Stem Cell Biology and Regenerative Medicine

David S. Allan
Dirk Strunk *Editors*

Regenerative Therapy Using Blood-Derived Stem Cells

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Preface

Blood has long been viewed as a conduit for therapy, stemming from the ancient days of phlebotomy to remove evil humors to the development of successful blood transfusions to replace missing blood components. The identification and characterization of hematopoietic stem cells by Drs. Till and McCulloch revolutionized the field and soon after, non-hematopoietic stem and progenitor cells were characterized from the blood and bone marrow. Some of these cell types and various blood-derived cell lineages are involved in the repair of various types of tissue damage that span the spectrum of medical disorders. The goal of this book is to provide an up-to-date review of the various types of blood-derived cells with regenerative capacity, identify opportunities for intervention by examining specific clinical applications, and recognize the regulatory environment that will encompass future therapies in regenerative medicine.

Through the contributors to this volume, we have succeeded in providing insight on numerous blood-derived cell types, including endothelial progenitors, mesenchymal stromal/stem cells, umbilical cord blood-derived undifferentiated somatic stem cells and others. Further, the concept of using umbilical cord blood is discussed throughout the book and several authors describe the current status of regenerative therapy for cardiac disease and neurological disorders. Technical and conceptual issues such as ex vivo expansion and the generation of induced pluripotent stem cells are covered and regulatory insight from various jurisdictions provides a degree of clinical relevance that may shape the immediate future of regenerative medicine.

We wish to thank the many contributors for their tremendous commitment and their precious time in preparing the insightful chapters that comprise this book. Some are long-time friends and contacts while others are new and welcome collaborators. All the contributors are dedicated to advancing our collective knowledge regarding the field of regenerative therapy. The cooperation and contributions from our colleagues and fellow authors has been inspirational. The guidance and support from the series editor, Dr. Kursad Turksen has been most valuable and the staff at Springer has been especially helpful in making this project a reality. In particular, we are indebted to the administrative assistance and invaluable editing performed by Monica Farrell and Stéphanie Rochette.

We hope this book will stimulate enquiring minds and future investigation in this exciting and evolving field of research. The community of dedicated researchers and health care providers will need to engage at all levels to continue the push towards viable treatments that improve the lives of patients around the globe.

Ottawa, Canada David S. Allan
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Chapter 1
Undertaking Regenerative Medicine Studies with Blood Stem Cells

Sowmya Viswanathan and Armand Keating

Abstract In this chapter, we provide a perspective on the advances achieved to date in regenerative medicine, identify some of the challenges confronting the field, and make specific recommendations aimed at hastening the translation of research to effective clinical practice. Regenerative medicine is well positioned to address many of the urgent unmet medical needs of the global community. The stakes are high, but success will come only from the collaboration and mindfulness of specialists from diverse fields and from the focused attention of funding agencies.

1.1 Cells, Secreted Factors, and Mechanisms of Repair

Stem cells have been used to treat a variety of malignant and nonmalignant hematological disorders since the first bone marrow transplantation in 1959 (Thomas et al. 1959). Interest in regenerative medicine, however, increased considerably after the identification of diverse populations of stem/progenitor cells from different tissues and was propelled further by promising results in animal models of injury and disease. Although numerous preclinical studies and early phase clinical trials have shown encouraging results, underlying mechanisms remain poorly understood. The discrepancy in efficacy among various cell sources, clinical trials, indications, and preclinical studies remains challenging and requires further investigation.

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The popular notion that cell therapy is synonymous with cell *replacement* therapy persists despite the lack of convincing evidence for the transdifferentiation of adult cells and limited evidence for prolonged donor cell engraftment or cell retention at sites of injury. In most cases, there is no correlation between improvements in functionality and cell dose, suggesting that beneficial effects may not arise solely from the local involvement of donor cells but may be due to other factors such as paracrine effects (Gnecchi et al. 2008). For example, conditioned medium from endothelial progenitor cells (EPCs) can ameliorate hind limb ischemia in rat models (Yang et al. 2010). Also, soluble factors from CD133+ bone marrow cells are neuroprotective in a murine model of brain ischemia (Bakondi et al. 2009). Thus, there is growing interest in the field to move from cell therapy to cell-free therapy using secretomes from stem/progenitor cells.

In some instances, cell replacement therapy is still needed. Embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) can generate tissue-specific, differentiated cells to replace absent or injured cells. For example, human iPSCs can generate fully functional human platelets (Takayama et al. 2010) that in the future could provide a much needed alternative to costly volunteer donor platelets that can lead to complications such as sepsis (Kruskall 1997). iPSCs may also be useful in treating monogenic diseases by replacing cells harboring disease-causing mutations by gene targeting and correction technology as demonstrated in a mouse model of sickle cell anemia (Hanna et al. 2007).

In many cases, the therapeutic cell of interest may not be a stem cell at all. This is particularly evident in our evolving understanding of the therapeutic role of mesenchymal stromal cells (MSCs) from cells that engraft and give rise to differentiated cells (i.e., stem cells) to cells that secrete anti-inflammatory, antiapoptotic, angiogenic, antifibrotic, and immunomodulatory factors (Singer and Caplan 2011). The therapeutic effects of MSCs can often be reproduced by MSC-conditioned medium (Oh et al. 2008; Ma et al. 2006; Ye et al. 2006), which contains proteins secreted in response to injury signals such as TNF-α–stimulated gene/protein 6 (TSG-6) (Milner et al. 2006; Milner and Day 2003) able to directly promote corneal (Oh et al. 2010) and myocardial (Milner et al. 2006; Milner and Day 2003) tissue repair.

Another issue to consider is the heterogeneity of cell populations used for preclinical and clinical investigations. Bone marrow cells which contain a mixture of hematopoietic stem/progenitor cells, EPCs, and MSCs are most often used in clinical applications as they are easy to obtain and isolate. However, the variable clinical outcomes obtained with these heterogeneous cells are difficult to interpret. This is underscored by the results of several clinical trials with bone marrow–derived cells for treating cardiac diseases including acute myocardial infarction (AMI) and chronic ischemic heart disease (Martin-Rendon et al. 2008; Kang et al. 2008; Donndorf et al. 2011). Systematic and meta-analyses of several clinical trials show slight to modest improvements in hemodynamic parameters including left ventricular ejection fraction (LVEF) (2.99% in Martin-Rendon et al. 2008; 2.88% in Kang et al. 2008, 5.90% in Donndorf et al. 2011) without concomitant changes in short-term clinical events such as arrhythmias, rehospitalization for heart failure, or performance status.

It is unclear whether or not these modest improvements are the result of a functional heterogeneity in the treatment cell population. Might some cell subpopulations give better outcomes? There are some hints that this might indeed be the case. When a more homogeneous population of culture-expanded MSCs were used to treat 34 AMI patients, LVEF improved significantly more (from 49% ± 9% to 67 11%) (Chen et al. 2004) compared with the use of heterogeneous population of mononuclear bone marrow cells to treat AMI patients (LVEF increased from 48.3% ± 9.2% to 53.8% ± 10.29% over the same period) (Schachinger et al. 2006).

If a certain purified cell population is indeed better than a heterogeneous mixture of cells, there is yet no indication which cell type might be best suited for a given indication. A systematic and comparative approach with different cell populations is needed to address this issue. There are two approaches that can be taken clinically: prospective randomized clinical trials to compare different cell populations likely to be effective for a given indication and/or the development of suitable database registries to identify variables and prognostic factors that affect outcome for a given indication. In the cardiac field, both meta-analyses and clinical investigations have focused on specific subpopulations (umbilical cord blood cells, EPCs [CD133 and CD34 positive cells]) although most trials are performed with heterogeneous mononuclear cells from the bone marrow (Scacciatella et al. 2010).

1.2 Tracking the Cells That Facilitate Repair

A key question yet to be addressed is whether certain cell types can be mobilized endogenously to act on target tissues and organs or whether isolation, ex vivo purification, manipulation, and reinfusion (in the case of autologous therapies) are required. We also need to know the fate of the cells at the injury site and whether they are donor-derived or endogenously mobilized. Cells can be tracked with radioisotope imaging techniques including positron emission tomography (PET) and single photon emission computed tomography (SPECT), magnetic resonance (MR) imaging techniques, optical imaging (OI) and fluorescence, and bioluminescent imaging (BLI). PET and SPECT imaging provide immediate clinical applicability as they can capitalize on the use of FDA-approved labels including the radioisotope ^{111}In (Meller et al. 2004; Brand et al. 2004) and ^{18}FDG (Meier et al. 2008). However, there are limitations: images tend to be of low resolution and high cost, expose patients to radiation, and the tracers decay quickly, within hours for ^{18}GDF and days for ^{111}In. Other tracers with longer half-lives are available but have not been exploited for donor cell tracking (Oude Munnink et al. 2009). Alternatively, cells labeled with iron oxide nanoparticles including FDA-approved ferumoxides (for liver imaging) and ferumoxytol (for iron deficiency) may be tracked by MR (Frank et al. 2003; Wu et al. 2007) to yield relatively high-resolution images with longer persistence of the signal (2–4 weeks). Cells can also be tracked with optical imaging if they are labeled with fluorescent dyes such as the FDA-approved indocyanine green (ICG) (Sutton et al. 2008).

While human cell tracking has been studied predominantly in immunodeficient animal models, biodistribution, homing, and tissue retention in clinical scenarios are largely unknown. For biologics and drugs, pharmacokinetics (PK) studies provide information such as the area-under-the-curve (AUC), biologic or drug bioavailability, and clearance from the bloodstream after a single dose. In the case of cellular "pharmacokinetics," very few such studies have been undertaken. For example, while MSCs have been used clinically to support hematopoietic engraftment in multiple studies (Koc et al. 2000; Le Blanc et al. 2007; Ball et al. 2007), only two studies looked at cell retention and distribution in patients (Le Blanc et al. 2007; Ball et al. 2007). Lazarus at et al. showed that only 2 of 19 adult patients had detectable MSCs in their bone marrow, 6 to 18 months postinfusion (Lazarus et al. 2005). This would suggest that MSC localization may not be critical, at least in this particular application. MSCs have similarly been shown to have a multi-organ clinical response in reducing graft-versus-host disease (Le Blanc et al. 2008; Kebriaei et al. 2009), but again, due to the lack of cellular tracking studies, it remains unclear whether or not localization to specific tissues or lymph nodes is needed for a clinical response.

In other cases, such as treating central nervous system (CNS) diseases, localization may be more relevant; we need to know if cells or soluble factors secreted by them can reach affected areas. A clinical trial in Israel is using iron oxide–labeled MSCs that are intrathecally or intravenously injected into multiple sclerosis patients to obtain just this kind of biodistribution information (NCT00781872 at clinicaltrials.gov). Dendritic cells were similarly labeled with iron oxide particles and ^{111}In and tracked after intranodal administration in eight stage III melanoma patients scheduled for lymph node dissection, providing information on biodistribution to the lymph nodes and interaction with T cells (de Vries et al. 2005). Overall, however, there are very few clinical studies with labeled cells; the limited published studies reflect a gap in our understanding of clinical trafficking, and in the absence of this knowledge, clinical protocols cannot be designed to optimize cell dosing and schedule.

1.3 Building on the Experience in Hematopoietic Stem Cell Therapy

An iterative approach is important to optimize and refine clinical protocols and requires concomitant preclinical studies with appropriate animal models. Selecting the best animal models for safety and efficacy testing of therapeutic cells is critical to the development of new therapies. It is difficult to strike a balance among small, immune compromised rodent models that are convenient but less physiologically relevant, large animal models which take significantly longer to develop and validate and may need immunosuppressive agents, and bedside to bench clinical studies. An excellent illustrative example of this iterative approach is the concomitant clinical investigations and preclinical studies performed in appropriate lethally irradiated mice and canine models to understand bone marrow transplantation. Early

studies with murine models showed that murine leukemia could be treated by sublethal irradiation and marrow grafting (Barnes et al. 1956). This concept was successfully translated only to human leukemic patients who received grafts from an identical twin donor (Thomas et al. 1959); 200 patients who received allogeneic grafts in the 1950s and 1960s did not survive (Bortin 1970), leading to pessimism about this approach. The important discovery of human leukocyte antigens (HLA) occurred around the same time (Dausset 1958; van Rood et al. 1958), but it was not until the field returned to preclinical studies using canine models and discovered that dogs that received marrow grafts from dog leukocyte antigen (DLA)-matched, but not mismatched littermates survived, did this translate into the now-accepted clinical practice of using marrow from HLA matched human siblings (Storb et al. 1968, 1970, 1971). In 1968, the first successful allogeneic transplantations were performed in children with immune deficiency diseases (Gatti et al. 1968; Bach et al. 1968). The field has grown exponentially since those early clinical and preclinical studies with over 400,000 annual bone marrow transplantations now routinely performed worldwide.

1.4 Promise for the Future

The future of regenerative medicine remains bright despite its many challenges, and is beyond what can be accomplished by any single discipline alone (Haseltine 2001). It requires a combination of technological approaches from many experts including stem cell biologists, molecular biologists, developmental biologists, material scientists, engineers, physicists, imaging experts, clinicians, veterinarians, pathologists, and others. Interactions among these specialists are needed as more complex solutions are required to meet the unmet medical needs of patients. For example, the development of 3-D tissue constructs may necessitate the use of cell bioreactors, scaffolds, cells, and microfluidic and electronic controls systems (Domansky et al. 2010). Increasingly, the development of these complex projects also requires multiple types of institutions including academic institutes, industrial partners, hospitals, and nongovernmental funding organizations (Daar and Greenwood 2007).

The future of regenerative medicine will also depend on how the funding model changes with time to meet the evolving need of the community. Funding for cell therapy research, especially translational research that focuses on bridging basic discoveries and novel clinically relevant therapeutics, remains a major bottleneck. This is true despite increased NIH funding in basic research from US$170.9 million in 2002 to US$340.8 million in 2010 for human nonembryonic stem cell research projects (NIH Stem Cell Research Funding 2011), which tend to focus on hypothesis-driven research projects rather than the more routine but critical, safety and validation studies. New initiatives, such as California Institute of Regenerative Medicine with US$3 billion funding over a 10-year period, are helping to overcome the bottleneck. The creation of new grants panel/study sections specifically focused on translational and clinical studies will further help to develop this exciting field.

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Chapter 2
Defining Endothelial Progenitor Cells

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Abstract Human cord blood-derived endothelial progenitor cells (EPC) have been defined as circulating cells that express a panel of cell surface markers similar to those known to be expressed by vascular endothelial cells, that home to sites of hypoxia/ischemia upon infusion into experimental animal models, and participate in blood vessel formation (as analyzed by in vitro and in vivo methods). Although no specific marker for an EPC has been identified, a group of markers has been consistently utilized as a surrogate marker for cells purported to display vascular regenerative capacity. Since both hematopoietic and vascular endothelial subsets display many of the same cell surface antigens and both participate in new blood vessel formation, recent analyses have stressed the need to reconsider the use of the term EPC. This chapter reviews our current approaches to identifying human EPC and provides a brief summary statement for a new definition of an EPC.

2.1 Introduction

The identification of circulating cells that displayed the potential to attach to matrix-coated tissue culture plates, lose expression of hematopoietic antigens and upregulate expression of antigens typically thought to be endothelial specific during in vitro differentiation, demonstrate in vitro colony-forming ability, and home to sites of ischemia/hypoxia upon injection into experimentally injured immunodeficient mice, served to herald the existence of human endothelial progenitor cells (EPC) (Asahara et al. 1997). These observations were paradigm shifting for the

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cardiovascular field which had come to understand that new blood vessel formation in the adult organism is restricted to angiogenesis: the formation of new blood vessels from existing vasculature (Risau and Flamme 1995). The existence of an EPC in circulating blood suggested that some aspects of vessel regeneration may rely upon vasculogenesis: the formation of blood vessels from angioblastic precursor cells that is most typical for the developing embryo (Sabin 1917). Since the original description of an EPC (Asahara et al. 1997), more than 9,500 original papers and reviews (PubMed as of March 1, 2011 using search term "endothelial progenitor cell") have been written about EPC identification, enumeration in healthy and ill subjects, roles in various developmental and disease-related processes, and potential use as a cell therapy for treatment of cardiovascular disorders. Many excellent reviews have been recently published on the topic of EPC (Diller et al. 2010; Dudek 2010; Fadini and Avogaro 2010; Kusuma and Gerecht 2010; Lenk et al. 2011; Luo et al. 2011; Melero-Martin and Dudley 2010; Psaltis et al. 2011; Sen et al. 2011; Yoder 2010). This chapter will focus on a brief overview of the methods to identify an EPC and summarize a call for a change in EPC definition.

2.2 Methods to Identify Human EPC

Despite the vast number of papers written on EPC biology, methods for identifying these cells have largely been restricted to 3 general paradigms. One method for EPC enumeration relies upon the ability of umbilical cord blood or adult peripheral blood mononuclear cell subsets to adhere to extracellular matrix-coated culture dishes (Hill et al. 2003; Ito et al. 1999). The assay requires that mononuclear cells are plated in specific culture medium containing certain endothelial growth factors. Following 5–7 days in culture, the adherent cells are termed EPC.

Two common molecules tested as EPC markers include oxidized acetylated low-density lipoprotein (Ac-LDL) and the plant lectin *Ulex europaeus* (Ulex). The Ac-LDL is recognized and bound by the cell surface scavenger receptor expressed by the putative EPC. While endothelial cells throughout the body are known to express this receptor and to bind Ac-LDL (Voyta et al. 1984), it is now understood that this receptor is also quite widely distributed among hematopoietic cell subsets, particularly of the myeloid lineage (Rohde et al. 2006). Thus, the use of this particular molecule does not discriminate an EPC from many other cells of the cord blood or peripheral blood that may have adhered to the culture dishes.

One cannot rely upon Ac-LDL ingestion to define an EPC since adhesion of circulating hematopoietic cells to the extracellular matrix is a well-recognized method for isolating certain cell subsets, particularly monocytes (Hassan et al. 1986). Is Ulex binding to the putative EPC a more specific endothelial marker than Ac-LDL uptake? Ulex is bound by many circulating blood cells and a variety of epithelial cells in addition to vascular endothelial cells (Graziano et al. 2001; Holthofer et al. 1982; Liu and Li 1996; Schwechheimer et al. 1984). Thus, neither Ac-LDL uptake nor Ulex binding is specific markers for an EPC.

An additional complicating matter to consider when examining the sensitivity and specificity of this assay is that cord blood and adult peripheral blood mononuclear cells are known to be contaminated with blood platelets during isolation (Prokopi et al. 2009). Platelets are known to become activated upon in vitro culture and to disintegrate with fragments of the platelet membrane becoming incorporated into the plasma membrane of any cells adherent to the culture dish. Thus, the adherent cells display a variety of membrane proteins that are platelet-derived and often not even transcribed by the adherent cells (Prokopi et al. 2009). In sum, the use of this adhesion-based method for isolating, expanding, and enumerating EPC is flawed as the most prominent cells cultured by this method are hematopoietic derivatives.

Nonetheless, some hematopoietic cells cultured under the above conditions may display the potential to promote angiogenesis even if they do not turn into endothelial cells. Of interest, hematopoietic stem cells, myeloid progenitor cells, and certain monocyte/macrophage subsets all play specific roles in different aspects of angiogenesis (Fadini and Avogaro 2010; Hirschi et al. 2008; Psaltis et al. 2011; Sen et al. 2011; Yoder 2010; Estes et al. 2010a, b).

The most common method for EPC enumeration is based upon the use of monoclonal antibodies to bind to certain cell surface antigens on the putative EPC and to determine the frequency of the population using flow cytometric protocols. An ongoing limitation to this approach is the lack of a unique identifying marker for a circulating EPC. Indeed, the ability to discriminate the EPC from a circulating endothelial cell (CEC) or a hematopoietic stem/progenitor cell (HSPC) is quite difficult and requires multiparameter cell sorting. Given the rare frequency of all these subsets in the circulating blood, additional challenges of this approach include the requirement of counting beads and high numbers of sampled cells to accurately detect these rare events. An analysis of the use of flow cytometric approaches for EPC detection has recently been reviewed (Mund and Case 2011).

The choice of cell surface antigens that might distinguish an EPC was originally proposed to include antigens that coidentified hematopoietic and endothelial lineage cells, based upon the known close emergence of these lineages during embryonic development (Asahara et al. 1997; Peichev et al. 2000). In the mouse embryo, a putative hemangioblast precursor that gives rise to the blood and endothelial cells that form the blood islands has been identified as an early event in mesoderm specification during gastrulation (Huber et al. 2004). A similar clonal precursor has been identified during the differentiation of mouse and human embryonic stem cells (ESC) into blood and endothelium (Choi et al. 1998; Kennedy et al. 2007; Zambidis et al. 2008). CD34 is a sialomucin expressed on the cell surface of HSCP and angioblasts in the developing human and mouse embryo (Tavian et al. 2010). CD133 is another molecule known to be present on human and mouse HSCP and on a variety of cancer stem cell populations (Hirschi et al. 2008). KDR (human vascular endothelial growth factor 2 receptor) is known to be required for the expansion and differentiation of both hematopoietic and endothelial lineages in murine embryos and differentiating ESC (Coultas et al. 2005). Thus, this panel of molecules became an early choice of markers that were

used to attempt to discriminate EPC from circulating HSCP and CEC (Peichev et al. 2000). Numerous other choices of cell surface antigens have been examined as markers of the EPC in murine cells and are reviewed elsewhere (Mund and Case 2010; Mund et al. 2009; Nolan et al. 2007).

Much of the evidence to support the use of CD34, CD133, and KDR as a human EPC marker was derived from studies examining murine embryonic development. As mentioned above, KDR (or Flk1 in the mouse system) is a cell surface receptor expressed on the hemangioblast and early blood and endothelial derivatives. Mesoderm cells emerging from the posterior primitive streak on embryonic day (E)7.0 in the mouse embryo express Flk1 during specification to the hemangioblast stage. Stimulation of the hemangioblast with vascular endothelial growth factor (VEGF) is an early and necessary step in commitment of the hemangioblast to the hematopoietic lineage (Choi et al. 1998). On E8.0, clusters of definitive hematopoietic progenitor cells emerge within the nascent vasculature and herald the onset of delivery of the blood cells into the beating heart at E8.25 (Ferkowicz et al. 2003). By E9.0, CD34 is widely expressed on vascular endothelial cells throughout the embryo and extraembryonic yolk sac and on all the definitive hematopoietic progenitor cells. At this stage, CD41 alone can be used to discriminate various blood cell subsets from endothelial cells (Ferkowicz and Yoder 2005). Both blood and endothelial populations also express Flk1, c-Kit (CD117), CD105, CD31, and Tie2 and neither population express Sca-1 or CD45 at this stage of development. Only later in development (>E10.5) do HSPC express Sca-1, CD45, c-Kit, CD105, and Tie2, but begin to downregulate CD41, Flk1, and CD31 expression or restrict these antigens to certain progenitor cell subsets (Dzierzak and Speck 2008; Mikkola et al. 2003; Mikkola and Orkin 2006).

Recent studies have identified several potential novel blood cell markers, including certain transcription factors or gene promoter/enhancer elements that serve to delineate specific cell lineage commitments during embryonic development (Bee et al. 2010; Swiers et al. 2010). However, this brief overview highlights the difficulty in discriminating a blood cell from an endothelial cell during murine development; in all cases, a variety of co-expressed molecules could have been used to identify both lineages, but at least one specific marker was required to clearly validate the executed divergence of hemangioblast progeny to the blood (CD41^{+}) or endothelial lineage (CD41^{-}) (Ferkowicz and Yoder 2005; Yoshimoto et al. 2011). Thus, the principle of cell discrimination may require not only finding a unique marker to highlight a difference (gain of expression of distinct marker) between cells, but finding additional proofs that one cell type does not share the cell marker or unique morphologic features or functions with another cell.

Given the lack of a unique human EPC identifier, how can one approach the use of cell surface markers to identify an EPC from a CEC or HSPC? We cannot compare and contrast the many EPC publications in detail, but will attempt to identify some common principles. The CEC can be identified with an assay that has gained international acceptance (Woywodt et al. 2006). This method relies upon the use of a monoclonal antibody to CD146 attached to an immunomagnetic particle and the retrieval of the circulating elements with multiple attached particles when exposed

to a magnetic field. Further staining of the enriched elements with a fluorescent lectin is subsequently performed, and the positive CEC are directly counted in a fluorescent microscope. Evidence has been presented that essentially all the CEC identified with this method are dead or dying mature endothelial cells, and in most healthy adult subjects, fewer than 10 CEC are counted per mL of peripheral blood (Woywodt et al. 2006). A more automated version of this general procedure results in CEC frequencies similar to the gold standard direct counting method (Rowand et al. 2007; Widemann et al. 2008).

Use of traditional flow cytometric approaches generally enumerate many fold more "events" when attempting to count CEC in the peripheral blood of human subjects with various diseases or disorders (Duda et al. 2007). In some cases, the flow cytometric methods proposed to identify CEC inadvertently identify platelets or cellular microparticles and exosomes (Dignat-George and Boulanger 2011; Strijbos et al. 2007). Thus, differences in counting CEC may result from the method chosen, but a gold standard method is available for comparison and standardization.

HSPC are isolated and infused clinically to rescue hematopoiesis in human subjects requiring a bone marrow transplant (Deeg and Sandmaier 2010; Tung et al. 2010). For clinical purposes, HSPC are mobilized into the circulation using administration of certain growth factors and/or CXCL12-binding antagonists, and $CD34^{+}$ cells are collected in specialized columns under GLP conditions (Mohty et al. 2011; Steinberg and Silva 2010). The isolated cells can then be infused intravenously into the conditioned host, and peripheral blood sampling of reconstituted blood cell lineages can be monitored.

For research purposes, more elaborate combinations of cell surface markers have permitted enrichment of HSPC from cord blood, mobilized peripheral blood, and bone marrow cells with the use of immunodeficient mouse models that serve as hosts in which the HSPC engraft and repopulate human hematopoietic lineages (McDermott et al. 2010; Notta et al. 2010). Quite specific and elaborate methods have evolved to quantitate marrow repopulation in the murine hosts by the donor HSPC (Doulatov et al. 2010). Of interest, human HSPC that engraft in immunodeficient mice are enriched in $CD34^{+}CD133^{+}KDR^{+}$ cells (Doulatov et al. 2010). Essentially, all of these cells also express CD45. Thus, the identification of EPC as $CD34^{+}CD133^{+}KDR^{+}$ cells (with or without CD45 expression) is problematic since this subpopulation would contain HSPC. Indeed, isolation of $CD34^{+}CD133^{+}KDR^{+}$ cells from mobilized human peripheral blood or cord blood enriches for HSPC and not cells that form endothelium in vitro or in vivo (Case et al. 2007; Timmermans et al. 2007, 2009). One recent protocol has devised a method for separating hematopoietic from endothelial cells based upon the use of polychromatic flow cytometric principles (Estes et al. 2010a, b). This kind of approach may permit clarification to the field by separating hematopoietic cells with proangiogenic properties from endothelial cells with in vivo vessel-forming ability and thus, allow restriction of the term EPC to the latter.

Finally, the third approach to isolate and enumerate EPC is to plate the cord blood or adult peripheral blood mononuclear cells in a colony-forming assay. In the

original report by Asahara et al. (Asahara et al. 1997), isolated human CD34+ cells formed distinct colonies of round cells with stellate-shaped cells emigrating from beneath the colony late in the first week of culture. Subsequent modifications of this assay led to the addition of initial plating steps to deplete the mononuclear cells of potentially contaminated adherent CEC and hematopoietic cells and then to identify the number of colonies that emerged from the nonadherent cells (Hill et al. 2003). The fact that these colonies emerge with 4–9 days of plating led to the term, early outgrowth EPC. A commercialized version of this assay has been called the colony-forming unit-Hill (CFU-Hill) assay and has been used to examine the correlation between the frequency of circulating CFU-Hill in a patient and the risk for developing an adverse cardiovascular outcome (Hill et al. 2003). A series of recent studies have determined that this method of identification of the EPC enriches more for HSPC cells (myeloid and lymphoid) than for cells that turn into endothelial cells (Medina et al. 2010a, b).

Plating of cord blood and adult peripheral blood mononuclear cells onto type 1 collagen-coated tissue culture plates leads to the outgrowth of endothelial cells from endothelial colony-forming cells (ECFC) when using specific tissue culture medium and added growth factors (Ingram et al. 2004). Since these colonies typically emerge later than the CFU-Hill (indeed the CFU-Hill disappear by day 7–9 of culture and do not become ECFC), the ECFC have also been referred to as late outgrowth EPC (Yoder et al. 2007). However, the fact that ECFC from cord blood can generate visible colonies within 5–6 days after plating and thus, the timing of each kind of colony may not predict the type of colony that emerges, has diminished the use of the early and late EPC nomenclature usage. ECFC in cord blood display higher clonal proliferative potential than ECFC derived from adult blood and are enriched 50–100-fold (Ingram et al. 2004; Reinisch et al. 2009). Cord blood-derived ECFC display higher telomerase activity and more replating potential than adult peripheral blood-derived ECFC. ECFC from either source display in vivo vasculogenesis upon suspension in an extracellular matrix and implantation into immunodeficient mice, though the cord blood-derived ECFC may form more vessels per area than the adult blood-derived ECFC (Ingram et al. 2004; Melero-Martin et al. 2007, 2008). In a variety of in vivo preclinical model systems, ECFC display both proangiogenic paracrine and in vivo vessel-forming potential that may herald great potential for these cells as a cell therapy for human subjects requiring vascular repair or regeneration (Medina et al. 2010a; Moubarik et al. 2011; Yoon et al. 2005). Perhaps not surprisingly, co-infusion of “early” and “late” EPC into a murine model of hindlimb ischemia resulted in synergistic improvement in the blood flow to the injured extremity when both cell types were administered (Yoon et al. 2005). Thus, clarification of the EPC field to recognize that both proangiogenic hematopoietic cells and in vivo vessel-forming ECFC may participate in vascular repair via different mechanisms may now permit identification of the use of the proper subset to rescue whichever one of these lineages is deficient in a given patient population and disease state.

2.3 Summary Statement with Call for New Definition of an EPC

What is the future for the field of EPC biology? While a vast amount of information has been derived from studies that measure circulating EPC concentrations in healthy or ill human subjects, translation of the positive results of human EPC infusion into animal model systems has not led to as promising results as may have been predicted. There are many variables that may have contributed to this disappointing translational impact (Gulati and Simari 2009). Many of the animal models of human disease are conducted as acute interventional studies to interrupt or disturb blood flow to an organ system in an otherwise healthy animal. Since the inherent proliferative and reparative potential of vascular endothelium is age dependent in many species, including rodents, performance of vascular injury in young versus old as well as healthy versus systemically ill animals may be an important variable that has been underappreciated (Liu et al. 2008). Lack of robust assays that can detect abnormalities in the proangiogenic functions of circulating hematopoietic cell subsets versus the circulating number and in vivo vessel-forming ability of ECFC also hampers the identification of which cell subsets are most abnormal in a given animal model system of human disease (Sieveking et al. 2008). The recent identification of resident ECFC in the pulmonary microvasculature of rats and mice has provided new tools to assess underlying vascular reparative properties that may be investigated in a particular animal model system of human disease to determine whether this intrinsic reparative system is impaired (Alvarez et al. 2008; Clark et al. 2008; Schniedermann et al. 2010). One would predict that infusion of human proangiogenic hematopoietic subsets is unlikely to enhance new vessel formation in an animal model system in which the resident ECFC population is impaired in vessel-forming ability. Thus, tools are emerging that may help to better define the animal models used to predict human disease.

Use of improved methods of rare event analysis and polychromatic flow cytometry to isolate cells via multiple cell surface antigens may also improve the distinction of which circulating cells are contributing to new blood vessel repair (Estes et al. 2010a, b). Defining the specific roles that each of these circulating blood cell subsets play in various types of vascular events in human subjects with disease and via the use of better defined animal model systems of human disease will also be required. Since many human subjects suffer from diseases strongly associated with aging, the metabolic syndrome and diabetes, and atherosclerosis, improved models of similarly affected animal models would be of greatest interest. Of course, an ongoing search for a unique antigen that identifies the rare circulating ECFC would greatly enhance research into this cell type that has lagged behind that of the more frequent and readily recovered proangiogenic hematopoietic cells.

The term EPC, as currently used, fails to reflect the complexity of cell lineages participating in vascular repair, and this lack of specificity may account for the largest portion of the confusion among scientists and clinicians as to the potential clinical benefit of these cells. If the term EPC is to be reserved for a progenitor cell for

the endothelial lineage, we would propose that there are fundamental properties that this cell should display: a circulating cell that gives rise to progeny displaying clonal proliferative potential and differentiation restricted to the endothelial lineage with cells displaying the ability to form lumenized capillary-like tubes in vitro, ability to form stable human blood vessels when implanted into tissues that become an integrated part of the host circulatory system, and potential to undergo remodeling to form the intima of arterial, venous, and capillary structures. Whether contribution to lymphatic endothelial repair or replacement is an additional aspect of an EPC definition remains to be determined, though evidence has been presented to support this contention (Schniedermann et al. 2010).

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Chapter 3
Blood-Derived ALDH^{hi} Cells in Tissue Repair

David M. Putman, Gillian I. Bell, and David A. Hess

Abstract Cell sorting based on high aldehyde dehydrogenase (ALDH) activity has emerged as a clinically applicable method to purify human bone marrow (BM) and umbilical cord blood (UCB) progenitors based on a conserved stem cell function. Although rare, ALDH^{hi} cells are highly enriched for progenitors of hematopoietic, endothelial, and mesenchymal stromal cell (MSC) lineages. Transplanted ALDH^{hi} progenitors are under investigation in clinical trials to enhance UCB engraftment in adults undergoing transplantation. Transplanted BM ALDH^{hi} cells also recruited to areas of tissue ischemia and augment endogenous revascularization and recovery after femoral artery ligation. Moreover, ex vivo expanded MSCs from ALDH-purified cells stimulated the neogenesis of small beta cell clusters in models of pancreatic injury. Understanding how angiogenic and regenerative programs are stimulated by ALDH^{hi} progenitor subsets may provide new approaches in progenitor cell therapy for tissue repair.

Abbreviations

ALDH	aldehyde dehydrogenase
BM	bone marrow
EC	endothelial cell
EPC	endothelial progenitor cell
FACS	fluorescent-activated cell sorting

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HSC	hematopoietic stem cell
MNC	mononuclear cells
MSC	multipotent stromal cell or mesenchymal stem cell
NOD/SCID	nonobese diabetic/severe combined immunodeficient
SRC	NOD/SCID-repopulating function
UCB	umbilical cord blood

3.1 Introduction

Adult or postnatal progenitor cells obtained from human bone marrow (BM) or umbilical cord blood (UCB) are comprised of a heterogeneous array of regenerative cell subtypes and represent transplantable cells used to reconstitute hematopoiesis or to facilitate the repair of damaged or diseased tissues (Deans and Moseley 2000). However, with the exception of hematopoietic stem cells (HSC) expressing CD34, prospective purification of infrequent BM- or UCB-derived stem and progenitor cells for use in targeted regenerative therapies is currently inefficient. Indeed, non-hematopoietic stem cell subtypes demonstrate a paucity of specific markers of differentiation *in situ* (Cai et al. 2004), and stem cell surface markers can also vary between species, source, and cell cycle progression (Hess et al. 2004). In order to simultaneously isolate stem and progenitor cells from multiple lineages, BM or UCB mononuclear cells (MNC) can be purified based on a conserved stem cell function, aldehyde dehydrogenase (ALDH) activity, an intracellular enzyme first reported to be highly expressed in primitive hematopoietic (Storms et al. 1999), and neural progenitors (Corti et al. 2006a, b).

ALDH1A1 activity is involved in oxidation of vitamin A to retinoic acid and is predominantly implicated in the protection of long-lived cells from oxidative damage (Riveros-Rosas et al. 1997; Storms et al. 1999). Most notably, ALDH activity is downregulated as primitive cells differentiate to maturity, making ALDH activity a unique function to distinguish regenerative precursors from expendable cells. Our group (Capoccia et al. 2009) and others (Gentry et al. 2007b) have recently shown that high ALDH activity is also a property shared by regenerative progenitors of endothelial and mesenchymal lineages. The purpose of this chapter is to discuss how ALDH purification of BM or UCB can be used to simultaneously isolate adult stem and progenitor cell subtypes for the preclinical development of regenerative therapies inducing tissue repair. Focusing on transplantation studies using human ALDH-expressing progenitor cells for hematopoietic reconstitution, blood vessel formation, and islet regeneration in immunodeficient mice, we aim to understand how multiple progenitor subtypes act together to coordinate complex regenerative processes.

3.2 FACS Purification of Multiple Human Progenitor Subtypes Using ALDH Activity

Intracellular ALDH activity can be quantified using a fluorescent substrate for ALDH, termed Aldefluor™ reagent (Storms et al. 1999). First synthesized by Clayton Smith's group in 1999, Aldefluor™ reagent represents a Bodipy fluorochrome conjugated to an aminoacetaldehyde molecule, an uncharged moiety that can freely transverse the cell membrane. Once inside the cell, cytoplasmic ALDH1A1 converts Aldefluor™ into a metabolized anion that becomes trapped in the cell due to its negative charge. Under pharmacological inhibition of ABC transporters with Aldefluor™ buffer, cells with high ALDH activity retain Aldefluor™ substrate and fluoresce brightly, while cells with lower ALDH activity are more dimly fluorescent. Thus, high-speed fluorescence-activated cell sorting (FACS) can efficiently purify UCB or BM MNC with low side scatter and low versus high ALDH activity. The integrity and function of the cell are not compromised by this procedure since upon removal of the Aldefluor™ buffer, ABC transporters become reactivated, and Aldefluor™ is pumped out, returning the cell to its original state. Thus, the Aldefluor™ purification procedure is clinically applicable for the efficient sorting of functional human progenitor cells based on a highly conserved stem cell function.

The amount of ALDH activity in all viable cells falls along a spectrum from low ALDH activity ($ALDH^{lo}$) to high ALDH activity ($ALDH^{hi}$), where $ALDH^{lo}$ versus $ALDH^{hi}$ cells are distinguished by cluster gating using diethylaminobenzaldehyde or DEAB, a pharmacological inhibitor of ALDH1A1. Purified $ALDH^{hi}$ cells highly coexpressed stem cell-associated surface markers (CD34, CD133, c-kit) and were enriched for multipotent hematopoietic and mesenchymal stromal progenitors, as well as precursor cells with endothelial colony forming cell (CFC) capacity in vitro (Hess et al. 2004; Gentry et al. 2007b; Capoccia et al. 2009). In contrast, $ALDH^{lo}$ cells were primarily comprised of more mature leukocytes (primarily T- and B-cells) and demonstrated minimal progenitor function in vitro (Capoccia et al. 2009). Therefore, high ALDH activity simultaneously purifies multiple progenitor cell subtypes ideal for lineage-specific expansion in vitro (Fig. 3.1). Subsequently, purified ALDH-purified mixed progenitor cells or ex vivo expanded progeny can be assayed for potential regenerative functions after xenotransplantation into a variety of immunodeficient models of tissue damage (Fig. 3.1).

