growth factors as well as their effects on the injured tendon are presented below (summary is based on the review articles from Docheva and Branford (Docheva et al. 2015; Branford et al. 2014)).

bFGF (basic fibroblast growth factor): Promotes early events in tendon healing during proliferation and remodeling phase. Stimulates cellular migration and angiogenesis.

BMP (Bone morphogenetic proteins): BMP's are elevated during early tendon healing process. BMP-12, -13, and -14, are tenogenic factors with the potential of driving differentiation of MSC. Additionally BMP-12, -13, and -14 stimulate mitogenesis during early regenerative events of the tendon.

CTGF (connective tissue growth factor): CTGF is a downstream mediator of TGF-β1 signaling and acts as a co-factor in fibrosis. Furthermore, it has been shown that CTGF plays a significant role in the accumulation of collagen type I in mechanically loaded fibroblasts. Additionally, CTGF gene expression is increased during acute and reparative phase of tendon healing.

IGF I (Insulin-like growth factor I): IGF I is most active during the inflammation and remodeling phase of tendon healing. It stimulates proteoglycan, collagen and non-collagen synthesis in tendon derived cells. Additionally, IGF-1 enhances synthesis of the ECM, stimulates tenocyte migration into the injured tendon and causes cell proliferation in both the epitenon and endotenon.

VEGF (vascular endothelial growth factor): VEGF is important in tendon degeneration as well as regeneration and is most active immediately after the inflammation phase. VEGF Induces angiogenesis, migration and mitosis of endothelial cells during tendon healing.

TGFβ (transforming growth factor β): TGFβ is most active during the inflammation phase. TGFβ promotes cell migration and mitogenesis, stimulates production of the ECM as well as collagen types I and III.

PDGF (platelet derived growth factor): PDGF enhances tendon cell proliferation and increases collagen production. It plays an important role during the entire tendon repair period

EGF (epidermal growth factor): EGF regulates cellular proliferation and differentiation through its receptor EGFR. Furthermore, exogenous application of EGF stimulates migration of tenoblasts from endotenon derives cells.

HGF (hepatocyte growth/scatter factor): HGF has been shown to antagonize the action of TGF-β1 in fibroblasts by inhibiting TGF-β1 induced fibrosis.

Scx (Scleraxis): The Scx protein is a member of the basic helix-loop-helix superfamily of transcription factors. Scx mutant mices show severe tendon defects. Scx regulates also the tendon differentiation and distinguishes force-transmitting tendons from muscle-anchoring tendons (Murchison et al. 2007).

Platelet-rich plasma (PRP) has been considered as a therapeutic method for tendon regeneration after injury. It has been suggested, that the components of PRP (like PDGF, VEGF, TGF, FGF and IGF) could regenerate injured tendons. However, recent randomized controlled trials failed to show that PRP treatment improves outcomes after rotator cuff repair (Rodeo et al. 2012).

Some experimental settings used both viral and non-viral gene transfer techniques as well as antisense oligodeoxynucleotides in order to deliver growth factor genes into a cell or to modulate the mRNA expression of genes (Hildebrand et al. 2004). As a result, therapeutic genes have been introduced into tendon tissue with evidence of corresponding functional alterations.

11.3.2 Cell Based Therapy

Cell-mediated tendon engineering therapies are promising alternatives to traditional graft/scaffold treatments. The used methods for a cell based therapy includes an injections of cell mediated suspensions into the site of injury alone or in combination with a scaffolding material. Cell based therapies were usually performed with tenocytes, dermal fibroblasts, muscle-derived cells, stem cells, bone marrow stem cells, adipose-derived stem cells, tendon stem cells, perivascular stem cells or embryonic stem cells. Additionally, engineered cells were introduced and their function was analyzed predominantly in vitro. This genetically modified cells overexpress Scleraxis, iPSCs, IGF-1, GDF-5, PDGF, FGF-2, VEGF, BMP-2 or Smad8. Till now, there is no consensus about the best cell population that should be used for translational research in terms of "bench to bedside" for tendon research. Tenocytes, dermal fibroblasts and BMSCs are up to now the best candidates for this purpose (Gaspar et al. 2015).

11.3.3 Scaffold Based Tendon Treatment

The scaffolds can be divided into two types: biologic or synthetic. Biological scaffolds can further be categorized depending on the used cell type (like small intestine submucosa, dermis, pericardium, and fascia lata). Depending on the cell source scaffolds can be classified in autograft, allograft or xenografts. Tissue engineering of tendon includes artificial polymers, biodegradable films, and biomaterials derived from animals or human. Goal of the scaffold use is to restore mechanical function as well as to repair the ECM of the damaged tissue and to induce an integrative tendon repair. Synthetic scaffolds are composed of macromolecules that do not have a well-defined structure and not bind well into the injured tendon. Biological scaffolds are more bioactive and have shown favorable results during in vitro culture trials, as well as in relevant in vivo models (Lomas et al. 2015). Major concern about biological and synthetic scaffolds is the biocompatibility, the cell cell interaction and cell matrix interaction was well as the inflammatory response associated with foreign body rejection.

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Chapter 12
Regenerative Therapies

Christina Irene Günter, Augustinus Bader, and Hans-Günther Machens

Abstract Skin is by far the largest organ in the human body. Its surface ranges between 1.5 and 1.8 m^2 and the thickness varies between 0.5 (lower eyelid) and 15 mm (foot sole) in a young average adult, resulting in a tissue volume of 7500–27,000 mm^3. The wide range of tissue thickness already indicates that skin has to fulfill a variety of physiological organic tasks, including mechanistic, metabolic, energetic and immunologic aspects. Skin also is the first organ which has been tissue engineered in vitro and translated back into clinical application. It is a prime target for regenerative therapies, not only due to its obvious easy clinical accessibility but also, because skin already is a continuously regenerating organ and therefore a fascinating model to learn more about the human body's intrinsic regenerative mechanisms.

This book chapter focuses on the regenerative capacities of skin tissue and its comprising cell compartments and explains how the principles of skin regeneration may be translated into clinical practice.