3.3 ALDH-Purified Cells Possessing Hematopoietic Repopulating Capacity

Hematopoietic stem cells (HSC) and lineage-specific hematopoietic progenitors are responsible for the replenishment and maintenance of blood after BM transplantation (Jordan and Lemischka 1990; Lemischka 1991). These cells can be isolated

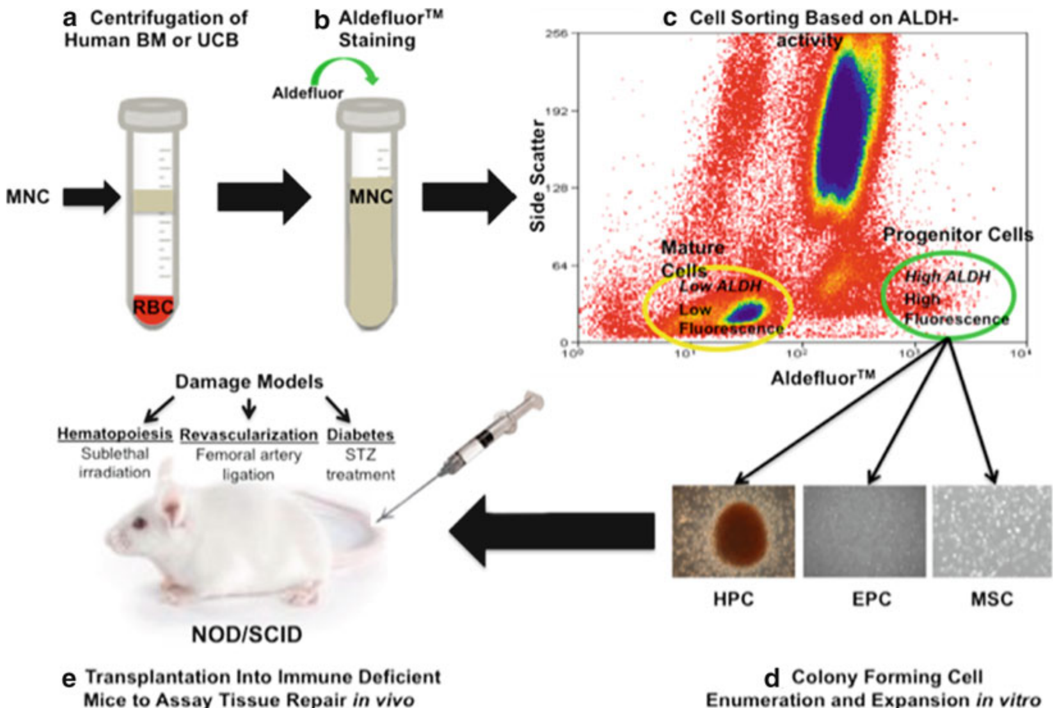

Fig. 3.1 *Isolation of progenitor cells from human bone marrow and umbilical cord blood based on ALDH activity for endogenous regeneration.* (**a**) UCB and BM samples are layered onto Hypaque-Ficoll for centrifugation to isolate mononuclear cells. (**b**) Mononuclear cells are incubated with Aldefluor reagent, which will become trapped within stem and progenitor cells expressing high levels of ALDH. (**c**) Using fluorescence-activated cell sorting, mononuclear cells are sorted into populations of cells with low ALDH activity, or high ALDH activity. (**d**) Progenitor cells of hematopoietic, endothelial, and mesenchymal lineages are enriched in the $ALDH^{hi}$ cell population and expanded in culture. (**e**) Isolated $ALDH^{hi}$ cells or culture expanded $ALDH^{hi}$ cells are transplanted into irradiated NOD/SCID mice to assay hematopoietic reconstitution, or into mice with critical limb ischemia, or STZ-induced hyperglycemia to induce endogenous revascularization and regeneration

from BM, cytokine-mobilized peripheral blood, or UCB based on expression of the cell surface markers CD34 and CD133, and assayed for hematopoietic repopulating function after transplantation into nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (Bhatia et al. 1997; Gallacher et al. 2000; Yin et al. 1997). Storms et al. first established that the ALDH^{hi} fraction of human UCB was enriched for primitive hematopoietic progenitors in vitro and was depleted of lineage-committed hematopoietic cells (Storms et al. 1999). Subsequently, our group in collaboration with Jan Nolta used prospective lineage depletion in combination with the commercially available Aldefluor™ reagent (Stem Cell Technologies, Vancouver, Canada) to demonstrate that transplantation of human UCB ALDH^{hi} cells into NOD/SCID mice resulted in multilineage human hematopoietic engraftment after sublethal irradiation (300–350 cGy) (Hess et al. 2004). Greater than 70% of the UCB ALDH^{hi} cell population coexpressed the HSC-associated cell surface markers CD34 and CD133 (Hess et al. 2004). ALDH^{hi}CD34^{+} cells were highly enriched for short-term myeloid progenitors while ALDH^{hi}CD34^{-} cells represented precursors to the CD34^{+} population that also demonstrated NOD/SCID-repopulating cell (SRC) capacity (Storms et al. 2005; Bhatia et al. 1998; Shojaei et al. 2004).

Our group has also shown that while both ALDH^{hi}CD133^{-} and ALDH^{hi}CD133^{+} demonstrated clonogenic hematopoietic progenitor function in vitro, only the ALDH^{hi}CD133^{+} population was able to engraft the murine BM after intravenous injection (Hess et al. 2006). Furthermore, prospective selection based on both high ALDH activity and CD133 increased the frequency of SRC by tenfold compared to selection by CD133 alone. Notably, ALDH^{hi}CD133^{+} cells demonstrated enhanced hematopoietic repopulating function in serial secondary transplants while maintaining primitive hematopoietic phenotypes (CD34^{+}CD38^{-}) (Hess et al. 2006). In addition to long-term hematopoietic repopulating function, human UCB ALDH^{hi}CD133^{+} cells also showed previously unrecognized engraftment in nonhematopoietic tissues using the highly sensitive human cell-tracking NOD/SCID MPSVII model, including deposition of CD45^{-} cells in the liver of transplanted mice (Hess et al. 2008).

Later studies established that hematopoietic engraftment after human UCB transplantation in immunodeficient mice occurs faster with increasing ALDH^{hi} cell doses (Liu et al. 2010). Indeed, clinical reconstitution rates following transplantation of BM or mobilized peripheral blood can be directly correlated with the number of ALDH^{hi} cells infused (Lioznov et al. 2005; Fallon et al. 2003). As a result of these promising data, there are currently ongoing clinical trials designed to assess the safety and efficacy of transplanted human UCB ALDH^{hi} cells to enhance the rate of engraftment in the treatment of adult leukemia (ALD-151). In summary, high ALDH activity is now well established as a functional characteristic of repopulating hematopoietic cells, and ALDH activity appears to be a superior indicator of the quality of BM or UCB samples for transplantation compared to standardized CD34^{+} counts (Lioznov et al. 2005).

3.4 ALDH-Purified Cells Stimulating Vascular Regeneration

Asahara et al. originally identified endothelial progenitor cells (EPC) as a population of circulating precursor cells in human peripheral blood that differentiate into mature endothelial cells (EC) in vitro and contribute to vessel formation after transplantation into mice (Asahara et al. 1997). Later studies showed that these cells expressed the primitive cell markers CD34, CD133, and KDR (VEGFR-2) (Peichev et al. 2000) and can be obtained from other sources such as BM and UCB (Murohara et al. 2000; Quirici et al. 2001). However, this cell phenotype was shared by hematopoietic progenitor cells, making discrimination of hematopoietic versus endothelial lineage precursors controversial. Because both myeloid hematopoietic and endothelial progenitor cells have been shown to promote angiogenesis in mouse models (Takakura et al. 2000; Urbich et al. 2003), Yoder et al. functionally demonstrated that true EPC are plastic-adherent blood-derived cells propagated in strict EC growth conditions that form proliferative colonies of $CD45^{-}$ ECs capable of forming perfused vessels in gel implants in vivo (Yoder et al. 2007). In contrast, the nonadherent $CD45^{+}$ blood-derived cells that coexpressed typical EC markers (CD31) were not EC precursors but myeloid/macrophage lineage cells, which do not incorporate into newly formed vessels, yet can contribute to angiogenesis through proposed paracrine signaling to activated vessel-derived EC (Yoder et al. 2007; Urbich et al. 2003; Capoccia et al. 2006).

We have shown that the $ALDH^{hi}$ fraction of BM is significantly enriched for endothelial CFC compared to $ALDH^{lo}$ cells and that high ALDH activity was downregulated as a result of culture in vitro (Capoccia et al. 2009). Thus, consistent with the classification by Yoder et al., true endothelial CFC initially possesses elevated ALDH activity. Nagano et al. expanded EPC in culture from UCB and subsequently sorted the $CD45^{-}$ EPC progeny based on ALDH activity (Nagano et al. 2007). Interestingly, they found that the more differentiated $ALDH^{lo}$ outgrowth population was more proliferative in vitro and showed higher expression of hypoxia-inducible factors (HIF1α), VEGF, and the chemokine receptor CXCR4 under hypoxic conditions. They further showed that the cultured $ALDH^{lo}$ outgrowth represented more mature EC that recruited effectively to the site of ischemia and reduced necrosis in a mouse skin flap model of ischemia (Nagano et al. 2007).

3.5 Roles of ALDH-Purified MSC in Vascular Regeneration

With accumulating evidence indicating the promise of BM-derived cells for vascular regeneration, clinical trials were initiated investigating the transplantation of heterogeneous BM MNC to treat limb ischemia (Tateishi-Yuyama et al. 2002) and promote cardiac repair (Schachinger et al. 2006; Assmus et al. 2006; Janssens et al. 2006). However, these trials demonstrated variable efficacy in promoting revascularization, prompting a closer look at the specific progenitor cell fractions that show vascular regenerative promise (Rosenzweig 2006).

As discussed, both hematopoietic and endothelial progenitor cells play a role in blood vessel remodeling and regeneration. In addition, multipotent mesenchymal stromal cells (MSC) have been shown to augment cardiomyocyte (Mirotsou et al. 2007) and EC (Hung et al. 2007) survival, and MSC have been recently identified as vascular pericytes that help stabilize newly formed vessels in vivo (Au et al. 2008; Crisan et al. 2008). Consistent with the idea that high ALDH activity simultaneously purifies stem and progenitor cells from multiple lineages, our group and others have shown that in addition to HSC and tubule-forming EPC, the BM ALDH^{hi} fraction was highly enriched for MSC that efficiently differentiated into fat, cartilage, and bone in differentiation cultures (Capoccia et al. 2009; Gentry et al. 2007a). Capoccia et al. went on further to describe that transplanted human BM ALDH^{hi} mixed progenitor cells transiently recruited to areas of ischemia and augmented the recovery of blood flow by stimulating the endogenous revascularization of ischemic limbs in mice with acute unilateral hind limb ischemia induced by femoral artery ligation (Capoccia et al. 2009). Even without permanent engraftment at the site of ischemia, a low dose of ALDH^{hi} cells were more effective at inducing revascularization than transplantation of unsorted BM nucleated cells containing the equivalent of fourfold more ALDH^{hi} cells.

Although the detailed mechanisms by which ALDH^{hi} cells induced endogenous revascularization remains an active area of investigation, our working hypothesis describes that high ALDH activity simultaneously depletes for inflammatory immune cells and enriches for multiple proangiogenic progenitor subtypes. After transplantation, ALDH^{hi} progenitor subtypes recruit transiently to areas of regional hypoxic damage and contribute to the generation of a proangiogenic microenvironment by providing both structural and paracrine support (Fig. 3.2).

Recent work by Sondergaard et al. has shown that intravenously transplanted UCB ALDH^{hi} cells recruited specifically to the ischemic myocardium where they augmented vascular density in a murine model of myocardial infarction (Sondergaard et al. 2010). Despite the paucity of mechanistic details regarding the vascular regenerative potential of ALDH^{hi} cells, clinical trials have been initiated to explore the safety and efficacy of the use of autologous BM ALDH^{hi} cells in improving outcomes after myocardial infarction/ischemic heart failure (ALD-201), critical limb ischemia (ALD-301), and ischemic stroke (ALD-401) (Keller 2009). Early phase 1 results demonstrated that ALD-301 was well tolerated in the study population, improved blood flow and clinical status in ischemic limbs, and a more extensive phase 2 study is pending (Keller 2009).

3.6 ALDH-Purified Cells Stimulating Islet Revascularization and Repair

In the context of regenerative therapies for the treatment of diabetes, the potential contributions of transplanted stem cells are not limited to the direct replacement of damaged beta cells. As an alternative, the endogenous repair of damaged islets or

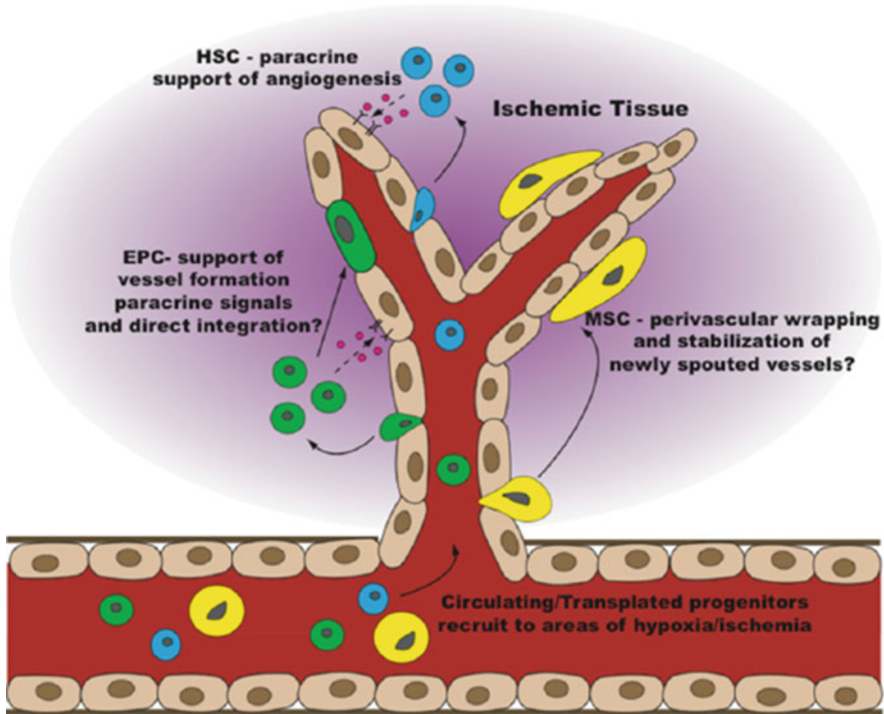

Fig. 3.2 *Human bone marrow and umbilical cord blood progenitor cells support the formation of a proangiogenic niche*. The ALDH^{hi} subpopulation of cells in UCB and BM is enriched for progenitors from the hematopoietic, endothelial, and mesenchymal lineages. Each of these progenitor cell types recruits to and coordinates a proangiogenic regenerative microenvironment in ischemic tissues

the generation of new islets in vivo has also been proposed (Bonner-Weir and Weir 2005). Hess and colleagues were the first to show that transplantation of murine BM-derived MNC or further purified c-kit$^+$ progenitor cells stimulate the recovery of streptozotocin (STZ)-damaged islets by inducing proliferation of recipient beta

cells and augmenting glycemic control via the endogenous regeneration of beta cell function (Hess et al. 2003). Donor mouse cells with both hematopoietic and endothelial phenotypes were recruited to ductal regions and surrounded damaged islets, subsequently stimulating beta cell proliferation and insulin production in recipient-derived beta cells. Several groups have extended these findings to show that islet recovery can be induced by the induction of hematopoietic chimerism in overtly diabetic NOD mice (Zhang et al. 2007) and that simultaneous infusion of murine BM MNC with allogeneic MSC optimize islet repair and protection against T-cell-mediated beta cell deletion (Urban et al. 2008). Although transplantation of murine BM-derived cells have shown proof of principle that MNC can impact endogenous beta cell regeneration, further purification and transplantation of human BM progenitor subtypes is warranted to study the actions of specific cellular populations relevant to islet regeneration.

Toward this end, we have performed studies transplanting human ALDH-purified BM stem cells into STZ-treated hyperglycemic NOD/SCID mice to promote islet regeneration. Transplantation of BM ALDH^{hi} cells led to a significant reduction in blood glucose and increased serum insulin due to an increase in endogenous beta cell proliferation resulting in increased islet size and total insulin content (Bell et al. 2011). Transplanted BM ALDH^{hi} cells recruited to damaged islets and stimulated functional capillary formation in islets. Similar to hyperglycemic mice transplanted with BM cells, mice transplanted with UCB ALDH^{hi} cells had increased islet size and vascularization compared to controls. However, blood glucose reductions were transient, returning to severe hyperglycemia after several weeks. Unlike ALDH-purified BM progenitor populations, ALDH-purified UCB did not consistently establish MSC cultures, indicating a requirement for MSC to maintain islet regeneration. Fortunately, adherent MSC can be liberated from UCB after collagenase treatment (Secco et al. 2008), and inclusion of these cells within the transplanted population may improve islet regeneration.

In the past, clinical applications of UCB-derived stem cells have been limited to transplantation in the fields of hematology or oncology; however, an increasing number of studies support the use of these cells for nonhematopoietic disorders, including diabetes (Harris 2009; Haller et al. 2008). UCB MNC transplantation into diabetic mice has shown delayed onset of autoimmunity and insulitis in a model of type 1 diabetes (Ende et al. 2004a) and improved in hyperglycemia and survival rates posttransplantation (Ende et al. 2004b). A clinical trial using autologous UCB cells is currently underway in children with recently diagnosed type 1 diabetes (Haller et al. 2008). Delayed loss of endogenous insulin production and enhanced glucose control have been reported and found to be due to a highly functional population of regulatory T-cells within UCB (Asano et al. 1996; Salomon et al. 2000). However, the functional mechanisms conferring beta cell protection after UCB transplantation require further preclinical experimentation.

3.7 Roles of ALDH-Purified MSC in Islet Regeneration

MSC possess properties beneficial in the repair of tissues damaged by autoimmunity. MSC have been shown to modulate the microenvironment after injury and stimulate a shift from an inflammatory to a regenerative response (Semedo et al. 2007), and to aid in tissue repair by exerting antifibrotic and neoangiogenic effects (Ninichuk et al. 2006). MSC have also been shown to migrate toward areas of damage and inflammation through their expression of a variety of chemokine receptors and adhesion molecules. In the pancreas, islets attract MSC in vitro and in vivo mediated by CX3CL1-CX3CR1 and CXCL12-CXCR4 interactions (Sordi et al. 2005). MSC may also play an important role in modulating the immune response, an important consideration in the treatment of type 1 diabetes. In damaged tissues, MSC stimulate reduced T-cell (Di Nicola et al. 2002) and B-cell proliferation (Corcione et al. 2006), inhibit maturation as well as differentiation of dendritic cells (Nauta et al. 2006), and decrease the production of inflammatory cytokines by immune cells (Aggarwal and Pittenger 2005). MSC can exert these effects by secreting immunosuppressive molecules such as TGF-β, IL-10, IDO, and PGE_2 (Aggarwal and Pittenger 2005).

Collectively, these characteristics make transplanted MSC an attractive target for the development of cellular therapies for autoimmune diabetes (Madec et al. 2009). As proof of principle, Lee et al. have shown that multiple high-dose intracardiac infusion of *human* BM-derived MSC into STZ-treated immune-deficient NOD/SCID mice repaired islets and improved hyperglycemia with only minimal engraftment in the pancreas (Lee et al. 2006).

Transplantation of ALDH-purified MSC into the tail vein of STZ-treated hyperglycemic mice also stably reduced blood glucose and increased serum insulin. Rather than stimulating increased islet size and vascularization, MSC transplantation stimulated an increase in the number of small islets present in the pancreas. Newly formed beta cell clusters were small in size with direct proximity to the ductal epithelium. Although Ngn3, an endocrine progenitor cell marker (Xu et al. 2008), was not detected in ductal regions, the regenerative patterns were suggestive of a neogenic mechanism, whereby transplanted ALDH-purified MSC stimulated new islet formation (Bell et al., in press). Similar to findings by Lee et al., transplanted ALDH-purified MSC showed low-level or transient recruitment to the pancreas, but initially lodge in the liver and lung capillaries (Hess et al. 2008; Meyerrose et al. 2007), presumably exerting their effects on islet regeneration via the release of unknown paracrine regenerative factors into the bloodstream.

Phase 2 clinical trials are currently underway using intravenous infusion of the drug PROCHYMAL®, in which the active ingredient is ex vivo cultured adult human MSC, to treat patients with newly diagnosed type 1 diabetes. The trial, performed by Osiris Therapeutics in collaboration with the Juvenile Diabetes Research Foundation, involves administration of expanded MSC at three time points 30 days apart. The aim of the study is to determine whether transplanted MSC can protect the pancreatic tissue from further autoimmune attack and/or stimulate the repair of damaged pancreatic tissue compared to placebo-treated controls. Results from this trial are currently pending.

3.8 Future Perspectives

BM- or UCB-derived ALDH-purified mixed progenitor cells and ALDH-purified MSC demonstrate a variety of regenerative functions that impact tissue repair and revascularization. However, differences in progenitor frequencies and functions should not be overlooked when considering the optimal progenitor cell populations for regenerative therapies. In addition, the regenerative mechanisms induced by ALDH-expressing progenitors and the paracrine mediators that comprise the regenerative niche require further elucidation. Provided with this foundation of critical information, scientists will be able to design rational regenerative therapies using a combination of regenerative small molecules or directed administration of multiple progenitor cell subtypes to synergize endogenous tissue repair.

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Chapter 4
Mesenchymal Stem Cells and Tissue Repair

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Abstract Mesenchymal stem cells (MSCs) are somatic stem cells endowed with extraordinary regenerative and immunomodulatory properties that have been used in over 100 clinical trials worldwide to date. Their accessibility from autologous sources and safety when used in allogeneic transplantation make them ideal candidates from a wide variety of cell therapies. On the other hand, our limited understanding of their ontogeny, physiological role, and in vivo behavior currently limits the efficacy of MSC-based therapies. More specifically, MSC-like cells can be procured from many organs and tissues, but it is still unclear how these relate to the traditional marrow-derived MSCs in terms of developmental origin, behavior, and therapeutic efficacy. Furthermore, there is still some uncertainty about the mechanisms by which MSCs home to sites of injury and participate in tissue repair. This chapter will review our current understanding about these various issues, including the characterization and ontogeny of marrow-derived MSCs, alternative MSC sources, trafficking and homing of MSCs, and mechanisms of tissue repair.

4.1 Introduction

The first evidence of the existence of mesenchymal stem cells can be traced to the 1860s, when Julius Cohnheim injected a dye in the circulation of animals and observed adherent fibroblastoid cells extravasating at sites of tissue injury (Cohnheim 1867).

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Cohnheim hypothesized that nonhematopoietic, bone marrow–derived cells could migrate through the bloodstream to sites of injury and participate in tissue regeneration. Around the same period, Goujon discovered that the bone marrow of rabbits and chickens possessed osteogenic potential when transplanted in chick muscle, although bone formation varied with the age of the donor marrow and the site of implantation (Goujon 1869). At this time, the concept of stem cells (*Stammzelle*) had just been developed by Haeckel (1868), who would later extend its use to the fertilized egg of multicellular organisms (Ramalho-Santos and Willenbring 2007). Extending on Haeckel's ideas, Pappenheim (1896) and Maximov (1909) would soon propose the existence of bone marrow–resident stem cells giving rise to all blood cells. Maximov also observed that blood cells differentiated hierarchically by interacting with marrow stromal cells or adventitial reticular cells (Friedenstein 1989).

Maximov's ideas were well ahead of their time as it would take more than half a century before Becker, Till, and McCulloch provided the first experimental evidence of the existence of marrow-resident HSCs (Becker et al. 1963). Other experiments recapitulating Goujon's observations suggested that bone tissue and marrow stromal cells originated from a different stem cell compartment than HSCs (Friedenstein et al. 1968; Tavassoli and Crosby 1968). Alexander Friedenstein and colleagues were the first to successfully isolate osteoprogenitor cells capable of transferring the hematopoietic microenvironment when transplanted in vivo (Friedenstein et al. 1974). These clonogenic, plastic adherent cells (termed colony-forming unit fibroblasts, CFU-Fs) recapitulated bone and marrow stroma development but could not give rise to vascular or hematopoietic tissue.

In the 1980s, several groups reported that cells isolated using Friedenstein's simple plastic adherence protocol possessed osteogenic, adipogenic, and chondrogenic potential in vitro and could be isolated from most mammalian species with small differences in growth requirements (reviewed in Prockop (1997)). In 1991, developmental bone biologist Arnold Caplan coined the term *mesenchymal stem cells* (MSCs) to describe these cells, as a means to emphasize their putative developmental origin, role in bone formation and repair, and therapeutic potential (Caplan 1991). The use of *mesenchymal stem cells* would then be widely used within the research community, especially following the observation that these cells could engraft in various mesenchymal (and nonmesenchymal) tissues after systemic infusion. It also appeared that MSC-like cells could be isolated from other mesenchymal tissues such as synovium, adipose, and muscle tissues (Prockop 1997). However, the term *mesenchymal stem cell* also met severe criticism because not all bones are derived from embryonic mesenchyme during development (the exceptions being neural crest-derived facial bones), and the true stem cell nature of MSCs remains disputed (Bianco et al. 2010; da Silva et al. 2008).

Despite these controversies, the extraordinary properties of MSCs (regenerative, angiogenic, immunomodulatory, etc.) have served as a basis for over 100 clinical trials registered to date using MSCs to treat a wide variety of acquired or inherited diseases (http://clinicaltrials.gov, searched March 2011). It is now accepted that both autologous and allogeneic MSCs can be safely administered to patients in large

doses without significant adverse effects. However, the efficacy of MSC therapies remains somewhat mitigated, mostly owing to our lack of understanding of basic MSC biology including their in vivo identity and behavior and the mechanisms by which endogenous and culture-expanded MSCs migrate through the circulation, home to sites of injury, and participate in tissue repair (Prockop et al. 2010; Prockop 2009). The present chapter will review our current understanding of MSC characteristics, sources, trafficking, and regenerative mechanisms. The immunomodulatory properties of MSCs and their use in neural repair are not discussed in detail in this chapter but are mentioned in other chapters of this book.

4.2 Mesenchymal Stem Cells: Identification and Characterization

Mesenchymal stromal cells have historically been isolated from bone marrow by their ability to adhere to and proliferate on tissue culture plastic at clonal density. Their capacity to recapitulate bone and marrow stroma formation and transfer the hematopoietic microenvironment in vivo is also one of their key features (Friedenstein et al. 1974). There are now well-established isolation protocols and in vitro assays to determine MSC multipotentiality including differentiation into osteoblasts, chondrocytes, adipocytes, and smooth-muscle cells (Pittenger et al. 1999) with little interspecies variation. Immunophenotyping methods by flow cytometry have also been used to identify the molecular signature of MSCs, albeit with less success than with HSCs. The first significant advance was the development of the monoclonal antibody STRO-1 against human stromal cells which, together with a glycophorin A–negative phenotype to exclude erythroid progenitors, enriches for CFU-Fs about 100-fold (Simmons and Torok-Storb 1991). This is still probably the most useful method to enrich human MSCs. However, there is no known equivalent to the STRO-1 antibody in other species, precluding its use in animal models. Moreover, for most therapeutic purposes, isolating MSCs by prospective isolation (immunoselection) or by clonal expansion after plastic adherence makes little difference because high numbers of MSCs are needed in most therapeutic applications. Because of their low frequency in human marrow (about 1 in 10^6 nucleated cells), ex vivo expansion of the initial cell population is unavoidable. Nevertheless, the capacity to enrich, identify in situ, or prospectively isolate MSCs without culture-induced modifications would be a major step toward the study of MSCs in mouse models, to study them in their native niches in vivo and to clarify their physiological role and role in normal tissue regeneration and repair.

Other cell surface markers that have been used to characterize MSCs by flow cytometry include CD44 (hyaluronic acid receptor), CD73 (5′-ecto-nucleotidase), CD90 (Thy-1), and CD105 (endoglin) (Pittenger et al. 1999; Horwitz et al. 2005), although none of these molecules are specific to MSCs or useful for their prospective isolation when used on their own. Indeed, in addition to the expression of these molecules, MSCs should also be negative for hematopoietic (CD45), endothelial

(CD31 or von Willebrand factor), and macrophage (CD11b/MAC-1) markers. Although this phenotype can be used to prospectively isolate MSCs by multicolor fluorescence-activated cell sorting (FACS), it is not useful to identify MSCs in situ on tissue sections even with the most advanced multicolor fluorescence microscopes.

Morphological criteria have also been used to describe MSCs in vitro (Colter et al. 2001). In this study, the authors clearly demonstrate that human MSC cultures contain at least three different cell types based on morphological criteria in vitro. These cells varied in proliferation potential, multipotentiality, and expression of cell surface markers. Interestingly, the cells with the best capacity to proliferate and differentiate were found in a very small, round, and poorly adherent fraction of cells. Also, these cells did not express STRO-1 or platelet-derived growth factor receptor but expressed c-Kit, a phenotype opposite to more mature cells.

More recently, it was reported that cells contained in the CD146+/CD45− fraction of human marrow possessed CFU-F capacity, recapitulated bone and marrow formation in vivo on calcium phosphate ceramic (where some cells adopted a perivascular location), and could be transplanted in secondary recipients (Sacchetti et al. 2007). CD146, also known as melanoma-associated cell adhesion molecule (MCAM), is typically expressed by pericytes but is also expressed in endothelial, melanoma, and breast cancer cells where it is believed to play a role in cell migration (Zabouo et al. 2009). Another group demonstrated the existence of rare CD146+ pericytes in many human organs including bone marrow (Crisan et al. 2008). This group demonstrated that some of these cells possessed MSC properties (clonogenicity, multipotentiality, expression of cell surface markers), thus partially supporting the hypothesis by Sacchetti et al. (2007) that tissue pericytes can behave as true MSCs. The expression of CD146 by MSCs of species other than humans has not been reported so far.

Other markers have recently been reported to enrich for MSCs including platelet-derived growth factor receptor (PDGFR), activated lymphocyte cell adhesion molecule (ALCAM), stem cell antigen-1 (Sca-1 or Ly-6), and nestin (Morikawa et al. 2009; Arai et al. 2002; Mendez-Ferrer et al. 2010; Bonyadi et al. 2003). However, the identity of the cells described in those studies has not yet been tested using rigorous assays nor replicated by other groups. Interestingly, however, it appears that cells expressing either STRO-1, CD146, PDGFR, or nestin are mostly found in the perivascular area, supporting the idea that there is interdependence between primitive skeletal stem/progenitor cells and the vasculature, at least during some steps of bone development, homeostasis, and repair. However, a restricted localization of MSCs to the perivascular space contradicts other reports suggesting MSC-like cells may also be found embedded in human trabecular bone, perichondrium, and periosteum (Tuli et al. 2003; Arai et al. 2002; Diaz-Flores et al. 1992).

In summary, much of what we know today about MSCs relates to their in vitro phenotype after culture expansion using Friedenstein's protocol, and as a consequence, MSC preparations are inevitably heterogeneous. A set of criteria has been proposed to broadly define MSCs (Horwitz et al. 2005), but these may not reflect the actual heterogeneity of MSCs in vitro and in vivo.

4.3 Ontogeny of Bone Marrow–Derived Mesenchymal Stem Cells

Understanding the true in vivo identity of any somatic stem cell type and its role in tissue development and homeostasis requires a profound knowledge of the developmental processes leading to tissue formation and maintenance, both at the molecular and cellular levels. Although the focus of MSC research during the last decade has been mostly translational, it now appears that a better understanding of the fundamental biology of MSCs may be required to achieve therapeutic benefit. This is emphasized by the fact that most of what we know today about MSCs relates to their in vitro phenotype or characteristics after culture expansion. Thus, there has been a renewed interest in studying MSCs from a developmental perspective (Schipani and Kronenberg 2009). Most studies to date have aimed at elucidating regulatory pathways governing embryonic bone development and comparing their effects on MSC fate decisions in vitro (Karsenty et al. 2009), but uncertainty remains about the true developmental origin and in vivo identity of postnatal MSCs.

Most bones such as those from the axial skeleton and limbs are formed during embryonic development through the process of endochondral ossification (Kronenberg 2003). Marrow-containing skeletal elements are always formed through endochondral ossification, and MSCs have almost exclusively been studied in such bones. In the limbs, all skeletal elements derive from a small number of undifferentiated mesenchymal cells in the lateral plate mesoderm that are covered by the apical ectodermal ridge. These mesenchymal cells receive signals (probably mediated by FGF-8) from elements medial of the lateral plate mesoderm as well as the AER and aggregate to form a mesenchymal condensation that will eventually give rise to all skeletal elements in the limb through expansion (cell proliferation), segmentation, and branching (Mariani and Martin 2003). Cells at the center of the condensation express a gene program driven by SOX-9 and differentiate into chondrocytes, giving rise to the growth plate. Cells at the periphery form the perichondrium containing undifferentiated mesenchymal cells. There ensues a highly orchestrated set of events involving cell proliferation, migration, differentiation, and apoptosis that will eventually lead to bone and marrow formation (Kronenberg 2003).

Current evidence suggests that osteostromal progenitors remain in the perichondrium until growth plate chondrocytes begin to hypertrophy and trigger vascular invasion (Kronenberg 2007; Colnot et al. 2004; Maes et al. 2007). Signals from the growth plate and blood vessels then induce commitment of the perichondrial cells by inducing a gene program directed by Osterix and Runx2. Activated lymphocyte cell adhesion molecule (ALCAM) may play a role in the interaction between endothelial cells and undifferentiated perichondrial cells (Arai et al. 2002). These cells adopt a perivascular localization and migrate along the vasculature to colonize other bone compartments including cortical bone, marrow stroma, and trabecular bone (Maes et al. 2010). What is not known is whether cells found in these various compartments are identical, but recent evidence suggests they are not (Colnot 2009).

Moreover, the precise developmental hierarchy downstream of the perivascular MSCs is not known, and many subsets of stromal cells exist (Nagasawa 2006; Tokoyoda et al. 2004). The precise location of postnatal MSCs within bone tissues thus remains elusive to date.

In summary, skeletal MSCs appear to originate in the perichondrium during embryonic development and migrate along blood vessels as specialized pericytes to colonize other bone compartments. This is consistent with the observations that MSCs share many markers with vascular pericytes in many organs (Crisan et al. 2008).

4.4 Alternative Mesenchymal Stem Cell Sources

In addition to bone marrow, MSC-like cells have also been described in other organs including adipose tissue, synovium, muscle, blood/cord blood, and others (Phinney and Prockop 2007). However, it must be noted that very few studies have so far performed an extensive characterization of these cells and comparison with bone marrow MSCs. For example, expression of some cell surface markers and the capacity to accumulate lipid droplets or mineralized calcium phosphate after treatment with supraphysiological levels of PPAR-γ agonists (isobutyl-methyl-xanthine) or phosphate donors (β-glycerol-phosphate), respectively, does not qualify a cell population as true stem cells or MSCs. Thus, caution must be observed before ascribing the name *mesenchymal stem cell* to any cell population capable of ex vivo expansion and in vitro mesenchymal plasticity. Nevertheless, some promising alternative MSC sources do exist and will be briefly discussed in this section.

4.4.1 Adipose Tissue

The adipose tissue is thought to harbor MSC-like cells and has received much attention from cell therapists within the last decade. It is considered an attractive MSC source since it can be easily obtained from lipoaspirates, and cells are easily processed by collagenase digestion. Adipose-derived MSCs are believed to be equivalent to the stromal vascular fraction of cells in adipose tissue (Schaffler and Buchler 2007), a specialized pericyte population, which are progenitors for preadipocytes and are known to be capable of clonal expansion. These cells express MSC and pericyte markers, including CD44, CD73, CD90, CD105, and possibly STRO-1 and CD146, although the latter show more variability between studies (Gimble and Guilak 2003). Numerous studies have reported that adipose MSCs are capable of ex vivo expansion and multilineage differentiation similar to bone marrow MSCs in vitro (adipose, osteoblasts, chondrocytes); however, the few studies directly comparing adipose MSC with bone marrow MSC in terms of proliferative and differentiation potential report mixed results (Yoshimura et al. 2007; Sakaguchi et al. 2005; Im et al. 2005; Wagner et al. 2005; Izadpanah et al. 2006). Some studies also suggested they may

have a broader differentiation capacity including the capacity to form neurons, hepatocytes, and pancreatic beta cells. However, their capacity to functionally engraft and repair damaged tissues in vivo has not been investigated yet.

Only four studies so far have tested the osteogenic potential of adipose MSC in vivo by transplantation on calcium phosphate ceramic, and the results are mixed (Hayashi et al. 2008; Scherberich et al. 2007; Muller et al. 2010; Hattori et al. 2004). Moreover, none of these studies have reported the establishment of hematopoiesis within the osseous constructs. The immunomodulatory properties of adipose MSC have also not been extensively tested yet, although some case reports suggest they may be effective for the treatment of multiple sclerosis and some inflammatory disorders (Riordan et al. 2009).

In conclusion, adipose tissue contains progenitor cells capable of ex vivo expansion that possess some characteristics of MSCs. Whether these cells are developmentally or functionally related to bone marrow MSCs remains unresolved. More studies are definitely needed to clarify their potency, capacity to repair tissues, and immune properties.

4.4.2 Peripheral Blood

The presence of regenerative marrow-derived cells of nonhematopoietic origin in peripheral blood was postulated by Cohnheim and Maximov over 100 years ago. However, the precise identity and origin of these cells remains disputed to this day. Numerous studies have described low levels of nonhematopoietic cells expressing MSC or osteoblast markers in steady state peripheral blood of humans and other mammals (Eghbali-Fatourechi et al. 2005; Kuznetsov et al. 2001). These cells typically constitute less than 1% of the total peripheral blood mononuclear cells (they may be more abundant in rodents than humans) and are characterized by the absence of hematopoietic (CD45) and endothelial (CD31) markers but the presence of osteoblast (alkaline phosphatase, osteocalcin) and MSC (CD90, CD44, CD105) markers (He et al. 2007). Some studies demonstrated multilineage differentiation of these cells. Interestingly, circulating MSC-like or osteogenic cells appear to be increased in animal models of bone fractures (Alm et al. 2010), skin damage (Mansilla et al. 2006), obstructive sleep apnea (Carreras et al. 2009), chronic hypoxia (Rochefort et al. 2006) as well as in human teenagers (Eghbali-Fatourechi et al. 2005), in patients with osteoporosis (Dalle et al. 2009), and after myocardial infarction (Wang et al. 2006).

More recent evidence that MSCs can migrate in the peripheral circulation was reported in a study using a parabiotic mouse model (Otsuru et al. 2008). In this study, the authors first performed a bone marrow transplant from GFP transgenic mice into irradiated wild type host. After marrow reconstitution, the transplanted animals were paired to normal mice, and a shared circulatory system was established. Subcutaneous BMP-2 pellets were then implanted into the wild type animal of each parabiotic pair, and evidence was shown that GFP positive osteoblasts could

be found in the ectopic ossicles and in peripheral blood and that their recruitment depended on the CXCR4-SDF1 pathway. Although this study suggests that marrow-derived osteoprogenitor cells can be recruited by SDF1 to exogenous sites of bone formation, the authors did not report the level of osteopoietic engraftment in the irradiated host. Thus, it is possible that the transplanted cells engrafted in tissues other than bone marrow (e.g., lungs, liver, spleen, skin; see next section) before colonizing the ectopic ossicles.

Definitive proof that endogenous marrow-resident MSCs could be recruited to peripheral blood by soluble signals was recently provided by Pitchford et al. (2009). In this elegant model, the femoral artery and vein are cannulated in situ, thereby isolating the femoral and tibial circulation. Using this model, the authors were able to demonstrate that MSCs could be mobilized from the marrow to peripheral blood by the administration of a CXCR4 antagonist. Of note, this study also demonstrated mobilization of hematopoietic cells by G-CSF treatment and of endothelial progenitors by VEGF administration. Thus, it appears that bone marrow harbors different types of progenitor cells that can be mobilized by specific soluble signals.

Taken together, these studies suggest that MSCs or MSC-like cells can be found at low frequency in peripheral blood. However, tissue injury, peripheral ischemia, and other soluble signals may also lead to an increase in blood-borne MSCs most probably by antagonizing the CXCR4-SDF1 pathway which is thought to sequester CXCR4 positive cells to the marrow due to the abundance of SDF1-secreting stromal cells. Whether MSCs mobilized in this way directly participate in tissue repair as part of their normal pathophysiologic role remains to be proven.

4.4.3 Umbilical Cord Blood

Mesenchymal stem cells or MSC-like cells have also been isolated from a variety of embryonic or fetal tissues including amniotic fluid, the outer mesenchymal layer of the amniotic membrane, Wharton's jelly (the connective tissue surrounding umbilical cord blood vessels), and umbilical cord blood (Pappa and Anagnou 2009). Cells from fetal tissues are considered attractive because of their availability and because they are thought to possess a broader differentiation potential and proliferation capacity than their adult counterparts (Montesinos et al. 2009). Moreover, they have never been shown to produce teratomas when transplanted in vivo and are thus safer than embryonic stem cells. Cord blood is probably the most attractive source of fetal MSCs to date because private and public cord blood banks already exist that could be used therapeutically.

The umbilical blood typically contains 60–80 cc of cord blood (CB) which contains embryonic stem cells, mesenchymal stem cells, hematopoietic stem cells, and endothelial progenitor cells (Francese and Fiorina 2010). The CB-MSCs are isolated by negative selection and/or density gradient centrifugation followed by plastic adherence and growth in selective media (Wagner et al. 2005; Laitinen and Laine 2007). These cells lack expression of hematopoietic and endothelial markers

(CD45, CD31) but express the MSC markers CD44, CD73, CD90, and CD105 (Flynn et al. 2007). When compared to marrow-derived MSCs, CB-MSCs showed limited differentiation into adipocytes (Bieback et al. 2004; Rebelatto et al. 2008) and proved more difficult to isolate than from the marrow (Wexler et al. 2003). However, their osteogenic and chondrogenic potentials in vitro appear intact (Lu et al. 2010). CB-MSCs were also found in the brain, liver, and heart after transplantation in fetal sheep (Kogler et al. 2004).

CB-MSCs also possess immunomodulatory properties and can suppress T cell proliferation by both direct and indirect mechanisms in vitro (Cutler et al. 2010; Girdlestone et al. 2009; Chen et al. 2010; Wang et al. 2009). They have also been shown to improve treatment-refractory lupus erythematosus in humans by stimulating regulatory T cell (T_{reg}) production and modulating cytokine imbalances (Sun et al. 2010). In summary, CB is a viable source of MSCs that can be used for immunotherapy, but more studies are needed to test their use in tissue repair.