Keywords Skin • Wound healing • Erythropoietin • Regenerative medicine

Abbreviations

BAD	Bcl-2-antagonist of cell death
Bcl-xL	Transmembrane molecule involved in signal transduction pathways of several anti apoptotic proteins (B-cell lymphoma-extra large)
Ca	Calcium
EPO	Erythropoietin

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EPOR2	EPO Receptor 2
EPOβ 1/2	EPO Receptor β 1 or 2
GSK-3β	Glykogen Synthase Kinase 3
IL-2	Interleukine 2
IL-6	Interleukine 6
IL-8	Interleukine 8
JAK-1/2	Janus Kinase 1 or 2 (protein tyrosine kinase)
MAPK	Mitogen-activated protein kinases
NFκB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NO	Nitric oxide
PDGF	Platelet-derived growth factor
PI3K	Phosphoinositide 3-kinase
PI3K-Akt	Signalling pathway regulates several cellular functions including cellular proliferation, growth, survival and mobility
PKB	Proteinkinase B
PLC	Phospholipase C
STAT	Signal Transducer and Activators of transcription
TEN	Toxic epidermal necrolysis
TGFβ 1-3	Transforming growth factor beta 1–3
TNF-α	Tumour Necrosis Factor α

12.1 Introduction

Skin provides a natural barrier between the human body and its environment. Therefore, it is the primary target for extrinsic noxes of any kind, but also intrinsic factors may induce damage to the skin (e.g. auto immunologic noxes). Furthermore, skin is in a continuous process of regeneration due to permanent loss of tissue and cell fractions within its surface. It is the only organ which privileges even the medically untrained to give an accurate diagnosis about its morphological and functional status (e.g. aging). But what is the cellular and molecular motor behind the fascinating regenerative capacities of skin tissue? The following book chapters will give insight into the developmental and structural principles of skin, its repair and regeneration tools after damage and the resulting therapeutic modalities, which may result from a better understanding of skin regeneration.

12.2 Skin Development and Stem Cell Function

Skin tissue can be structurally divided into two major components: dermis and epidermis. The lower dermal part is the functionally important tissue and is mainly comprised of matrix collagen type I, elastin, fibroblasts and skin appendices (capillary, sweat and sebaceous glands, sensory corpuscles, blood vessels). The upper

epidermis consists mainly of keratinocytes in different developmental stages and represents the most superficial layer of skin, a squamous epithelium with several strata: the stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum, and stratum basale (Freedberg et al. 2003) Cellular nutrition is provided to these layers via diffusion from the dermis, since the epidermis has no direct blood supply. The epidermis contains four cell types: keratinocytes, melanocytes, Langerhans cells, and the Merkel cell. Of these, keratinocytes constitute about 95 % of the epidermis (Burns et al. 2006). This stratified squamous epithelium is maintained by constant cell division within the stratum basale, in which differentiating cells slowly migrate outwards through the stratum spinosum to the stratum corneum, where cornified cells are continually shed from the surface. In normal skin, the rate of production equals the rate of loss; it takes an average of 2 weeks for a cell to migrate from the basal cell layer to the top of the granular cell layer, and an additional 2 weeks to cross the stratum corneum (Bolognia et al. 2007).

The dermis is the layer of skin between the epidermis and subcutaneous tissue, and comprises two sections, the papillary and reticular dermis. The superficial papillary dermis interdigitates with the overlying rete ridges of the epidermis, between which the two layers interact through the basement membrane zone (Rapini 2005). Structural components of the dermis are collagen, elastic fibers, and extrafibrillar matrix. Within these components are the pilosebaceous units, arrector pili muscles, and the eccrine and apocrine glands. The dermis contains two vascular networks that run parallel to the skin surface—one superficial and one deep plexus—which are connected by vertical communicating vessels (Freedberg et al. 2003). The function of blood vessels within the dermis is twofold: to supply nutrition and to regulate temperature. These blood vessels are also crucial for regaining rapid reconnection with underlying blood vessels after split skin transplantation on wounds by a process called 'inosculation' within 24 h after transplantation (Converse et al. 1975) (Fig. 12.1 and Table 12.1).

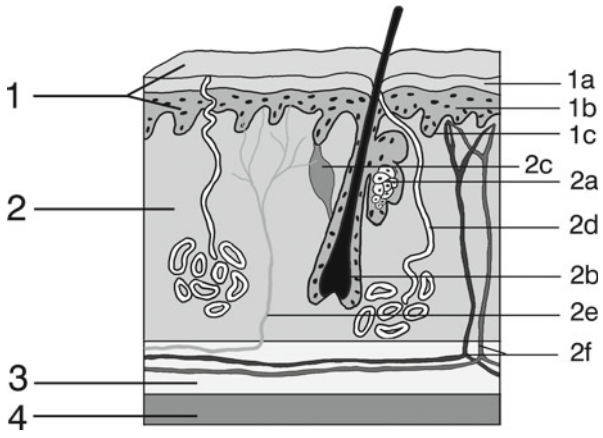

Fig. 12.1 Anatomical structure of the human skin. *1* Epidermis: *a* Stratum corneum, *b* Stratum germinativum, c Basement membrane. *2* Dermis: *2a* Sebaceous gland, *2b* Hair follicle, *2c* Arrector pili muscle, *2d* Sweat gland, *2e* Nerve, *2f* Capillaries and vascular plexus. *3* Subcutaneous fatty tissue

Table 12.1 Skin: anatomical structure and function

Layer	Structure	Function
Epidermis	Epithelial cells	Barrier, protect against injury, contamination and moisture loss
	Melanocytes	
	Langerhans cells	Protect against UV-light, origin for skin pigmentation and tanning
		Antigen presenting, immuncompetent cells
Dermis	Collagen	Strength and support
	Elastin	Elasticity
	Nerves	Sensors for: pain, temperature, touch, vibration
	Capillaries	Nutrition and oxygen supply, waste removal, thermoregulation
	Fibroblasts	Mesenchymal derived cells producing the extra cellular matrix
Epidermal appendages	Hair follicles	Produce epidermis and hair, epidermis regeneration
	Sweat glands	Produce sweat: thermoregulation
	Sebaceous glands	Produce sebum: antimicrobial, maintains: pH, skin and hair condition
Subcutaneous tissue	Fat cells	Isolation, energy storage
	Stem cells	Pluripotent cells in the fat tissue enabling regeneration
	Connective tissue	Attaches skin, divides tissue into compartments

Skin tissue covers the surface of the embryo at the earliest embryologic stages and has contributions from two germ layers: Ectoderm forms the surface epidermis and the associated glands, whereas mesoderm forms the underlying connective tissue of the dermis. Ectodermally derived neural crest cells also migrate into the forming epidermis to populate with melanocytes and specialized sensory endings. There are numerous detailed developmental overviews of all these specific structures (McGrath et al. 1971) (Fig. 12.2).