4.5 Trafficking and Homing of MSCs

In the 1990s, several investigators studied the biodistribution of systemically delivered MSCs [reviewed in Prockop (1997; 2003)]. Not surprisingly, MSCs were found to be able to partially reconstitute marrow stromal elements in irradiated animals. Interestingly, some investigators also found MSC-derived cells in other tissues such as lung, liver, spleen, thymus, skin, bone, and cartilage. Importantly, MSC progeny found in cartilage tissue seemed to express genes in a tissue-specific manner, suggesting that the cells functionally engrafted in some tissues. The capacity of MSCs to engraft in multiple tissues was later confirmed when early passage human MSCs were injected in utero in sheep (Liechty et al. 2000). These experiments not only confirmed that MSCs could engraft in many different tissues after systemic delivery, they also suggested that MSCs were immune privileged as they seem to be tolerated in xenotransplantation in immune-competent (although prenatal) animals.

More detailed analyses of the kinetics of MSC homing, migration, and engraftment were later performed. Gao et al. (2001) studied the biodistribution of MSCs very shortly after systemic infusion in rats. They show that MSCs first appear to get trapped in the lungs immediately after infusion. At 48 h, a large fraction of the cells had reached the liver, but they were also detected in bone marrow and spleen. The use of a vasodilator reduced MSC "homing" to the lungs and increased marrow engraftment. In another study, GFP-labeled MSCs were infused in baboons after total body irradiation, and the presence of the transgene was analyzed in various organs 9–21 months later. This study showed that a large number of cells engraft in the gastrointestinal tract, but they are also found in high numbers in the kidneys, liver, lungs, thymus, and skin (Devine et al. 2003). More recently, it was confirmed that human MSCs engraft at low levels in the marrow and lungs in nonirradiated NOD-SCID mice (Mouiseddine et al. 2007; Francois et al. 2006). However, after total body irradiation, MSCs were also found in the brain, heart, muscles, and liver.

Interestingly, it was also found that giving an additional localized irradiation increased MSC homing to the irradiated site.

Homing of MSCs to specific sites after intravenous injection was also reported in animal models of cardiac infarct (Pittenger and Martin 2004; Price et al. 2006; Nagaya et al. 2004; Lee et al. 2009), although cell entrapment into the lungs may limit this approach (Barbash et al. 2003). Recruitment of MSCs to the infarcted heart probably involves signaling by SDF-1 through CXCR4 expressed on MSCs (Yu et al. 2010; Tang et al. 2009). Indeed, MSC homing to site of injuries implies that MSCs can sense and migrate toward factors released by tissue damage. Several chemokine and cytokine receptors have been identified in human and murine MSCs (Deak et al. 2010; Chamberlain et al. 2008). mRNA expression analysis and flow cytometry analysis in human MSCs identified CC chemokine receptors CCR1, CCR7, and CCR9 as well as CXC chemokine receptors CXCR4, CXCR5, and CXCR6. All of these receptors responded to their cognate ligand by inducing migration of human MSCs in chemotactic transwell chambers (Honczarenko et al. 2006). Ponte et al. (2007) confirmed these results but were also able to detect CCR2, CCR4, and CCR5 expression by Western blot analysis. In addition, they demonstrated that human MSCs also expressed TNF receptor 1 and 2. Prestimulation of the MSC with recombinant TNF-α increased the expression of CCR2, CCR3, and CCR4. TNF-α stimulation alone was able to induce spontaneous migration of MSC and increased MSC migration toward RANTES, CCR3 ligand.

Trafficking of MSCs may also be influenced by molecules other than CC or CXC receptor ligands. For instance, Sackstein et al. (2008) demonstrated that MSC homing to bone or marrow could be drastically improved by modifying the glycosylation pattern on CD44 using an enzymatic treatment. This generated E-selectin binding sites on CD44 allowing MSCs to adhere and migrate through specialized blood vessels within the marrow. This capacity to modulate the homing of MSCs and target them to specific sites without genetic modification may prove very useful for clinical applications.

The mechanisms by which MSCs transmigrate the endothelial wall from the blood stream to the tissue are unclear (for an exhaustive review on MSC homing and trafficking, see Karp and Leng Teo 2009). Due to the enlargement of the MSC size following cell culture, the MSCs could simply be trapped in smaller capillaries and subsequently translocated through the endothelium in a passive manner. Alternatively, MSCs could actively home to a specific tissue by an active mechanism similar to leukocyte endothelial transmigration. The process of transendothelial migration by MSC would involve several steps: (1) cell rolling, (2) activation, (3) adhesion, and (4) diapedesis (Fox et al. 2007). Active transmigration by human MSCs has been demonstrated by (Ruster et al. 2006) using flow chamber assays and real-time video imaging. Their results indicated that interactions of unknown P-selectin ligands and α4β1-integrin VLA-4 on MSCs with P-selectin and VCAM-1 on endothelial cells, respectively, allowed MSCs to roll onto and firmly adhere to endothelial cells. Moreover, Ries et al. (2007) demonstrated with an in vitro study that human MSCs expressed metalloproteinase-2 (MMP-2), membrane type 1-MMP (MT1-MMP), and tissue inhibitor metalloproteinase-2 (TIMP-2) and that specific blockage of

MMP-2, MT1-MMP, and TIMP-2 using inhibitors and RNA interference (RNAi) blocked the ability of MSCs to migrate across an artificial basal membrane composed of a combination of extracellular matrixes (laminin, collagen type IV, and proteoglycans). A similar study using Matrigel-based transmigration assay with human MSCs also identified the enzymatic activity of MMP-2 and TIMP-3 as responsible for MSC transmigration (De Becker et al. 2007). These results do not directly demonstrate the transmigration potential of MSCs but do suggest that MSCs could use proteinases to breakdown the basal membrane of the endothelial wall in order to extravasate out of blood vessel.

Thus, we can conclude that culture-expanded MSCs have the capacity to home to various organs. This tissue-specific homing is, however, influenced by tissue damage and probably involves paracrine mechanisms. Even though MSC migration mechanisms have been partially clarified in vitro, the mechanisms of MSC homing in vivo have not yet been fully elucidated.

4.6 Tissue Repair Mechanisms

The capacity of MSCs to home to various organs (in particular to damaged tissues) and the fact that tissue injury increases the number of MSCs in peripheral blood prompted researchers to test their use in a wide variety of congenital and acquired diseases in preclinical studies. The idea was to take advantage of the tropism of MSCs, their apparent plasticity, and their extraordinary immunomodulatory properties. Targeted conditions thus included bone diseases, myocardial infarction, Parkinson's disease, amyotrophic lateral sclerosis, stroke, multiple sclerosis, Crohn's disease, and acute lung injury, among others. The promising results obtained in animal models served as a basis for over 100 clinical trials registered worldwide to date using MSCs (http://clinicaltrials.gov). However, despite the vast amount of literature on the subject, surprisingly little is known about how MSCs promote tissue repair. It was initially assumed that MSCs could directly engraft and replace lost cells within damaged tissues. However, observations of therapeutic benefit without significant functional engraftment of MSCs suggested that MSCs probably act in a paracrine manner, indirectly modulating tissue repair (Prockop 2007; Prockop et al. 2010). This section will briefly review the mechanisms by which MSCs participate in tissue repair. For a discussion regarding brain repair by MSCs, the reader is referred to another chapter of this book (see Chap. 9).

The first human clinical trial using MSCs aimed at using allogeneic MSCs to correct the congenital bone disease *osteogenesis imperfecta* (Horwitz et al. 1999). The cells were systemically infused in children suffering from the disease and resulted in increased bone deposition and growth concomitant with decreased fracture occurrences. However, the effect was short lived due to limited homing and engraftment of the cells in bones. Thus, it is not clear how MSCs promoted tissue repair in this study. MSCs have also been considered to repair bone lesions caused by cancer, trauma (nonunion fractures), for spine fusion, revision total joint arthroplasty,

maxillofacial reconstruction, and segmental bone defects. In particular, to improve cell survival after transplantation and promote osteogenesis, MSCs have been coupled with osteomimetic calcium phosphate ceramics (Ohgushi and Caplan 1999). In general, MSC-seeded scaffolds have been shown to be more effective than scaffolds alone in promoting bone repair (Srouji et al. 2006). Current research primarily aims at increasing MSC survival within large constructs and improving the mechanical properties of the scaffolds (Coutu et al. 2009).

Cardiovascular regenerative medicine is one of the most promising avenues for MSC-based cell therapy. We have already mentioned that systemically infused MSCs can home to the normal heart and are recruited to the infarcted myocardium by SDF-1 and that circulating MSCs are increased after myocardial infarction. MSCs are thought to improve heart function after infarction by various paracrine mechanisms involving neovascularization, protection of cardiomyocyte death (and/or stimulation of cardiac stem cell proliferation), and prevention of inflammation, cardiac remodeling, hypertrophy, and fibrosis (Laflamme and Murry 2005; Segers and Lee 2008). Relevant cytokines and growth factors that are produced by MSCs and could explain these effects include SDF-1/CXCL12, hepatocyte growth factor (HGF), insulin-like growth factor 1 (IGF-1), fibroblast growth factor 2 (FGF-2), vascular endothelial growth factor (VEGF), interleukin 1 and 6, and monocyte chemoattractant protein 1 (MCP-1). Current challenges in cardiovascular MSC therapy are to identify the best timing and delivery route for the cells and also to identify the best MSC subtype to use in order to improve efficacy and limit potential side effects such as arrhythmias and unwanted differentiation of the MSCs (Psaltis et al. 2008; Prockop and Olson 2007).

Lung repair is another field that has been targeted by MSC therapy based on the observations that MSCs tend to get trapped in the lungs following systemic infusion. However, this entrapment does not result in significant lung engraftment of the MSCs, which tend to home to other organs within a few days (Gao et al. 2001; Weiss et al. 2008). Lung injury by intratracheal administration of bleomycin appears to increase MSC homing to the lungs, which ameliorates the outcome by decreasing lung fibrosis and collagen accumulation (Ortiz et al. 2003). Some of these effects can be attributed to the secretion by MSCs of an interleukin 1 receptor antagonist (Ortiz et al. 2007). On the other hand, the increased homing of MSCs to the lungs in this model has been attributed to reduced macrophage migration inhibitory factor (MIF) within the lung, which is an inhibitor of MSC migration (Fischer-Valuck et al. 2010). Although promising, these studies still await confirmation in larger animal models and in human clinical trials.

In summary, MSCs stimulate tissue repair mostly by producing paracrine factors affecting the endogenous regenerative processes in various tissue, with the exception of bone repair where they may functionally engraft by differentiation into osteoblasts and chondrocytes. In general, molecules secreted by MSCs can be classified as: (1) hematopoietic support, (2) anti-inflammatory, (3) antifibrotic, (4) chemotactic, (5) immunomodulatory, and (6) mitogenic/antiapoptotic. Because culture-expanded MSCs are highly heterogeneous, it is probable that specific subtypes of cells are responsible for these various effects. A better understanding of the lineage hierarchy of MSCs would help in designing more efficient therapies by selecting the appropriate subtype of MSCs required for specific applications.

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Chapter 5
Animal Protein–Free Expansion of Human Mesenchymal Stem/Progenitor Cells

Katharina Schallmoser, Nathalie Etchart, Dirk Strunk, and Eva Rohde

Abstract Human mesenchymal stem/progenitor cells (MSPCs) are currently used in a large number of clinical trials aimed at regeneration or immune modulation. Despite a growing body of knowledge about MSPC biology and function, fundamental questions regarding the optimal manipulation procedure still remain unresolved. Due to biological, ethical, and safety reasons, the use of fetal bovine serum as a standard cell culture supplement has come under dispute inducing a search for comparable alternatives. A humanized culture system requires not only animal serum–free supplements for basal media but also animal protein–free additives like coating reagents or trypsin preparations. In comparison to other blood-derived substances, pooled human platelet lysate (pHPL) has been proven to be the most efficient stimulant for MSPC proliferation enabling the expansion of sufficiently high cell yields within a suitable timeframe. In this chapter, we introduce alternatives to avoid animal-derived factors in the clinical-grade MSPC production with a special emphasis on pHPL as a novel and promising cell culture supplement.

5.1 Introduction

Mesenchymal stem/progenitor cells (MSPCs) are currently the focus of research due to their potential role in tissue regeneration, hematopoiesis or vasculogenesis support, and immune regulation. These fibroblast-like spindle-shaped cells were first described as nonhematopoietic stem cells of the bone marrow (BM) in 1867

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(Cohnheim 1867). A century later, Friedenstein defined osteogenic precursors as being plastic-adherent and able to form colonies in culture (Friedenstein et al. 1974). In the 1990s, Pittenger and Prockop first reported about mesenchymal stem cells with their long-term self-renewal capacity and the potential to differentiate along osteogenic, adipogenic, and chondrogenic lineages (Prockop 1997; Pittenger et al. 1999). Since then, the term *mesenchymal stem cells* has been uncritically used for a rising number of nonhematopoietic stem and progenitor cells. For this reason, a group of researchers with the International Society for Cellular Therapy (ISCT) aimed to characterize mesenchymal stromal cells (MSCs) by defining the minimal criteria of plastic adherence, in vitro differentiation capacity into three mesodermal lineages, and a distinct combination of surface markers (Dominici et al. 2006). We suggest using the more accurate term *mesenchymal stem/progenitor cells* (MSPCs). The classical definition of a stem cell comprises capacities of self-renewal and differentiation into mature cell lineages. Because MSPCs probably represent heterogeneous populations of cells at best containing a minor subpopulation of true stem cells, we suggest adapting the expression *stem/progenitor cell* as has been established to describe hematopoietic stem/progenitor cells. The mixed population of human $CD34^+$ blood cells is mostly comprised of hematopoietic progenitor cells with a minor subfraction of true stem cells. MSPCs are considered to originate from the mesodermal germ layer to be an integral part of the stroma in every organ and perhaps related to pericytes (da Silva et al. 2006, 2008; Sacchetti et al. 2007; ; Crisan et al. 2008; Bianco et al. 2010). Multiple variables such as species, donor, tissue origin, isolation technique, and culture conditions may influence the composition of cellular subpopulations and genomic stability (Wagner and Ho 2007; Schallmoser et al. 2010). It is a matter of debate whether the capacity to develop into three mesodermal lineages (adipo-, chondro- and osteogenic potential) in fact justifies the attribution of "multipotential differentiation capacity" to MSPCs. As molecular standards for identifying homogenous MSPC subtypes are still missing, the prospective development of consistent MSPC isolation and expansion procedures is essential for standardized cell therapy strategies.

5.2 Isolation and Bioprocessing of MSPCs

5.2.1 MSPC Sources and Starting Material

Samples of BM, white adipose tissue, or umbilical cord blood are most frequently used for the isolation of MSPCs. In addition, umbilical cord, amniotic fluid, placenta, tendons, and muscles are possible sources for the isolation of MSPCs (Kern et al. 2006; Reinisch and Strunk 2009; Tolar et al. 2010; Mosna et al. 2010). A quite practical possibility to gain BM-derived MSPCs is to wash out remnant cells from bags and filters used for BM transplantation (Capelli et al. 2007). Although the further enrichment of the BM mononuclear cells by density gradient centrifugation is a common technique (Mosna et al. 2010), it is not mandatory for efficient expansion

(Schallmoser et al. 2007; Schallmoser et al. 2008). A clinical MSPC production must comply with good manufacturing practice (GMP) and, in our view, should avoid density gradient centrifugation or other extensive manipulation of the primary material. Notably, when seeding unfractionated BM aspirates, one is recommended to remove the nonadherent hematopoietic cell layer after 2–3 days by repeatedly rinsing with PBS and a complete medium change (Rohde et al. 2008). When starting MSPC isolation from umbilical cord, the material can be cut into 1–2 mm pieces. Sticking to the plastic surface, these cord fragments are the source of outgrowing MSPCs (Reinisch and Strunk 2009). Adipose tissue is readily available from liposuction procedures, and MSPCs are isolated after digesting the lipoaspirate by collagenase type I, which can be produced completely devoid of animal-based components (www.worthington-biochem.com/cls/default.html). After several washing steps, final filtration, and seeding of the resuspended cells, nonadherent cells are removed after 72 h by washing and medium change (Zuk et al. 2002; Kern et al. 2006; Mosna et al. 2010). New technologies for the concentration of mononuclear cells including MSPCs from BM aspirates are under investigation. For instance, a closed system known as the BMAC (bone marrow aspirate concentrate) method enables the immediate application of MSPCs on biomaterials for bone regeneration as a basis for dental implants (Sauerbier et al. 2010).

5.2.2 Coating of Culture Vessels

The adhesion of MSPCs as well as the proliferation, migration, and function depend on the particular characteristics of the culture dishes. To some extent, cells are more likely to attach to specific adsorbed proteins than to plain plastic or glass surfaces (Cooke et al. 2008; Lindner et al. 2010). To mimic in vivo conditions, some protocols comprise the coating of cell culture glass or plastic with purified animal-derived extracellular matrix (ECM) components prior to seeding. Additionally, ECM substances like collagens, laminin, thrombospondin, vitronectin, or fibronectin may be produced by the cells themselves or are present in the serum added to the culture medium (Kleinman et al. 1987). Our own observations with the focus on MSPC adhesion or proliferation indicate that coating is not a strict requirement for efficient isolation (unpublished results, Schallmoser). Therefore, we recommend avoiding this additional manipulation step in a GMP-compliant cell expansion protocol, especially with regards to proteins of animal origin.

5.2.3 Cell Culture Medium and Seeding Density

When testing optimal culture conditions for clinical-scale expansion of MSPCs, Sotiropoulou and co-workers compared different basal media like Dulbecco's modified Eagle's medium (DMEM) with low or high glucose and L-glutamine or DMEM

with Glutamax instead of L-glutamine. Furthermore, Iscove's modified Dulbecco's medium (IMDM) with L-glutamine, alpha-modified Minimum Essential Medium (α-MEM) with L-glutamine or Glutamax®, and Opti-MEM® were tested. These media were all substituted with 10% fetal bovine serum (FBS) (Sotiropoulou et al. 2006). These authors concluded that MSPC isolation and expansion was most efficient in media with an α-MEM base. Additionally, a glucose concentration of 1,000 mg/L compared to 4,500 mg/L in DMEM increased the MSPC yield. Glutamax®, a dipeptide L-alanyl-L-glutamine, was consistently superior with regard to cell proliferation compared to L-glutamine. This effect may be attributed to the greater stability of Glutamax® in aqueous solutions. L-glutamine degrades at 4°C leading to ammonia formation and even cell growth inhibition as previously shown (Butler and Christie 1994). Notably, Dipeptiven®, which is approved for intravenous therapy, is an efficient, stable, and GMP-compliant alternative to L-glutamine for clinically expanded MSPCs. The influence of various culture conditions and seeding densities on MSPC proliferation and clonogenicity has been shown, indicating a higher proliferative capacity for MSPCs cultured at low seeding densities defined as less than 100 MSPCs per cm^2 (Colter et al. 2000, 2001; Sotiropoulou et al. 2006; Bartmann et al., 2007). However, cell colonies after low density seeding evolve sporadically and never reach confluence (Both et al. 2007). When starting with more than 1,000 MSPCs per cm^2, the distribution of attached cells is quite homogenous. Before reaching the state of replicative senescence (>40–50 cumulative population doublings), MSPCs reach 80–90% confluence after as few as 7 to 10 days depending on individual donor variations and the respective medium supplement (Reinisch et al. 2007).

5.3 Human Alternatives to Fetal Bovine Serum

In the last few years, a remarkable change from the use of the standard medium supplement FBS to human alternatives has emerged. The circumvention of the risks of xenoimmunization against bovine antigens and the transmission of pathogens are major goals of this development along with ethical issues concerning crude methods of FBS collection (Selvaggi et al. 1997; Mackensen et al. 2000; Horwitz et al. 2002).

5.3.1 Human Serum

The efficiency of autologous and allogenic human serum has been tested in several studies. In one report, autologous serum was shown to be superior to allogenic serum and to FBS for the stimulation of BM-MSPC proliferation (Shahdadfar et al. 2005). In another study, a higher proliferative effect of allogenic AB serum over FBS was reported in the culture of adipose tissue–derived MSPCs (Kocaoemer et al. 2007).

However, in many applications, autologous serum or serum-free media have not yet proved to be suitable alternatives for FBS (reviewed in Mannello and Tonti 2007; Tonti and Mannello 2008). The preparation and use of human serum derived from allogeneic cord blood may also be an alternative to FBS but the number of studies to date is rather small (Phadnis et al. 2006; Jung et al. 2009).

5.3.2 Serum-Free Media

The replacement of serum in cell culture requires a defined mixture of factors stimulating cell attachment and growth. Also, transport proteins and stabilizing and detoxifying factors should be added in an optimal ratio to the basal medium. Recently, a freely accessible interactive online database (http://www.goodcellculture.com/) facilitating the search for serum-free supplements was created (Brunner et al. 2010). Notably, the term "serum free" does not consequently mean "animal component free," a fact that has to be taken into account when using commercially available supplements for a clinical-grade expansion of MSPCs (Chase et al. 2010). The comparison of a commercial serum-free medium to α-MEM-containing FBS for BM-MSPC expansion revealed an advantage for serum-free culture conditions with regard to cell growth (5.25 compared to 4.25 cumulative population doublings in 18 days) and amount of colony-forming progenitor cells (88 compared to 68 colonies per 5,000 seeded BM mononuclear cells) (Meuleman et al. 2006). In our own experiments, the proliferation and clonogenicity of human umbilical cord–derived MSPCs was lowest in a commercial serum- and animal protein–free medium compared to basal medium α-MEM with either 10% FBS or pooled human platelet lysate (pHPL) (Fig. 5.1).

5.3.3 Platelet-Derived Growth Factors

The use of human platelet lysate (HPL) containing multiple growth factors has been proven by a number of researchers to be superior to FBS or other human serum preparations regarding MSPC proliferation (Doucet et al. 2005; Muller et al. 2006; Capelli et al. 2007; Schallmoser et al. 2007; Reinisch et al. 2007; Lange et al. 2007; Bernardo et al. 2007; Carrancio et al. 2008; Schallmoser et al. 2008; Perez-Ilzarbe et al. 2009; Bieback et al. 2009; Blande et al. 2009; Crespo-Diaz et al. 2010). A plethora of growth factors, hormones, and cytokines are released following several freeze and thaw cycles from fragmented platelets into the supernatant during the production of HPL. We speculate that the successful application of platelet-derived substances to support in vitro cell proliferation is an obvious consequence of their known function in vivo. Factors such as platelet derived growth factors (PDGFs), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), transforming growth factor-β (TGF-β), hepatocyte growth factor (HGF), insulin-like growth

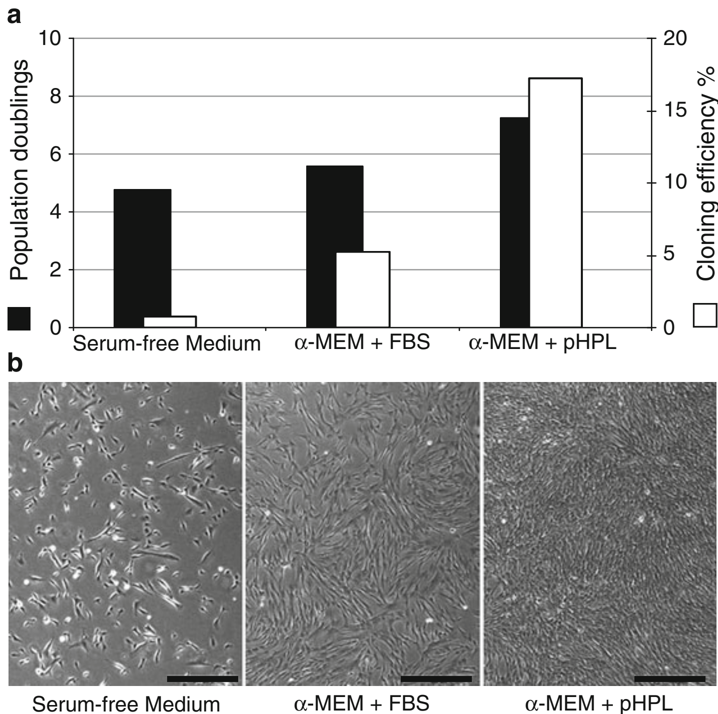

Fig. 5.1 *Proliferation and clonogenicity of MSPCs in serum-free medium compared to fetal bovine serum or pooled human platelet lysate***.** Human umbilical cord–derived MSPCs were seeded at a density of 1,000/cm^2 to test cell proliferation and at 3/cm^2 to determine clonogenicity. (**a**) Cells were harvested after 6 days. Colonies were stained and counted after 13 days. Black columns indicate the population doublings and white columns, the cloning efficiency. (**b**) Representative microphotographs were taken on day 6 before harvest, (40× magnification, size bar 500 μm). Abbreviations: FBS (fetal bovine serum), pHPL (pooled human platelet lysate), α-MEM (alpha-modified Minimum Essential Medium), MSPCs (mesenchymal stem/progenitor cells)

factor-1 (IGF-1), and also vascular endothelial growth factor (VEGF) can act as mitogenic substances and are essential for in vivo wound healing (Nurden et al. 2008; Barrientos et al. 2008). In a current review, the complex biological effects of platelet-derived components on cell migration, proliferation, and differentiation via specific receptors and intracellular signal transduction pathways have been described in detail, emphasizing their role in development and regeneration (Mazzucco et al. 2010). Currently, beneficial effects of platelet preparations are tested in clinical trials for the treatment of chronic ulcers; bone, tendon, and nerve regeneration; and for tissue engineering and cell therapy (www.clinicaltrials.gov) (Anitua et al. 2006; Martinez-Zapata et al. 2009; Foster et al. 2009; Giusti et al. 2009). Some publications even report the combined application of platelet-derived factors with MSPCs (Yamada et al. 2004; Kitoh et al. 2007; Pieri et al. 2009). However, clinical outcomes are often inconclusive probably due to a lack of standardization in in vitro

and in vivo experiments. Prospective randomized clinical studies could provide us with more reliable information regarding MSPC and HPL functionality in vivo (Martinez-Zapata et al. 2009; Borzini and Mazzucco 2005). Platelet concentrates from single donors have been shown to contain varying amounts of growth factors (Weibrich et al. 2002; Frechette et al. 2005). Consecutively, platelet lysate units from individual donors used as supplements for cell cultures differentially stimulated MSPC proliferation (Horn et al. 2010). To overcome this limitation, the development of platelet lysates with the highest possible level of standardization would be preferable for further clinical studies. Recently, a GMP-compliant procedure for the pooling of lysed platelet concentrates from up to 60 individual blood donors has been established (Schallmoser and Strunk 2009, 2011). After a final centrifugation step and the sterile filtration through a 0.2-μm filter, the amount of platelet fragments in cell cultures is decreased to a minimum. This step may help to prevent a putative alloimmunization against platelet antigens and to reduce the tendency for fibrin clot formation. Due to economic concerns, the use of pHPL prepared from outdated platelet concentrates is a possible alternative to FBS and has been proven to be an efficient cell culture supplement comparable to fresh pHPL in our own studies (Fig. 5.2). The efficient utilization of pooled human platelet lysate (pHPL) has not only been proven for human MSPC expansion but also for the culture of human endothelial colony-forming progenitor cells (Reinisch et al. 2009; Reinisch and Strunk 2009; Hofmann et al. 2009)

5.4 MSPC Harvest and Immunophenotyping

Trypsin/EDTA composed of 0.5 g/L porcine trypsin and 0.2 g/L EDTA in Hank's Balanced Salt Solution is a widely used enzyme/chelator combined preparation for detaching adherent cells. Alternatively, there are at least two commercially available biological substitutes for porcine trypsin provided along with certificates for GMP-grade production (TrypZean, Sigma-Aldrich, ref. T3449 and TrypLE™ Express, Invitrogen ref. 12605–028). Whereby the first consists of bovine trypsin expressed in recombinant maize, the latter is not trypsin but a "trypsin-like enzyme," extracted from a nongenetically modified microorganism (not defined in detail). The manufacturers have adapted the concentration of recombinant trypsin as well as the protease activity of TrypLE in the ready-to-use solutions so that it matches that of conventional trypsin/EDTA. Therefore, protocol changes to conventional harvesting methods can be kept to a minimum (Carvalho et al. 2011). Our own validation experiments were performed with BM-derived MSPCs cultured in α-MEM/10% pHPL until they reached approximately 70% confluence. Trypsin/EDTA, TrypZean, and TrypLE were applied for 2, 5, or 10 min at 37°C, 5% CO_2. Cell count and viability data indicated a very similar performance of all three enzymatic/chelator preparations with a slightly increased toxicity after prolonged exposure to trypsin/EDTA (Fig. 5.3). The cumulative effect of reattachment, lag phase, and subsequent proliferation was tested by reseeding MSPCs from the nine previously described conditions

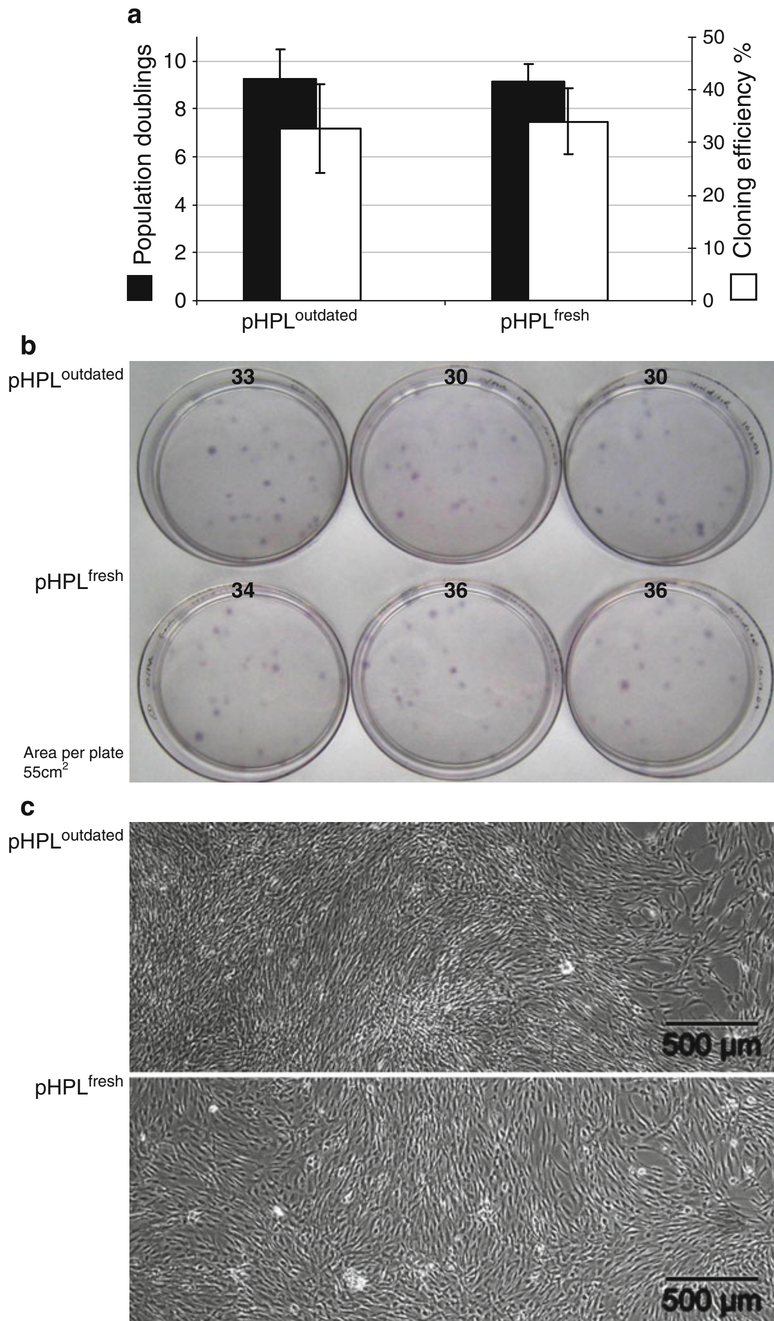

Fig. 5.2 *Proliferation support and cloning efficiency is retained in pooled human platelet lysate generated from outdated platelet concentrates*. We compared the proliferation and clonogenicity support of pHPL generated from either fresh PRP (pHPL^{fresh}) or PRP stored for 6 to 7 days at room

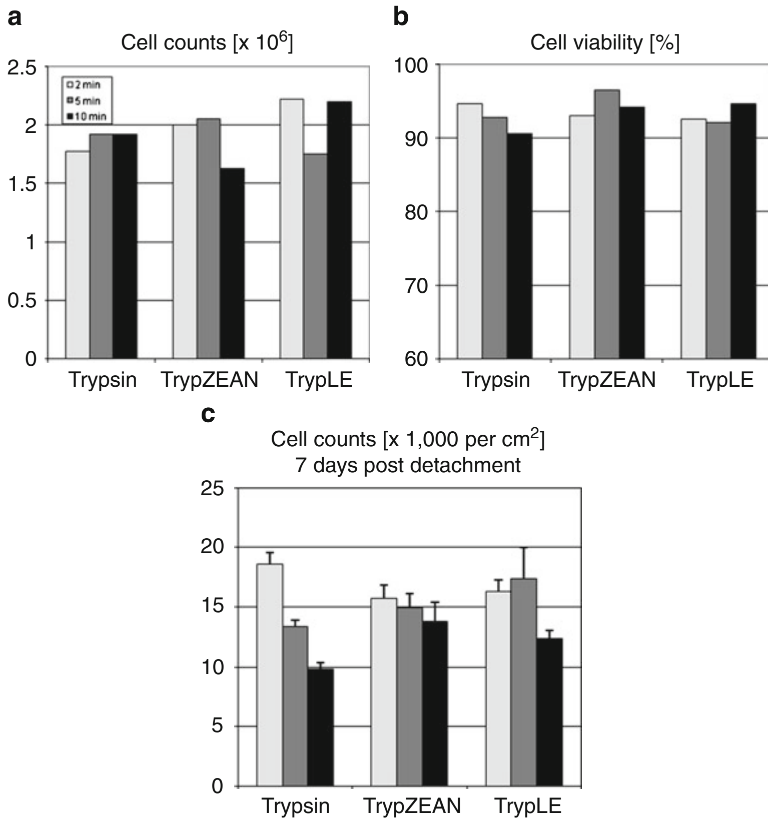

Fig. 5.3 ***Impact on MSPC characteristics of animal protein–free enzymatic treatment compared to porcine trypsin.*** BM-MSPCs were harvested after culture in α-MEM/10% pHPL at approximately 70% confluence. A primary treatment with trypsin/EDTA, TrypZean, and TrypLE for 2, 5, or 10 min revealed no difference regarding cell counts or viability (**a** and **b**). After reseeding and 7 days of culture, a considerable reduction of the cell count per cm^2 was found only in the prolonged treatment group with porcine trypsin/EDTA (**c**). Abbreviations: MSPCs (mesenchymal stem/progenitor cells), pHPL (pooled human platelet lysate), α-MEM (alpha-modified Minimum Essential Medium)

Fig. 5.2 (continued) temperature ($pHPL^{outdated}$) from identical starting samples. BM-derived MSPCs of three donors were seeded in α-MEM/10% pHPL at a density of 30/cm^2 for testing cell proliferation and 3/cm^2 for analyzing clonogenicity (**a** and **b**). Cells were harvested after 10 days. Colonies were stained and counted after 11 days. There were no significant differences detectable with regards to cell or colony count. (**c**) Representative microphotographs were taken on day 10 before harvest, (40× magnification, size bar 500 μm). Abbreviations: PRP (platelet-rich plasma), pHPL (pooled human platelet lysate), α-MEM (alpha-modified Minimum Essential Medium), BM (bone marrow), MSPCs (mesenchymal stem/progenitor cells)

in triplicates. After 7 days of culture and before reaching confluence, cells were harvested using conventional trypsin/EDTA. Cell proliferation was comparable in all conditions with the exception of the prolonged exposure to conventional trypsin/EDTA. This phenomenon indicating a negative effect on MSPC proliferation was not observed in either animal protein–free enzyme preparations (Fig. 5.3). In addition, the surface antigens CD3, CD13, CD14, CD15, CD19, CD29, CD31, CD34, CD44, CD45, CD49a, CD56, CD63, CD73, CD90, CD105, CD146, CD166, CD271, HLA-ABC, HLA-DR, MSCA-1, and Stro-1 were tested after the different harvest procedures by flow cytometry. The antigenic profile was identical after all tested harvest conditions, and the classical markers such as CD13, CD29, CD73, CD90, CD105 were expressed on all viable MSPCs (data not shown). Significant differences in efficiency of detachment, cell viability, and preservation of surface antigens could not be found among the different enzyme preparations. However, a slight decrease in proliferation and viability was observed after prolonged treatment with porcine trypsin/EDTA, a fact that justifies even more a switch to animal protein–free enzymatic preparations.

5.5 Differentiation Potential In Vitro

MSPCs are key candidates for cell therapy in regenerative medicine. Therefore, their potential for differentiation into chondroblasts and osteoblasts for articular cartilage and bone repair, and also adipocytes for plastic surgery, is of high interest. Notably, the differentiation profile of MSPCs may vary depending on the tissue origin (da Silva et al. 2006; Kern et al. 2006; Baksh et al. 2007; Rebelatto et al. 2008) and culture conditions. We and others have previously shown a comparable multilineage differentiation capacity in vitro for MSPCs expanded in FBS and human platelet-derived supplements (Capelli et al. 2007; Schallmoser et al. 2007; Reinisch et al. 2007; Bernardo et al. 2007; Perez-Ilzarbe et al. 2009; Bieback et al. 2009; Prins et al. 2009) (see also Table 5.1). On the other hand, accelerated osteogenic and chondrogenic differentiation and up-regulated osteoblastic gene expression of MSPCs induced by the culture in HPL were reported (Zaky et al. 2008; Chevallier et al. 2010). However, as summarized in a recent review (Augello and De Bari 2010), the findings of in vitro studies may not be translated one-to-one into the in vivo situation. So far, three reports indicate that bone mineralization in vivo of MSPCs is enhanced for cells cultured in HPL (Zaky et al. 2008; Prins et al. 2009; Chevallier et al. 2010); however, the long-term persistence of transplanted cells is proven in only one report (Zaky et al. 2008).

5.6 Genomic Stability of MSPCs

The putative risk of malignant transformation during the process of large-scale MSPC expansion for clinical application is a major concern. Until now, a few studies have analyzed long-term cultured human MSPCs under animal protein–free

Table 5.1 Toward clinical-grade MSPC generation: Selected studies comparing the biologic and functional characteristics of MSPCs expanded with animal protein–free or animal-derived factor using protocols.

MSPC Characteristics:	Animal protein–free protocols	Protocols using animal-derived factors	Reference:
Proliferation Support	↑	↓	
Immunophenotype profile	$CD73^+/90^+/105^+/14^-/45^-$	$CD73^+/90^+/105^+/14^-/45^-$	
T cell alloreactivity (MLR)	Suppressed	Suppressed	
Differentiation capacity	Osteo-, chondro- and adipogenic lineage	Osteo-, chondro- and adipogenic lineage	Doucet et al. 2005
Proliferation support	↑	↓	
Immunophenotype profile	$CD90^+/105^+/45^-$	$CD90^+/105^+/45^-$	
T cell alloreactivity (MLR)	Suppressed	Not tested	
Karyotyping	FISH and GTG-banding	Not tested	Lange et al. 2007
Proliferation support	↑	↓	
Immunophenotype profile	Basic phenotype, $HLA\text{-}ABC^+ /13^+/29^+/105^+/146^+/31^-/34^-/56^-$	Basic phenotype, $HLA\text{-}ABC^+ /13^+/29^+/105^+/146^+/31^-/34^-/56^-$	
Differentiation capacity	Osteo- and adipogenic lineage	Osteo- and adipogenic lineage	
In vivo tumorigenicity	No tumor found in immune incompetent mice	No tumor found in immune incompetent mice	Schallmoser et al. 2007
Proliferation support	↑	↓	
Immunophenotype profile	Basic phenotype, $HLA\text{-}ABC^+/ CD44^+/31^-$	Basic phenotype, $HLA\text{-}ABC^+/ CD44^+/31^-$	
Differentiation capacity	Osteo- and adipogenic lineage	Osteo- and adipogenic lineage	Kocaoemer et al. 2007
Proliferation support	↑	↓	
Immunophenotype profile	Basic phenotype, $CD105^+/34^-$	Basic phenotype, $CD105^+/34^-$	
T cell alloreactivity (MLR)	Suppressed	Suppressed	
Differentiation capacity	Osteo- and adipogenic lineage	Osteo- and adipogenic lineage	Capelli et al. 2007

(continued)

Table 5.1 (continued)

MSPC Characteristics:	Animal protein–free protocols	Protocols using animal-derived factors	Reference:
Proliferation support	↑	↓	
Immunophenotype profile	Basic phenotype, HLA-ABC$^+$/ CD13$^+$/105$^+$/31$^-$/34$^-$/80$^-$	Basic phenotype, HLA-ABC$^+$/ CD13$^+$/105$^+$/31$^-$/34$^-$/80$^-$	
T cell alloreactivity (MLR)	Suppressed	Suppressed	
Differentiation capacity	Osteo- and adipogenic lineage	Osteo- and adipogenic lineage	
Karyotyping	Array-CGH ⊥	Array-CGH ⊥	Bernardo et al. 2007
Proliferation support	Equal	Equal	
Immunophenotype profile	Basic phenotype, HLA-ABC$^+$/ CD13$^+$	Basic phenotype, HLA-ABC$^+$/ CD13$^+$	
Differentiation capacity	Osteo-, chondro- and adipogenic lineage	Osteo-, chondro- and adipogenic lineage	
			Reinisch et al. 2007
Proliferation support	↓ (!)	↑ (!)	
Immunophenotype profile	CD73$^+$/90$^+$/105$^+$/45$^-$/ HLA-DR$^+$ < 20%	CD73$^+$/90$^+$/105$^+$/45$^-$/ HLA-DR$^+$ < 50%	
T cell alloreactivity (MLR)	Not tested	Suppressed	
Karyotyping	FISH and G-banding: Aneuploidy in 1/5 samples at passage 1 and aneuploidy in 1/4 samples at passage 2	Aneuploidy in 4/15 samples at passage 1 and aneuploidy in 1/7 samples at passage 2	
In vivo tumorigenicity	Not tested	No tumor found in immune incompetent mice No tumor formation in 1 patient after 2-year follow-up	Tarte et al. 2010

Abbreviations: Basic phenotype: CD73$^+$/90$^+$/HLA-DR$^-$/CD14$^-$/45$^-$

MLR mixed lymphocyte reaction, *FISH* fluorescence in-situ hybridization, *Array-CGH* array-comparative genome hybridization

conditions and could prove genomic stability despite extensive proliferation rates (Table 5.1) (Schallmoser et al. 2007, 2008; Bernardo et al. 2007, 2009). One recent study reported chromosomal instabilities after MSPC expansions with both animal protein–free and conventional FBS cultures. Cells with aneuploidy were transplanted into immunocompromised mice. Accidentally, one patient received MSPCs with chromosomal alterations. Authors argued that a progressive growth arrest and the reaching of senescence without evidence for transformation was the reason for the absence of tumor formation in a 2-year follow-up period (Tarte et al. 2010).