During early embryologic development the ectodermal sheath becomes intrinsically important since it provides environmental protection already in the early gestational weeks. The further progress into epidermal and dermal layers arises at a much later time, but the immanent stem cell population remains active within the later formation of the hair bulges. From here, pre-keratinocytes grow out and form the epidermal layers, losing subsequently its differentiating capacity while slowly migrating from the lower, more undifferentiated towards the outer, more differentiated cell layers and finally squamous epithelial.

It has been shown recently that both epidermally and dermally derived stem cells can differentiate into all three germ layers, indicating the intrinsic potential of these cell sources within the skin (Rolletschek and Wobus 2009). Meanwhile laboratory protocols are available for isolation and cultivation of human keratinocytes from skin or plucked hair for the generation of induced pluripotent stem cells (Aasen and Belmonte 2010). A further resource of stem cells is located within the dermal layer,

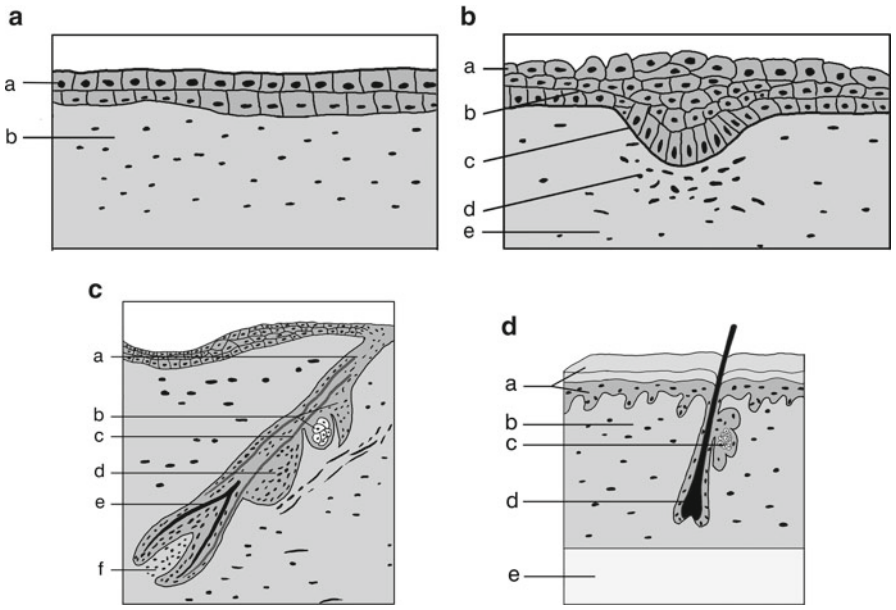

Fig. 12.2 Different embryological phases of skin development. (**a**) 4 weeks: *a* Surface ectoderm, *b* Mesenchyme. (**b**) 7 weeks: *a* Periderm, *b* Germinal layer, *c* Hair Germ, *d* Mesenchyme cells, *e* Mesenchyme. (**c**) 16 weeks: *a* Hair canal, *b* Sebaceous Gland, *c* Inner Root Sheath, *d* Bulge, *e* Hair, *f* Mesenchyme Cells. (**d**) Birth: *a* Epidermis, *b* Dermis, *c* Sebaceous Gland, *d* Hair Bulb, *e* Subcutaneous fatty tissue

where fibroblasts present the main population of resident cells (Fernandes et al. 2008). Pluripotent stem cells can be recruited from glandular cells including sweat glands (Petschnik et al. 2009). Therefore, it seems worthwhile to take a closer look into the relation between skin development and stem cell function (Fig. 12.3).

Stem cells play a continuous and dominant role in postnatal skin maintenance, since they provide permanent recruitment of important functional tissue in the dermal and epidermal compartment. Within the different stem cell compartments, epidermal stem cells may play an exceptional role: since the epidermis continually renews itself by sloughing a layer of cells every day, it is in a constant state of cellular turnover and requires continual cell replacement for life. Thus, maintaining a vital epidermal stem cell population is of prime importance, even during aging. Unlike stem cells from internal tissues, epidermal stem cells show little response to aging (Racila and Bickenbach 2009). They do not appear to decrease in number or functionality with age, and do not show changes in gene expression, developmental responsiveness, or age-associated increases of reactive oxygen species. While human skin grows older, the stem cells within loose in numbers and in their regenerative capacity. It is tempting to hypothesize that the process of aging is strongly related to stem cell reservoir and functional capacity.

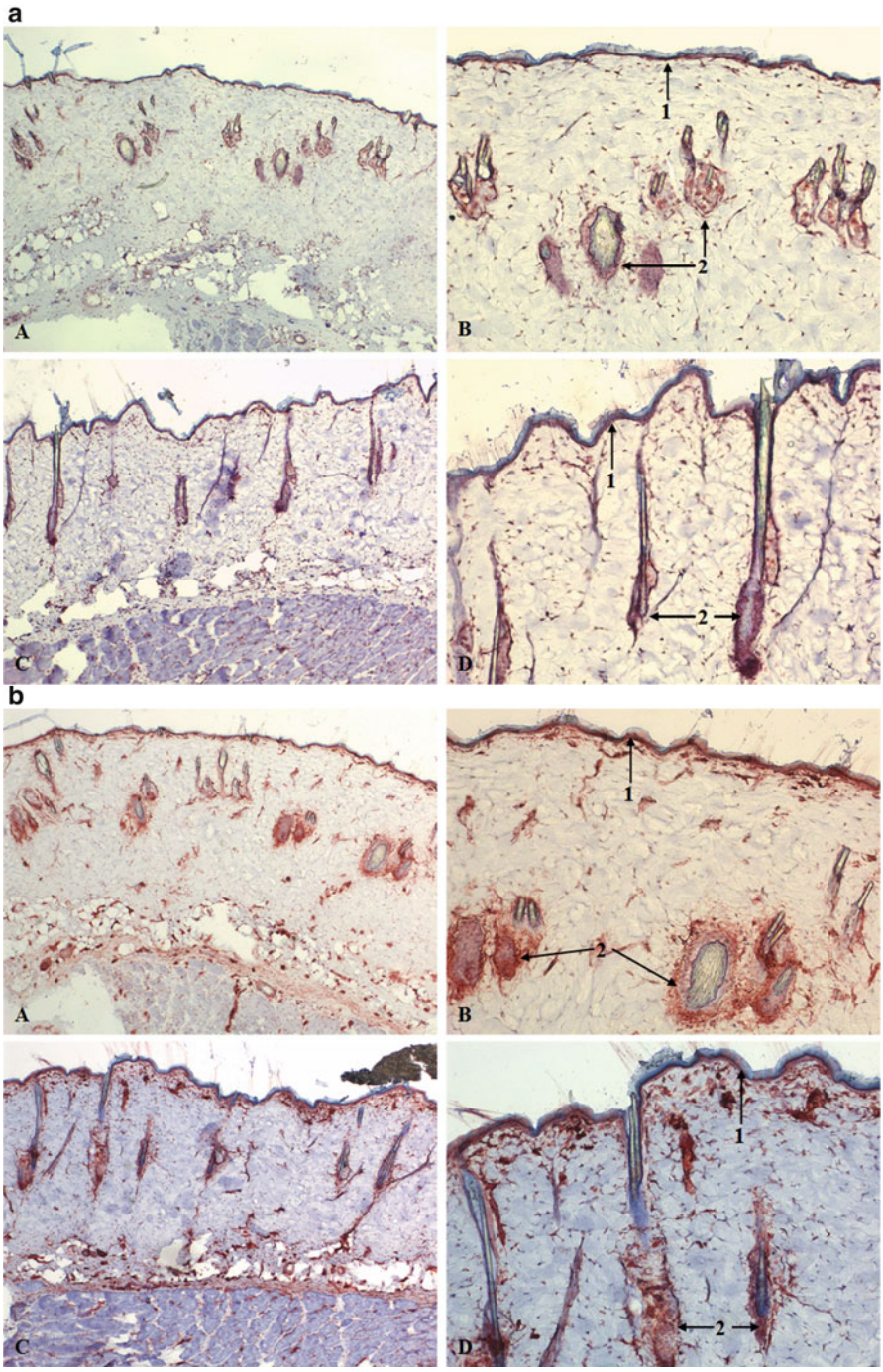

Fig. 12.3 Stem cells in the adult skin. (**a**) Immunohistological images of rat skin tissue sections, stained with monoclonal antibody against nestin for identification of neuronal stem cells, *A*/*C* magnification × 40, *B*/*D* magnification × 100; *1* basement membrane (basal lamina); *2* hair follicle. (**b**): Immunhistological images of rat skin tissue sections, stained with antibody against CD 90 for identification of mesenchymal stem cells and fibroblasts, *A*/*C* magnification × 40, *B*/*D* magnification × 100; *1* basement membrane (basal lamina); *2* hair follicle

Table 12.2 Skin diseases, affected skin compartment and intrinsic regenerative tools

Disease	Affected compartment	Regenerative tool	Healing	Scars
Epidermiolysis bullosa	Epidermo-dermal junction	Dermal stem cells	Spontaneous in 14–21 days	No
Toxic epidermal necrolysis	Epidermo-dermal junction	Dermal stem cells	Spontaneous in 14–21 days	No
Burn Grade 1	Epidermal	Dermal stem cells of the stratum basale	Spontaneous in 5–10 days	No
Burn Grade *2 a*	Superficial dermal	Dermal stem cells in the stratum basale and the skin appendages	Spontaneous in 10–21 days	No
Burn Grade *2 b*	Deep dermal	Dermal stem cells in the skin appendages and remaining dermal components	Spontaneous in >21 days	Yes
Burn Grade 3	Sub dermal	None	No spontaneous healing	Yes
Pressure sore	Sub dermal	None	No spontaneous healing	Yes

12.3 Skin Diseases and Regenerative Principles

There is an abundant number of endo- and exogenous noxes, affecting the skin in its different cellular components and as a whole organ. Certainly, thermal injury is the most devastating traumatic cause for total loss of all skin components at a single incident, while auto immunologic agents mostly affect the epidermal part or the epidermo-dermal junction (e.g. epidermolysis bullosa, toxic epidermal necrolysis (TEN)).

As long as parts of skin tissue survive, regenerative tools are recruited to commence repair and regeneration. In general, it may be stated that the regenerative capacity of skin directly depends on three factors: the amount of surviving epidermo-dermal tissue, the regenerative capacity of local tissue in the trauma zone and ability of the organism to recruit new cells from other restorative compartments (e.g. bone marrow). In principle, loss of epidermis may be fully compensated and restitutio ad integrum achieved, as long as the dermis and its regenerative cellular departments are still intact and vital enough to reproduce the necessary tissue (Table 12.2).

12.4 Clinical Principles, Diagnostics, Indications of Skin Regenerative Therapies

Skin may regenerate even after total loss of the epidermo-dermal junction, depending on the regenerative capacity of the underlying tissue and the whole organism. It is sometimes clinically difficult to estimate the correct amount of surviving tissue,

especially after partial loss of dermal tissue. In such instances, younger patients develop more regenerative activity compared to elderly. Therefore, dermal hypertrophy can result on the one hand and total loss of remaining dermal tissue on the other. Clinical experience with thermally injured patients allows a more accurate estimation of wound depth (Fig. 12.4).

A typical clinical example for a non-thermal first degree wound surface is TEN (Fig. 12.5).

While the regeneration of epidermis is in process, the dermis and underlying tissue are prone to infection and injury. Therefore, it is mandatory to protect the integument both immunological and mechanically with a temporary epidermal skin substitute. Meanwhile, there are many industrial products available, which fulfill these requirements.

Partial loss of dermis may still be regenerated, as long as enough dermal tissue and especially the sub dermal vascular plexus are preserved. A typical example is given by a 2a thermal injury, resulting in preservation of the deep dermal tissue. Clinically, after removal of bullae and blisters, a reddish wound bed appears. Recapillarisation occurs after gentle pressing on the wound surface, indicating an intact subdermal and partially intradermal vascular plexus with vital dermal capillaries. In this clinical picture, dermal regeneration can occur and sufficient nutrient and cellular supply transported into the wound area. The major complication in superficial dermal lesions, however, is systemic infection, especially in the elderly. The regenerative capacity becomes insufficient, when too much of body surface is involved or other systemic conditions which jeopardize cellular repair and regeneration mechanisms exist (diabetics mellitus, autoimmune disease, chronic dialysis patients…).