5.7 Conclusion

The development of further optimized serum-free formulations consisting of well-defined substances for cell culture is an imperative goal in the growing field of clinical stem cell research and regenerative medicine. Notably, the influence of human platelet-derived growth factors on MSPC function in vitro and in vivo is observed in an increasing number of preclinical and clinical studies. To date, FBS is still the most frequently used culture medium supplement, but a change to protocols employing platelet-derived factors or pHPL may represent an initial milestone toward controlled clinical-grade, animal protein–free MSPC expansion.

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Chapter 6
Defining Hierarchies of Unrestricted Somatic Stem Cells and Mesenchymal Stem Cells in Cord Blood

Gesine Kögler

Abstract Multipotent cells termed *unrestricted somatic stem cells* (USSC) were recently described with the potential to differentiate into mesodermal cells, cardiomyocytes under specialized circumstances, ectodermal neural cells, and endodermal hepatic cells. More recently, it has become clear that USSCs are heterogeneous with regard to their differentiation potential, and distinct functional differences in differentiation potential suggests USSCs are at different developmental stages. Expression of particular genes and differentiation toward the adipogenic lineage can discriminate USSCs from other related cell types such as CB-derived mesenchymal stromal cells. Current work regarding the characterization and differentiation of USSC and their distinct differentiation potential in comparison to other cell populations may enable us to distinguish several distinct multipotent progenitor cell populations.

In 2004, our group described multipotent cells, which were called *unrestricted somatic stem cells* (USSC). USSC were described with the potential to differentiate into mesodermal cells (osteoblasts, chondroblasts, but also to adipocytes), cardiomyocytes – however only in the preimmune fetal sheep model and not in porcine, to ectodermal neural cells in vitro and to endodermal hepatic cells. During the recent years it has become clear that the so-called homogeneous population of USSC was homogenous with regard to the immunophenotype, but heterogeneous with regard to their differentiation potential, since it was composed of distinct cell population/clones. Functional differences in the differentiation potential suggest different developmental stages. Expression of genes and differentiation toward the adipogenic lineage can discriminate between these two populations. USSC, including clonally derived cells lacking adipogenic differentiation, strongly expressed delta-like

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1/preadipocyte factor 1 (DLK-1/PREF1) correlating with high proliferative potential, while CB MSC showed a strong differentiation toward adipocytes correlating with a weak or negative DLK-1/PREF1 expression. Based on the adipogenic differentiation potential, the inversely correlated DLK1 expression and the HOX profile we are now able to clearly distinguish USSC from CB MSC. Current work regarding the characterization and differentiation of USSC and CB MSC by DLK1 and HOX-expression, and their distinct differentiation potential in vitro and in vivo also in comparison to other cell populations, such as BM MSC, will enable us to distinguish several different multipotent progenitor cell populations.

6.1 Why Do We Find Nonhematopoietic Progenitors in Cord Blood?

It has already been shown that hematopoietic cells (HSC) develop during embryogenesis and fetal life in a complex process involving multiple anatomic sites and niches (yolk sac, the aorta-gonad-mesonephrons region, placenta, and fetal liver), before they colonize the bone marrow at birth. As fetal and neonatal hematopoietic cells are markedly different from adult HSC with respect to their cell cycle status and proliferative capacity, it was conceivable that different mechanisms/niches control engraftment and self-renewal of HSC during fetal and adult life. Since fetal blood is formed in close association with organs, the search for cell functions as niches similar to cell types present in the adult BM environment (reticular cells, osteoblasts, adipocytes, endothelial cells, fibroblasts) was a logical consequence. In 2000, Erices described multipotent mesenchymal stromal cells (MSC) from cord blood which revealed an immunophenotype (CD45−, CD13+, CD29+, CD73+, CD105+) (Erices et al. 2000) similar to bone marrow (BM)-derived mesenchymal stroma cells, followed by many other publications summarized by Kögler et al. (2009). In 2004, our group was able to detect cells in CB with an even broader differentiation potential, the so-called unrestricted somatic stem cells (USSC) (Kogler et al. 2004, 2005) and the data were confirmed by other groups (Jansen et al. 2010; Ghodsizad et al. 2009; van den Berk et al. 2009). In a 2004 publication (Kogler et al. 2004), USSC were described as a homogenous cell population with respect to their phenotype. However, as of today further characterization revealed distinct adherent stem cell populations within cord blood (Kluth et al. 2010; Liedtke et al. 2010) which are not distinguishable by their phenotype detected by FACS analysis (Fig. 6.1). Markers described in the literature for defined subtypes of MSC, e.g., CD271, CD140b, STRO-1, GD2, or NG2, could not be translated for cord blood into functional subpopulations (data not shown).

The concept that one progenitor in cord blood can give rise to multiple tissues of different germ layers was not proven in vivo, and in vitro data using artificial culture systems (Wernet et al. 2010) should be discussed with a great deal of skepticism. During prenatal organogenesis, the series of tissues are generated by a system of different progenitors, rather than a common ancestor. Endodermal cells, skeletal

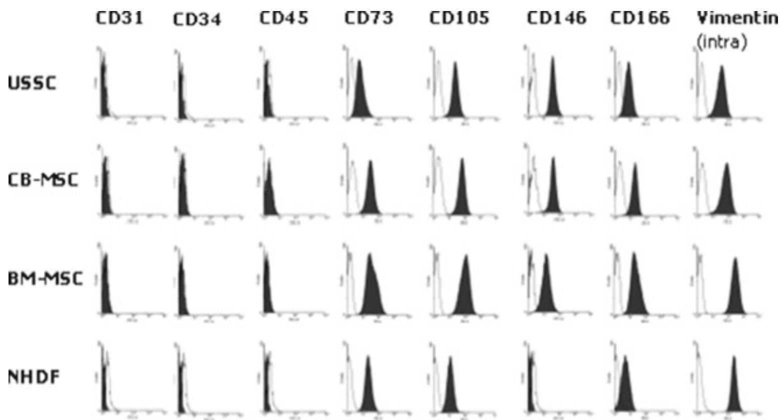

Fig. 6.1 Representative FACS analysis displaying the phenotype of cord blood–derived adherent stem cell populations (USSC and CB MSC) in comparison to bone marrow–derived mesenchymal stem cells (BM MSC) and normal human dermal fibroblasts (NHDF). Note: normal human dermal fibroblasts (NHDF) are negative for CD146

cells, and neural cells are derived from different progenitors. Therefore, multipotency (a property of a single cell or a defined cell population) must include both clonal and nonclonal cell populations and subsequent analysis in vitro and in vivo.

6.2 Isolation, Expansion, and Characterization of Cord Blood–Derived Adherent Cells

USSC and CB MSC cultures were generated by the same method. Classification of the adherent cells into USSC and CB MSC was only possible after generation by determining the adipogenic differentiation potential and DLK1 expression (Kluth et al. 2010). CB was collected from an umbilical cord vein with the informed consent of the mother. Mononuclear cells (MNC) were obtained by ficoll (Biochrom, density 1.077 g/cm^3) gradient separation followed by ammonium chloride lysis of RBCs. Five to seven 10^6 CB MNC/ml were cultured in DMEM low glucose (Cambrex) with 30% FCS (Perbio), 10^{-7} M dexamethasone (Sigma-Aldrich), penicillin/streptomycin and L-glutamine (PSG; Cambrex). Over the last few years, we have initiated adherent cell cultures from 860 CB samples, from which 43% ($n=370$) gave rise to an average of 1–11 colonies per CB (Fig. 6.2). After trypsinization, these spindle-shaped cells grew into monolayers within 2–3 weeks. Cell lines that did not reach more than two passages ($n=196$; 53%) were not characterized. Once established, 10% ($n=37$) of these cell lines reached passage (P) 3–4, 6% ($n=22$) P5–6, 21% ($n=77$) P7–8, and 10% ($n=37$) yielded more than nine passages (Kluth et al. 2010). As early as passage 4, 1.5×10^9 cells were obtained after expansion.

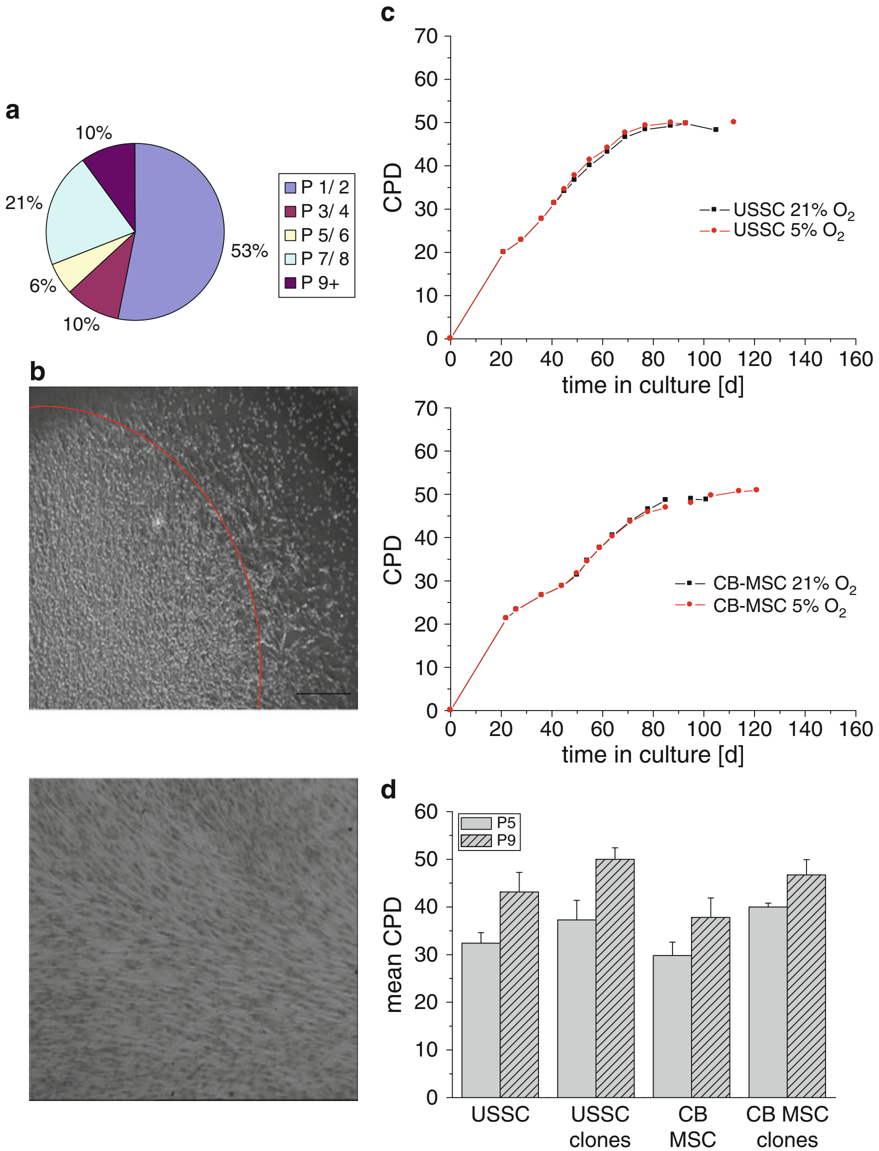

Fig. 6.2 Isolation, expansion, and characterization of USSC and CB MSC. (**a**) Generation frequency of cord blood–derived stem cells. Fifty-three percent of primary cultures did not reach more than two passages. Only 10% reached passage 10 and more. (**b**) Morphology of primary USSC colonies and USSC at passage 5. (**c**) Representative growth curves of USSC and CB MSC at 21% and 5% O_2. (**d**) Cumulative population doublings of USSC, CB MSC and corresponding clonal populations after passage 5 and 9

If cells reached passage 9 ($n=37$), they could be further expanded to more than 20 passages, theoretically yielding up to 10^{15} cells. USSC and CB MSC were always generated from fresh cord blood (<36 h after delivery). Variation of culture conditions as hypoxia (3% and 5% O_2) did not result in higher generation and expansion capacity of USSC and CB MSC (Fig. 6.2). USSC and the respective clones reached higher cumulative population doublings in passage 9 compared to CBMS.

6.2.1 GMP-Conform Generation and Cultivation of USSC and MSC from Fresh Cord Blood

Generation and expansion under GMP conditions is mandatory for potential use in clinical application (and also for preclinical data validation). The automated cell-processing system Sepax (BIOSAFE) with the CS900 separation kit was used for mononuclear cell separation from cord blood in a similar way as described for bone marrow MNC[61]. For the subsequent generation of USSC/MSC colonies, 30% GMP-grade fetal calf serum, low-glucose DMEM-medium/10^{-7} M dexamethasone was used. Expansion of USSC/CBMSC was performed in a closed system applying cell stacks (Costar Corning). Results achieved so far indicate that the generation frequency and quality of generated USSC under GMP conditions are equal to manual generation under laboratory conditions. One hundred and twenty-four cord blood units were processed, resulting in USSC-colony formations in 53 of the samples within 14–28 days. The closed system applied was perfectly suitable to ensure safe and easy handling of the USSC/CBMSC, including seeding, trypsinization, and harvesting. The combination of this procedure together with the cell stack system (1, 2, 5, and 10 layers) yielded cell numbers of 1×10^9 USSC obtained within four passages (Aktas et al. 2010). These USSC products were cryopreserved, thawed, and expanded further in clinical grade quality.

6.2.2 Generation of Cell Clones and Clonal Populations

Generation of cell clones and clonal populations was established during the last 3 years. Clonal populations were obtained from established cell lines and, as an additional approach, special cloning cylinders were applied on the generated colonies. In this case, cell lines were generated as described above and, if distinct, separate colonies were observed (Fig. 6.2), a cloning cylinder was attached on a single colony, and cells were trypsinated according to the standard protocol. Cells of one colony were subsequently plated at low density into six-well cell culture plates, and single cells were picked using the AVISO CellCelector™ (Kluth et al. 2010). Remaining cells were expanded as a whole bulk culture and referred to as initial cell line. With this approach, clonal lines were established from the youngest cells.

Clonal cells were plated at low density (166 cells/cm^2) in six-well cell culture plates and after allowing the cells to become adherent again, distinct single cells were selected, picked and transported to a defined destination well of a 96-well cell culture plate and cultured with preconditioned medium. For verification, pictures were taken before and after each picking process to document successful single cell selection (Kluth et al. 2010).

6.3 Hematopoiesis-Supporting Activity of GMP-Grade-Produced USSC and MSC from Cord Blood In Vitro and In Vivo

USSC and CBMSC produce significant amounts of hematopoiesis-supporting cytokines and are superior to BMMSC in supporting the expansion of CD34$^+$ cells from CB (Kogler et al. 2005; Jeltsch et al. 2010). USSC are therefore a suitable candidate for stroma-driven ex vivo expansion of hematopoietic CB cells for short-term reconstitution. In vivo data of Chan et al. (2007) have shown that USSC induced a significant enhancement of CD34$^+$ cell homing to both bone marrow and spleen.

In a recent publication in cooperation with the group of the DKFZ in Heidelberg, it was shown that 4 weeks after transplantation, homing of human cells (CD45+) to the bone marrow of NOD/SCID mice was significantly increased in mice cotransplanted with CD34+ cells and USSC (median 30.9%; range from 7% to 50%), as compared to the CD34+ cells only control group (median 5.9%; range from 3% to 10%; $p = 0.004$) (Jeltsch et al. 2010). An in vivo tumorigenicity assay showed no tumorigenic potential of USSC. This preclinical study clearly showed that USSC produced under GMP-grade conditions have an enhancing effect on engraftment of CD34+ cells and demonstrate a safe application.

6.4 Differentiation Potential of USSC and CBMSC

6.4.1 In Vitro Differentiation into Osteoblasts and Chondroblasts

Differentiation into osteoblasts and chondroblasts was performed as described previously. Differentiation of CB-derived cells (USSC and CB MSC) into osteoblasts was induced by culturing the cells in the presence of dexamethasone, ascorbic acid, and β-glycerol phosphate (Kluth et al. 2010). Cells formed nodules, which were stained with alizarin red or von Kossa (Fig. 6.3), an indication for osteoblast-typical calcification and functional competency of the differentiated cells. Bone-specific alkaline phosphatase (ALP) activity was detected, and continuous increases in Ca^{2+} release was documented (data not shown). Osteogenic differentiation was further

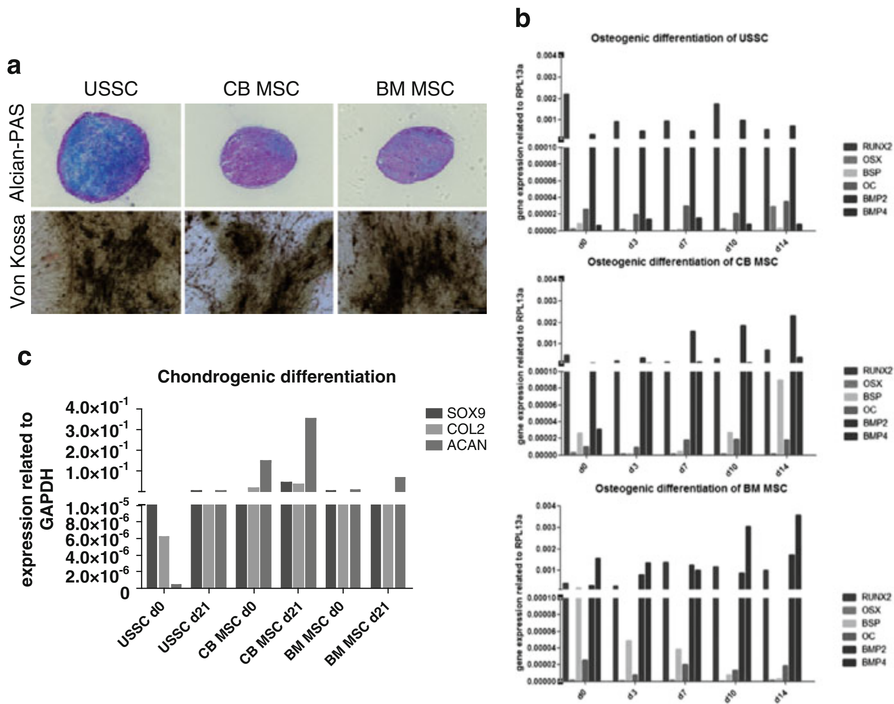

Fig. 6.3 (**a**) Exemplified Alcian-PAS and von Kossa staining displaying osteogenic differentiation. (**b**) Osteogenic markers *Runx2*, *OSX*, *BSP*, *OC*, *BMP2*, and *BMP4* were evaluated by qPCR at different time points during osteogenic differentiation in USSC, CB MSC, and BM MSC. (**c**) Chondrogenic markers *SOX9*, *COL2*, and *ACAN* were evaluated by qPCR at different time points during chondrogenic differentiation in USSC, CB MSC, and BM MSC

confirmed by the detection of Runx2, osterix (OSX), bone sialo protein (BSP), osteocalcin (OC), BMP2, and BMP4 and detected by RT-PCR/qPCR (Fig. 6.3). A pellet culture technique in the presence of dexamethasone, ascorbic-acid-2-phosphate, sodium pyruvate, ITS + premix, and TGFβ1 was employed to trigger CB-derived cells (USSC and CB MSC) toward the chondrogenic lineage (Kluth et al. 2010). The chondrogenic nature of differentiated cells was assessed by Alcian blue staining (Fig. 6.3) and by expression analysis of the cartilage extracellular protein collagen type II. Chondrogenesis was further confirmed by RT-PCR showing expression of the cartilage-specific mRNAs encoding Sox9, collagen type II, and aggrecan (Fig. 6.3). Size and area of chondrogenic pellets were determined by applying the AVISO CellCelector™ (Kluth et al. 2010). All CB lines tested were capable of differentiating along the osteogenic and chondrogenic differentiation pathways in vitro, however the qPCR data clearly revealed major differences between USSC, CB MSC, and BM MSC.

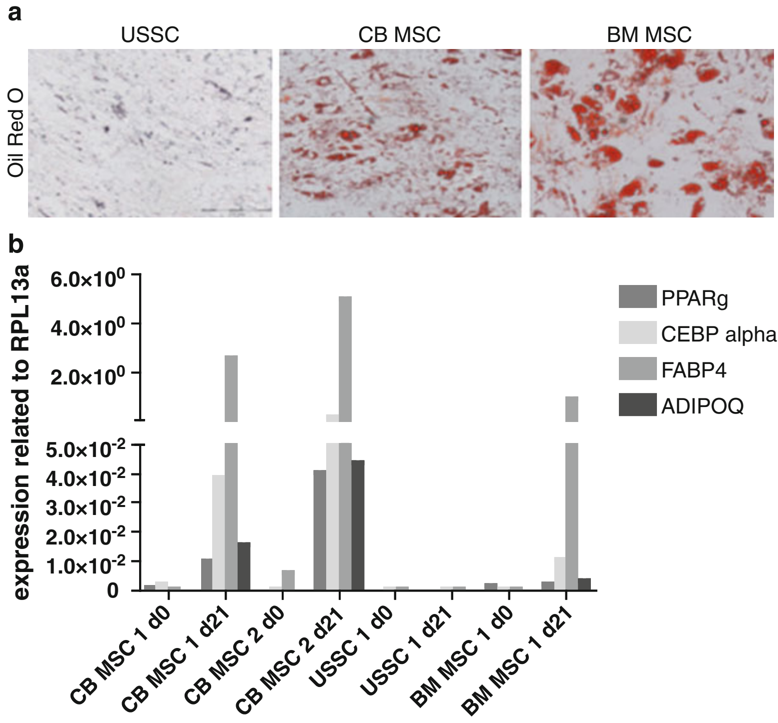

Fig. 6.4 (**a**) Exemplified Oil Red O staining displaying adipogenic differentiation. (**b**) Adipogenic markers *PPARγ*, *CEBPβ*, *FABP4*, and *AdipoQ* were evaluated by qPCR at different time points during adipogenic differentiation in USSC, CB MSC, and BM MSC

6.4.2 Adipogenic Differentiation as a Distinguishing Feature Between USSC and CB MSC

For induction of adipogenic differentiation, CB-derived cells and BM MSC were cultured in the presence of dexamethasone, insulin, IBMX, and indomethacin. Adipogenic differentiation was documented by the Oil Red O staining of lipid vacuoles (Fig. 6.4). On day 14 after induction, 43.1% of 65 CB cell lines and 37.7% of 98 cell clones showed the adipogenic phenotype, while no lipid vacuoles were ever detected in the noninduced control cells. Adipogenic differentiation was documented by expression of peroxisome proliferator activator γ 2 (PPARγ2), adiponectin (ADIPOQ), fatty acid binding protein 4 (FABP4), and CCAAT/enhancer-binding protein alpha (CEBPα) in USSC, CB MSC and BM MSC by qPCR (Fig. 6.4b) and by Western blot analysis as well as immunohistochemical staining for PLIN after 21 days of induction (Kluth et al. 2010). Cell lines capable of differentiating toward the adipogenic lineage (as judged by Oil Red O staining and detection of adipogenic markers) were defined as CB MSC and cell lines that did not show any adipogenic characteristic were defined as USSC.

6.5 Definition of Differences Between USSC and CB MSC on the Basis of DLK

During the last 3 years, we defined differences/hierarchies between the USSC and CB MSC compartment in cord blood in comparison to BM MSC based on several markers. DLK-1/PREF-1 (Delta-like 1/preadipocyte factor 1): In conjunction with the work of Kluth et al., we were able to show that USSC, in contrast to CB MSC and BM MSC, do not differentiate naturally toward the adipogenic lineage. DLK-1 was identified as the distinguishing transcription factor. DLK-1 in the adult controls the lineage commitment (osteogenic, chondrogenic and adipogenic lineage) and differentiation of MSC to adipocytes (Li et al. 2005; Nueda et al. 2008; Ruiz-Hidalgo et al. 2002). Moreover, prolonged expression has been shown to inhibit the differentiation of preadipocytes to mature adipocytes (Sul 2009). DLK-1 is an established player for adipogenesis and exists in two forms, as transmembrane and secreted protein, and is expressed in multiple embryonic tissues (Yevtodiyenko and Schmidt 2006). DLK-1 knockout mice display growth retardation, skeletal malformation, scoliosis, hypotonicity, and obesity (Moon et al. 2002). After birth, DLK-1 is downregulated in most cells of the body, except in preadipocytes, pancreatic β cells, thymocytes, and cells in the adrenal gland. DLK-1 expression was verified by RT-PCR in 31 of 54 CB-derived cell lines and 39 of 114 cell clones. In these cells, none of the adipogenic specific genes (PPARγ2, CEBPα, FABP4, and AdipoQ) were expressed after adipogenic induction, which correlates with a lack of lipid vacuole formation. These cells were therefore named USSC. After adipogenic induction, DLK-1 expression was downregulated in USSC, however not completely absent. Coinciding with the lack of adipogenic differentiation, DLK-1 was highly expressed in USSC as detected by immunohistochemistry and Western blot analysis CB MSC ($n=16$). Cultured preadipocytes expressed very low amounts of DLK-1 but exhibited a strong adipogenic differentiation capacity. DLK-1 expression of BM MSC varied depending on the age of the donors, correlating with a weaker adipogenic differentiation potential of BM MSC from young donors than BM MSC from older donors. With subsequent passages, DLK-1 was completely downregulated, and an adipogenic phenotype was detected (in P10) by Oil Red O staining. Preliminary data reveal that we can define USSC lines/clones with a very high DLK-1 expression profile above the limit of other USSC lines (Fig. 6.5) revealing in addition to the strong DLK-1 expression, overlapping features (cytokine repertoire, data not shown here) with defined progenitors in murine fetal liver. Our group has already been able to show that DLK-1$^+$ USSC can be differentiated into functional hepatic-like cells displaying a hepatocyte-like glucose metabolism (Waclawczyk et al. 2010). In summary, we were able to demonstrate that different nonhematopoietic stem/progenitor cell populations exist in CB (USSC and CB MSC) that can be distinguished by their adipogenic differentiation potential and by their DLK-1 expression profile (Kluth et al. 2010).

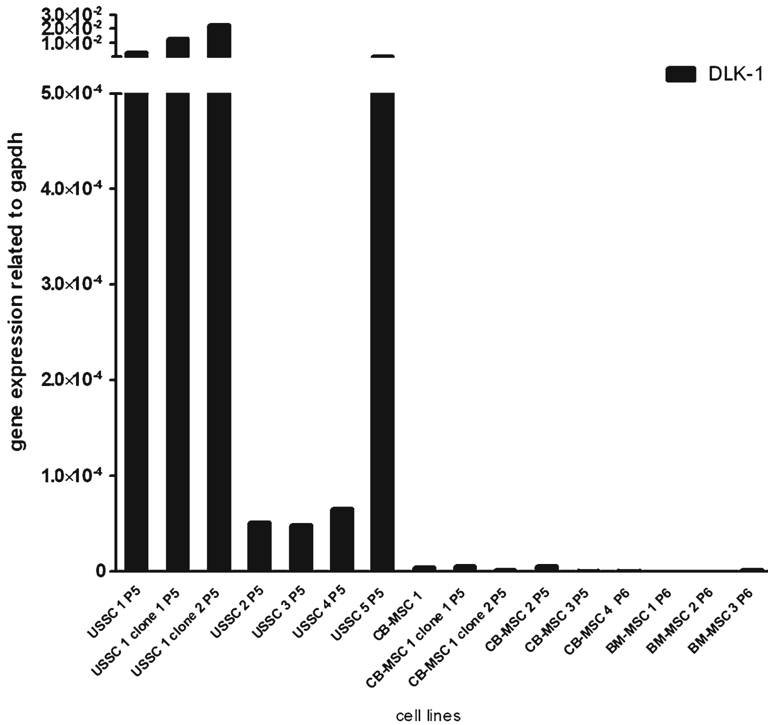

Fig. 6.5 Quantitative *DLK-1* expression of USSC, CB MSC, BM MSC, and respective clones

6.6 Endodermal Differentiation

Organ transplantation provides a definitive cure for pancreatic or liver diseases. Although technical improvements are continuously being made, procedures employed still carry considerable risks and limitations. More importantly, the limited availability of transplantable organs remains a growing impediment to this form of therapy. In comparison, stem cell therapies might be a minimally invasive procedure and could offer a potentially unlimited source of cells for tissue replacement.

To evaluate the potential of USSC to differentiate into liver cells, in utero transplantation into fetal sheep was employed, and livers of the sheep were taken 14 months post USSC transplantation. Of total liver cells, 21.1% ± 3.2% were stained positive by the antibody that specifically recognizes human hepatocytes. Some of the liver cells showed a very strong pattern of albumin staining. A Western blot demonstrated a specific human albumin band, and thus the functional production of this human protein in vivo in serum of the sheep obtained 17 months after transplantation of the USSC in utero. No fusion to host cells occurred (Kogler et al. 2004). Since the in vivo results were very encouraging, our group examined whether it was possible to trigger USSC in vitro into the endodermal differentiation pathway,

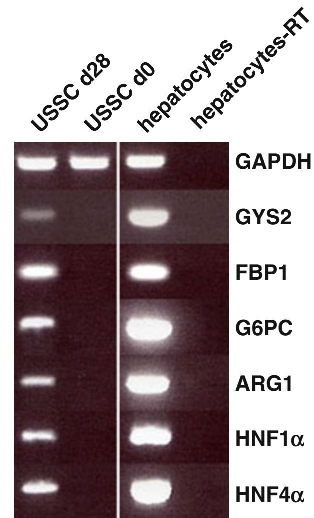

Fig. 6.6 Hepatic differentiation of USSC for 28 days results in a hepatic-like cell type expressing multiple hepatic enzymes and transcription factors. Gene expression changes of undifferentiated (USSC d0) and differentiated USSC (USSC d28) in comparison to primary cultured human hepatocytes. GYS2: glycogen synthase 2; FBP1: fructose-1, 6-biphosphatase 1; G6P: glucose-6-phosphatase; ARG1: arginase 1; HNF1α and HNF4α: hepatocyte nuclear factor 1α and 4α

applying protocols described for both embryonal as well as adult stem cells (Sensken et al. 2007; Waclawczyk et al. 2010).

For this approach, individual USSC with a high DLK-1 expression and corresponding clonal populations were analyzed after applying a three-stage protocol resembling embryonic developmental processes of hepatic endoderm. Hepatic pre-induction was performed by Activin A and FGF4, resulting in enhanced SOX17 and FOXA2 expression demonstrated by real-time PCR and immunohistochemical analysis. Hepatic differentiation was achieved sequentially by retinoic acid, FGF4, HGF, EGF, and OSM resulting in gene expression of glycogensynthase2 (GYS2), glucose 6-phoshatase (G6PC), fructose 1,6-bisphosphatase (FBP1), arginase1 (ARG1) hepatocyte nuclear factor 1α (HNF1α), and hepatocyte nuclear factor 4 α (HNF4α) after differentiation (Fig. 6.6), thus indicating a mature state. Functional testing specified the hepatic-like nature of differentiated USSC by albumin secretion, urea formation, and cytochrome-p450-3A4 (CYP3A4) enzyme activity (Waclawczyk et al. 2010). In order to characterize the differentiated cells at a metabolic level, USSC were incubated with [1-^{13}C] glucose, and neutralized perchloric acid (PCA) extracts were analyzed by NMR spectroscopy (Waclawczyk et al. 2010). Corresponding to GYS2, G6PC, and FBP1 expression, formation of both glycogen and some gluconeogenetic activity could be observed providing evidence of a hepatocyte-like glucose metabolism in differentiated USSC; however, the degree of activity was much lower as compared to adult liver cells (Waclawczyk et al. 2010).

Endodermal differentiation in in vitro MSC from CB was also confirmed by Lee et al. (Lee et al. 2004) following a two-step protocol with hepatocyte growth factor and oncostatin M resulting in in vitro functions of liver cells as phenobarbital-inducible cytochrome p450 activity. Besides USSC and MSC, there are no further substantial data available concerning in vitro endodermal hepatic differentiation.

In light of the encouraging data available with regards to an endodermal liver cell phenotype, one clinical application today could be the regeneration of liver after portal vein embolization in patients with large liver tumors. Preliminarily data applying USSC in an adult sheep model focusing on this approach look promising (personal communication A. Ruhparwar, Medical Center, University of Heidelberg).

6.7 A Hierarchy Based on the Specific HOX Expression Pattern

Based on the adipogenic differentiation potential and the inverse correlated DLK-1 expression (Fig. 6.7), USSC ($n=3$), CB MSC, and BM MSC were subjected to a Chip analysis (Affymetrix) comparing the distinct cell populations. This array (Affymetrix) revealed the HOX genes as the most prominent differentially expressed gene group between USSC and CB MSC (Liedtke et al. 2010). The specific *HOX* code of a cell reflects a continuation of embryonic and fetal patterning and reveals an organ-specific topographic localization during development.

Homeobox genes encode homeodomain-containing transcription factors. In humans, the 39 known *HOX* genes are distributed among four clusters *HOX*A to *HOX*D, located in chromosomes 7, 17, 12, and 2, respectively, and are expressed sequentially 3′ to 5′ along the anterior–posterior axis during embryogenesis, called "temporal and spatial colinearity" (Kmita and Duboule 2003). The typical *HOX* code of a cell describes the specific expression of functionally active *HOX* genes in distinct tissues (Kessel and Gruss 1991). Recent findings revealed that this intrinsic *HOX* code of a cell reflects a continuation of embryonic patterning (Morgan 2006), and several studies have reported on specific *HOX*-gene expression in adult human tissues. A study by Chang et al. revealed that fibroblasts from different anatomic sites across the human body express distinct *HOX* patterns (Chang et al. 2002). In addition, Chang et al. (2002) presented data that more than 1,000 genes are differentially expressed due to the anatomical origin of the cell. Others were able to confirm in mice that the typical *HOX* code can be sufficient to indicate the positional identity of a cell and that the position-specific *HOX* code is independent of the age of the donor (Rinn et al. 2008).

The expression of all 39 human HOX genes in BM MSC, CB MSC, and USSC was further determined by conventional RT-PCR. This analysis of the HOX code revealed a high similarity between BM MSC and CB MSC, which are both HOX-positive, whereas USSC resembled HOX-negative H9 embryonic stem cells/iPS. Based on these results, we defined HOXA9, HOXB7, HOXC10, and HOXD8 as molecular markers to distinguish USSC and CB MSC and validated these markers by quantitative RT-PCR (Liedtke et al. 2010). In addition, several tissues and cell lines were tested as well, leading to a hypothetical hierarchy based on the specific HOX expression pattern (Fig. 6.7). Based on these results, the USSC can be grouped together with the embryonic stem cell line H9/iPS-USSC and HOX negative tissues

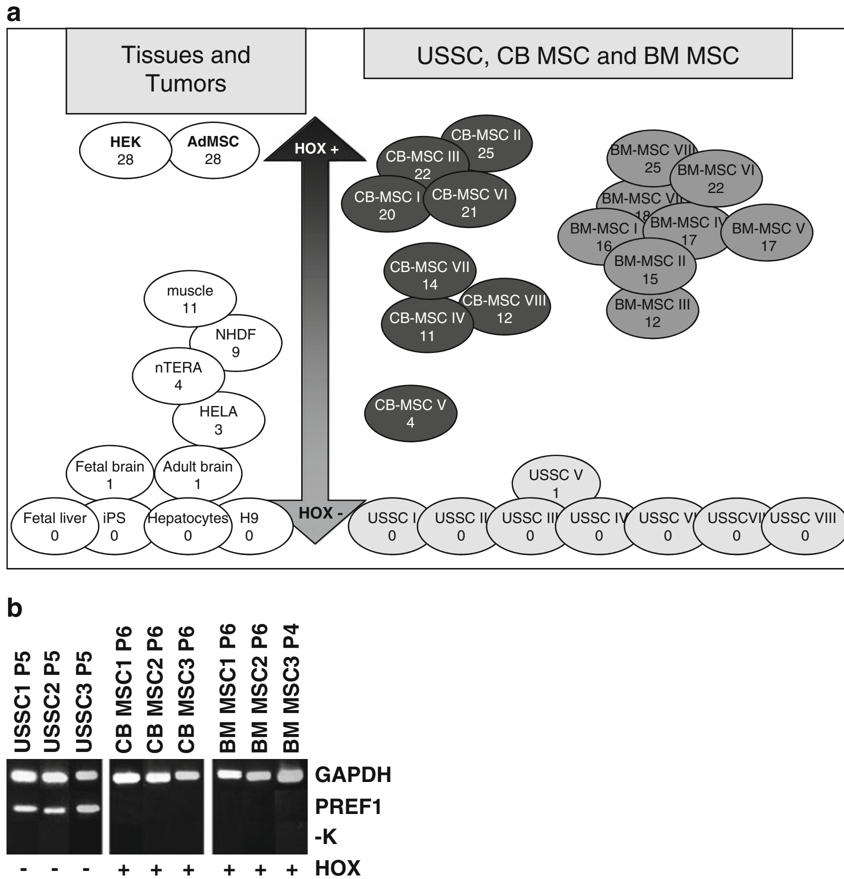

Fig. 6.7 (**a**) Hierarchy based on different *HOX* expression patterns. This schematic view represents complete RT-PCR analysis (39 *HOX* genes) of different tissues and tumors in comparison to the adult stem cell populations USSC, CB MSC, and BM MSC. This hierarchy may reflect the higher immaturity of USSC cells, which are on the same level of embryonic stem cells (H9). CB MSC and BM MSC are more mature maybe reflected by a higher number of expressed *HOX* genes. (**b**) Representative RT PCR displaying *DLK-1* expression in USSC, CB MSC, and BM MSC

like fetal liver and brain. CB MSC revealed a much higher divergence of the expression status of HOX genes as compared to BM MSC. It can be hypothesized that this broader spectrum of divergent HOX expression pattern might reflect additional subtypes of adherent cells derived from cord blood. By analyzing HOX^{low} and HOX^{high} subpopulations, a more accurate hierarchy/definition of subpopulations will be achieved to provide a basic characterization of the cell populations used in further experiments.

6.8 Perspectives and Recommendations

Expanding the use of cord blood–adherent cells (USSC and MSC) for regenerative applications to treat patients would be an enormous practical advancement in stem cell therapeutics. This requires an understanding of the unique properties of each of the presented cell populations, choosing indications where the application of cells is safe, and combining cell populations or amplifying cellular effects in combination with various drugs. Very qualified preclinical animal models together with a state of the art monitoring will be needed in advance of the first in-human clinical trials.

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Chapter 7
Induced Pluripotent Stem Cells from Blood

Ulrich Martin

Abstract The induction of pluripotency in somatic cells is widely considered to be a major breakthrough in regenerative medicine because this approach provides the basis for individualized stem cell–based therapies. Initial reports demonstrated the generation of induced pluripotent iPS cells (iPSCs) from fibroblasts. Since then, pluripotency has been induced in a variety of cell lineages suggesting that the majority of somatic cell types, if not all cells, can be reprogrammed. For broad clinical application, easily accessible cell sources that allow for efficient derivation of patient-specific iPSCs of high biological quality are required. Clearly, blood represents one of the most easily accessible cell sources, and techniques for the induction of pluripotent stem cells from different blood cell types are summarized in this chapter.

7.1 Induction of Pluripotency and Reprogramming of Somatic Cells

In contrast to adult stem cells, pluripotent stem cells, such as embryonic stem cells (ESCs) are characterized by their unlimited potential to grow in vitro and to develop into virtually any cell type. Pluripotent cells of several species including mouse and human can be isolated from early embryos by collecting blastomeres or by isolating the inner cell mass (ICM) of blastocysts and subsequent cultivation in appropriate

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cell culture conditions. Interestingly, these conditions differ distinctly between various mammalian species, and to date, we are still not able to derive true ESCs from species other than mice (Evans and Kaufman 1981), nonhuman primates (Thomson et al. 1995, 1996), humans (Thomson et al. 1998), and rats (Buehr et al. 2008). However, various issues need to be considered with respect to human ESCs for clinical therapies. Besides strong ethical concerns regarding the destructive use of human embryos, the major limitation for clinical use may be an immunologic rejection of allogeneic ESC-derived grafts, which accounts for the latest efforts to explore patient-derived pluripotent stem cells.

Recently, it has been described that somatic cells can be reprogrammed to a pluripotent state through over-expression of several transcription factors including *OCT4* (also known as *POU5F1*), *SOX2, NANOG, MYC, KLF4,* and *LIN28* (Meissner et al. 2007; Park et al. 2008; Takahashi et al. 2007; Yu et al. 2007). These induced pluripotent stem (iPS) cells were largely identical to ESCs, and in vitro differentiation into derivatives of all three germ layers was demonstrated.

The recent reprogramming of somatic cells into pluripotent ES-like cells (Takahashi et al. 2007; Takahashi and Yamanaka 2006) is generally considered to be a revolutionary breakthrough for the development of novel regenerative therapies. However, the initial technique was very inefficient and was restricted to embryonic and adult fibroblasts as source cells. With respect to the generation of clinically useful cells, the classic technology based on retroviral overexpression of several reprogramming factors poses risks including the potential for insertional mutagenesis (Li et al. 2002) and malignant transformation resulting from activation of oncogenic transgenes.

Early reports on the induction of murine and human-induced pluripotent stem cells reported reprogramming efficiencies of about 0.01–0.1% resulting in relatively few fully reprogrammed cell clones. In the meantime, major improvements in reprogramming efficiencies have been achieved. Recent results demonstrated reprogramming of a variety of cell types (e.g., (Aoi et al. 2008; Kim et al. 2008)), including keratinocytes, hair cells (Aasen et al. 2008), and blood cells (Hanna et al. 2008). These results suggest that the majority of somatic cell types if not all cells can be reprogrammed. The efficiency of reprogramming could be dramatically increased up to ~2% for human cells (Lin et al. 2009) and up to 28% for secondary mouse iPS cells in an inducible transgenic mouse model (Eminli et al. 2009). In addition, it has been shown that depending on the cell type, and although very inefficient, iPS cells can be generated using only two (Kim et al. 2008) and even one reprogramming factor (Kim et al. 2009b, c).

These remarkable improvements have been achieved mainly through optimized reprogramming protocols, the use of siRNAs/shRNAs against p53/p21/UTF-1/DNA methyltransferase (Kawamura et al. 2009; Mikkelsen et al. 2008; Zhao et al. 2008) and application of different small molecules for inhibition or activation of different factors and pathways (for a review, Feng et al. (2009) is recommended). These include inhibitors of histone deacetylase (Huangfu et al. 2008), the G9a histone methyltransferase (Shi et al. 2008b), the TGFß- and MEK-ERK pathways (Lin et al. 2009), and an agonist of L-type calcium channels (Shi et al. 2008a). Inhibitors

of GSK-3 (Li et al. 2009; Silva et al. 2008), MAP kinase (Silva et al. 2008), and TGF-ß (Ichida et al. 2009; Maherali and Hochedlinger 2009) have been used to replace KLF4 (Lyssiotis et al. 2009) or SOX-2 (and c-Myc) (Maherali and Hochedlinger 2009; Shi et al. 2008b; Silva et al. 2008). MicroRNA (miR)-based approaches may represent another way to replace integrating vectors, and recent publications have indicated the usefulness of miR 302 and miRs of the 290er cluster, the latter being downstream effectors of c-Myc, for reprogramming of somatic cells (Judson et al. 2009; Lin et al. 2008).

Since all of the typically applied reprogramming factors including OCT4, SOX2, KLF4, MYC, NANOG, and LIN28 can be considered oncogenes and may lead to malignant transformation of iPS-derivatives, the permanent presence of those transgenes in the reprogrammed cells should be avoided and the development of transgene-free iPS cells is mandatory. In addition, insertional mutagenesis associated with integrating vectors may result in malignant transformation and loss of function. Thus alternative approaches are desired for the production of clinically applicable iPS cells, and very recent studies have demonstrated the possibility to use conventional plasmids (Okita et al. 2008), nonintegrating adenoviral (Stadtfeld et al. 2008) and episomal vectors (Yu et al. 2009) as well as protein transduction (Kim et al. 2009a; Utikal et al. 2009) instead of integrating vectors. Another paper demonstrated the generation of transgene-free human iPS cells by means of a vector system based on Sendai virus, an RNA virus without DNA state (Fusaki et al. 2009). Very recently, efficient reprogramming of human cells was shown through simple transfection with synthetic mRNAs (Warren et al. 2010).

Although alternative approaches to induce pluripotent stem cells that avoid integration of transgenes into the host genome have now been demonstrated as generally feasible, most methods are currently largely far from being technically mature: episomal approaches are extremely inefficient, genomic integration is not excluded and oncogenes such as MYC and large T-antigen are required (Yu et al. 2009). Protein transduction is so far extremely inefficient, also, and importantly, requires huge amounts of recombinant proteins (Kim et al. 2009a; Zhou et al. 2009). Whether the reported high efficiencies and robustness of Sendai virus transduction and transfection of synthetic mRNA can be confirmed remains to be demonstrated. Clearly, the above techniques are extremely promising, nevertheless, further significant improvement and development of novel and modified techniques is required.

7.2 iPS Cells from Bone Marrow–Derived and Peripheral Blood–Derived Hematopoietic Cell Lineages

Initial attempts to reprogram hematopoietic cell lineages suggested that induction of pluripotency in these cells is more difficult than in fibroblasts. Hanna et al. realized the impact of stimulating cell proliferation through suitable cytokines and provided initial evidence for the reprogrammability of hematopoietic cells through the induction of pluripotency in murine bone marrow–derived pre- and pro-B-cell subsets

(Hanna et al. 2008). However, in this initial study the reprogramming of mature spleen-derived murine B cells required previous transdifferentiation into adherent macrophage-like cells through retroviral overexpression of the myeloid transcription factor CCAAT/enhancer-binding protein-beta (Hanna et al. 2008).

Subsequent studies demonstrated that pluripotency can be induced in murine spleen–derived mature B cells similar to bone marrow–derived granulocytes (Polo et al. 2010), and that stimulation with Il-2 and anti-CD3 allows for generation of iPS cells from murine spleen–derived T-cells (Hong et al. 2009).

Based on the presumption that reprogramming of less mature hematopoietic cell types would be easier to achieve, several groups attempted the reprogramming of bone marrow–derived hematopoietic cells (Kunisato et al. 2010; Loh et al. 2010; Okabe et al. 2009). Kunisato et al. successfully reprogrammed murine bone marrow–derived mononuclear cells although they were not able to exclude the possibility that pluripotency was induced in mesenchymal rather than hematopoietic cells (Kunisato et al. 2010). However, using an elegant single hematopoietic stem cell (HSC) transplantation model, Okabe et al. were able to definitively prove the reprogramming of HSC-derived hematopoietic cells towards pluripotency (Okabe et al. 2009). Finally, Loh et al. generated human iPSCs from in vitro stimulated CD34^{pos} hematopoietic cells obtained from peripheral blood after G-CSF mobilization (Loh et al. 2010).

The vast majority of iPS cell lines described thus far were isolated from skin fibroblasts or other cell types that require harvesting via surgical interventions. For clinical application, it is therefore desirable to identify alternative reprogrammable cell types that can be collected either without or by means of minimally invasive procedures, such as hair-derived keratinocytes (Aasen et al. 2008).

Peripheral blood represents another cell source that can be easily obtained from most patients. However, reprogramming of predominantly nonadherent and slow-cycling peripheral blood cell types has initially been unsuccessful (Hanna et al. 2008) and G-CSF-mobilization of HSCs as utilized by Loh et al. (Loh et al. 2009) is expensive, time-consuming and may have detrimental effects on individual donors. For these obvious reasons, several groups attempted the reprogramming of various cell types that can be obtained from human peripheral blood without prior stem cell mobilization. In the meantime several groups have succeeded with the reprogramming of different mouse (Watarai et al. 2010) and human blood lineages (Brown et al. 2010; Carette et al. 2010; Kunisato et al. 2011; Loh et al. 2010; Seki et al. 2010; Staerk et al. 2010; Ye et al. 2009). Among these are CD34^{pos} cells (Kunisato et al. 2011; Loh et al. 2010,; Ye et al. 2009), myeloid cells (Carette et al. 2010; Staerk et al. 2010), and lymphocytic cells such as natural killer cells (Watarai et al. 2010), and especially T-cells (Brown et al. 2010; Loh et al. 2010; Seki et al. 2010; Staerk et al. 2010). While stimulated T-cells are obviously easier to reprogram than other cell types from peripheral blood, the clinical use of T cell–derived iPSCs with their clonal TCR rearrangements is probably restricted in the case of hematopoietic reconstitution. Nevertheless, although the observed reprogramming efficiencies for different blood lineages were typically lower than for fibroblasts, these reports clearly demonstrate that iPSCs can be obtained from peripheral blood as an easily accessible cell source.

7.3 iPS Cells from Cord Blood

Despite recent pioneering developments there is still a need to further improve the efficiency of reprogramming and even more important, to resolve certain safety issues. For instance, as is the case for ESCs, there are issues of teratoma formation after transplantation of iPS cell derivatives and of chromosomal abnormalities that could arise during stem cell expansion (Lefort et al. 2008; Spits et al. 2008). Another aspect regarding the production of clinically useful iPS cells concerns the quality of iPS cells derived from somatic cells of aged individuals. In fact, nuclear and mitochondrial mutations in adult stem cells and differentiated somatic lineages appear to accumulate over a lifetime and have been suggested to contribute to aging and cancer formation (Ono et al. 2002; Trifunovic and Larsson 2008; Turker 2000).

Whereas epigenetic changes and loss of telomerase activity in cells of aging individuals may be reversed during the induction of pluripotent stem cells (Marion et al. 2009), acquired chromosomal abnormalities and/or point mutations are not corrected during reprogramming. In addition, somatic cell clones with acquired mutations that result in higher reprogramming efficiency and increased proliferation rates are likely to become enriched during expansion of the primary cell source. This is further enhanced during the reprogramming and proliferation of the resultant iPS cells, thereby supporting an increased cancer risk.

Obviously, human cord blood (CB) represents an easily accessible source of autologous and allogeneic young cells. To date, CB is routinely collected for public and commercial blood banks. CB can be obtained noninvasively and is expected to be superior to cells isolated from aged individuals with respect to the frequency of accumulated somatic mutations. In view of these clinically meaningful features of human CB, several groups have focused on establishing CB-derived iPSC lines. Our group has developed a straightforward methodology to produce iPS cells from human CB (Fig. 7.1). Since recent reports suggested that generation of iPSCs is easier to achieve from adherent and actively dividing cells, we sought a cell type present in CB in sufficient numbers that could be easily obtained by well-established and straightforward protocols. In addition, it was crucial that the cells were adherent under standard culture conditions and maintained proliferation ex vivo. Based on these criteria, we identified CB-derived endothelial cells, as a superior cell type for the induction of pluripotent stem cells (Haase et al. 2009).

In contrast, Giorgetti et al. utilized CB-derived CD34^{pos} cells for reprogramming (Giorgetti et al. 2009) and succeeded with overexpression of OCT4 and SOX2 only (Giorgetti et al. 2010). In addition to fresh blood, Giorgetti et al. demonstrated the generation of iPSCs from frozen cord blood (Giorgetti et al. 2009). Further studies successfully induced pluripotency in CD34^{pos} cells (Takenaka et al. 2010) or unrestricted somatic stem cells from cord blood (Zaehres et al. 2010).

Importantly, the use of CB for the generation of iPS cells sheds new light on the customized collection and storage of autologous CB by commercial CB banks. Thus far, the usefulness of commercially stored autologous CB stem cells has been controversial. The utility of autologous stem cells in the treatment of genetically-based

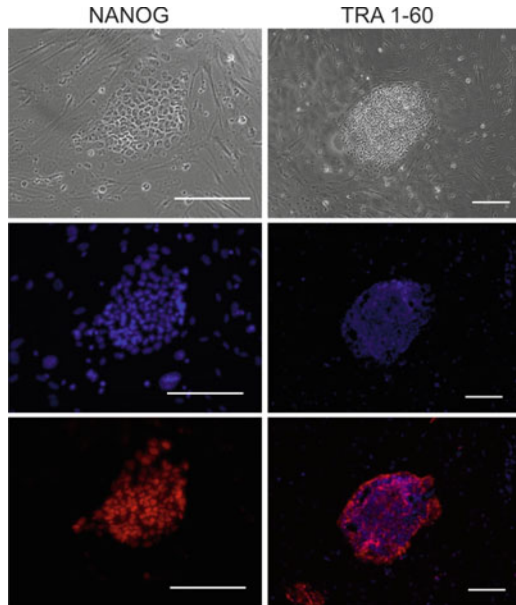

Fig. 7.1 *Phenotype of iPS cells derived from human cord blood.* Embryonic stem cell markers NANOG and TRA 1–60 (*both red*) are shown for human cord blood iPS cells in passage 15. Nuclei are stained with DAPI (*blue*). *Scale bars* represent 100 μm

diseases, such as many types of leukemias, seems to be limited. In addition, there are issues of restricted plasticity and ex vivo proliferative capacity (Hofmeister et al. 2007). In contrast, CB can be used for future therapeutic production of juvenile iPS cells, providing a cell source with biological superiority that could instantly be made available for thousands of pediatric patients, and is of particular relevance for newborns with congenital malformations. For example, a significant number of children born with cardiac malformations could benefit from CB-iPSC-derived tissue transplants in the future. As clinicians frequently have evidence of these defects in utero, CB samples have been prospectively collected and stored in public CB banks with the idea that suitable therapeutic options will become available. In addition, HLA matching of allogeneic CB from public banks could be done, similar to current clinical practice in the treatment of leukemia.

7.4 Conclusions

In conclusion, hematopoietic cells especially from cord blood and peripheral blood represent easily accessible cell sources for the derivation of clinically useful iPSCs. Recent studies provided feasible strategies to reproducibly induce pluripotent stem cells from human blood. In view of the questionable quality of cells of aging individuals, allogeneic and autologous CB from public and commercial CB banking may provide a superior and almost unlimited juvenescent cell source for the production

of clinically useful iPSCs. Certainly, recent progress in the derivation of iPSCs from human blood establishes an important basis for the development of novel cellular therapies eventually addressing a variety of different diseases.

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Chapter 8
Endothelial Progenitors and Repair of Cardiovascular Disease

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Abstract Since their initial description in 1997, considerable effort has been expended defining subsets of endothelial or vascular progenitor cells with the capacity to modulate a host of cardiovascular diseases. Indeed, the expansion of regenerative medicine as a field has led to a paradigm shift from pharmaceutical or surgical intervention to the potential use of cell-based therapies. While preliminary clinical studies have shown promise, conflicting results from both preclinical animal models and small clinical trials reflect, in part, a lack of consensus regarding the characteristics, isolation methods, and definitions of what constitutes an endothelial progenitor cell (EPC). Moreover, as our understanding of the mechanisms by which EPCs modulate cardiovascular repair progress, novel strategies are emerging to either enhance the function of transplanted cells or modulate endogenous progenitor-mediated repair. Herein we will review highlights of the preclinical and clinical data underlying the therapeutic potential of EPCs for repair following arterial injury.

8.1 Defining Endothelial Progenitor Cells

The earliest studies to suggest the existence of vascular progenitor cells described implantation of Dacron grafts or silastic tubing into the vasculature of various animal models (Stump et al. 1962, 1963; Florey et al. 1961; Jordan et al. 1962; Pasquinelli et al. 1987; Feigl et al. 1985; Campbell et al. 1999; Shi et al. 1998). These experiments demonstrated the formation of a rudimentary vessel wall or "pseudoneointima," comprised of mature endothelial (ECs) and vascular smooth muscle cells

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(SMCs), independent of input from the native vasculature – leading the authors to postulate a blood-derived source of mature vascular cells. However, it was not until the isolation and characterization of endothelial progenitor cells (EPCs) in 1997 (Asahara et al. 1997) that an explosion in the study of vascular progenitors as both markers and mediators of cardiovascular disease truly began. Consequently, the last decade has seen a rapid progression in our understanding of the fundamental biology that underlies this endogenous repair mechanism, with results from early small trials showing significant promise for therapeutic potential.

Paradoxically, as research in the field progresses at an exponential rate, the very definition of what constitutes a true EPC continues to evolve (Urbich and Dimmeler 2004a, b; Hirschi et al. 2008; Yoder 2009). The lack of a uniform understanding of the most fundamental question – what is an EPC – has hampered both preclinical and clinical studies as conflicting results, at least in part, reflect a lack of consensus regarding definitions. While it is beyond the scope of this chapter to review all of the literature regarding the definition of an EPC, for the purpose of this chapter we will discuss the topic using in vitro culture definitions ascribed in a recent review by Hirschi and colleagues (Hirschi et al. 2008). Briefly, fibronectin adherence depleted colony-forming cells (CFU-Hill), adherent early cultured angiogenic cells (CACs), and late outgrowth proliferative endothelial colony-forming cells (ECFCs) will be specifically referenced.

Similarly, the characterization of an EPC by FACS analysis of peripheral blood has evolved with an ongoing debate regarding the appropriate gating strategy and panel of markers needed to ensure both reproducibility and specificity (Schmidt-Lucke et al. 2010; Schmidt-Lucke et al. 2005; Fadini et al. 2010; Bearzi et al. 2009). While CD45 negative/dim, CD34+, and VEGFR2/KDR + markers are generally accepted to be present on EPCs, numerous authors have suggested inclusion of CD133, CD117 (c-kit), CXCR4, and CD31 as additional markers of putative EPCs (Bearzi et al. 2009; Brehm et al. 2009; Grundmann et al. 2007; Wojakowski et al. 2004; Estes et al. 2010; Peichev et al. 2000; Duda et al. 2007). In the absence of clear evidence favoring the use of any one panel of markers, we have elected to use the term circulating EPC (cEPC) to describe vascular progenitors enumerated by flow cytometry. While cultured and cEPC populations undoubtedly represent distinct lineages of cells, each has been termed "EPC" in the literature and as such will be discussed in the context of cardiovascular repair.

8.2 Endothelial Progenitor Cells and the Coronary Artery

Atherosclerosis and development of obstructive coronary artery disease (CAD) remains a leading cause of mortality and morbidity in developed nations (Manuel et al. 2003). In addition to pharmacotherapy, the mainstay of treatment for ischemic heart disease includes revascularization whereby blood flow is restored to ischemic myocardium. With well over one million procedures being performed annually, percutaneous coronary interventions (PCI) with deployment of stents have become

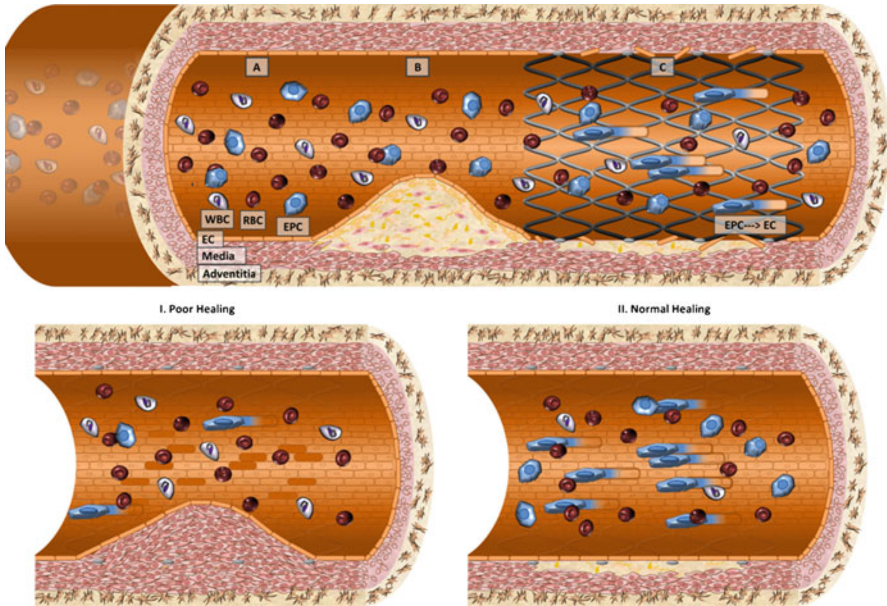

Fig. 8.1 *Role of EPCs in vascular healing.* Arterial cross section (*upper panel*) displaying vascular anatomy composed of outer adventitial layer with fibroblasts and extracellular matrix (ECM), medial layer with smooth muscle cells, and inner endothelial cell (EC) layer. Erythrocytes (RBCs), leukocytes (WBCs), and endothelial progenitor cells (EPCs) circulate within the vessel's lumen. *Region (A)* Normal arterial vessel with intact endothelium. *Region (B)* Atherosclerotic lesion containing a fibrous cap and cholesterol lined by endothelium. *Region (C)* Atherosclerotic lesion treated via insertion of metal stent, compressing the plaque while denuding the endothelial layer in this region. Reendothelialization then occurs via EPCs adhering to the vessel wall where they differentiate into endothelial cells (EPC--->EC) or secrete paracrine factors which promote reendothelialization by local mature ECs. *Lower panels* illustrate time lapse healing of stented region (C). *I. Poor healing* due to diminished EPC numbers and/or function results in incomplete reendothelialization with exposed stent struts and neointima formation. This delayed arterial homeostasis results in in-stent restenosis. *II. Normal healing* displays completely reendothelialized stented segment with no exposed struts or neointima formation owing to EPC numbers and/or function stimulating normal arterial homeostasis (Adapted from Padfield et al. 2010)

the preferred revascularization strategy for patients with CAD (Froeschl et al. 2004) (Fig. 8.1). While coronary stenting has greatly reduced the rates of arterial renarrowing at the site of intervention, in-stent restenosis (ISR) still occurs in 10–30% of patients receiving bare metal stents (Serruys et al. 2006). As the development of an intima was initially thought to arise through proliferation of local SMCs, current therapies target SMC proliferation and have achieved reduced rates of ISR ranging from 5% to 12.5% (Marx et al. 1995; Marx and Marks 2001; Wessely 2010; Stone et al. 2007; Moses et al. 2006; Kirtane et al. 2008). However, while these therapeutic devices have improved outcomes dramatically, they may increase the risk of late stent thrombosis – a catastrophic acute closure of the stent due to clot formation on exposed stent struts – by delaying regrowth of the endothelium (Mauri et al. 2007;

Luscher et al. 2007). Thus, the ongoing challenge remains to devise therapies that enable vascular homeostasis, maintaining endothelial integrity and function, at sites of arterial injury while simultaneously inhibiting neointimal formation.

8.2.1 Preclinical Models of Vascular Injury and EPC-Mediated Arterial Repair

Interventions designed to increase vessel lumen patency with either balloon angioplasty or PCI result in focal denudation of the endothelium and subsequent development of a neointima (NI) (Fig. 8.1). Rapid reconstitution of the endothelium is necessary for reducing the risk of thrombosis, but evidence suggests that an intact endothelium abrogates SMC and extracellular matrix (ECM) accumulation in the NI (Kirton and Xu 2010). The first evidence that EPCs may play a role in repairing an injured artery originated from studies on the effects of statins in rodent models. Both cEPCs and CACs were independently reported to home and incorporate into sites of injury, mitigating NI formation, when animals were treated with statins (Werner et al. 2002; Walter et al. 2002). However, because of the concomitant statin therapy, improvements in reendothelialization could not be attributed to the EPCs alone, and thus subsequent studies were performed using isolated spleen-derived CACs (Werner et al. 2003). The authors isolated CACs from mice and, following carotid wire injury, injected 1×10^6 cells at the time of injury and 1 day later resulting in a 30% reduction in the intima to media ratio. Interestingly, these cells homed only to the injured vessel and were not found in the contralateral uninjured artery suggesting a specific homing mechanism. Recently, our group replicated the findings of this seminal paper using CACs isolated from patients with CAD and demonstrated that improving CAC function could further improve reendothelialization (Hibbert et al. 2009). Finally, estrogens (Strehlow et al. 2003), G-CSF therapy (Kong et al. 2004), and leptin (Schroeter et al. 2008) can mobilize cEPCs or CACs resulting in enhanced arterial repair in various models of vascular injury. These findings have supported the hypothesis that interventions which improve either EPC number or function may enhance vascular repair and ultimately clinical outcomes. Thus, results from these preclinical models have provided the impetus to investigate clinical interventions specifically designed to modulate EPC biology.

8.2.2 Clinical Interventions and EPC-Mediated Arterial Repair

8.2.2.1 Statin Therapy

Early studies in patients with CAD noted both higher levels of cEPCs and CACs with statin treatment as well as a robust mobilization during initiation of therapy (Llevadot et al. 2001; Vasa et al. 2001). Since then, increased numbers of EPCs have

been observed with statin therapy in patients with stable CAD (Schmidt-Lucke et al. 2010), pre-PCI, in the setting of acute myocardial infarction (Leone et al. 2008), and post coronary arterial bypass grafting (Spadaccio et al. 2010). In addition to simply increasing the number of cEPCs and CACs isolated from patients, statin therapy also appears to regulate qualitative properties of circulating progenitors. Notably, members of the integrin family of extracellular matrix receptors have been shown to be upregulated by statin therapy which may in part improve adhesion at the site of arterial injury (Walter and Dimmeler 2002; Chavakis et al. 2005). This is of particular importance as cEPCs and CACs from patients with CAD can have impaired functional capacity which may bear importance on their ability to maintain vessel wall homeostasis. For example, it is known that not only are fewer CFU-Hill (George et al. 2003) and CACs (Hibbert et al. 2004) found in patients with ISR but also that a decrease in their adhesive properties is an independent risk factor for development of neointima following stent implantation (George et al. 2003). Overall, these small observational studies demonstrate convincingly that statin therapy is associated with both improved number and function of EPCs in patients undergoing PCI, but all have lacked the power to address meaningful clinical outcomes.

Statins have a number of effects independent of LDL lowering, including improving endothelial function, enhancing atherosclerotic plaque stability, and modulating inflammation (Liao and Laufs 2005). These pleiotropic effects may in part explain benefits above and beyond what might be expected by LDL cholesterol lowering alone. For example, in the PCI substudy of the PROVE IT trial, there was a marked reduction of target vessel revascularization in patients treated with high-dose atorvastatin, an effect which remained after adjusting for on-treatment LDL cholesterol (Gibson et al. 2009). Similarly, the Atorvastatin for Reduction of Myocardial Damage during Angioplasty (ARMYDA) study group has demonstrated important early reductions in periprocedural infarction by either initiating (Pasceri et al. 2004) or reloading patients with high-dose statin immediately prior to performing PCI (Di Sciascio et al. 2009). While these larger trials do not specifically test the hypothesis that EPCs can improve clinical outcomes following PCI, it is attractive to postulate a link between the early and robust mobilization of EPCs with statin therapy and the observed clinical benefit.

8.2.2.2 Stent Design

Coronary stents have evolved from mere scaffolds designed to prevent elastic recoil of the artery into highly sophisticated drug delivery systems in which every aspect – from the alloy used to the polymer coating – is designed to facilitate arterial healing (Garg and Serruys 2010a, b). Thus, it is only natural that as the potential impact EPCs play in restoring an intact endothelium following PCI became apparent, researchers have attempted to enhance attachment and proliferation of EPCs at the site of deployment (Padfield et al. 2010).

The first stent designed to exploit endogenous EPC reparative mechanisms was the Genous CD34 antibody coated stent (Aoki et al. 2005). In this First-in-Man

registry, 16 patients underwent successful coronary stenting with only 1 patient requiring target vessel revascularization at 9 months of follow-up. To date, this stent has been studied in randomized studies of ST elevation myocardial infarction patients (Co et al. 2008; Lee et al. 2010) and in a prospective registries of stable CAD patients (Miglionico et al. 2008; Duckers et al. 2007b) with satisfactory efficacy and safety profiles out to 1 year. However, most recently (Beijk et al. 2010), data comparing the Genous EPC capture stent to the Taxus Liberte paclitaxel-eluting stent showed more than double the late lumen loss (1.14 vs 0.55 mm). In these 193 patients at high risk of restenosis, there was also a nonsignificant trend toward an increased need for target vessel revascularization with the CD34 antibody coated stent. Thus, while the use of an EPC capture stent is promising, data from larger randomized control trials will be needed before use of this device is widely adopted.

Perhaps the most interesting results from the clinical studies of the Genous stent is the post-hoc analysis in which patients who appear to benefit most are those with higher circulating EPC levels (Duckers et al. 2007a). In this study, cEPCs defined as CD45+/CD34+/KDR+/7AAD- were approximately two-fold higher in patients treated with a statin and inversely correlated with late lumen loss. As patients with CAD have both fewer EPCs and decreased EPC function (Werner et al. 2005, 2007; Hill et al. 2003), this has led researchers to mandate statin therapy in studies looking at the Genous EPC capture stent in an attempt to enhance reendothelialization (Beijk et al. 2010). However, these findings may underscore a major limitation in therapies targeting EPCs. That is, in the patient populations in whom these therapies are designed to function, EPC number may be too low or dysfunctional to derive the benefits observed in preclinical models. Further studies into molecules which mobilize EPCs, such as statins, or targets which improve survival and paracrine function of the recruited cells, such as glycogen synthase kinase 3β (Ma et al. 2010; Hibbert et al. 2009), may ultimately improve the performance of stents designed to improve EPC-mediated arterial repair.

8.2.2.3 Cell-Based Therapies

To date, there are no clinical studies investigating either systemic or intracoronary injection of EPCs for reduction in rates of ISR or late lumen loss following coronary stenting. Moreover, the relationship between EPCs, PCI, and ISR continues to yield discordant and sometimes confusing results likely reflecting both the inconsistencies by which EPCs are defined as well as limitations in study design. Banerjee et al. (Banerjee et al. 2006) looked at 38 patients and enumerated ECFCs. While they observed a mobilization of ECFCs induced by PCI, the authors noted that this seemed to be independent of serum VEGF levels and was restricted to those patients undergoing elective procedures. In contrast, Garg et al. noted a 37% increase in Hill-CFUs in 20 patients having PCI with all of their patients having had a recent non-ST elevation myocardial infarction (Garg et al. 2008). Finally, Egan et al. (Egan et al. 2009) compared 10 patients undergoing PCI with 13

patients having only angiography and found that CXCR4 positive cEPCs were mobilized in response to PCI alone, suggesting that vascular injury is necessary to induce mobilization. While these findings strongly support the notion that the vascular injury induced by PCI mobilizes EPCs, none of the aforementioned studies were adequately powered nor designed to assess the association between EPCs and ISR.

The first study to examine the relationship between EPCs and ISR was performed by Inoue et al. (Inoue et al. 2007). They enumerated cEPCs (defined as CD34+ cells), CACs, and outgrowth of smooth muscle progenitor cells and noted an association between mobilization of CD34+ cells, outgrowth of SMCs, and the ultimate development of ISR. Interestingly, others had previously noted a strong correlation between CD34+ cell levels and late lumen loss following PCI (Schober et al. 2005). However, by limiting their flow cytometry to a single marker, these investigators included all early bone marrow-derived cells including hematopoietic progenitors making it difficult to draw conclusions. The largest study to date was conducted by Pelliccia et al. in 155 patients undergoing PCI for stable angina (Pelliccia et al. 2010). Paradoxically, they too noted an association between elevated cEPCs (defined as CD45-/CD34+/KDR+) and clinical ISR which developed in 30 patients. Thus, while animal models purport regenerative benefit with regards to the vascular endothelium, in humans, there seems to be at least an association between mobilization of CD34+ cells and development of ISR. Thankfully, clinical studies using intracoronary injection of cultured cells have not demonstrated the adverse events that may have been predicted by these observational trials. Specifically, the ASTAMI trial (Lunde et al. 2006; Beitnes et al. 2009), the REPAIR-AMI trial (Schachinger et al. 2006), and the study by Janssens et al. (Janssens et al. 2006) all performed intracoronary injection of mononuclear-derived cells without increased rates of ISR. While these studies do not directly address the potential benefit of a cell-based therapy, the experimental data provides a rationale while these clinical trials demonstrate an acceptable safety profile in patients undergoing PCI.

8.3 Future Directions

Therapies which augment re-endothelialization following vascular injury, such as in the context of PCI, are necessary to improve arterial healing and ultimately enhance clinical outcomes. Clearly, we are only beginning to better define the involved cell populations and the underlying mechanisms which promote their mobilization, homing, and incorporation into the arterial wall. While it is conceivable that cell-based therapies may show benefit, it stands to reason that as our understanding of the biology of EPCs improves, pharmacologic interventions or devices which modulate this endogenous repair mechanism represent the most likely manner in which EPCs will make the leap from bench to bedside.

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Chapter 9
Bone Marrow–Derived Cells as Treatment Vehicles in the Central Nervous System

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Abstract Substantial research has focused on the potential for bone marrow–derived cells (BMDCs) to function as vehicles to transport pharmaceuticals into the diseased central nervous system (CNS). By employing bone marrow–chimeric models, investigators have determined that BMDCs retain their hematopoietic identity within the CNS with a majority of cells acquiring macrophage phenotypes. Although the use of irradiation in creating bone marrow chimeras is believed to be necessary for the engraftment of BMDCs within the CNS, further investigations into alternative conditioning regimens will improve the clinical potential for this treatment modality.

9.1 Introduction

Neurodegenerative diseases are characterized by the death of specific populations of neurons, which due to a lack of regenerative potential, culminate in permanent and progressive disability. Pharmacological treatments for these disorders have been ineffective at slowing the disease process, due in part to the inability of drugs to reach sites of neurodegeneration. The CNS is surrounded by the blood–brain barrier

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(BBB), a specialized endothelial network that separates circulating blood from the extracellular space of the CNS. Tight junctions seal the apical surface between capillary endothelial cells and regulate the entry of blood-borne substances and cells into the CNS, largely preventing the influx of many drugs. The inefficacy of pharmacological treatments for neurodegenerative diseases has spurred investigations into whether bone marrow (BM) cells could function as vehicles to transport therapeutic substances into the CNS. It is well established that after receiving BM transplants, donor cells are observed in the CNS of patients at autopsy in limited numbers (Unger et al. 1993; Appel et al. 2008) demonstrating that BM cells are capable of entering the CNS under some circumstances.

9.2 Background

9.2.1 Hematopoiesis

Within BM, the pluripotent hematopoietic stem cell (HSC) is the predecessor of all cells within the hematolymphoid system including erythrocytes, lymphocytes, granulocytes, and monocytes. Differentiation of HSCs within the hematopoietic compartment follows a hierarchal path that descends from the HSC through multipotent progenitor cells of increasing lineage commitment until ultimately arriving at a unipotent progenitor cell (Fig. 9.1). HSCs and downstream progenitors are defined based on the expression of several cell-surface markers (Table 9.1).

Traditionally, the HSC population is identified as being c-Kit^{+}Thy1.1^{lo}Lin1$^{-/low}$Sca1^{+} (KTLS) and is further subdivided based on the expression of CD34. HSCs capable of long-term reconstitution of the BM compartment after myeloablation are CD34– while short-term reconstituting HSCs (also referred to as multipotent progenitors (MPPs)) are CD34+ (Zhu and Emerson 2002). More recently, the differential expression of members of the SLAM family of surface receptors, specifically CD48, CD150, and CD244 (Kiel et al. 2005), and endothelial protein C receptor (EPCR; Balazs et al. 2006) by hematopoietic stem and progenitor cells has also been employed to determine the primitiveness of hematopoietic progenitors (Kiel et al. 2005). The most primitive HSCs express EPCR and are identified as CD150^{+}CD48^{-}CD244^{-} while MPPs are CD150^{-}CD48^{-}CD244^{+} and EPCR negative; more restricted progenitor populations are characterized by CD150^{-}CD48^{+}CD244^{+} expression (Kiel et al. 2005; Balazs et al. 2006).

Within BM, the determination between HSC self-renewal versus differentiation is dictated both through the production of cytokines and by receptor/ligand interactions with surrounding stromal cells (Seita and Weissman 2010). The cellular events that direct HSC differentiation toward a single lineage during hematopoiesis are not fully understood, however, the expression of transcription factors (TFs) by hematopoietic progenitors plays a central role in cell fate determination and lineage commitment. The two main theories regarding control of TF expression differ in whether control is exerted intrinsically or extrinsically and are not mutually exclusive.

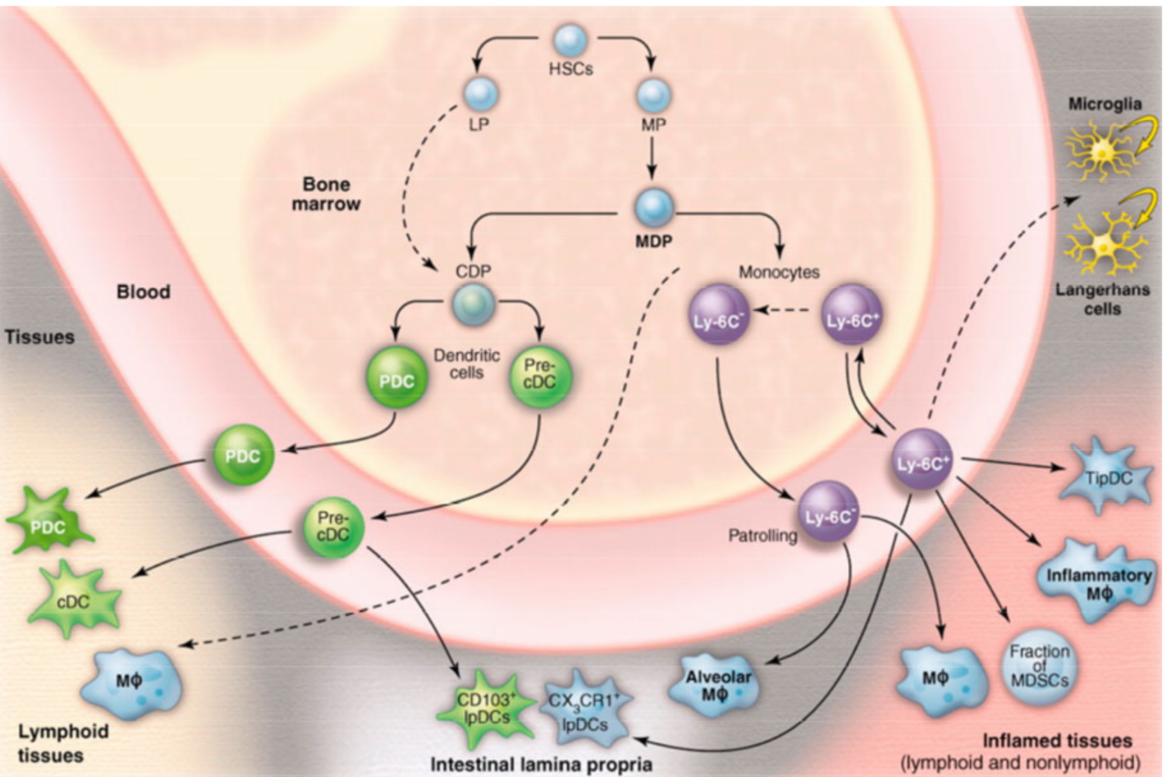

Fig. 9.1 Within the bone marrow compartment, HSCs are pluripotent progenitors giving rise to all myeloid and lymphoid cell types. The first step in lineage commitment is either the myeloid precursor (MP) or the lymphoid precursor (LP). Cell differentiation within BM moves through downstream precursor populations of increasing lineage restriction eventually arriving at unipotent precursor cells (reproduced from Geissmann et al. 2010 with permission from AAAS)

Table 9.1 Antigenic identification of BM progenitor populations. Progenitor cell populations within BM are discriminated based on the expression of a variety of antigenic markers (Iwasaki and Akashi 2007)

Progenitor	Antigenic expression
Long-term HSC	$c\text{-Kit}^{+}\text{Thy1.1}^{lo}\text{Lin}^{-/lo}\text{Sca-1}^{+}\text{CD34}^{-/lo}$
Short-term HSC/MPP	$c\text{-Kit}^{+}\text{Thy1.1}^{-}\text{Lin}^{-/lo}\text{Sca-1}^{+}\text{CD34}^{+}$
Common myeloid progenitor	$\text{IL-7R}^{-}c\text{-Kit}^{+}\text{Lin}^{-/lo}\text{Sca-1}^{-}\text{Fc}\gamma\text{RII/III}^{lo}\text{CD34}^{+}$
Common lymphoid progenitor	$\text{IL-7R}^{+}c\text{-Kit}^{lo}\text{Lin}^{-/lo}\text{Sca-1}^{lo}$
Megakaryocyte/erythrocyte progenitor	$\text{Lin}^{-/lo}\text{Fc}\gamma\text{RII/III}^{lo}\text{CD34}^{-}$
Granulocyte-macrophage progenitor	$\text{Lin}^{-/lo}\text{Fc}\gamma\text{RII/III}^{hi}\text{CD34}^{+}$
Monocyte-dendritic cell precursor	$\text{Lin}^{-/lo}\text{Fc}\gamma\text{RII/III}^{hi}\text{CD34}^{+}\text{CX3CR1}^{+}$

The intrinsic theory claims that the expression patterns of TFs are initiated stochastically and that spontaneous alterations in levels of deterministic TFs tip the balance in favor of one lineage commitment over another. The extrinsic theory claims that the induction of transcriptional programs is the result of signaling from external components of the progenitor cell (Zhu and Emerson 2002).