12.4.1 Regenerative Repair Mechanisms (Trauma: Dependent Activation of ß1-EPO Receptors)

In recent years a further player in wound regeneration has been explored and described. It is a well known protagonist, which is used for the treatment of anaemia for more than two decades: Erythropoietin (EPO). The tissue-protective effects of EPO seem to be mediated by a special EPO receptor-sub-type, which is postulated to be different from the EPOR2 of the erythropoietic system (Brines et al. 2004). In nearly all tissues which have been examined so far its expression is triggered by trauma, injury and metabolic stress. EPO β 1/2 receptors are described as heteroreceptors containing an α and a β chain, whereby the β chain is phosphorylised by EPO (Hanazono et al. 1995).

The signalling via this receptor initiates multiple, coordinated functions that counteract the collateral damage caused by the stereotype injury response which occurs after trauma. The organism reacts in this stereotypic manner to primarily prevent the invasion of pathogens and generalised infection; it does not differentiate between sterile and infectious pathogens, or between external trauma and internal stress reactions (Lotze et al. 2007).

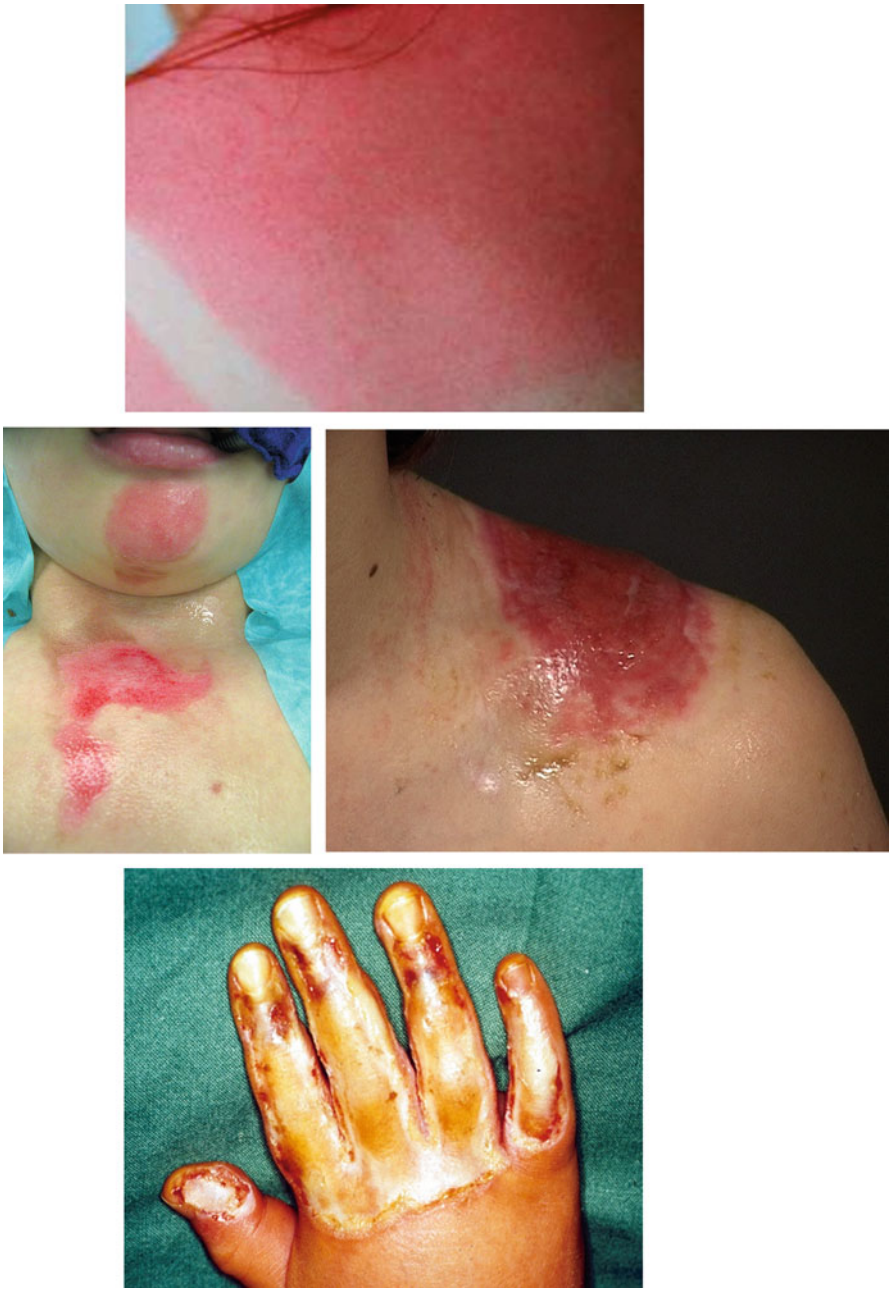

Fig. 12.4 Four stages of thermal injury to the skin. *1*. Degree burn (Spontaneous healing in 5–10 days, no scar formation): *2a* Degree burn, superficial (Spontaneous healing in 10–21 days, no scar formation). *2b* Degree burn, 2 weeks old, without adequate therapy, therefore granulation tissue occured (Spontaneous in >21 days, scar formation). *3*. Degree burn (No spontaneous healing, scar formation)

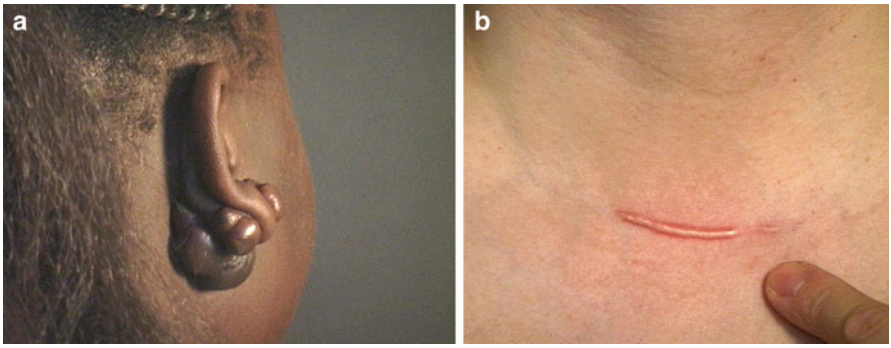

Fig. 12.5 (**a**) Keloid, (**b**) Hyperthropic scar

Initially, the reaction is mediated via members of the pro-inflammatory Type I cytokine family such as TNF-α (Takeuchi et al. 2007). In turn multiple players like 'free radicals' and other highly reactive molecules are released. Herewith, pathogens which potentially have penetrated the organism are destroyed, whereby healthy cells are destroyed as well (Brines et al. 2008).