9.2.2 Monocytes

In humans, monocytes (MOs) represent approximately 10% of peripheral leukocytes and have a half-life of 3 days in the circulation (Yona and Jung 2010). Based on the differential expression of cell surface markers, human MOs can be divided into three discrete populations. $\text{CD14}^{++}\text{CD16}^{-}$ "classical" monocytes comprise up to 95% of circulating MOs, $\text{CD14}^{+}\text{CD16}^{++}$ "nonclassical" or "patrolling" comprise 5% of circulating MOs, and a small population of $\text{CD14}^{++}\text{CD16}^{+}$ "intermediate" MOs has also been described (Tacke and Randolph 2006). Murine MOs can be divided into two populations based on their expression of Ly6C and the chemokine receptors CCR2 and CX_3CR1. Inflammatory or "classical" MOs express high levels of Ly6C, CCR2, and low levels of CX_3CR1 while "resident" MOs lack or express low levels of Ly6C and CCR2 but express high levels of CX_3CR1 (Geissmann et al. 2008). These MO subpopulations correspond to the classical and nonclassical MOs of humans, respectively. In mice, peripheral MOs constitute approximately 4% of circulating leukocytes and have a half-life in the circulation of approximately 24 h (Yona and Jung 2010).

Along with distinct expression of antigenic markers, MO subpopulations also exhibit differential physiological activities. Inflammatory MOs are recruited to tissues after injury or infection and are associated with the promotion of inflammation while resident MOs are associated with patrolling the vasculature and the resolution of inflammation (Kamei and Carman 2010). A recent study also identified an extramedullary population of MOs located in the splenic red pulp of mice which may enable the rapid mobilization of MOs into the circulation in response to injury or infection (Swirski et al. 2009).

9.2.3 The Mononuclear Phagocyte System

The mononuclear phagocyte system (MPS) is composed of hematopoietic progenitors, blood MOs, dendritic cells, and tissue macrophages (Hume 2006). During inflammation, and to a lesser degree under steady state conditions, circulating MOs are recruited to tissue compartments where they infiltrate and differentiate into macrophages. Macrophages are key players in the innate immune response and under inflammatory conditions their functions include phagocytosis, pro-inflammatory cytokine production and antigen presentation (Davoust et al. 2008). Similar to Kupffer cells in the liver or alveolar cells in the lungs, microglia are considered the tissue-resident macrophages of the CNS. Microglia are highly ramified, stellate-shaped cells that reside within the parenchyma of the CNS. This location can be used to distinguish microglia from other populations of CNS-associated macrophages such as perivascular macrophages located between the glia limitans and basal lamina of blood vessels, and the meningeal macrophages located in the leptomeninges surrounding the brain and spinal cord. Although it is well accepted that in nonneuronal tissues endogenous macrophage populations are maintained to a variable degree by recruitment of MOs from the circulation, whether or not this is also true for microglia has been greatly debated (Lawson et al. 1992).

9.3 BM Cell Entry into the CNS

Investigations of BM-derived cell (BMDC) migration into the CNS employ BM-chimeric models in which the host's BM is replaced by BM that expresses a label such as green fluorescent protein (GFP), enabling the discrimination of BMDCs from host cells. These models are typically created by exposing host animals to myeloablative levels of radiation to deplete BM, followed by BM reconstitution via intravenous injection of donor BM cells. The successful reconstitution of the host's BM compartment is dependent on both the level of myeloablation achieved and the number of long-term reconstituting HSCs introduced into the host's circulation (Nevozhay and Opolski 2006).

9.3.1 BM Cell Entry into the Healthy CNS

In rats and mice, irradiated/BM transplanted chimeras have been employed to investigate the migration of BM cells into the healthy brain. In the brains of BM-chimeric rats, elongated BMDCs are found on the abluminal surface of blood vessels in a perivascular position, as well as within the surrounding leptomeninges (Hickey and Kimura 1988). These cells, commonly called perivascular cells or perivascular

microglia, are positively immunolabeled with OX-42, an antibody-identifying macrophage/microglia in rats, indicating these BMDCs were perivascular and meningeal macrophages, respectively (Hickey and Kimura 1988). Only very rare OX-42-positive, stellate-shaped donor BMDCs are observed in the brain parenchyma, indicating that rarely BMDCs can acquire a microglial phenotype (Hickey and Kimura 1988).

Similarly, in the brains of murine BM chimeras, BMDCs are observed in perivascular and meningeal locations one month after transplantation, and these cells are labeled with the monocyte/macrophage marker F4/80 (Kennedy and Abkowitz 1997). At one year posttransplant, 30% of F4/80-positive cells in the brain were of donor origin. However, investigators only rarely observed donor BMDCs in the brain parenchyma, suggesting that the vast majority of BM-derived macrophages were confined to perivascular and meningeal locations, with BMDCs making only limited contributions to the parenchymal microglial population (Kennedy and Abkowitz 1997). More recent investigations have shown that 2 weeks post-irradiation and transplantation BMDCs are observed within the leptomeninges (Chinnery et al. 2010), and at 3 months post-transplant, roughly 40% of the leptomeningeal macrophage population was comprised of BMDCs; by 6 months this number increased to 70% (Vallieres and Sawchenko 2003), suggesting that leptomeningeal macrophages, like those in most other tissues, are slowly replaced by BM-derived cells. All BMDCs in both the leptomeninges and the cerebral cortex express the pan-hematopoietic marker, CD45, and the vast majority of BMDCs also express the macrophage/microglial marker Iba-1 (Vallieres and Sawchenko 2003). Consistent with previous results however, only ~0.1–0.25% of BMDCs within the cerebral cortex were observed in parenchymal regions, the remainder being found in association with blood vessels (Massengale et al. 2005; Chinnery et al. 2010).

Although numerous reports have claimed that BMDCs make only modest contributions to parenchymal microglial populations, there have been occasional reports that BMDCs constitute up to 40% of the microglial pool in the healthy CNS (Hess et al. 2004; Simard and Rivest 2004). Despite isolated reports of BMDCs making substantial contributions to the parenchymal microglia population in healthy mice, this has not been our observation, nor that of many others (Hickey and Kimura 1988; Kennedy and Abkowitz 1997; Vallieres and Sawchenko 2003; Solomon et al. 2006; Lewis et al. 2009; Chinnery et al. 2010).

In general, BMDC entry into CNS appears to produce at least five specific types of cell populations within the CNS; these include the parenchymal microglia, perivascular microglia, leptomeningeal macrophages, and other CD45-positive CNS populations that may express lineage markers at levels too low to be detected by immunohistochemistry, as well as T cells. Currently, the general consensus among most investigators is that while BMDCs make significant contributions to the maintenance of CNS macrophage populations in areas lacking BBB (i.e., perivascular and leptomeningeal areas), BMDCs make only modest contributions to the parenchymal microglial pool in healthy irradiated BM chimeras.

9.3.2 Transdifferentiation of Bone Marrow Cells

Previous studies have claimed that BM-derived cells in the CNS acquired the phenotypes of astrocytes (Eglitis and Mezey 1997), cardiac myocytes (Jackson et al. 2001), hepatocytes (Lagasse et al. 2000), and neurons (Brazelton et al. 2000; Mezey et al. 2000). Based on these observations, it was postulated that BMDCs had the potential to transdifferentiate or cross lineage boundaries and acquire nonhematopoietic lineage fates. Frequently, the results of these studies could not be duplicated, nor could the functionality of transdifferentiated BM cells be verified, creating skepticism as to the validity of these observations. It was suggested that tissue damage played an integral role in inducing BMDC plasticity (Abedi et al. 2004) and studies employing CNS injury models such as aspiration injury to the cerebral cortex (Hess et al. 2004), middle cerebral artery occlusion (MCAO, a stroke injury model; Vallieres and Sawchenko 2003), and others were carried out to determine if neuronal injury provided a microenvironment necessary to induce BMDC transdifferentiation. Examinations of BMDC phenotypes in these disease models were consistent with observations in the healthy CNS, namely that the vast majority of BMDCs acquired a macrophage phenotype (Vallieres and Sawchenko 2003). Notably, although NeuN (a neuronal marker)-positive BMDCs were observed after MCAO, closer examination of these cells using confocal microscopy indicated that the immunostaining pattern was consistent with BMDC phagocytosis of NeuN-positive cells (Hess et al. 2004).

Further investigations into the transdifferentiation potential of BMDCs demonstrated that in BM chimeric mice, fusion events between BMDCs and Purkinje neurons, cardiac myocytes, and hepatocytes occur (Alvarez-Dolado et al. 2003). Rather than transdifferentiating, BMDCs form heterokaryons with host cells, resulting in cells that demonstrate immunolabeling of both donor and host epitopes (Weimann et al. 2003; Corti et al. 2004). Furthermore, a recent study demonstrated that surface antigens of donor BM cells can be transferred to endogenous cell populations in vivo in a process termed trogocytosis, resulting in nonhematopoietic cells within the host that express the donor BM label (Yamanaka et al. 2009). The current consensus is that in vivo, BMDC differentiation is restricted to hematopoietic cell fates (Rodić et al. 2004).

Interestingly, an in depth investigation into the fusogenic nature of BMDCs with Purkinje neurons demonstrated that under conditions of systemic inflammation, such as during dermatitis and after induction of experimental allergic encephalitis (EAE), significantly greater numbers of fusion events occur (Johansson et al. 2008). Investigators also employed parabiosis to connect the circulations of genetically distinct mice to determine if irradiation induced the fusion of BMDCs with Purkinje neurons and further demonstrated fusion events occur in the absence of both irradiation and BMDC contributions to microglia (Johansson et al. 2008). Analysis of the gene expression pattern by the donor nuclei of heterokaryons indicated that donor nuclei were reprogrammed to express Purkinje neuron-specific genes while hematopoietic gene expression was repressed. Further investigation into the phenomena of BMDC fusion events may elucidate an additional therapeutic role for these cells (Johansson et al. 2008).

9.4 BM Cell Entry into the CNS in Models of Neurodegenerative Disease

Injuries and neurodegeneration in the CNS are both associated with microgliosis, the activation and proliferation of microglia. Numerous studies employing BM-chimeric mice have demonstrated that in various models of neurodegenerative disease the number of BMDCs that populate the CNS is significantly increased in affected areas.

9.4.1 Parkinson's Disease

Parkinson's disease is associated with the selective degeneration of dopaminergic (DA) neurons that project from the substantia nigra to the striatum. Clinically this culminates in bradykinesia, leading to akinesia with progressive degeneration of DA neurons and, consequently, loss of independence. A commonly used murine model for PD utilizes treatment of mice with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin affecting DA neurons (Jackson-Lewis and Przedborski 2007). Mice with BM chimerism treated with MPTP have been used to study the migration of BMDCs into the brain in this PD model. Analysis of affected brains has consistently demonstrated significantly increased BMDC density in areas of DA innervation, compared to non-DA sites and DA areas in untreated controls (Kokovay and Cunningham 2005; Keshet et al. 2007; Rodriguez et al. 2007). The numbers of BMDCs observed correlated with the severity of neurodegeneration and suggests that neuronal degeneration enhanced either the migration or proliferation of BMDCs within affected areas (Rodriguez et al. 2007). Identification of BMDC phenotypes using either CD68 or CD11b, yielded dissimilar results with approximately 50% of BMDCs in MPTP-treated mice immunolabeling with CD68 (Rodriguez et al. 2007) compared to up to 90% of BMDCs labeling with CD11b (Kokovay and Cunningham 2005), suggesting a disparity between the sensitivities of these antigenic markers.

9.4.2 Alzheimer's Disease

The most common neurodegenerative disease of the elderly, Alzheimer's disease (AD), is characterized by the formation of amyloid plaques, neurofibrillary tangles, and neuron loss within gray areas of the cerebral cortex (El Khoury and Luster 2008). The vast majority of AD cases have a sporadic etiology; however, genetic variations and mutations in amyloid precursor protein (APP) and presenilin (PSEN) genes are involved in the familial forms of the disease. The identification of these genes associated with the development of AD has enabled the creation of transgenic

mice that express one or both mutated genes, and these mice typically develop a phenotype similar to AD and provide a model with which to study the disease. Studies employing BM chimeric APP23 mice (Stalder et al. 2005) and APP/PSEN1 double transgenic mice (Malm et al. 2005) have shown that the total number of BMDCs observed in cortical regions was significantly increased in AD model chimeras compared to controls (Malm et al. 2005; Stalder et al. 2005). Furthermore, in the brains of the APP23 chimeras, a significant portion of BMDCs were observed in brain parenchyma with increased BMDC density in cortical areas containing greater numbers of amyloid plaques, suggesting a positive correlation between BMDC migration and/or proliferation and levels of amyloid deposition (Stalder et al. 2005). This interpretation was supported by the observation that increased numbers of BMDCs were observed in APP23/PSEN1 mice transplanted at a time point when mice were symptomatic (21 months) compared to mice that received BM transplants presymptomatically (2.5 months) that were analyzed at symptom onset (9 months; Malm et al. 2005).

9.4.3 Amyotrophic Lateral Sclerosis

Characterized by the progressive degeneration of brain and spinal cord motoneurons, ALS is a fatal neurodegenerative disease of humans typically diagnosed during the fifth decade of life. Although primarily sporadic in etiology, there is a familial form of ALS seen in 10% of ALS cases, where 20% of these familial cases are attributed to mutations in the gene encoding superoxide dismutase 1 (SOD1). Transgenic mice that over-express mutant SOD1 (mSOD) develop motoneuron pathology similar to ALS and provide a model with which to study this disease.

Several groups have employed BM chimeras to study the migration of BMDCs into the spinal cord of the mSOD mouse model (Corti et al. 2004; Solomon et al. 2006; Chiu et al. 2009; Lewis et al. 2009). Increased numbers of BMDCs were observed in the lumbar spinal cords of mSOD mice compared to control mice at both the symptomatic and end-stages of disease (Solomon et al. 2006). Furthermore, the numbers of BMDCs in the mSOD spinal cord significantly increased between the symptomatic and disease end-stages, suggesting either continuous recruitment of circulating BM cells or the proliferation of BMDCs within the spinal cord (Solomon et al. 2006). BMDCs acquired a variety of morphologies and were observed in association with blood vessels in a perivascular position, and within the spinal cord parenchyma (Fig. 9.2). The majority of BMDCs were observed to express the macrophage/microglial markers F4/80, CD11b, and Iba-1; however, BM-derived CD3+ T-lymphocytes were also observed (Solomon et al. 2006; Lewis et al. 2009). Although it has been reported that transplantation of wild-type BM into mSOD hosts resulted in significantly increased lifespan (Corti et al. 2004), this result has not been observed by other groups (Solomon et al. 2006; Chiu et al. 2009).

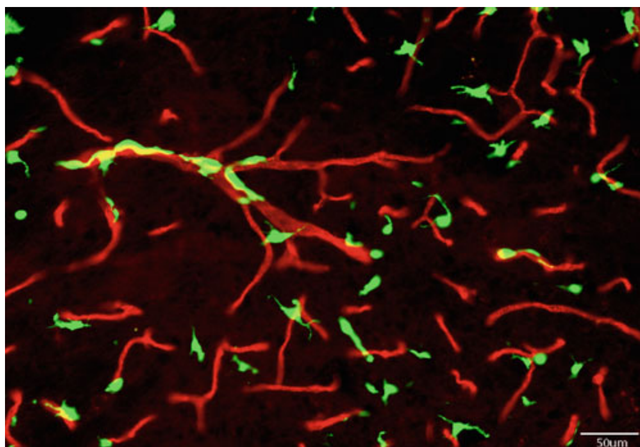

Fig. 9.2 GFP+bone marrow–derived cells in the spinal cord of the mSOD mouse. Green GFP+BMDCs in an mSOD mouse lumbar spinal cord section immunolabeled with antibody to PECAM1 (*red*). BMDCs were observed to have a variety of morphologies and are found in association with blood vessels and within the spinal cord parenchyma

9.5 BMDCs as Treatment Vehicles in Neurodegenerative Disease

The treatment of progressive neurodegenerative diseases using pharmacological agents is significantly impaired by the limited permeability of the BBB. The vast majority of small-molecule drugs and virtually all large drugs cannot traverse the BBB, enter the CNS, and exert therapeutic effects at sites of neurodegeneration (Pardridge 2003). Those drugs that are capable of entering the CNS are typically lipophilic and of small molecular weight (less than 500 Da) but current members of this class of drugs have not demonstrated therapeutic effects on the progression of neurodegenerative diseases (Pardridge 2002). The limited treatment efficacy of pharmacological agents raises the possibility that greater therapeutic success might be achieved by the use of BMDCs as treatment vehicles, by exploiting their ability to circumvent the BBB and to deliver neurosupportive substances into the CNS.

Recently Biju and colleagues employed lentiviral vectors to transfect donor HSCs in vitro with rat glia-derived neurotrophic factor (GDNF) genes driven by a macrophage-specific promoter (MSP) that restricted the expression of the vector to BM-derived macrophages (Biju et al. 2010). Transfected HSCs were transplanted into lethally irradiated mice that were subsequently treated with MPTP to induce DA neurodegeneration. In control MPTP-treated mice, there was a 50–55% reduction in DA within the substantianigra, while in the MPTP GDNF-BM mice only a 15–20% reduction in DA was observed (Biju et al. 2010). This reduction in DA degeneration was also associated with a reduced decline in activity levels compared to sham-treated mice.

Similarly, Lebson and colleagues exploited the ability of monocytes to home to sites of amyloid deposition in the brain of a transgenic mouse model of AD by transfecting CD11b-positive BM cells with constructs for three isoforms of neprelysin, a membrane-bound ectoprotease (Lebson et al. 2010). Cells were injected into the left ventricle of mice and researchers determined the half-life of the injected CD11b-postive cells to be roughly 90 min, with total cell clearance from the circulation by 24 h postinjection. Analysis of brain tissue indicated that the cells homed to areas of amyloid deposition and the majority of cells expressed Iba-1. Furthermore, over the two-month observation period, amyloid loads in treated mice did not increase while in untreated mice, there was a considerable increase in levels of amyloid deposition.

These studies demonstrate the therapeutic potential of BM cells for treating CNS injuries or disease. Particularly promising are the results of Lebson and colleagues who demonstrated that CD11b-positive BM cells can populate the diseased CNS in the absence of replacement of the host's BM.

9.5.1 Identification of BMDC That Enter CNS

Although experiments that employ BM-chimeric models provide valuable information on the migration of BM cells into the CNS and the functional fates they acquire, the clinical validity of this approach is questionable. Reconstituting patients' BM with genetically modified stem cells is certainly undesirable and could potentially have deleterious effects systemically. Therefore, identification of specific populations of BM cells capable of differentiating into microglia in the absence of whole BM transplantation might improve the clinical potential of BM cells as treatment vehicles.

To identify specific BM cell populations capable of migrating to the CNS, Hess and colleagues (2004) fractionated donor BM into isolated single HSCs which were expanded in culture and delivered into irradiated mice. When systemic injections of 100 HSCs were given, BMDCs were subsequently observed within the CNS where cells were histologically identified as perivascular cells and microglia (Hess et al. 2004). Given that the majority of BMDCs observed within the CNS of BM chimeras exhibit macrophage phenotypes, it appears likely that these cells derive from BM MOs or their precursors. To determine which populations of circulating MOs (i.e., inflammatory Ly-6C^{hi} or resident Ly-6C^{lo}) enter the CNS, Mildner and colleagues transplanted irradiated control mice with CCR2-negative GFP-positive BM. The ablation of CCR2 expression is hypothesized to hinder inflammatory MOs from leaving the BM compartment into the circulation (Serbina and Pamer 2006) and 4 weeks after BM transplant, significantly reduced numbers of circulating Ly-6C^{hi}MOs were observed (Mildner et al. 2007).

Analysis of the healthy brain of CCR2-GFP + BM chimeras demonstrated substantially reduced BM-derived microglial engraftment. Similarly, in a cuprizone-induced model of demyelination within the corpus callosum, significantly greater

numbers of BMDC engraftment was observed in mice that received CCR2 + GFP + BM transplants compared to mice that received CCR2-GFP + BM after irradiation. The results of these studies support the hypothesis that CCR2 expression is a requisite for the emigration of Ly-6C^{hi} MOs from the BM compartment, and that the primary source of BM-derived microglia is the Ly-6C^{hi} (inflammatory) population of MOs (Mildner et al. 2007).

9.6 Remaining Questions and Limitations

A significant caveat associated with the irradiation/BM transplantation protocol used to create BM chimeras is the widespread effects that myeloablative levels of radiation have on the host. Studies have indicated that in the two weeks after lethal irradiation of hosts, there is apoptosis of endothelial cells within the rat blood–spinal cord barrier, permitting serum albumin to enter the CNS (Li et al. 2004). Furthermore, in mice, treatment of the brain with 25 Gy of radiation is associated with a neuroinflammatory response that persists for 2 to 3 months after treatment (Gourmelon et al. 2005). Although the level of exposure in this experiment is significantly greater than that necessary to achieve high levels of BM chimerism, it has been demonstrated that 16 days after exposure to 10 Gy of radiation, there is increased cytokine and chemokine expression within the CNS, including increased levels of the myeloattractant and CCR2 ligand CCL2 (Mildner et al. 2007).

Experiments aimed at teasing out the effects of irradiation on BMDC migration into the CNS have employed parabiosis to connect the circulations of genetically distinct mice, resulting in 50% chimerism of peripheral blood cells (PBCs). In the absence of radiation/BM transplantation, essentially no BMDCs are observed in both the healthy and injured/diseased murine CNS (Massengale et al. 2005; Ajami et al. 2007; Mildner et al. 2007). Although these results suggest that the effects of irradiation are requisite for the migration of PBCs into the CNS, Ajami and colleagues created parabiotic pairs of GFP + and GFP – mice and subjected the GFP- parabiont to myeloablative doses of radiation while protecting the GFP + mouse from radiation exposure by using a lead shield. Five weeks after irradiation, an average of 78% of PBCs were GFP + and the irradiated mouse was subjected to facial nerve axotomy (Ajami et al. 2007). However, similar to results from nonirradiated parabionts, very few GFP + cells were observed near the inured facial nucleus, and those that were observed appeared to be intravascular (Ajami et al. 2007). The results of this study suggest that not only is radiation necessary for BMDC engraftment into the CNS but so too is the presence of circulating BM progenitors that in that absence of whole BM transplantation, would not normally enter the blood stream (Ajami et al. 2007). The necessity of preconditioning patients by exposing them to radiation in order to enhance the migration of BMDCs into the diseased CNS may limit the clinical potential of these cells.

9.7 Conclusion

Considerable research has demonstrated that under certain conditions, BMDCs engraft within the CNS, and this engraftment is enhanced in models of neurodegenerative disease. Although the majority of BMDCs acquire the phenotypes of perivascular or leptomeningeal macrophages, a proportion of cells also contribute to the parenchymalmicroglial pool. However, there is evidence suggesting that the conditions under which this migration is permitted are manifested by both the irradiation of hosts and the introduction of BM progenitor populations into the circulation. Further investigation into alternative preconditioning regimens that will similarly enable the migration of BMDCs into the CNS and the specific populations of BMDCs capable of this migration are necessary to exploit the therapeutic potential of these cells

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Chapter 10
Regenerative Potential of Blood Stem Cell Products Used in Hematopoietic Stem Cell Transplantation

Laura Labonté and David S. Allan

Abstract In this chapter, we address the role of nonhematopoietic progenitors with regenerative potential that are present in blood stem cell grafts used in hematopoietic transplantation. In particular, we discuss the potential role of nonhematopoietic progenitors in reducing transplant-associated tissue toxicity. We discuss specific cell types including endothelial progenitors and mesenchymal stromal cells that may be associated with tissue repair, and we discuss some potential strategies for augmenting the regenerative capacity of blood stem cell products which could have more widespread applicability to a broad range of acute tissue injuries.

10.1 Introduction

Hematopoietic stem cell transplantation (HSCT) has evolved as a critical treatment strategy for several hematologic disorders including aplastic anemia, acute leukemia, lymphoma, and multiple myeloma. Stem cell products used in HSCT may be from patients themselves, termed autologous transplants, or cells may be harvested from an allogeneic donor such as a sibling or unrelated donor that is compatible in terms of key genes regulating immune recognition, the human leukocyte antigen complex (HLA). Peripheral blood stem cells (PBSCs) can be collected from the peripheral circulation by leukapheresis after stimulation with molecules including granulocyte colony-stimulating factor (G-CSF) and the CXCR4 antagonist, plerixafor,

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to mobilize progenitor cells from the bone marrow into the peripheral blood. Bone marrow can be harvested from the iliac crests through repeated aspiration in the operating room, and stem cell products for transplantation can also be collected from umbilical cord blood following delivery and stored in cord blood banks for later use. Both autologous and allogeneic transplant approaches typically involve toxic chemotherapy and/or radiation treatment to eliminate residual cancer cells and/or to sufficiently suppress the immune system to prevent graft rejection and graft versus host disease in the allogeneic setting. This high-dose conditioning treatment causes widespread tissue damage and contributes significantly to transplant-related morbidity and mortality. Although reduced intensity allogeneic transplant methods have evolved in recent years, which are primarily immunosuppressive and allow more gradual engraftment of the donor hematopoietic system with a marked reduction in acute transplant-related toxicity, this approach is not applicable in all situations. HSCT, therefore, provides an ideal opportunity for assessing recovery from acute tissue injury caused by the chemotherapy and radiation conditioning regimen. Both the timing and nature of the toxicities observed after HSCT can be reasonably predicted. Moreover, we are gaining important insight regarding specialized nonhematopoietic cells within blood stem cell products which can facilitate repair of damaged tissues and makes HSCT particularly interesting as a model to better understand the regenerative capacity of blood-derived stem cells.

In this chapter, we address the role of nonhematopoietic progenitors with regenerative potential that are present in blood stem cell grafts and we discuss their potential role in reducing HSCT-associated tissue toxicity. We discuss specific cell types that may be associated with tissue repair and discuss some potential strategies for augmenting the regenerative capacity of blood stem cell products which could have more widespread applicability to a broad range of acute tissue injuries.

10.2 Nonhematopoietic Cells with Regenerative Potential in Blood Stem Cell Products

Blood stem cell products including bone marrow, mobilized peripheral blood, and umbilical cord blood include variable numbers of cells with regenerative capabilities including mesenchymal stem or stromal cells, endothelial-like vascular progenitor cells (VPCs), and angiogenic monocytes. These blood-derived cells have demonstrated promise in facilitating tissue repair following ischemic injury such as myocardial infarction, stroke, and vascular injury such as trauma and burns. As a result, increasing attention has been directed to these nonhematopoietic precursors in recent years and has contributed immensely to furthering our understanding of the mechanisms involved in cell-mediated tissue repair and regeneration. In particular, grafts enriched for cells capable of vascular repair may allow the reduction of transplant-associated tissue injury after HSCT. Studying vascular repair in the HSCT setting could allow regenerative therapy using blood-derived cells to be translated to other clinical models of acute tissue damage.

10.2.1 Mesenchymal Stromal Cells (MSCs)

Mesenchymal stromal cells, also referred to as mesenchymal stem cells (MSCs), are discussed in more detail in other chapters of this book (see Chaps. 4 and 5). Briefly, MSCs are nonhematopoietic oligopotent progenitors that reside in mesenchymal tissues including bone, cartilage, muscle, and fat. Furthermore, MSCs reside in the bone marrow microenvironment to provide critical stromal support for hematopoietic development (Calvi et al. 2003; Mendez-Ferrer et al. 2010). MSCs can give rise to fibroblasts, adipocytes, and other nonhematopoietic stromal cells that provide growth signals and contact for hematopoietic stem and progenitor cells in the stem cell niche. One recent clinical trial reported that co-infusion of MSCs facilitated more rapid platelet engraftment following allogeneic haploidentical HSCT (Liu et al. 2011). Additionally, allogeneic bone marrow-derived MSCs have been transplanted following bone marrow transplantation to accelerate bone growth in patients with congenital bone disorders such as osteogenesis imperfecta and have even been administered in utero to achieve greater donor chimerism during fetal development for the same disorder (Le Blanc et al. 2005). Marrow-derived osteogenic progenitors can persist in the marrow in endosteal niches, although the capacity for new bone growth in the diaphysis of long bones may be limited by intrinsic defects in patients with congenital bone disorders (Marino et al. 2008). Furthermore, MSCs have immune modulatory and anti-inflammatory properties that have particular relevance in the HSCT setting and have been studied extensively as a means of inducing immune tolerance in patients with graft versus host disease (GVHD) and also in limiting tissue damage caused by the profound inflammatory response associated with GVHD (Le Blanc et al. 2004; Le Blanc et al. 2005). The immunosuppressive and anti-inflammatory effects of MSCs have also been investigated in autoimmune conditions such as Crohn's disease (Ciccocioppo et al. 2011) and multiple sclerosis (Karussis et al. 2010). Several large studies have been conducted in North America and Europe, and the approval of MSCs as a form of immunosuppressive therapy for specific clinical applications appears likely.

Although MSCs can be expanded quite readily from bone marrow, they are rare and may be transplanted in very small numbers when bone marrow harvests are used in HSCT. MSCs, however, do not circulate readily in the peripheral blood, even after conventional cytokine stimulation with G-CSF and are present only at negligible levels in PBSC grafts. It is possible to expand MSCs from umbilical cord blood, although only 20–30% of UCB units can support MSC expansion, making it likely that MSCs are infrequently transplanted in UCB units used in HSCT (Flynn et al. 2007). Biomarkers associated with the capacity to expand MSCs from UCB units remain under active investigation and could be useful in selecting units for MSC expansion. The relevance of MSCs, therefore, in the HSCT setting appears restricted to the use of bone marrow grafts. Many studies comparing BM to PBSC transplantation have not reported major differences in terms of toxicity and long-term complications, and quality of life appears similar, at least in the allogeneic setting (Friedrichs et al. 2010). Overall, the role of MSCs in reducing the toxicity of

HSCT appears minimal and is likely restricted to the acceleration of hematopoietic engraftment in certain settings such as haploidentical transplantation or umbilical cord blood transplantation and to their use as third party cells for the treatment of GVHD.

10.2.2 Endothelial-like Vascular Progenitor Cells

Endothelial-like vascular progenitor cells (VPCs and also termed EPCs) are discussed in more detail in several chapters in this book (see Chaps. 2 and 8). Briefly, VPC-like cells encompass a number of cell types that can be collected from peripheral blood, bone marrow, and umbilical cord blood and consist of endothelial precursors that are recruited to sites of tissue injury where they facilitate vascular repair and recovery of organ function by inducing and modulating angiogenesis. In one study, endothelial progenitor cells were able to support the reendothelialization of injured blood vessels through the replacement of dysfunctional endothelial cells (Rabelink et al. 2004), whereas many studies report that infused VPCs can home and adhere to sites of vascular injury and facilitate endogenous repair through paracrine secretion of putative cytokines and other factors. Many aspects of the mechanisms involved in VPC-mediated vascular repair remain incompletely understood and under active study.

Cells that give rise to VPCs can be enriched and enumerated using flow cytometry to identify cells expressing specific surface markers such as CD34, CD133, and vascular endothelial growth factor receptor 2 (VEGFR-2) (Asahara et al. 1997; Peichev et al. 2000). Many additional markers have also been described, and the precise identity of cell populations most enriched for vascular repair remains under investigation. It appears likely that multiple cell populations with angiogenic immunophenotypes contribute to the process of vascular repair. While some markers are shared by hematopoietic stem/progenitor cell populations (Verfaillie 2002; Shizuru et al. 2005; Bryder et al. 2006), other markers appear distinct and suggest the persistence of hemangioblast-type precursors that are independent of the hematopoietic hierarchy (Verfaillie 2002). VPC clusters can also be enumerated using cell culture–based progenitor assays that identify "endothelial cell colony-forming units" (CFU-ECs). CFU-ECs are grown by replating nonadherent cells following an adherence depletion step in culture to remove mature endothelial cells. Initial spindle-shaped cells attach to fibronectin-coated plastic plates and attract other cells to cluster, including monocytes and lymphocytes under specific cytokine stimulation. The clustering of nonclonal immune cells, including monocytes expressing angiogenic surface markers may reflect the various cell types that contribute in vivo to vascular repair and may be targets for further understanding the paracrine effects that may direct endogenous repair following ischemic vascular injury.

Importantly, VPC cluster levels have particular clinical relevance and correlate strongly with markers of cardiovascular health (Hill et al. 2003), where increased VPC levels are associated with favorable cardiovascular risk profiles. In their study,

Hill et al. (2003) suggest that low levels of circulating VPCs may account for the persistence of endothelial dysfunction following repeated endothelial injury associated with cardiovascular risk factors such as elevated cholesterol, hypertension, and diabetes.

The therapeutic role of various VPC-like cells has been demonstrated in several animal models of ischemic tissue damage (Asahara et al. 1999; Zhang et al. 2002) and in clinical studies of ischemic injury such as myocardial infarction and stroke where favorable outcomes related to tissue repair and improved organ function have correlated with increased levels of circulating VPCs (Shintani et al. 2001; Ghani et al. 2005) or with the infusion of VPC-like cells. However, the administration of VPCs or bone marrow-derived cell populations enriched for VPCs into patients with cardiovascular disease does not necessarily lead to new vessel formation (Yoder et al. 2007). It appears that paracrine effects of infused VPCs are responsible for the modest clinical improvements seen in patients, without long-term VPC engraftment in newly formed vessels (Hristov and Weber 2006; Badorff and Dimmeler 2006; Dimmeler et al. 2005).

In the context of HSCT, there are few studies addressing the infusion of VPC-like cells, although VPCs can be mobilized in autologous and allogeneic PBSC products (Allan et al. 2007), and one study from our group that addressed the role of VPCs in the autologous HSCT setting suggested that high VPC cluster levels in PBSC grafts were associated with reduced transplant-related toxicity (Iqbal et al. 2008). We hypothesized that re-infusing grafts with greater numbers of VPC clusters may better protect against organ dysfunction caused by the conditioning treatment regimen. The investigation of strategies designed to increase VPC levels in stem cell grafts is ongoing. Taken together, these findings support the important role that VPCs may play in tissue repair and suggest that VPCs may be relevant in the HSCT setting.

10.2.3 Endothelial Colony-Forming Cells

An important source of therapeutic blood-derived endothelial progenitors is endothelial colony-forming cells (ECFCs), also termed late outgrowth EPCs or blood outgrowth endothelial cells (BOEC) (Lin et al. 2002; Lin et al. 2000). ECFCs have gained much attention due to their clinical relevance and therapeutic potential and are distinct from VPC-like cells discussed previously. They can be expanded from human peripheral blood and from umbilical cord blood and likely originate from somatic vascular precursors within the vasculature (Ingram et al. 2004; Ingram et al. 2005) or from precursors within the bone marrow. ECFCs are organized into a hierarchy of progenitor states that have varying levels of proliferative potential that can be identified in clonal plating conditions (Ingram et al. 2004). These *ex vivo* expanded cells have robust proliferation in vitro and significant vessel-forming capacity in vivo making them attractive candidates for vascular regenerative therapy (Yoder et al. 2007). However, cells that differentiate into ECFCs are found at very

low frequency of less than one per million nucleated cells in steady-state peripheral blood (Ingram et al. 2004; Reinisch et al. 2009). These cells and their progeny express endothelial but not hematopoietic cell surface markers and appear to be clonally distinct from hematopoietic stem cells (Yoder et al. 2007). Interestingly, ECFCs can be efficiently expanded from CD146-selected cells from umbilical cord blood and co-express CXCR4. Higher CXCR4 surface expression has been associated with increased vascular repair capacity, suggesting that SDF-1 signaling via CXCR4 may be a dominant pathway for angiogenic signaling in these cells (Oh et al. 2010). Interestingly, the co-injection of VPCs and ECFCs has been shown to synergistically improve neovascularization in mice following hind limb ischemic injury (Yoon et al. 2005). Whether ECFC capacity of peripheral blood increases following cytokine stimulation used in the collection of grafts for HSCT is unknown, and to our knowledge, there are no studies of infusing ECFCs as part of HSCT. Cells that give rise to ECFCs may be present within blood stem cell grafts but more research is needed to understand the role of ECFCs or ECFC-initiating cells in HSCT.

10.2.4 Circulating Angiogenic Cells and Angiogenic Monocytes

Circulating angiogenic cells (CACs) and angiogenic monocytes can also contribute to vascular repair. CACs arise from peripheral blood cultured on fibronectin-coated plates under conditions that favor endothelial differentiation for 4 to 7 days using specific tissue culture medium and growth factors (Critser and Yoder 2010). Nonadherent cells are typically removed, and the remaining adherent cells, or CACs, acquire an angiogenic phenotype with the majority of cells taking up acetylated LDL and ulex-lectin and manifesting endothelial cell surface antigens such as CD144 and von Willebrand factor. CACs appear to arise from the monocyte/macrophage lineage and possess limited endothelial differentiation potential and minimal proliferative capacity but have been reported to have important angiogenic function (Kalka et al. 2000). It is possible that CAC culture methods include platelets that co-fractionate with mononuclear cells (Prokopi et al. 2009) and become attached to adherent mononuclear cells in culture. Consequently, platelet membrane proteins may be transferred to adherent cells, thereby conferring an angiogenic phenotype and possible angiogenic properties to these adherent cells (Critser and Yoder 2010). Although some studies have reported that CACs can increase blood vessel perfusion and capillary density in athymic nude mice with hind limb ischemia (Kalka et al. 2000), CACs are likely cells of hematopoietic origin that are unable to form de novo blood vessels in vivo, but may still contribute to vascular repair and the regulation of normal and abnormal blood vessel formation (Asosingh et al. 2008). It is also likely that proangiogenic effects of CACs are due to the secretion of growth factors (Kalka et al. 2000) and the ability of monocytes to differentiate into endothelial-like cells (Fernandez et al. 2000; Schmeisser et al. 2001). Culturing blood mononuclear cells on fibronectin is known to activate monocytes and their differentiation into macrophages (Wesley et al. 1998). Moreover, CD163, a cell

surface marker of monocyte-to-macrophage differentiation, and the activation marker CD11c are upregulated on CACs after 4 days in culture in comparison to circulating monocytes of the same donor (Buechler et al. 2000). Consequently, the specific angiogenic culture conditions may be required to amplify the proangiogenic effects of CACs. Prospective isolation of specialized monocytes expressing the cell surface protein Tie-2, an angiopoietin-2 receptor, has also proved promising in preclinical models of vascular injury. In the context of HSCT, the role of infusing stem cell products enriched in monocytes is unclear. When considering the role of monocytes, it may require more insight to balance the benefits of their angiogenic potential with the risk of provoking more profound and damaging inflammatory responses at sites of tissue injury. Measurement of specialized subsets of monocytes such as Tie-2(+) monocytes will be required to better appreciate opportunities for reducing the toxicity associated with HSCT. The relative abundance of monocytes in PBSCs in particular, makes the prospect of vascular repair using PBSCs worthy of further study.

10.3 Mobilizing Cells with Regenerative Properties in PBSCs

PBSC grafts have evolved as the most common source of stem cells used in adult HSCT, in both the autologous and allogeneic setting. PBSCs may be ideal for applications in regenerative therapy given the opportunity to manipulate the conditions of mobilizing cells into the peripheral circulation and the ability to control the parameters of PBSC collection during leukapheresis, including timing, duration, and postcollection interventions. Among cell types with regenerative capacity that are associated with PBSCs, VPCs have demonstrated particular promise. VPCs can be mobilized from quiescent vascular zones of the marrow (Ribatti et al. 2002) and blood, and cells that give rise to VPC clusters can be readily collected in PBSCs. Although standard mobilization schemes involving chemotherapy and cytokines such as G-CSF can yield an eightfold increase in circulating VPCs (Allan et al. 2007), the optimal conditions for collection of graft VPCs remain unclear. Additional cytokine stimulation using granulocyte-monocyte colony-stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF) (Aicher et al. 2003), erythropoietin (Heeschen et al. 2003), estrogen (Strehlow et al. 2003), and HMG-CoA reductase inhibitors (Walter et al. 2004) may also influence VPC mobilization and collection. In this section, we will briefly discuss the potential role of common cytokines in current use that may influence the graft content of VPCs.