The inhibition of the initial reaction is of utmost importance for a normal healing process. EPO has this key-role of the inhibitory mediator, although EPO and TNF-α are antagonists. Both inhibit their mutual production and biological activity (Bernaudin et al. 1999) additionally EPO receptor expression is enhanced by TNF-α, creating a balance between the initial injury response and the inhibitory EPO system.

Additionally, EPO recruits stem cells and thus ensures that they are present in the area of regeneration, it also triggers the release of specific growth factors (Viviani et al. 2005).

EPO acts protectively on capillary endothelial cells which, under hypoxic stress would become apoptotic, thus providing improved blood supply to surrounding areas (Peterson et al. 2007).

In addition, an inhibitory influence is impacted upon specialised cells for the prevention of infection, such as leukocytes and macrophages: thus the production of IL-2, IL-6, IL8, γ-Interferon and TNF-α is inhibited (Schultz et al. 2008; Yazihan et al. 2008).

Different cell protective cascades exist, which are active on an intracellular level, they are triggered via the phosphorylation of JAK-1/2. Subsequently, STAT, Bcl-xL, PI3K-Akt and MAPK pathways are activated. STAT is activated via phosphorylation as well, and translocates into the nucleus where it induces the synthesis of Bcl-xL, a central apoptosis inhibitor (Wen et al. 2002). PI3 is being phosphorylated via JAK-2, which in turn activates Akt-B, an important factor for cell survival (Siren et al. 2001).

Today it could be shown that EPO and EPO receptors are produced in the skin, where the typical cell protective effects could be demonstrated. The same has been examined for hair follicles; here a protective effect against chemotherapy-induced apoptosis of hair follicle cells could be revealed (Bodó et al. 2007).

Unfortunately all clinical trials (known to the authors) investigating rhEPO as a pleiothrop, pro-regenerative treatment after traumata, like burn wounds, myocardial infarction or stroke did miss the primary endpoints or reveal inconclusive results. There exists an enormous discrepancy between the very promising results of a large number of pre-clinical investigations and the so far generated clinical experience. So far no scientific proof arguments could be found but one very likely explanation is the difference between the young and healthy, very standardized animal models and the multimorbid, elderly patients treated in most of the clinical trials.

12.4.2 Hypoxia Preconditioned Plasma

Autologous hypoxia preconditioned plasma is an innovative approach mimicking the bodies own regenerative mechanisms. As many clinical trials examining only a single growth factor have failed to prove its efficacy in the clinical setting, this approach uses the bodies own regenerative cocktail. Therefore the patient's blood is preconditioned ex-vivo under hypoxic conditions an applied back onto for example a chronic wound. Here it triggers a pro-regenerative and a pro-angiogenetic stimulus, which enables the chronic wound to heal. Pre-clinical studies have shown excellent results. A proof of concept clinical trial is to be started soon (Hadjipanayi and Silling 2013, 2014).

12.4.3 Cultured Keratinocytes

An other initially very promising product has been cultured keratinocytes. But cultured keratinocytes need, if transplanted onto a wound, to be able to develop an intact epidermis, an existing dermis, which ensures sufficient nutrient support and growth factor supply and other interactions such as formation of a stable epidermo-dermal junction. Otherwise these keratinocytes are prone to apoptosis or at least they do not continue to grow and do not form an epidermal layer. Thus it is not surprising that early attempts transplanting keratinocytes onto full thickness skin defects without a neo-dermis or dermis remnants allowed primary survival of severely burned patients but showed unsatisfying results after longer observation periods due to unstable wounds and secondary infection.

12.4.4 Split Skin Grafts

Therefore until today the gold standard to cover large skin defects are split skin grafts. Split skin grafts contain dermal and epidermal parts, thus they can be transplanted directly onto a full skin defect. But if the destroyed area of skin comprises more than 60 % body surface area there are not sufficient amounts of split-skin grafts to be taken, to cover the defect in one operation. Therefore, a variety of dermal substitutes or combined dermal and epidermal substitutes have been developed. They can be permanent or temporary, biological or synthetic or combined (Table 12.3).

Xeno- and heterologic-split-skin-transplants and Epigard are temporary substitutes, which are subject to phagocytosis or have to be surgically removed. Therefore a second-step operation is necessary for permanent closure of the wound. Alloderm™, Biobrane™ and Integra™ need a second-step operation as well and a thin split-skin-graft transplantation as epidermal layer or a keratinocyte transplantation. However, previous to that a neo-dermis has to develop by invasion of the collagen matrix via fibroblasts and angiogenesis must have occurred. Suprathel® is a temporary wound dressing for superficial dermal lesions. After application it gets transparent, so the wound controls are still possible. After complete re-epithelialization the rests of Suprathel® dressings just fall off. So no further interventions are needed. In addition wound dressings with Suprathel® display statistically significant less wound infections, faster re-epithelialization and a merely complete pain reduction during wound healing and dressing changes (Rapp).

Some indications require full thickness skin grafts. Especially if areas which do not tolerate the high rate of contracture of split skin grafts are involved (face, hands, joints). Here foll thickness skin grafts might be a solution. Here it is important to remember, that the full thickness grafts requires a much better vasculatization and perfusion than a split skin graft.

Table 12.3 Gives an overview of some of the commonly used skin substitutes

Biological substitutes	Synthetic substitutes/combined substitutes
Xeno-split-skin-transplants (porcine) temporary	Biobrane® (nylon, silicone, bovine collagene) permanent/temporary
Heterologic-split-skin-transplants (human) temporary	Integra® (shark hyaluronic acid, bovine collagen, silicone) permanent/temporary
Alloderm® (a-cellular, de-epidermalised human skin, with collagen and elastin) permanent	Epigard® (gore-tex, polyurethane) temporary
Autologus split skin grafts (autologous human) permanent	Suprathel® (Polylactate) temporary
Amnion (heterologe or xenologe) temporary	Veloderm (hemicellulose) temporary
Apligraf® (bovine collagen, heterologe fibroblasts and keratinocytes) temporary/ permanent	–
Dermagraf® (collagen-glycosamninoglycan matrix, heterologe fibroblasts) temporary/ permanent	–

12.4.5 Hypertrophic and Keloid Scars

Scars develop, if the dermis is completely divided and the cellular connections in the regenerative layers are interrupted. A typical clinical example is given after a clean cut with a sharp instrument, also after meticulous surgical repair. Interestingly, the tendency to form scar tissue is different in the human population and codepending on age, sex and race. Hypertrophic scarring and keloid formation are found more often in children and young adults than in the elderly. Persons who developed keloids in their childhood may not exhibit this tendency at an older age. Keloids and hypertrophic scars are more often found in more intensely pigmented skin than in less or no pigmented skin; this can be seen within one person as well as in different individuals. The incidence of keloid formation is 6–16 % higher in darker pigmented populations as compared to Caucasians. Gender seems to be a risk factor too, as the female to male ratio is 2:1. Keloids can occur in every region of the body; they are most commonly located on the upper trunk (chest, upper back and shoulders). The etiology remains unclear. Hormone (estrogen) and growth factor influences (melanocyte stimulating hormone, MSH) may play a role, as well as different immunological influences and genetic predisposition. The diagnosis is mainly clinical: hypertrophic scars remain on the borders of the initial injury, whereas keloids grow over these boundaries like a pseudo tumor (Rockwell et al. 1989). Histologically, hypertrophic scars and keloids show both stretched and aligned collagen bundles, but fewer cells and capillaries are found in keloids. Today we know that although the macroscopic result of this scarring disorder cannot be seen for weeks or month, it is the result of very early dysregulation during wound healing (Meenakshi et al. 2005). Satisfactory, predictable and reliable therapies do not exist so far. Future therapies will have to be based on our improved understanding of regenerative mechanisms in the injured skin and will therefore lead to more specific therapeutic interventions.

Meanwhile two very effective treatment options, which might be combined for even more effective results, have entered the clinical routine: medical needeling and autologous fat injection.

Medical needeling, also called percutanious collagen induction is a minimal invasive method providing excellent results even in large hyperthropic post burn scars. A sufficient narcosis is necessary but in many cases a local or even topical application of an anesthetic is sufficient.

The basic mechanism is to apply a new trauma into the scar region, which is on the one hand so small that no new scar occurs on the other hand is so invasive that the regenerative cascade is started anew. As a result the scar tissue remodels and displays more “normal skin” attributes. If necessary the treatments can be repeated after a time period of 3 month, until the desired effects are achieved (Aust et al. 2008).

Autologous fat injections: are more invasive, because it contains of two steps: harvest of autolougus fat and injection of the prepared autologous fat into the lesion. Fat harvest might be done with any of the published methods, so far no scientific

sound data exist preferring any of the existing methods. Fat preparation might be done again with any of the formerly published methods. Fat injection is performed must successfully with Cooleman-Needels, the application should be directly within the sub-dermal layers of the skin. Here we have a mixture of effects. Fist we have analogue to the medical needeling method a new (micro) trauma, with consecutive remodeling. In addition we inject a highly potential mixture of stem cells and multiple growth factors, which take pro-regenerative action in the new location. In addition the injected, surviving cells (fat and stem cells and fibroblasts) start to grow there and build a neo dermis/sub-dermal tissue, thus a formerly adherent scar gets more mobile and less attached (Bollero et al. 2014).

12.4.6 Scar-Free Healing in the Embryo

A very interesting aspect is the scar-free healing of mammalian embryos. Several studies have been carried out to investigate adult and embryonic wound healing and scarring. In the meantime, most of the involved factors in adult and in embryonic skin regeneration are known. One important factor is the fact that in embryos the immune system and the inflammatory cascade are not sufficiently developed. Thus, the resulting inflammatory reaction in an embryo is much smaller and of a shorter time period than in more advanced developmental stages and adults. Additional key roles are played by TGFβ 1-3 and PDGF. If PDGF and TGFβ1 and 2 are neutralized and TGFβ3 is added to adult wounds, embryonic scar-free healing can be achieved. This was demonstrated in rodents, pigs and healthy human volunteers. Following these promising results new scarring-improving drugs are being developed and clinical trials are carried out (Ferguson et al. 2004). It could be shown that locally administered TGFbeta3 is well tolerated and improves skin regeneration and thus reduces scarring after trauma (Occleston et al. 2008). Unfortunately a multy-national, multy-centre, double-blind clinical phase III trial testing two different dosing regimes against placebo was interrupted after 350 patients had been enrolled and neither the primary nor the secondary study end-points could be meet (Renovo 2011).

12.5 Standardized Treatment and Technologies

12.5.1 Loss of Epidermis (First Degree)

The classical example for a first degree wound is a sun-burn. Usually, as the dermis is intact this wound heals completely without scar formation within 4–8 days. It is moderately painful and generally there is usually no need for any analgetic therapy. There are abundant therapeutically options, most available products have a cooling and local analgetic effect and some just keep the wound moist.

12.5.2 Loss of Superficial Dermis (2a Degree)

Superficial second degree thermal dermal injuries usually result in blister formation. To prevent infections the blisters should be punctured carefully; as exposure of the open blister to air is extremely painful. Therefore, blister removal should be considered carefully and only performed if necessary. Additionally an adequate analgetic therapy or especially in children a short narcosis should be taken into account.

Basically there are two possible treatment options: the occlusive (removing the blisters and starting an occlusive local therapy) or the exposure (laving the blisters intact as long as possible as a natural wound dressing) method. In Europe and Northern America the occlusive method is more commonly used. In southern regions the exposure method is seen more frequently, because it is cheap and as long as the scab is intact, the wound heals pain-free and very often without scar formation.

Concerning the occlusion method there exist a multitude of treatment options (silver nitrate, Mafenid, Vinegar, iodine, silver sulfadiazine, etc.). Their most important characteristics are the microbiological control and the preservation of a moist wound environment to enable undisturbed wound healing.

A broadly used product is Flammacine® (silver sulfadiazine), which is simple to handle and has a favourable cost-effectiveness ratio. Disadvantageous are the dressing changes which have to be performed at least daily and which might be painful.

Our favourite method is to cover the wound surface with Suprathel® under strictly sterile conditions after careful cleaning and debridement of the wound. Suprathel® stays in place until complete wound healing. This has the advantage that no deep dressing changes are necessary, although frequent wound controls have to be performed (Rapp).