10.3.1 G-CSF

Granulocyte colony-stimulating factor (G-CSF) has been used clinically for more than 15 years to mobilize hematopoietic stem cells into the peripheral circulation, facilitating the collection of PBSCs for both autologous and allogeneic HSCT

(Mikirova et al. 2009). G-CSF appears to induce matrix metalloproteinase–dependent alteration of the stromal cell–derived factor-1 (SDF-1) gradient in the bone marrow (Jin et al. 2008; Carion et al. 2003) as well as remodeling the extracellular matrix of the bone marrow (Ratajczak et al. 2008; Lee et al. 2009). In addition to mobilizing hematopoietic stem cells, G-CSF can mobilize VPCs through similar mechanisms (Pitchford et al. 2009). In vivo, G-CSF releases the proteinases elastase and cathepsin G from neutrophils which induce the cleavage of cell-cell adhesive bonds on stromal cells, which include integrin-mediated cellular interactions with hematopoietic stem cells (Levesque et al. 2001). These proteinases cleave SDF-1, releasing it from its receptor CXCR4 on stem and progenitor cells (Levesque et al. 2003) and increasing SDF-1 levels in circulation. Greater peripheral mobilization of stem and progenitor cells ensues (Hattori et al. 2001). However, G-CSF also promotes inflammation by inducing an increase in circulating mature granulocytes (Aicher et al. 2005). In patients undergoing G-CSF-stimulated mobilization of PBSCs, VPCs also increase in the peripheral blood and can be enumerated in the graft as VPC clusters. There is no apparent correlation, however, between the number of VPCs enumerated using the cell cluster assay and hematopoietic progenitors, measured by flow cytometric enumeration of CD34(+) cells or by CFU colony numbers, suggesting that the mobilization kinetics of HSCs and VPCs may be under separate control. Furthermore, cytokine profiling of plasma from patients undergoing G-CSF-stimulated PBSC mobilization has demonstrated that MIP-1alpha is increased in the plasma prior to mobilization in patients with greater capacity to mobilize VPCs. Plasma EPO levels, however, are significantly increased on the day of PBSC collection in patients with greater VPC mobilization, suggesting a role for EPO in the mobilization of VPC cluster-producing cells.

10.3.2 EPO

Erythropoietin (EPO), a glycoprotein that stimulates the proliferation of early erythroid precursors and the differentiation of the red blood cell lineage (Krantz 1991), and has also been shown to mobilize vascular progenitors (Urao et al. 2006; Bahlmann et al. 2004). Mature endothelial cells in culture express EPO receptors, and when EPO binds, a proangiogenic response is induced resulting in endothelial cell proliferation and greater in vitro migration (Anagnostou et al. 1990; Carlini et al. 1995). EPO administration in mice was associated with increased hematopoietic stem cell proliferation and a marked increase in circulating VPCs with enhanced neovascularization function in vivo (Heeschen et al. 2003). Furthermore, Heeschen et al. (2003) identified EPO as an independent predictor of VPC levels and function in patients with coronary heart disease and suggest that the stimulatory effect of EPO on vessel formation is related to the stimulation of vasculogenesis by inducing proliferation and differentiation of VPCs and by activating angiogenesis via mature endothelial cells. They suggest that reduced EPO serum levels may help identify patients who have an impaired ability to recruit or mobilize VPCs. Consistent with

these observations, Labonté et al. (2009) found that in patients undergoing autologous hematopoietic stem cell transplantation, plasma EPO levels on the day of PBSC collection correlated well with graft VPC levels. The possibility of using EPO as a biomarker for the collection of increased VPCs in PBSCs, in the same way peripheral blood CD34(+) cell numbers can guide the collection of hematopoietic progenitors, is appealing and introduces the possibility of using exogenous EPO or interventions that serve to increase endogenous EPO levels to increase graft VPC levels and the regenerative capacity of PBSC grafts (Labonté et al. 2008). At least one study where EPO was used to support chemotherapy treatments before PBSC collection was associated with reduced toxicity after transplantation, supporting the notion that EPO could influence graft composition although graft VPC levels were not reported in this study (Hart et al. 2009). EPO-induced VPC mobilization may act by stimulating nitric oxide synthase in regions of ischemic tissue injury (Burger et al. 2006) where local increases in nitric oxide can induce greater number of circulating VPCs through increased recruitment and mobilization (Urao et al. 2006). Caution is warranted, however, in the cancer setting where increased angiogenesis may lead to enhanced tumor growth (Lyden et al. 2001), although evidence from clinical trials supports the use of recombinant human EPO as a treatment option for patients with chemotherapy-associated anemia (Rizzo et al. 2002). In patients with multiple myeloma and concomitant disease–related anemia who were receiving chemotherapy treatment, EPO administration was associated with improved quality of life and longer survival (Mittelman et al. 1997). In several controlled clinical trials involving the use of EPO in the mobilization of PBSCs, insufficient information related to the post-transplant toxicity is available to make meaningful conclusions, and more study is required to understand the role of EPO in HSCT (Filip et al. 1999; Olivieri et al. 1995; Kessinger et al. 1995).

10.3.3 CXCR4 Antagonists

The use of CXCR4 antagonists to mobilize PBSCs has increased in recent years. Profound increases in circulating CD34(+) cells occur within hours after administration (Micallef et al. 2009). Furthermore, VPC-like cells are also mobilized but since CXCR4-expressing cells have been implicated in vascular repair processes through binding to damaged tissues that produce SDF-1 in response to tissue hypoxia, it remains unclear what effect the CXCR4-antagonist, plerixafor, may have on the regenerative function of these cells. Since plerixafor has a relatively short half-life and PBSC products are collected well in advance of autologous HSCT, deleterious effects of plerixafor would seem unlikely. The possibility that CXCR4 antagonists could be used to mobilize allogeneic PBSCs from healthy donors that are infused into recipients within several hours of collection may require closer scrutiny. In one study, the use of plerixafor was associated with rapid mobilization of VPC-like cells; however, prolonged administration limited their ability to adhere to sites of tissue damage (Shepherd et al. 2006).

10.4 Role of Anemia in Transplant Patients: A Surrogate Marker of Regenerative Capacity?

Transfused blood products are routinely required following high-dose preparative regimens and contribute to the intense resource utilization associated with transplantation. Moreover, transfusion has been associated with immune modulation (Haynes et al. 2001; Avall et al. 2002), delayed tissue healing (Weber et al. 2005a), longer hospital stays (Weber et al. 2005b; Vamvakas and Carven 1998), and increased mortality in some clinical settings (Hébert et al. 1999). Anemia has been identified as an important factor contributing to increased transfusion requirements and has been associated with greater transplant-related mortality following allogeneic transplantation (Xenocostas et al. 2003).

The role of anemia in transplant-related toxicity following autologous transplantation, however, has not been addressed in previous studies. Unlike allogeneic grafts from healthy volunteers, pre-transplant anemia in patients undergoing autologous HSCT may influence graft composition, including cells responsible for timely erythroid recovery and robust tissue regeneration. Further, a broad range of intervention strategies may be envisioned to address pre-transplant anemia in patients undergoing autologous HSCT which may not be possible in allogeneic donors.

We recently addressed the role of pre-transplant anemia on toxicity following autologous HSCT and on red cell transfusion requirements. Interestingly, multivariate modeling found reduced hemoglobin on the day of PBPC collection to be significantly associated with reduced toxicity following transplantation but not with post-transplant red cell transfusion requirements. In contrast, anemia in the unstimulated steady state prior to transplantation was associated with red cell transfusion requirements post-transplant but not toxicity.

Previous work from our group highlighted the presence of vascular progenitors in autologous stem cell grafts (Allan et al. 2007) and showed that increased levels of these cells in PBPC grafts were associated with reduced tissue injury after transplantation (Iqbal et al. 2008). Additional data supports an association between reduced hemoglobin on the day of PBPC collection and the graft content of VPCs (Kasbia et al. 2008). Taken together, it appears that anemia on the day of autologous stem cell collection has an important influence on the graft content of cells capable of vascular repair which may then influence organ toxicity after autologous HSCT.

Additional work is required to increase our understanding of the biological mechanisms that determine vascular repair processes and the relevance of anemia. Perhaps, anemia in the HSCT setting reflects a damaged marrow microenvironment and relative incapacity to accommodate rapid erythroid recovery in addition to depleted reserves of blood-derived cells with regenerative function. There are many additional concomitant factors which complicate our understanding of how anemia and red cell transfusion may influence cells involved in vascular repair and recovery after HSCT. In a pilot study addressing the impact of red cell transfusion on circulating VPC levels in critically ill patients, no apparent change in VPC levels was

observed (Abou-Nassar et al. 2009), but more work is needed in this area, and a clinical trial of red cell transfusion after HSCT is ongoing to address a possible association with increased toxicity [clinical trials.gov].

10.5 Future Applications of Blood Stem Cell Products for Vascular Repair and Tissue Regeneration

In summary, HSCT offers a unique clinical situation where the timing and nature of tissue damage can be easily predicted and where stem cell products that contain nonhematopoietic cells with regenerative capacity are infused, allowing us to study aspects of clinical regenerative therapy that could be applicable in other forms of clinical regenerative therapy. Nonhematopoietic cells including MSCs, vascular progenitors, and angiogenic monocytes are among the cell types involved in tissue regeneration and may be readily procured in blood products that are routinely used in HSCT. Further refinements in our understanding of the specific cell types that are most relevant will allow us to develop strategies that optimize the collection and reinfusion of cell products with enhanced regenerative potential.

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Chapter 11
Concepts to Facilitate Umbilical Cord Blood Transplantation

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Abstract Blood stem cell transplantation aims to regenerate the entire hematopoietic system. Until recently, a significant proportion of patients in need of a hematopoietic cell transplant could not undergo transplantation due to the lack of a suitable matched related or unrelated adult donor. With the advent of umbilical cord blood harvesting and processing technology, the majority of patients can now receive a suitable transplant. This chapter will review current cord blood engineering strategies with a particular focus on novel concepts to facilitate blood stem cell engraftment and hematopoietic reconstitution. Because blood stem cell transplantation serves as the model system for many novel regenerative stem cell therapy strategies, the concepts described are considered to be applicable to nonhematopoietic stem cell therapy as well. Groundbreaking laboratory-based cell processing technologies and strategies to improve engraftment will be discussed.

11.1 History of Hematopoietic Stem Cell Transplantation

Since the first successful transplantation (TX) of hematopoietic stem and progenitor cells (HSPCs) by E.D. Thomas in 1957 to rescue patients after intensive radiation and chemotherapy (Thomas et al. 1957), the use of autologous and allogeneic blood-forming stem cells (SCs) has become an increasingly routine treatment for malignant and selected nonmalignant hematological diseases. Constant improvement in TX regimens and new methods for SC mobilization into the circulation have resulted in more than 25,000 autologous and similar numbers of allogenic TXs becoming

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performed annually worldwide. The concurrent expansion of donor registries facilitates the search for optimal immunological matching of HSPCs and enables the ability to perform transplants within a reasonable timeframe. Up to 80% of patients will either have a matched sibling donor (MSD) or will find matched unrelated donors (MUD) using the donor registries. Unfortunately, there are still up to 20% of patients that lack an appropriate donor, many of whom belong to ethnic minorities.

After systematic analysis of cord blood HSPC content and its hematopoietic reconstitution potential (Broxmeyer et al. 1989), Eliane Gluckman and Hal E. Broxmeyer (Gluckman et al. 1989) used cord blood for the first time from human leukocyte antigen (HLA) identical siblings for the TX of a patient with Fanconi's anemia in 1989. This seminal work paved the way for the use of HSPCs from umbilical cord blood (UCB) for patients for whom no matched bone marrow (BM) or mobilized peripheral blood HSPC donor could be found. Since this time, public and private cord blood banks have begun to develop, which has led to the storage of more than 450,000 cord blood units worldwide. From 1989 to 2010, more than 20,000 UCB TXs were performed (Foeken et al. 2010).

The advantages of using UCB as an alternative HSPC source include the relative ease of procurement, the reduced risk of transmitting infections like CMV, and the storability of HLA-typed units ready for immediate use. For patients, UCB TX has the dual advantage of requiring less stringent immunohistocompatiblity matching combined with a lower incidence in graft versus host diseases (GvHD). Due to limited physical volumes and subsequently lower numbers of HSPCs (generally below 10% of the numbers of approximately $2–4 \times 10^6$ $CD34^+$ HSPCs per kg recipient body weight (BW) transplanted in conventional BM or mobilized peripheral blood [mPB] transplants), UCB is still considered as an alternative SC source for TX of adults with regular body weight with more predominant use in children and low weight adults. Cell dose, total nucleated cell (TNC) count, and $CD34^+$ cell dose are considered the most important predictors of UCB TX success (Gluckman et al. 2004; Cohen et al. 2010; Herr et al. 2010). Current recommendations are summarized by E. Gluckman and recommend $>3 \times 10^7$ TNCs/kg BW or $\geq 2 \times 10^5$ $CD34^+$ cells/kg of recipient body BW in cases where ≤ 2 HLA disparities exist in single unit UCB TX (Gluckman 2009). HLA matching for UCB TX is typically determined in low (two-digit) resolution for HLA A and B loci and high (four-digit) resolution for HLA DRB1. Many centers require identical matching at HLA DRB1, thus confining HLA disparities to HLA A or B among the six tested loci (Wagner et al. 2002). Lower cell number results in delayed recovery of white blood cells (WBCs) and platelets, leading to increased risk of infections and subsequent transplant-related morbidity and mortality. The lack of cells available for posttransplant therapies, such as donor lymphocyte infusions, has further been considered as a disadvantage of UCB TX. Nevertheless, after successful engraftment, UCB may provide better reconstitution of the hematopoietic reservoir compared to BM or mPB HSPC TX (Frassoni et al. 2003). The concurrent use of two partially matched cord bloods (Barker et al. 2001) could partially circumvent the problem of low cell numbers, but results in higher frequency of GvHD, is more cost intensive, and increases the time to find appropriately matched units. Other concepts to overcome the problem of

limiting cell numbers are promising and include cell expansion methods, but the optimal way to expand HSPCs in vitro without losing their immature state and long-term reconstitution potential could still be considered the finding of the "holy grail" of HSPC biology.

Within the last few years, the increasing knowledge about HSPC function, homing, and behavior in the BM microenvironment, including the regulatory cellular components of the BM niche, could help to develop several strategies that allow improved protocols for the expansion of repopulating cells with concurrent preservation of SC properties in vitro. These strategies have mainly been evaluated in murine preclinical models, but several approaches have already reached the translation into clinical trials.

11.2 Strategies to Improve UCB TX

In principle, five different approaches (Fig. 11.1) have been extensively tested to improve the outcome of UCB TX with the goal of enhancing engraftment and decreasing time to recovery of conditioning-induced cytopenias:

1. *Ex vivo* liquid culture of UCB-derived HSPCs in standard growth factor containing expansion media that includes newly discovered proteins (Zhang et al. 2008), synthetic receptor ligands (Delaney et al. 2005, 2010; Boitano et al. 2010), and copper chelators (de Lima et al. 2008; Petropoulos and Chan 2009) and other supplementary factors.
2. *Ex vivo* cellular conditioning during short-term pretreatment of the transplant graft prior to infusion with modulators such as prostaglandin E2 (North et al. 2007), CD26 (dipeptidylpeptidase VI) inhibitor (Christopherson et al. 2004; Campbell et al. 2007), or 1,3-fucosyltransferase (Xia et al. 2004) to achieve higher and/or faster BM engraftment.
3. Coculture with BM niche-derived auxiliary stromal cells like mesenchymal stem/progenitor cells (MSPCs) (Noort et al. 2002; Robinson et al. 2006) and endothelial cells (ECs) or their immature progenitors named endothelial colony-forming progenitor cells (ECFCs) (Butler et al. 2010). Stromal cells can mimic in vivo conditions and are therefore considered to be a promising concept in increasing the quantity of immature HSPCs. The major challenge of all in vitro expansion methods continues to be the preservation of the self-renewing immature stem and progenitor cell population responsible for long-term engraftment and life-long sustained hematopoiesis.
4. A further promising way that has already been translated into a clinical trial (ClinicalTrials.gov Identifier: NCT00393380, Study of Parathyroid Hormone Following Sequential Cord Blood Transplantation From an Unrelated Donor) is a modification of the recipients' hematopoietic BM niche with parathyroid hormone (PTH) (Adams et al. 2007). Instead of manipulating the composition and cellularity of the graft, this strategy aims to physically expand the HSPC niche.

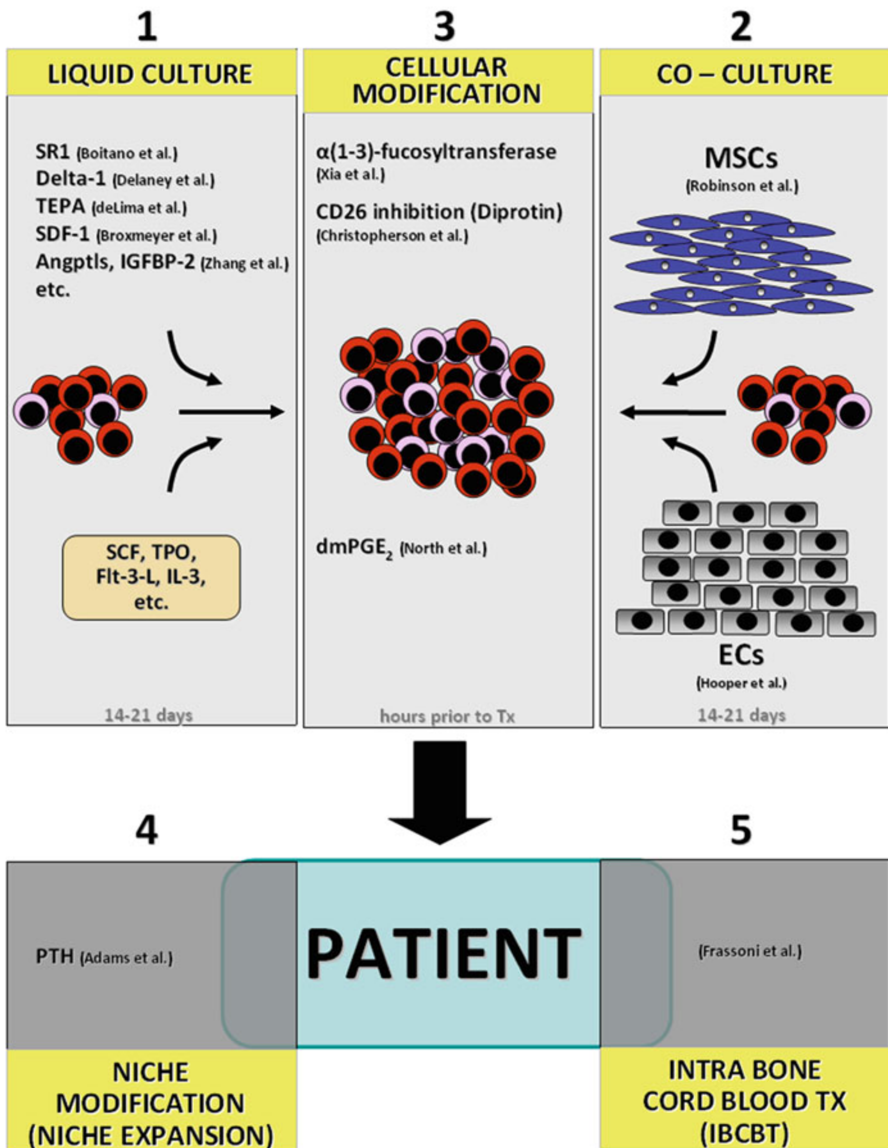

Fig. 11.1 Five strategies to improve stem cell engraftment. Different strategies have been developed over the recent years modifying the stem cells (1–3) or the conditions within the recipient (4+5) to boost engraftment and eventually aiming to result in functionally stable sustained (hematopoietic) organ regeneration. References within this illustration indicate prototype strategies and appear within the general reference list at the end of this chapter. Liquid suspension culture protocols without (1) or with (3) feeder cells mimicking conditions resulting in progenitor or even stem cell expansion have been widely tested and require action over longer time periods. More recently, rather acute (within hours) enzymatic or chemical modification of stem cell surface structures has been established mainly aiming to increase stem cell homing possibly accompanied by an elevated state of residence of the stem cells within their niche. Manipulation that directly influences the homing environment within the patient may include (4) therapeutic expansion of the niche size or (5) direct transplantation of stem cells into the target organ (e.g., intrabone), thus reducing the risk of stem cell trapping on the way to the target lesion

5. To minimize the loss of HSPCs through unintended cell trapping in organs other than the BM (mainly lung, spleen, and liver) that would further exacerbate issues related to low cell dose, direct intrabone UCB TX (IBCBT) (Castello et al. 2004; Frassoni et al. 2008) has emerged as a feasible strategy to overcome the problems of graft failure. Direct IBCBT has been associated with better engraftment rates and a lower incidence and severity of acute GvHD (Frassoni et al. 2008).

Successful treatment strategies may also be used in combination to overcome the limitations of single method use and maximize the benefit for the patients.

11.2.1 *Ex vivo* Expansion of Cord Blood

Regarding the preservation of the major HSPC features after *ex vivo* expansion, there are highly contradictory results described in literature. On the one hand, scientists have provided evidence of conserving stem cell self-renewal, long-term engraftment potential, and transplantability into secondary and even tertiary recipients after *ex vivo* expansion. Others argue that a selective proliferation of particular hematopoietic subsets, in particular short-term reconstituting HSPCs at the expense of long-term reconstituting "high-quality" HSCs, may occur. This could result in delayed hematopoietic exhaustion and subsequent graft failure despite initially supporting timely neutrophil recovery (Piacibello et al. 1999; Lewis et al. 2001; McNiece et al. 2002; Shpall et al. 2002). This earlier engraftment, facilitated through the expanded cells, could provide a clinical advantage and benefit for patients by limiting the duration of cytopenia, thereby reducing transplant-related mortality and morbidity. Combining expanded cells with unmanipulated UCB transplants either by using double cord TX (dUCB TX [one expanded, one unmanipulated]) or dividing a single UCB transplant into one fraction that is expanded and the other is left untouched (Pecora et al. 2000; de Lima et al. 2008) could allow for both rapid recovery after myeloablation together with long-term maintained hematopoiesis.

11.2.2 Traditional Liquid Culture

One strategy to mimic the cytokine and growth factor milieu naturally present around the BM niche is liquid culture. $CD34^+$ and/or $CD133^+$-enriched HSPCs are traditionally cultured in the presence of animal serum, culture supplements, growth factors, and cytokines. The proteins that are most commonly used include stem cell factor (SCF), thrombopoietin (TPO), granulocyte colony-stimulating factor (G-CSF), Flt-3-ligand, interleukin-3 (IL3), and interleukin-6 (IL6) (Lazzari et al. 2001a; Lazzari et al. 2001b; Yao et al. 2004; Mohamed et al. 2006). One additional step for improving safety in the human TX setting would be the reduction or general abandonment of xenogeneic proteins like animal serum in liquid culture media.

Several groups have already introduced animal serum-free culture conditions (Lazzari et al. 2001a; Yao et al. 2006). Nevertheless, the optimal combination of culture supplements has yet to be defined.

11.2.3 Improved Liquid Culture

Many different compounds (Fig. 11.1) have been tested to further optimize liquid culture conditions of human HSPCs in vitro. In addition to the various commonly used different growth factor cocktails, many newly discovered proteins and chemicals are currently under evaluation regarding their potential to further augment HSPC expansion and reconstitution potential. Most recently, Boitano et al. reported that antagonism of aryl hydrocarbon receptor (ARH) with a purine derivative called StemReginin 1 (SR1) resulted in a dramatic increase in CD34$^+$ cells and 17-fold increase in immature cells that were able to repopulate SCID mice (increasing SCID repopulating cells [SRCs]) (Boitano et al. 2010). A role of Notch signaling in hematopoiesis was suggested after the detection of *Notch1* gene expression in human CD34$^+$ cells and the enhancing effect of the Notch ligand Delta-1 on self-renewal and repopulating capacity of human hematopoietic precursors (Milner et al. 1994; Ohishi et al. 2002; Delaney et al. 2005). In 2010, Delaney and colleagues further showed Notch-mediated expansion of human UCB progenitors that were able to substantially shorten time to neutrophil recovery (ANC $\geq$ 500/μl) after allogenic TX in a phase 1 clinical trial (Delaney et al. 2010).

The reduction of cellular copper in *ex vivo* culture conditions enabled preferential proliferation of hematopoietic progenitors and immature CD34$^+$/CD38$^-$ HSPCs (Peled et al. 2002), thus establishing another strategy to augment UCB TX efficiency. Tetraethylenepentamine (TEPA; StemEx), a low-molecular-weight linear polyamine chelator with strong binding affinity to copper, could increase hematopoietic colony-forming units (CFUs) by 172-fold and immature CD34$^+$/CD38$^-$ HSPC number by 30-fold over input level (Peled et al. 2004a; Peled et al. 2004b; de Lima et al. 2008; Petropoulos and Chan 2009) and has already been translated into a clinical phase I/II trial conducted at MD Anderson Cancer Center, Houston, Texas (de Lima et al. 2008). Liquid culture of HSPCs with stromal derived factor 1 (SDF-1 or CXCL12) has been found to induce proliferation and enhance survival of human and mouse HSPCs that lead to increased engraftment of competitive repopulating cells (Broxmeyer et al. 2003; Broxmeyer et al. 2007). By transcriptional profiling, angiopoietin-like proteins (Angptls) and insulin-like growth factor binding protein 2 (IGFBP2) were identified as hematopoietic growth factors increasing SRCs up to 20-fold (Zhang and Lodish 2004; Zhang et al. 2006; Zhang et al. 2008). Other proteins that can potentially regulate hematopoietic stem cell function are reviewed by Zhang et al. (Zhang and Lodish 2008).

Short-term treatment of the transplant graft prior to infusion, thereby directly modifying HSPCs to increase engraftment, is another promising approach that could facilitate UCB use. CD26 (dipeptidylpeptidase IV [DPPIV]) is a surface

molecule that truncates SDF-1, thereby blocking its chemotactic activity. Inhibition of CD26 with small peptides (Diprotin A) prior to HSPC infusion into conditioned recipients can lead to enhanced homing and facilitated engraftment into the murine as well as human BM niche (Christopherson et al. 2002; Christopherson et al. 2004; Campbell et al. 2007; Christopherson et al. 2007). Another related approach is the short-term treatment (two hours pulse) of BM with prostaglandin E_2 (PGE_2) or its long-acting derivative 16,16-dimethyl PGE_2 ($dmPGE_2$) (North et al. 2007). This lipid compound of the eicosanoid family has been considered to increase the number of repopulating HSPCs without disturbing their differentiation. Enhanced BM engraftment after PGE_2 treatment could be associated with increased homing via upregulation of CXCR4 (Hoggatt et al. 2009). These findings have been translated into a clinical trial testing the effect of $dmPGE_2$ on hematopoietic reconstitution in a double UCB transplantation protocol (ClinicalTrials.gov Identifier: NCT00890500, Reduced Intensity, Sequential Double Umbilical Cord Blood Transplantation Using Prostaglandin E2 [PGE2]). In this specific trial, one of the cord blood samples is treated with the compound, the other one left untouched.

Another novel technology called "glucosyltransferase-programmed stereosubstitution" (GPS) has been introduced to modify cell surface glycans without affecting cell viability or immune phenotype. GPS is used to convert membrane CD44 into a potent E-selectin ligand glycoform known as hematopoietic cell E-/L-selectin ligand (HCELL) (Sackstein 2009). Exofucosylation with α(1–3)-fucosyltransferase enforces higher E-selectin ligand expression on HSPCs and can improve extravasation into the BM parenchyme and subsequent engraftment (Xia et al. 2004).

11.2.4 The Hematopoietic Niche Concept: Ex Vivo Coculture with Supporting Niche Cells

As early as the 1970s, Dexter and coworkers established HSPC culture systems comprising an adherent fraction of supporting stromal cells (reticular cells, endothelial cells, giant fat cells, and macrophages) and a nonadherent fraction of hematopoietic cells (Dexter et al. 1973; Dexter and Testa 1976; Dexter et al. 1977a; Dexter et al. 1977b; Dexter et al. 1977c). The progeny of these adherent cells was described by Friedenstein as colony-forming unit of fibroblasts (CFU-F) (Friedenstein et al. 1974a; Friedenstein et al. 1974b) and can transfer the hematopoietic microenvironment to secondary recipients. Later on others termed these cells mesenchymal stem cells (MSCs) (Caplan 1991; Prockop 1997).

Based on these early discoveries, Schofield hypothesized in 1978 the presence of cells in the BM that are in close contact to HSPCs and have the ability to directly or indirectly regulate HSPC behavior and number (Schofield 1978). These cells provide the structural components of physically discrete microenvironments within the BM and act as the basis for the BM SC niche concept. By further elucidating this complex anatomical site, a distinct SC supportive microenvironment has been identified at the (1) "endosteal niche," the cellular interface between the marrow cavity

and its surrounding bony structures (Calvi et al. 2003; Zhang et al. 2003; Arai and Suda 2007; Arai et al. 2009) as well as (2) in close proximity to the BM sinusoids and adjacent mesenchymal cells. This area was later termed the "(peri)vascular niche" (Avecilla et al. 2004; Kiel and Morrison 2006; Sugiyama et al. 2006; Kiel and Morrison 2008). So far, it is not clear whether the "endosteal SC niche" and "vascular SC niche" are indeed separately organized compartments within the BM or overlapping anatomical units (Lo Celso et al. 2009; Xie et al. 2009). The HSPC niche, in general, consists of heterogeneous populations of cells of mesodermal origin. These cells include mesenchymal stem and progenitor cells (MSPCs) with various functions in a variety of developmental stages (Mendez-Ferrer et al. 2010; Omatsu et al. 2010), mature sinusoidal ECs (SECs), and ECs with higher proliferative potential, reminiscent of endothelial colony-forming progenitor cells (ECFCs) (Ingram et al. 2004; Ingram et al. 2005; Reinisch et al. 2009). This cell population can potentially help to replenish the BM vasculature after toxic or radiation injury caused by conditioning therapy. Adult BM in particular is rich in adipocytes that can act as negative regulators of the hematopoietic microenvironment (Naveiras et al. 2009) (Fig. 11.2).

Highly complex cellular and molecular interactions between HSPCs and cells of the hematopoietic niche are responsible for tightly regulated HSPC maintenance, self-renewal, proliferation, and differentiation (Dexter et al. 1973; Allen and Dexter 1984; Allen et al. 1984; Lemischka and Moore 2003; Fuchs et al. 2004; Wilson and Trumpp 2006).

The historical but still valid concept of the BM hematopoietic stem cell niche provides the basis for virtually all currently tested coculture strategies. The most frequently used cellular niche components for coculture approaches are multipotent MSPCs. Coculture of HSPCs with MSPCs can restore at least some of the molecular clues that are thought to occur within the niche microenvironment and appear to be essential for the self-renewal and maintenance of HSPCs. MSPCs are known to secrete hematopoietic cytokines and support long-term hematopoiesis (Majumdar et al. 2000). Interactions of immature cells with MSPC feeder layers lead to their migration and proliferation, thereby allowing them to form cobblestone-like areas, which is considered one of the most powerful in vitro assays predicting precursor frequency within stem and progenitor cell bulk populations (tested in the cobblestone area-forming cell [CAFC] assay).

For HSPC expansion, MSPC monolayers are established before culturing enriched $CD34^+$ and/or $CD133^+$ HSPCs in medium containing growth factors and cytokines (Yamaguchi et al. 2001; McNiece et al. 2004; Robinson et al. 2006; Reinisch et al. 2007; Andrade et al. 2010). An increase of TNCs as well as hematopoietic precursor frequency evaluated by CFC assays has been reported after coculture. Remarkable expansion of $CD34^+$ cells and the more immature $CD34^+/CD38^-$ HSPCs was demonstrated (Reinisch et al. 2007). Due to additional immunomodulatory (Le Blanc 2003; Le Blanc et al. 2004; Tyndall et al. 2007) and engraftment supporting properties of MSPCs (Koc et al. 2000; Noort et al. 2002), systemic co-application together with the expanded hematopoietic cell fraction may even bear clinical benefits and provides a further strategy to be tested in clinical trials. Increased

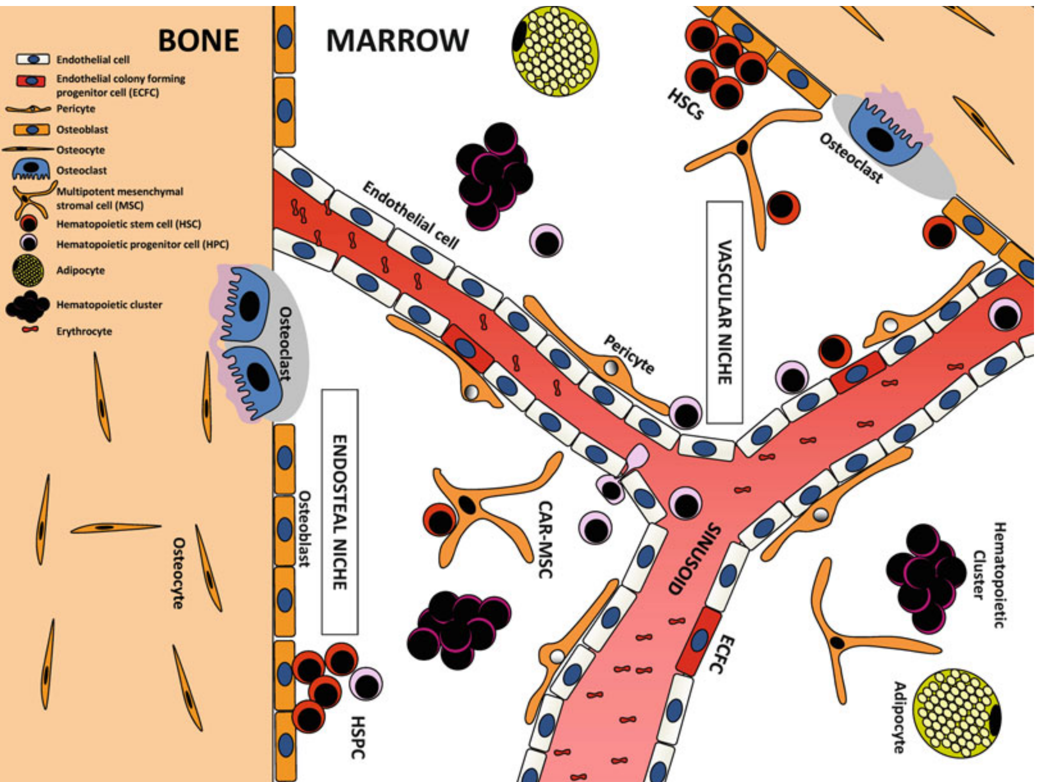

Fig. 11.2 The bone marrow microenvironment represents the prototype stem cell niche. Within the mammalian bone marrow, at least two anatomically but not functionally distinct vascular and endosteal niches have been introduced. At least in the murine marrow, there exists a peculiar chemokine CXC ligand (CXL12) abundantly expressing reticular (CAR) mesenchymal stromal cell possibly connecting different niche sites and/or serving as a mesenchymal stem/progenitor cell (MSPC) of mature niche components. Additional abbreviations point to a variety of interacting cell types and are explained in the symbolic legend

human engraftment was successfully shown in xenogeneic murine models after cotransplantation of Stro1⁻ (Bensidhoum et al. 2004) and even placental derived human MSPCs (Hiwase et al. 2009).

The fact that HSPCs in situ localize to BM endothelial cells raised the question of whether ECs can also regulate hematopoiesis. Intact BM sinusoidal vessels appear to have an impact on HSPC engraftment and hematopoietic reconstitution (Hooper et al. 2009; Kopp et al. 2009). Transplantation of ECs and endothelial progenitor cells mediated hematopoietic recovery and induced reconstitution in lethally irradiated mice (Chute et al. 2007; Salter et al. 2009). Microvascular ECs isolated from human BM have been demonstrated to be capable of supporting proliferation and differentiation of myeloid and megakaryocyte progenitors (Rafii et al. 1994; Rafii et al. 1995). In vitro EC-derived (angiocrine) growth factors could support the self-renewal and repopulation capacity of long-term HSCs (Butler et al. 2010). A tight balance of the intracellular signaling cascades of ECs is important to achieve self-renewal and HSPC expansion instead of differentiation and commitment into mature hematopoietic lineages (Kobayashi et al. 2010). The contribution of human ECFCs during hematopoietic reconstitution in a UCB Co-TX model is currently being investigated in our laboratory (Reinisch et al. unpublished data).

11.2.5 Direct Intrabone UCB TX

The limited number of HSPCs in UCB grafts and a further reduction after intravenous (i.v.) injection have prompted investigators to deliver CB grafts directly into the bone marrow (BM) cavity. After conventional application, most of the cells do not reach the marrow niche and are most likely sequestrated within other organs with large capillary beds. The strategy of intrabone cord blood transplantation (IBCBT) was first used in rats and mice (Kushida et al. 2001; Castello et al. 2004) and has most recently been translated in the human TX setting by F. Frassoni. A groundbreaking clinical trial underway in Italy could provide proof of the feasibility and safety of this procedure (Frassoni et al. 2008). A follow-up study of Eurocord and EBMT (Rocha et al. 2010) comparing single unit i.v. IBCBT versus double UCB TX (dUCB TX) showed in multivariate analysis adjusting for statistical differences between both groups, that recipients of single IBCBT had improved disease-free survival (DFS), faster platelet recovery and decreased acute GVHD compared to dUCB TX recipients.

11.2.6 Recovery of Superior HSPC Numbers

Since the collection of UCB from placental veins is strictly dependent on the skills, time, and motivation of midwives or other professionals performing the collections, appropriate education and training of these experts is probably the most reasonable

way to improve the quantity of transplantable UCB HSPCs. Aiming for 30 million TNCs per kg BW of a 70-kg recipient requires collection of 2.1 billion TNCs. This translates to a minimum volume of 210 mL UCB to be collected provided there is a TNC count of 10,000 white blood cells per μL (corrected after subtracting nucleated red cell precursors present in UCB). Alternatively, a quality control measure may be introduced for high-quality UCB banking aiming for a minimum TNC content of at least one billion TNC per unit before freezing [TNC total = TNC/μL x volume (mL) × 1,000].

11.3 Future Directions/Outlook

The valuable improvements in the field since the first UCB TX more than 20 years ago include better understanding of HSPC biology as a result of the continuous effort of basic scientists as well as innumerable clinicians and have established UCB as an almost equivalent source of HSPCs. The key to further improve UCB TX efficacy will be to translate new laboratory-based discoveries into clinical protocols for assessing their potential benefit for patients in prospective clinical trials. UCB TX serves as a model for future applications of blood stem cell therapies, including ones that involve other cell types and applications in regenerative therapy.

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Chapter 12
Cord Blood Banking for Regenerative Therapy

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Abstract The first successful umbilical cord blood (UCB) transplant in 1989 on a patient with Fanconi anemia sparked the creation of cord blood banks worldwide, including more than 100 public banks and nearly 500,000 banked units. Although cord blood units have been historically collected and stored for use in hematopoietic transplantation, there is increasing attention regarding nonhematopoietic cells within cord blood units and their important regenerative functions, making the prospect of using UCB for vascular repair or regenerative therapy most exciting. In this chapter, we provide an outline of various cell types with regenerative function that are present in UCB units and discuss the potential role of using banked cord blood cells for regenerative therapy.

12.1 Nonhematopoietic Stem Cells with Regenerative Function in Cord Blood

Various nonhematopoietic cell types with regenerative function have been discussed in greater detail in other chapters of this book (see Chaps. 2–4, 6, 7). Herein, we briefly describe specific cells types that are most relevant to umbilical cord blood and address practical issues related to the expansion, enumeration, and functional analysis of these cell types with respect to their use in regenerative therapy.

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12.1.1 Mesenchymal Stem Cells

Mesenchymal stem or stromal cells (MSCs) can differentiate to cell types of mesenchymal origin, including bone, cartilage, and muscle. MSCs can be harvested from several sources including bone marrow, adipose tissue, and umbilical cord blood. Many clinical trials have been conducted or are ongoing that address the immune modulatory, regenerative, and angiogenic properties of MSCs to treat a range of disorders that encompass cardiac disease, autoimmune disorders, and graft versus host disease that can occur after bone marrow transplantation. MSCs can also be co-infused with hematopoietic stem cells to accelerate engraftment after bone marrow transplantation. A total of 17 randomized clinical trials involving cord blood–derived MSCs are registered on www.clinicaltrials.gov (searched April 2011), including one study recruiting patients with type 1 diabetes to a trial of autologous cord blood–derived MSC therapy. One previously published report addressed the potential of utilizing autologous cord blood–derived MSCs that were expanded and then reinfused following myocardial infarction (Siepe et al. 2008). Another study has also addressed the use of autologous UCB-derived MSCs for the treatment of congenital heart valve defects (Nishiyama et al. 2007). Preliminary data has revealed significant migration of MSCs to the site of tissue damage in response to injury signals, and the cells appear to contribute to tissue remodeling and healing, suggesting that autologous MSCs expanded from UCB may be candidates for cell therapy to facilitate tissue healing. In other studies, MSCs derived from UCB engrafted in the lung in both preclinical mouse models and in preliminary clinical studies of acute and chronic lung injuries. Although modest benefit was reported in these studies, precise mechanisms remain unclear (Brody et al. 2010).