12.5.3 Loss of Deep Dermis (2b Degree)

After a deep second degree dermal thermal injury the necrotic superficial layers have to be removed surgically. Today's standard therapy is tangential necrectomy until sufficiently perfused layers are reached. This is easily recognised by little spot bleedings in the healthy dermis. After bleeding control, keratinocytes (as solution or as sheets) can be transplanted if enough dermal tissue is preserved or split skin grafts are used if deeper layers of dermis are involved. If after extensive thermal trauma the remaining non-damaged body surface does not allow for sufficient amounts of split skin grafts to be taken, temporary skin substitutes, such as heterologic or xeno split-skin grafts, amnion or Epigard can be used for a short period of time to prevent both infection and also hypertrophic granulation and later scar tissue formation.

In deep second degree injuries, the regeneration capacity of the skin will be exhausted in most cases. Without healthy split-skin transplantation, the remaining dermal tissue will create a protective layer of scar tissue as the most primitive, yet

effective way to protect the wound from external noxes. It may be stated that in younger patients the response mechanism of mature and stem cells in this tissue compartment is stronger compared to adults and elderly. Therefore, the production of scar tissue is also more rapid and pronounced. The therapeutic consequence in young patients is radical excision of the epidermo-dermal compartment down to the lowest dermal layers and split skin transplantation to prevent overgrafting on scar – developing tissue.

12.5.4 Loss of Full Skin (Third Degree)

When an acute full-thickness skin defect has occurred (for example third degree burn) the wound has to be carefully cleaned and all remnants of necrotic skin have to be removed cautiously. This means that the underlying tissue is also subjected to infection and trauma, since the biological barrier is lost.

If a clean wound bed is established, split skin grafts can be transplanted, or, after pre-treatment with a dermis substitute and neo-dermis formation, keratinocytes may be transplanted.

If a chronic full thickness skin defect exists (for example pressure sores, crural ulcer, diabetic foot) the therapeutic strategy has to be different. That is due to the fact, that in chronic wounds an “anti-healing environment” prevails, with a majority of inhibitory factors which prevent healing. Additionally, the wound area is colonized with a multitude of microorganisms, which have to be at least grossly, most often surgically, eradicated before a definitive wound closure can be performed. If the wound is thus cleaned and necrotic tissue remnants have been removed, first and foremost the environment has to be changed from an anti-proliferate to a pro- proliferate environment.

Therefore, chronic hypoxic conditions have to be changed into acute hypoxic conditions to enable angiogenesis anew and consecutively enable new formation of granulation tissue..

An innovative and very promising new concept is hypoxia preconditioned autologous plasma.

Therefore the patients own blood is preconditioned ex-vivo under hypoxic conditions and than transferred back into the chronic wound. The preconditioned plasma provides now a highly effective growth factor mix, which enables the wound to enter into a pro-regenerative status again (Hadjipanayi and Silling 2013, 2014).

Then granulation tissue formation can successfully take place or a neo-dermis can be grown using a dermis substitute. Split skin grafts can then be transplanted on these prepared new wound environments if necessary.

If a pro-proliferative environment cannot be created due to advanced loss of vital and vascularised tissue, plastic surgical techniques have to be employed by using local or free tissue transfers to substitute the previous tissue loss in an adequate manner (Table 12.4).

Table 12.4 All four stages of skin loss

Grade	Anatomical layer	Appearance	Pain	Under-laying tissue	Needle test	Recapillarisation	Healing	Scars
1	Epidermal	Red	Yes	Normal	Positive	Yes	5–10 days	No
2 a	Superficial dermal	Red ground bullae/ blisters	Yes	Oedema	Positive	Yes	2 weeks	No
2 b	Deep dermal	Big bullae and blisters	Moderate or none	Pronounced	Delayed or negative	Delayed or none	>3 weeks	Yes
		Ground white or red		Oedema				
3	Sub dermal	White or red	None	Dried	Negative	None	No-spontaneous healing	Yes
				Out				
				Coriaceous				

12.6 Clinical Studies and Outcome of Skin Regenerative Therapies

There are abundant clinical studies on cosmetic skin alterations and its therapeutical options. Only in recent years the regenerative trigger for such diseases has come into focus. Dermatological disorders of the skin have also been studied extensively in clinical prospective trials. Very few data, however, are available on live-threatening skin wounds. Mostly, trials on testing wound dressings after split-skin transplantation have been performed with only poor focus on skin regeneration. A major reason for the lack of evidence-based data in this field is probably the fact that each traumatic, also each thermally induced wound has its own special pattern and therefore cannot be readily standardized for clinical trials. Full skin defects have been treated with dermal substitutes within the last 30 years. Several products have reached routine clinical practice.

However, there is a classic exception from this statement: the surgically induced split-skin harvesting wound, which is created by a surgical instrument (dermatome), thus exactly defining depths and size of the surgically created wound. This, in fact, is the only standardized traumatic wound in clinical practice. More than 50 studies have been carried out to compare different strategies of locally applied therapeutics, especially dressings. None of these, however, has focused on the biological regenerative effects on a cellular level. Primary treatment target was always the time needed until complete reepithelialisation was achieved.

In a recent publication, a multi-layer tissue engineering approach to cover large full thickness defects was described. In this approach, keratinocyte and fibroblast primer cell cultures are established from autologous skin biopsies. Cells are grown on special hyaluronic acid matrices, with which they are transplanted in two-time, two-step operative procedures. If necessary, a thin split skin graft expanded to 1:6 can be added later on. That way, a completely autologous and biological fully active epidermal-dermal substitute is realized (Hollander 2004).

12.7 Conclusions and Future Perspectives on Skin Regenerative Therapies

It is obvious that regenerative therapies after skin loss have been executed especially with local topical approaches since a long time without focusing on the underling biological processes taking place. Only recently, with a better molecular biological understanding of stem-cell and protein- based principles, we are able to customize regenerative therapeutic strategies which respect such fundamental biological principles. Perhaps the triggering of hypoxia generates angiogenesis, may play a key role in future developments of new therapeutic therapeutics to trigger full skin regeneration after skin loss.

Full skin loss will still remain a therapeutic challenge for clinicians. Since total skin loss necessitates full skin transplantation or bioartificial generation of skin substitutes in such instances, three key problems need to be solved in the future to optimize skin tissue engineering and tissue regeneration: creating a stable epidermodermal junction between the two major compartments dermis and epidermis, implementing a vascular supply in the dermal layer and supporting the construct with its functional cells and appendices (e.g. melanocytes, sweat and sebaceous glands, hair bulges etc.).

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