One drawback of UCB-derived MSC therapy is the time required for MSC expansion which is typically several weeks or more. Furthermore, several groups have reported low rates of MSC growth from UCB units, ranging from 20–30% (Wexler et al. 2003), although cord blood plasma biomarkers or gene expression profiles may prove useful in allowing cord blood banks to predict in advance which UCB units can support MSC expansion. Alternatively, banks could expand MSCs at the time of collection from fresh units and store MSCs separately for later use. Increased rates of MSC expansion may also be possible from the cord itself or Wharton's jelly. Once the indications for MSCs become clearer with additional clinical studies and clinical trials, banks will need to adapt to the potential need to bank UCB-derived MSCs for regenerative applications and guidelines, and quality control measures will need to be standardized.

12.1.2 Unrestricted Somatic Stem Cells from Placental Cord Blood

Unrestricted somatic stem cells (USSCs) were recently described by Kogler et al. as rare but highly proliferative CD45-negative cells from placental cord blood that can give rise to endodermal and mesodermal tissues in animal models (Kogler et al. 2004).

In particular, USSCs may have relevance in the treatment of myocardial infarction, bone formation, and in neurological disorders (reviewed in Chap. 6). USSCs have been shown to express vascular endothelial growth factor (VEGF), the corresponding VEGF-Receptors (VEGFR), and CD31 (PECAM), which is shared with monocytes and endothelial cells (Jansen et al. 2010). Taken together, autologous cell therapy using USSCs may have broad application. Furthermore, the migratory capacity of USSCs seems to correlate positively with surface expression of CXCR4, which can be up-regulated and down-regulated, respectively, by VEGF and insulin-like growth factor (Ahmadbeigi et al. 2010).

Cord blood–derived MSCs and USSCs are distinct from adult bone marrow-derived MSCs in terms of gene expression profiles and neurodifferentiation potential (Greschat et al. 2008). Furthermore, MSC and USSC from cord blood can be distinguished based on cell surface antigens and in their differentiation response to exogenous factors. For example, overexpression of Delta Like 1/Preadipocyte Factor (PREF-1) in MSCs leads to reduced MSC adipogenic differentiation while underexpression in USSC was associated with increased differentiation potential (Kluth et al. 2010). In addition, MSCs can be distinguished from USSCs based on homeobox (HOX) gene expression patterns. In particular, gene expression of HOXA9, HOXB7, HOXC10, and HOXD8 were informative and demonstrated increased expression in both adult and cord blood MSCs in comparison with USSCs which resembled more closely the HOX gene expression pattern associated with human embryonic stem cells (Liedtke et al. 2010).

Although there is tremendous interest and excitement surrounding USSCs, much remains to be learned about these cells and substantial preclinical work remains to be done. Nevertheless, banking of umbilical cord blood units may prove critical for embracing the full potential of these rare cells. At present, autologous infusion of USSCs seems most practical until we learn more about their immunological properties. Furthermore, since they behave like embryonic stem cells in many ways, vigilance will be required to monitor for ectopic tissue or teratoma formation.

12.1.3 Endothelial-Like Vascular Progenitors

Endothelial-like vascular progenitor cells (VPCs and also referred to as EPCs) were first described in 1997 as CD34+ human circulating cells expressing endothelial cell characteristics (Asahara et al. 1997). Although the immunophenotype of true endothelial progenitors remains unclear (see Chap. 2), EPCs typically express CD31 and FlK-1 and can be enriched from CD34-selected or CD133-selected cells from blood, bone marrow, and umbilical cord blood (Peichev et al. 2000). EPCs can be distinguished from hematopoietic stem and progenitor populations which typically lose CD34 and FlK-1 expression during in vitro differentiation (Murohara 2010). Ongoing work is aimed at delineating a more precise hierarchy among endothelial and vascular progenitor populations. Monocytes that express Tie-2, however, retain angiogenic potential and are hematopoietic in origin which

may account for some of the observed overlap in hematopoietic and angiogenic function of blood-derived cells.

Expanding cells in culture with greater proliferative capacity and with endothelial-like function has been encouraging in several models of tissue injury. Endothelial colony-forming cells (ECFCs) or blood outgrowth endothelial cells (BOECs) (Lin et al. 2000, 2002) can be readily expanded from UCB and could be stored for later use, analogous to MSCs as discussed above. ECFCs or BOECs have been associated with improved organ function in several preclinical animal models and in early phase clinical studies (see Chap. 2 for more complete description). The degree of ECFC expansion is highly variable and biomarkers may be required to identify units that could support robust ECFC expansion. One can combine cell isolation strategies with cell expansion protocols as described recently which may increase the clinical potential of the cellular product (Delorme et al. 2005). Expansion protocols can be cumbersome, time consuming, costly, and require close regulatory scrutiny, which may pose challenges for some clinical situations and jurisdictions. Moreover, at least one report has described karyotypic aberrations that can occur with clinical scale ex vivo expansion of late outgrowth endothelial cells (Corselli et al. 2008). Whether third-party cells could be used in a non-HLA compatible manner, like MSC therapy for immune modulation, remains to be tested.

There may be particular advantages in using cord blood–derived EPC-like cells or ECFC cells compared with adult autologous cells given changes in progenitor cells that can occur with aging. While EPCs or ECFCs can be produced by adult blood and bone marrow, cord blood–derived progenitors have greater proliferative capacity and may be better suited to repair of vascular injury (Au et al. 2008). The greater number of passages that can be supported by UCB in comparison to adult-derived VPC cells makes UCB-derived cells particularly attractive for banking.

12.2 Clinical Applications for Regenerative Therapy Using Cord Blood Cells

Exciting preliminary work in animal models has highlighted the potential of human cord blood–derived cells for tissue regeneration. Examples include the initial reports that cells capable of vascular repair can improve hind limb ischemia, demonstrated in several animal models by numerous groups (Murohara 2001 for review), and a more recent observation that cord blood cells can be used to improve fracture healing in mice (Nagano et al. 2010) as well as many other examples of acute tissue injury. In addition to these models of acute injury, autologous cells have been used to improve chronic diseases associated with tissue and organ damage including the use of autologous MSCs to treat colitis (Duijvestein et al. 2010) and neurodegenerative disease (see Chap. 9). Taken together, these findings support the prospect that autolgous cord blood could be a potential source of cells for similar indications. Use of cord blood cells for tissue regeneration in human studies is

beginning to emerge. One exciting example is the use of autologous umbilical cord blood transplantation in patients with cerebral palsy with three active studies registered with www.clinicaltrials.gov (searched April 11, 2011).

The use of cord blood cells for regenerative therapy is complicated by several factors. Following hematopoietic stem cell transplantation, using cells from an allogeneic donor, conditioning therapy involving chemotherapy, radiation, and/or other immunosuppressive drugs is required to prevent the rejection of allogeneic donor cells and to prevent the development of graft versus host disease, whereas the use of intensive conditioning in patients undergoing cell therapy for vascular repair or tissue regeneration would be prohibitive. Less intensive immunosuppressive regimens used in solid organ transplantation may not be sufficient to prevent rejection and graft versus host disease. Until these important immunological barriers can be addressed, transplantation of UCB cells for regenerative therapy may be limited to autologous cord blood cells, HLA-identical allogeneic cells, or third-party non-HLA compatible cells that do not induce immune reactions against the host. There is, however, extensive experience with the infusion of third-party MSCs derived from bone marrow to treat refractory graft versus host disease in patients following HSCT (Sundin et al. 2009), suggesting that infusing third-party cells derived from umbilical cord blood may also be feasible from the perspective of immune privilege. The immunogenicity of USSCs and various EPC-like cells remains to be fully elucidated, and it remains uncertain how these cells may be applied in the allogeneic cell therapy setting. If HLA-matching is required, this will introduce a level of complexity and cost and could compromise the timeliness of therapeutic interventions. Once the immune hurdles have been addressed, however, there remains great promise and much excitement regarding the prospect of using cord blood–derived cells for regenerative therapy.

In a recent survey on the clinical use of cord blood for nonhematopoietic indications, the European Blood and Marrow Transplant (EBMT) group reported that no autologous cord blood transplants were performed in 2008 and that all of the 768 cord blood transplants were performed for hematological indications (Gratwohl and Baldomero 2010). Another recent survey from the EBMT indicates a growing number of mesenchymal stem cell transplants and HSCTs for nonhematopoietic indications, including cardiovascular disorders, neurological diseases, and for tissue repair (Gratwohl et al. 2009). It is possible that some transplants using cord blood–derived cells have been performed but were not captured in these surveys. The American Society for Blood and Marrow Transplantation also has a committee that is addressing the use of nonhematopoietic indications for HSCT, including the use of cord blood units, and the results of this survey should be available soon. It will be interesting to observe in the coming years whether the interest and excitement regarding cord blood–derived cells for regenerative therapy translates into greater activity captured by these surveys.

The development of highly immunoablative therapy for refractory autoimmune diseases such as multiple sclerosis, systemic lupus erythematosus, and Crohn's disease necessitates reinfusion of autologous peripheral blood stem cells to restore the blood system. Many protocols involve the use of CD34-selected cellular products to

limit immunological recovery. In general, the results have been encouraging in terms of arresting the immunological injury, particularly in multiple sclerosis (Atkins 2010; Marmont and Burt 2008). Although immune-mediated damage may be arrested, subsequent improvements in organ function have been described, and the role of reinfused CD34(+) cells or other cells present in the mobilized cellular graft cannot be excluded from the repair process. Autologous banking of cord blood may influence the choice of stem cells for the treatment of autoimmune disease in future protocols. It remains to be seen whether the immune-mediated damage can be arrested and whether endogenous repair can be facilitated within damaged organs after reinfusion of autologous cord blood.

12.3 Characterizing Cord Blood Units with Regenerative Capacity

The development of reliable biomarkers that can identify cord blood units enriched for cells capable of mediating vascular repair could greatly improve cell-based therapy for regenerative medicine. Applications in regenerative therapy could greatly expand the clinical utility of banked umbilical cord units, and powerful biomarkers of regenerative capacity may assist in the collection and ultimate selection of units best suited for applications in vascular repair. In addition, the ability to expand specific cell types in culture from umbilical cord blood units is highly variable, as reported recently for MSCs (Wexler et al. 2003). Alternatively, specific cell types such as MSCs, EPCs, or ECFCs could be expanded from selected units, and these specialized cell products could be banked for future use. More work is needed to improve our ability to quickly and selectively identify units that are optimal for regenerative applications, but insight in this area will likely be crucial to the cost-effective development of banks with cord blood units suitable for applications in regenerative therapy.

12.4 Banking Cord Blood for Regenerative Therapy

Another issue related to the use of cord blood cells for regenerative therapy relates to the need to cryopreserve CB units. Cryopreservation is associated with significant cellular injury during the freezing process and due to recrystallization injury that occurs while in the frozen state and during thawing. Recrystallization leads to ice reshaping that causes damage to cell membranes and cellular apoptosis. The hematopoietic function of cryopreserved human umbilical cord blood units can be augmented using inhibitors of recrystallization injury and may be a promising avenue to improve the storage of UCB for both hematopoietic and regenerative applications (Wu et al. 2011). Whether or not current methods can preserve the regenerative function of umbilical cord blood units remains under study (Vanneaux et al. 2010).

Many protocols utilizing MSCs from umbilical cord blood involve fresh samples. Whether storage conditions can be optimized to allow MSC or ECFC expansion from cryopreserved cord blood units remains unclear. Although the criteria for selecting units for HSCT principally focus on volume collected and the total number of nucleated cells in the stored unit (Shaw et al. 2009), the parameters that define cord blood units that are best suited to regenerative applications remain to be defined. Although public banks have emerged in most developed countries, private sector banking may offer some specific advantages for indications where autolgous cells appear most encouraging.

12.5 Practical Limitations and Challenges

In addition to the funding challenges facing both public and private banks, the development of cord blood banks that focus on regenerative applications faces several important hurdles. Strict regulatory requirements will be needed that are specific for cell types used in regenerative applications, building on regulatory requirements developed by the foundation for the accreditation of cellular therapy (FACT) and Netcord, a foundation of member cord blood banks. Both FACT and Netcord have developed widely adopted standards for the collection and storage of umbilical cord blood units. In addition, the labeling and record keeping required of cord blood banks and the approach to infectious disease testing, HLA testing and cord blood unit searching requirements provide an essential foundation for the development of requirements for the use of cord blood cells for regenerative therapy. The international community will be collectively able to develop and implement the appropriate standards to ensure a safe transition from preclinical studies to early phase studies of regenerative therapy in humans.

The role of private and public banks remains uncertain with regards to regenerative therapy. If cells from cord blood units need to be immune-compatible, then HLA-searching of international banks and/or the use of autologous or related units from private banks may both have something to offer. The use of third-party cells such as MSCs may allow greater ease of access to units from public banks and possibly the use of units that are not ideally suited for HSCT but remain efficacious for regenerative applications.

12.6 Summary and Conclusions

Banking readily infusible blood products for regenerative therapy using cells derived from umbilical cord blood may be part of mainstream cord blood banking in years to come. Keeping abreast of cell types, clinical applications, and regulatory, safety, and quality issues will challenge the organization and funding of cord blood banks. Resources and personnel will be needed to ensure the timely development of cord blood banks that are prepared for applications in regenerative therapy.

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Chapter 13
Regulatory Questions in the Development of Blood Stem Cell Products for Regenerative Therapy

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Abstract Blood stem cells have been used for decades to regenerate the bone marrow in patients with degenerative disorders of the blood system. Recent studies demonstrating the potential of blood stem cells to mediate the regeneration of tissues other than blood provide encouragement for their broader therapeutic use. Regulatory oversight plays a key role in ensuring the safety, quality and effectiveness of blood stem cells for regenerative therapy. This chapter outlines the current federal regulations governing therapeutic use of blood stem cells from a Canadian perspective.

13.1 Background

13.1.1 The Use of Blood Stem Cells in Regenerative Medicine

Blood is comprised of many cell types that differ in both their phenotype and function (Ogawa 1994). Most of these cells are relatively short lived, and their supply must be replenished on a frequent basis (Ogawa 1993). The task of blood system replacement is the responsibility of a single cell known as the blood stem cell or hematopoietic stem cell. Blood stem cells reside within the marrow portion of mammalian bones and are capable of replenishing the blood system over the entire lifespan of an individual (Morrison et al. 1995). In the late 1950s, it was determined that diseases of the blood system could be successfully treated by transplantation of bone marrow from a healthy donor (Ford et al. 1956). Since that time, bone marrow

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transplantation has been used countless times to treat marrow failure due to radiation and chemotherapeutic insults or diseases such as myelodysplasia, leukaemia and lymphomas. With the identification of blood stem cells in the early 1960s (Till and McCulloch 1961), it is now known that bone marrow transplantation represents the first use of stem cell–based regenerative therapy to treat disease. Scientific discoveries over the past few decades have allowed improvements to stem cell isolation and transplantation protocols and identified additional sources of blood stem cells for use in these procedures. Today, the use of blood stem cells for regenerative therapy of the bone marrow is an accepted and well-established medical procedure.

With the development of techniques to grow and characterize human embryonic stem cells came an explosion of interest in the therapeutic use of stem cells, of both somatic and embryonic origin. Many researchers began to examine whether stem cells from somatic sources may function in a non-homologous manner to regenerate tissues outside of the organ in which they reside (e.g. skeletal muscle stem cells differentiating into neurons) (Ferrari et al. 1998; Bjornson et al. 1999). As the most highly characterized type of somatic stem cell, and the only one accepted for use in humans at the time, blood stem cells were a prime research candidate for testing this theory. As a result, the ability of blood stem cells to regenerate tissue other than bone marrow has been studied extensively both in culture and in animal models (Gussoni et al. 1999; Brazelton et al. 2000; Lagasse et al. 2000; Jackson et al. 2001; Orlic et al. 2001a; Orlic et al. 2001b; LaBarge and Blau 2002; Hess et al. 2003). These studies have indeed reported the potential of blood stem cells to either directly differentiate into, or augment the formation of, neurons, skeletal muscle, cardiac muscle, liver and pancreatic islets. This pre-clinical evidence suggests that blood stem cells may have uses far beyond traditional therapies for blood disorders.

13.1.2 Pathways for Regulatory Oversight of Blood Stem Cell–Based Products and Therapies

The science of blood stem cells is certainly very intriguing, and their promise for treating diseases and disorders for which there is currently no known cure may be immense. An important aspect of utilizing blood stem cells for medicinal purposes and in regenerative therapies is ensuring that both the processes and products are safe and effective. In most developed countries, this task falls under the mandate of the National Regulatory Authority or the federal government. In the European Union, United States of America (USA) and Canada, the government bodies responsible for regulatory oversight to ensure the safety and effectiveness of therapies and health products are the European Medicines Association (EMA), the US Food and Drug Administration (FDA) and Health Canada, respectively. This chapter outlines the regulatory mechanisms used by Health Canada to review and evaluate blood stem cell–based products. With some exceptions, the principles underlying the need for regulatory oversight of cell-based medicines and regenerative therapies are similar to those utilized by the aforementioned agencies.

Health Canada is a department of the federal government that is overseen by the Minister of Health who reports to Canadians through parliament. Health Canada is responsible for regulating the safety, efficacy and quality of health interventions, including drugs and therapies, through the authority conferred under the *Food and Drugs Act*. *Drugs*, as defined in the *Food and Drugs Act*, include 'any substance', or mixture of substances, manufactured, sold or represented for use in: (a) the diagnosis, treatment, mitigation or prevention of a disease, disorder or abnormal physical state, or its symptoms, in human beings or animals; and (b) restoring, correcting or modifying organic functions in human beings or animals. Consequently, blood stem cells fall under this definition and are regulated as drugs under this Act. In general, Health Canada utilizes a risk-based regulatory framework when assessing drug products. This approach ensures the level of regulatory oversight applied corresponds to the level of risk associated with the product.

Consistent with the risk-based approach, blood stem cells are subject to two different regulatory pathways developed under the authority of the *Food and Drugs Act*. The pathway that is utilized to oversee the safety, quality and efficacy of a blood stem cell–based product or therapy is chosen based on two important parameters:

(a) The amount of manipulation used for processing the cells
(b) The intended therapeutic use for the cells

More specifically, the therapeutic use of blood stem cells obtained from allogeneic sources through well-established procedures for collection, distribution and storage, to regenerate bone marrow, is considered standard medical practice. Such stem cell products are regulated through the Safety of Human Cells, Tissues and Organs for Transplantation Regulations, referred to as the CTO Regulations henceforth. Alternatively, the use of blood stem cells which are more than minimally manipulated (e.g. ex vivo expansion, differentiation, gene modification, photodynamic treatment, encapsulation in biomaterials, tissue engineering), or for treatment of diseases other than hematologic in nature, is considered experimental and regulated under *Part C*, *Divisions 1A*, *2*, *5* and *8* of the *Food and Drug Regulations*. The following sections of this chapter highlight the areas of these regulatory frameworks that apply to the oversight of blood stem cells and therapies.

13.2 Regulation of Blood Stem Cells Used in Standard Medical Practice: The Safety of Human Cells, Tissues and Organs for Transplantation Regulations

In Canada, the therapeutic use of blood stem cells through protocols of standard medical practice does not require pre-market review and approval by Health Canada but is currently regulated under the CTO Regulations. The purpose of these regulations is to minimize the health risks associated with cells, tissues and organs that have been procured for transplantation. To accomplish this, the regulations outline requirements for safe processing, storage, distribution and importation of cells, tissues

and organs used in transplantation as well as specific requirements for error, accident and adverse reaction investigation and reporting. The CTO Regulations were created in response to a lack of consistent standards in the field of transplantation and the need for a comprehensive approach to safety evaluation and monitoring. They were developed by Health Canada through extensive consultation with transplantation experts, provincial and territorial governments and the public. The technical requirements of the regulations are based on standards of safety and were designed using a traditional risk management approach and harmonize well with the policies and regulations of federal authorities from other nations. Specific sections of the CTO Regulations mandate that the National Standard for Cells, Tissues and Organs for Transplantation and Assisted Reproduction: General Requirements be followed. This national standard and its subset standards are administered by the Canadian Standards Association (CSA) and are updated regularly based on new science. For blood stem cell transplantation specifically, CSA standards must be applied for the assessment of donor suitability as outlined by the National Standard of Canada entitled 'Lymphohematopoietic Cells for Transplantation'. By referencing the CSA standards, Health Canada has ensured that the CTO Regulations is a constantly evolving document that will incorporate new standards of safety as they arise.

13.2.1 What Is the Scope of CTO Regulations?

In general, CTO Regulations apply to cells, tissues and organs of human origin for which the transplantation has been well established as effective and safe through long-term, repetitive clinical use. More specifically, the regulations apply to blood stem cells that are as follows:

Minimally manipulated
Allogeneic
Used in a homologous manner

The terms 'minimally manipulated' and 'homologous' are key points in these regulations. Minimal manipulation, with respect to cells processed for transplantation, is defined within the regulations as 'processing that does not alter the biological characteristics that are relevant to their claimed utility'. A key concept of this definition is the necessity for sufficient evidence that methods used for retrieval, preparation and banking do not affect the capacity of blood stem cells to engraft and generate hematopoietic tissue. Minimal manipulation in cell processing includes cell selection, washing, formulation, preservation and cryopreservation. Blood stem cells that have been more than minimally manipulated fall outside of the scope of the CTO Regulations since these procedures introduce new safety, quality or efficacy concerns. Blood stem cell–based products derived through more than minimal manipulation are, therefore, regulated by Health Canada as a drug through the *Food and Drug Regulations*.

The concept of 'homologous use' is also important in determining whether a product to be used for transplantation is encompassed under the CTO Regulations

or regulated as a biologic drug. The regulations define 'homologous' as a cell, tissue or organ that is performing 'the same basic function after transplantation'. In the context of blood stem cells, 'homologous' refers to products that are used to regenerate the blood system after transplantation. By inference, the use of blood stem cells to treat or regenerate any tissue other than the bone marrow is considered 'non-homologous use' and would not fall under the jurisdiction of the CTO Regulations, at this time, but regulatory oversight would be provided under the authority of the *Food and Drugs Act and Regulations*.

In the case of blood stem cells, the CTO Regulations encompass cells derived from bone marrow, umbilical cord blood (UCB) or mobilized peripheral blood (MPB). Each of these sources has been well characterized and is known to provide blood stem cells that allow regeneration of the bone marrow following transplantation. They are, thus, considered safe for treating disorders of the blood based upon previous studies and empirical evidence when used in compliance with the CTO Regulations. When considering mobilized peripheral blood, it is important to note that only specific protocols for stem cell mobilization have received regulatory approval in Canada. Thus, a clinical trial is required in cases where novel methods of mobilization are employed in order to establish their safety and effectiveness, as well as to assess their potential impact on stem cell function.

13.2.2 How Are the CTO Regulations Applied?

A main component of the CTO Regulations, and an important tool for monitoring compliance with safety standards, is the registration of 'source establishments' with Health Canada. The criteria defining a source establishment, as outlined in the CTO Regulations, differ depending on both the type of cell, tissue or organ and the establishment responsible for determining that the material is safe for use in transplantation. Since allogeneic blood stem cells are derived from living donors when used for transplantation, their level of suitability for safe use is usually determined by the transplant facility. As such for blood stem cells, the transplant facility is usually designated as the source establishment. The CTO Regulations recognize that different activities related to blood stem cell transplantation are completed by multiple establishments on a continuum. Accordingly, when multiple establishments play a role in the processing of blood stem cells, the transplant facility is considered the source establishment and is responsible for determining the safety of the cells by reviewing results obtained by all participating facilities. The regulations also recognize that banked bone marrow, UCB or MPB can provide an additional source for transplantation. In the case of banked stem cells, the facility that is responsible for banking and distributing the cells is considered a source establishment.

Registration is a mandatory requirement for all source establishments in Canada. During the registration process, source establishments must provide to Health Canada a list of activities that it carries out as well as an attestation, signed by the medical director, certifying compliance with CTO Regulations and the underlying

national standards for each of these activities. Arguably, the most important aspect of monitoring regulatory compliance is the requirement that source establishments both audit their activities and submit to an inspection from Health Canada. Audits must be conducted every 2 years by an individual that is not directly responsible for the audited activities and should verify the compliance of those activities with CTO Regulations. Inspection of source establishments by Health Canada representatives is to be completed on a routine basis. Inspectors will review the facility and relevant documentation that pertains to the activities carried out by the source establishment in order to verify compliance with CTO Regulations.

Health Canada acknowledges that there are cases where blood stem cells that are suitable for transplantation are procured from donors outside of Canada and require importation. In such cases**,** exporting establishments do not require registration with Health Canada, regardless of whether or not they completed the suitability and safety testing. Given the lack of international requirements for suitability and safety assessments for cells, tissues and organs used in transplantation, and the challenges with finding compatible donors for potential stem cell recipients, the CTO Regulations allow for some flexibility on how imported cells are determined to be safe. However, the onus of ensuring that the imported stem cells are processed in compliance with CTO Regulations is on the registered Canadian source establishment that is using them for transplant.

13.2.3 What Are the Safety Standards Set Out in the CTO Regulations?

The requirements set out in Health Canada's CTO Regulations concentrate on five specific areas:

Processing (as defined in CTO Regulations and described later)
Errors, accidents and adverse reaction reporting
Record keeping
Personnel, facilities, supplies and equipment requirements
Quality assurance systems

The specifications for carrying out each of these activities must be defined in standard operating procedures that are readily accessible to any personnel that are completing the activities. Except for the methods by which blood stem cells are prepared for use in transplantation, the activities listed above are regulated by a common set of requirements regardless of the cell, tissue or organ type. In general, for the screening and preparation of blood stem cells, CTO Regulations refer to CSA standards outlined in Lymphohematopoietic Cells for Transplantation.

The term 'processing' as defined in the CTO Regulations encompasses a broad scope of activities beyond the simple isolation and purification of blood cells prior to transplantation. The regulations refer to accepted methods for donor screening, testing and assessment of suitability. This initial screening process is based on

medical/social history, physical examination and laboratory testing. These screening procedures are designed to identify donors of a suitable blood group, and leucocyte antigen match and exclude donors that are unsuitable due to an increased risk for transmitting infectious disease. Disease agents that are mandatory for testing include human immunodeficiency virus, types 1 and 2 (HIV-1, HIV-2), hepatitis B virus (HBV), hepatitis C virus (HCV), human T-lymphotropic virus type I and type II (HTLV-I, HTLV-II), cytomegalovirus and syphilis. These agents must be tested using licensed serological test kits by laboratories certified to perform the tests. In addition, nucleic acid tests for HIV-1, HCV and West Nile Virus are currently recommended. Mandatory testing must be completed within 30 days prior to collection of cells for transplantation in the case of bone marrow and MPB. Currently, for UCB donation, specimens for testing must be collected within 7 days of retrieval. Source establishments that have imported a stem cell source for use in transplantation are responsible for ensuring that the requirements of the regulations have been met. In the case of bone marrow and MPB, if the donor suitability process determines the donor cells are safe for transplantation, cell retrieval and preparation methods can commence. The methods themselves are not outlined in the CTO Regulations but must be described by the establishment in standard operating procedures. The blood stem cell source must be packaged and labelled accordingly and, if not being used for transplantation immediately, can be preserved or banked.

13.2.4 Rules of Exceptional Distribution

Circumstances can arise when stem cells from a compatible donor are available but do not meet the criteria for safety as outlined in the CTO Regulations. Health Canada recognizes that, because blood stem cell transplantation is often an urgent and life-saving measure used under conditions of last resort, situations may arise where it is necessary to utilize such donations. The mechanism of exceptional distribution, provided in the CTO Regulations, allows the distribution of cells that may not meet all the requirements of the regulations when no fully compliant CTO is available. The CTO Regulations outline three criteria that must be met in order to allow exceptional distribution:

(a) A cell, tissue or organ that has been determined safe for transplantation is not immediately available.
(b) The transplant physician, based on their clinical judgement, authorizes the exceptional distribution.
(c) The transplant establishment obtains the informed consent of the recipient.

Due to the often urgent nature of cell donation, the CTO Regulations do not set specific timelines for meeting these criteria, provided they are met prior to transplantation.

An example where exceptional distribution may be applied is when a matching donor has been identified that falls under exclusionary criteria for risk factors associated with HIV, HBV and HCV. Under such circumstances, cells from the high-risk

donor may be used for transplantation under exceptional distribution even though it would not meet the safety criteria set out in the CTO Regulations. The need for utilizing the exceptional distribution provision is often determined by the direness of the recipient's situation. As this is often determined by the transplant physician through their own clinical discretion, the CTO Regulations mandate that exceptional distribution be authorized by the physician and with informed consent of the patient.

13.2.5 Summary

Government regulation for the use of blood stem cells in clinically proven blood therapies is accomplished at Health Canada through CTO Regulations. Such therapies currently include the use of blood stem cells from allogeneic donors of bone marrow, UCB and MPB that have been minimally manipulated and are for homologous use. CTO Regulations outline requirements for determining and maintaining the safety of blood stem cells for transplantation and mandate the registration of transplant and cell banking facilities with Health Canada. Source establishment initiated audits of regulated activities and Health Canada initiated inspection of source establishments and their activities are used to monitor the continued compliance with CTO Regulations. This regulatory pathway provides an important and effective means for maintaining the safe use of blood stem cells for transplantation in the treatment of hematologic disorders.

13.3 Regulating the Novel Use of Blood Stem Cells for Regenerative Therapies

13.3.1 The General Scope of Drug Regulations in Canada

In Canada, the authority of the federal government to exercise regulations on drug products distributed in Canada is provided by the *Food and Drugs Act*. The government regulations that have been made pursuant to the Food and Drugs Act are outlined in the *Food and Drug Regulations* which consists of seven parts. Novel blood stem cell therapies are categorized as drugs and are, thus, subject to federal regulation through these statutes. The *Food and Drug Regulations* that apply to the case of novel therapies involving blood stem cells are outlined in *Part C: Drugs*. This part of the regulations describes the mechanisms by which Health Canada regulates the distribution of drugs and provides a description of the different categories of drugs covered by the *Food and Drugs Act*. The divisions in *Part C* of the regulations include the following:

(a) The various conditions that must be met for different categories of drugs in Canada, and include, among others, provisions regarding drug identification numbers, labelling, serious adverse drug reaction reporting and recalls (*Division 1*).

(b) The requirements for Establishment Licensing, as well as the categories of drugs and a list of regulated activities. The latter includes fabrication, packaging/labelling, testing, distribution, importing and wholesale (*Division 1A*).
(c) The requirements for Good Manufacturing Practices (*Division 2*).
(d) Specific requirements for the biologic drugs listed in *Schedule D* to the *Food and Drugs Act* (*Division 4*).
(e) The requirements for clinical trials involving human subjects, with clinical trials defined as *'an investigation in respect of a drug for use in humans that involves human subjects and that is intended to discover or verify the clinical, pharmacological or pharmacodynamic effects of the drug, identify any adverse events in respect of the drug, study the absorption, distribution, metabolism and excretion of the drug, or ascertain the safety or efficacy of the drug'* (*Division 5*).
(f) The requirements for a new drug, which is defined to include drugs that have not been sold in Canada for sufficient time and in sufficient quantity to establish in Canada, the safety and effectiveness of the drug, its use or condition of use (*Division 8*).

Health Canada has also developed policies and guidelines that interpret and provide details on how to meet the regulations. These policies and guidelines are not legally binding and cannot contradict or override regulations.

The essence of *Part C of the Food and Drug Regulations* is that drugs, including cell-based therapies, can only be marketed for use in Canada if Health Canada has issued the following to the sponsor:

(a) A Notice of Compliance (i.e. market authorization) after determining that the sponsor's drug submission contains adequate data to substantiate the drug's safety and efficacy
(b) An Establishment License based on provision of proof that the applicant's buildings, equipment and proposed practices and procedures meet the applicable requirements in *Divisions 2 and 4*

The data required to prove that drugs are safe, effective and of high quality are generated during clinical trials involving human subjects. Since novel blood stem cell therapies are mostly at the clinical trial stage, this chapter will focus on *Division 5* of *Part C*, which outlines the regulations pertaining to clinical trials involving human participants.

13.3.2 An Introduction to Clinical Trials

Clinical trials are investigations conducted in humans and are designed to answer specific questions pertaining to a disease or therapeutic intervention. Clinical trials can be broadly categorized as either observational or interventional studies. Observational studies do not involve the administration of treatment to the study participants and are usually used to gather information that is specific to a disease. These studies are quite useful for better understanding of diseases or identifying

useful disease biomarkers, but are not typically used for accumulating evidence of health product safety or efficacy. Interventional trials are prospective studies that involve the treatment of participants with a product or intervention for the purpose of understanding their effect in humans (Friedman et al. 2010). Properly designed interventional clinical trials provide an effective mechanism for demonstrating the safety and effectiveness of health products. This holds true for all health products, including cell-based therapeutics involving blood stem cells that are more than minimally manipulated or intended for non-homologous use. Interventional studies are designed to investigate specific outcomes associated with the administration of a drug or other intervention. Typical outcomes for interventional trials include the verification of product safety; the identification of any unintended effects on patient health associated with the drug (termed adverse effects); the determination of optimal product dosage; and measurement of product efficacy.

There are three separate phases of interventional clinical trials. Each of these phases is differentiated based on how much is already known about the effects of the intervention in humans (Wang et al. 2006). Early trials are considered Phase I and/or Phase II and usually involve the first administration of the product to humans (i.e. first in human study). These are exploratory trials which are designed to identify adverse events, determine safety and investigate side effects associated with increasing doses. Early phase trials can provide data that may be important to understanding the mechanism of action for a product or allowing further product development. Phase I and II trials typically recruit small numbers of participants and rarely provide an indication of product effectiveness. Data obtained from these early exploratory trials should produce sufficient evidence of safety to support the initiation of a Phase III trial. Phase III trials are considered confirmatory trials in that their primary outcome is to confirm that administration of the product at a specific dose can effectively treat the intended indication with minimal risks to patient health. Phase III trials involve a sufficiently large number of participants to complete a full risk/benefit analysis of the product. Overall, each phase of the clinical trial process should support the initiation of the next phase with the overall goal of obtaining a continuum of data that provides sufficient evidence of the safety and effectiveness of a therapeutic intervention.

Historically, clinical trials have been designed via several different methods; however, it has become accepted that the most scientifically sound mechanism for evaluating a drug or therapy is by comparison to a control group (Friedman et al. 2010). Control groups are intended to provide a baseline measurement to which safety and efficacy can be directly compared. The type of control used in a clinical trial will be dependent on the scientific basis of the clinical trial and the primary outcome being tested. For example, some clinical trials may be designed to determine whether a product or therapy provides superior effectiveness in treating a disease when compared to receiving no treatment. For such trials, a control group may consist of participants that have been administered a placebo. Other trials may be designed to compare superiority or equivalence to the current standard of care for a disease, or a therapy that is already on the market. In these trials, the appropriate control group may consist of participants that receive only the standard of care or the marketed therapy to which the new product is being compared. Regardless of the type of control used in the clinical trial, participants should be randomly selected into

control and treatment groups in order to reduce bias in the data. Clinical trials are most effective when completed under double-blind randomized conditions. This means that participants in control and treatment groups are chosen at random and that neither the investigators nor the patients are made aware of which treatment a participant has received until completion of the study. A double-blind randomized trial is an effective method to reduce potential bias that can be introduced either by the participant, clinician, trial investigator or treating physicians (Chow and Liu 2004).

The introduction of bias in clinical trials can affect the accuracy of conclusions made from the study. Clinical trials should be designed carefully in order to reduce bias wherever possible. Bias can be introduced from factors in the design and conduct of the trial as well as the analysis and interpretation of data obtained. In addition to lack of blinding and non-randomization of participants, sources of bias include protocol variations, patient withdrawals, the inclusion of multiple trial outcomes, insufficient recruitment and the use of inappropriate statistical models to analyze the data. The number of participants recruited and statistical methods used must be sufficient to allow accurate assessment of the primary outcome. Bias can also be reduced by utilizing an Independent Data Monitoring Committee (IDMC) for data analysis. The IMDC should analyze the data independently of the sponsor, investigators or staff conducting the trial using well-defined procedures set out during the design of the clinical trial. The concept of bias and strategies for reducing bias during clinical trials are appropriately addressed in the *ICH Topic E9: Statistical Principles for Clinical Trials*.

13.3.3 Canadian Federal Regulations Pertaining to Clinical Trials

When designing a clinical trial, it is important to be familiar with the regulations set out by the federal authority in the country where the trial will take place. In Canada, clinical trials are regulated under *Division 5* of the *Food and Drug Regulations*, which applies to the sale or importation of drugs to be used in clinical trials. As outlined in these regulations, prior to the initiation of a clinical trial, an application must be submitted to Health Canada for review. The provisions in *Division 5* regarding information to be provided in the Clinical Trial Application (CTA) and the sponsor's obligations include, among others:

(a) The requirement for a representative in Canada who is responsible for the sale of imported drugs.
(b) Provision of a copy of the protocol for the clinical trial and the informed consent form which outlines the risks and anticipated benefits of the trial.
(c) A clinical trial attestation signed and dated by the sponsor's representative in Canada and senior executive officer stating, among others, that the clinical trial will be conducted in accordance with good clinical practices and the regulations.
(d) The CTA must include an investigator's brochure that contains all known information on (1) the properties of the drug; (2) the pharmacological aspects of the drug, the pharmacokinetics and toxicological effects in animals; (3) the results of carcinogenicity and clinical pharmacokinetic studies and (4) information

on human-sourced excipients that indicates the excipient is a product that has already obtained market approval. In the case of excipients that have not yet received approval, sufficient information to support its identity, purity, potency, stability and safety must be provided.

(e) Chemistry and manufacturing information in respect to the drug, including its site of manufacturing, must be provided for drugs that have not been assigned a drug identification number or issued a notice of compliance.
(f) An indication that an approval has been obtained from a Research Ethics Board in respect of both the protocol and the informed consent form.
(g) The sponsor's obligation to conduct the trial in accordance with Good Clinical Practice and ensure the drug is manufactured, handled and stored in accordance with applicable sections of Good Manufacturing Practice as referred to in *Divisions 2 and 4*.
(h) The requirements for labelling and record keeping.
(i) The requirement for reporting of all serious unexpected adverse drug reactions to Health Canada.
(j) Health Canada has the authority to suspend or cancel the clinical trial.

The sponsor may sell or import a drug for the purpose of the clinical trial if Health Canada does not send the sponsor a notice within 30 days after the date of receipt of the CTA, indicating that the sponsor may not sell or import the drug.

Currently, Health Canada has adopted guidelines developed by the International Conference on Harmonization (ICH) which harmonize with the requirements in Japan, USA and the European Union (see below). These guidelines provide additional information on both the chemistry and manufacturing requirements for the drug and the pre-clinical studies involving animals. Together, Health Canada clinical trial regulations and the ICH guidelines outline important aspects of the clinical trial application and initiation process including the information that must be included and the procedures for filing the application. To further aid sponsors in the preparation of a CTA, Health Canada has prepared guidance documents providing interpretation of *Division 5* regulations and will provide input and advice to clinical trial sponsors prior to the submission of an application. Such input can be invaluable to the success of both the CTA and the outcomes of the clinical trial itself. In addition, sponsors are encouraged to utilize qualified individuals, including biostatisticians, clinical pharmacologists and physicians, during all aspects of the clinical trial design.

13.3.4 Pre-clinical Evidence to Support the Translation of Blood Stem Cells to Clinical Trials

The initiation of any clinical trial must be extensively supported by basic scientific research that provides evidence for both the safety and effectiveness of the product. Blood stem cells are unique compared to other potential cell-based therapies in that they have been researched extensively since the 1960s so that certain aspects of their safety can be reasonably assured. However, this research does not fully address the

level of safety or degree of effectiveness associated with blood stem cells that have been manipulated ex vivo or in the context of their use in the treatment of tissues outside of the hematopoietic system. Thus, there are still important issues that need to be addressed in a pre-clinical setting prior to the investigation of such products in humans. When designing studies to provide rationale for clinical trial investigation, researchers must carefully choose experimental models that can appropriately demonstrate the biological activity of the product and address all potential safety issues. The precise safety issues that must be addressed and type of experiments used to address them are dependent upon both the product and the manufacturing process.

A common question regarding pre-clinical testing of cellular therapies, including blood stem cells, is the extent to which animal models must be used to assess product safety and effectiveness. Specifically, investigators question in what situations are *in vitro* data sufficient, which disease models are the most appropriate and what necessitates the use of large animal models. Several guidance documents have been developed regarding pre-clinical evaluation of drug products that can provide investigators with the answers to some of these important questions (*FDA Guidance for Industry: Cellular Therapy for Cardiac Disease; ICH Topic S6: Guidance for Industry: Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals; ICH S8, Immunotoxicity Studies for Human Pharmaceuticals; ICH Topic M3 (R2) Non-Clinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals (Note for Guidance on Non-Clinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals*)). Some of the guidance provided by these documents is summarized below.

In vitro assay systems are valuable tools for pre-clinical research as, compared with animal models, they are relatively inexpensive and provide a closed environment that can be directly manipulated by the researcher. *In vitro* assays are useful for early experimentation to identify evidence of biological activity for a blood stem cell product. Data provided by *in vitro* assays can be used to design experiments in animal models and may allow insight into the appropriate animal models to be used for assessing biological activity and safety *in vivo*. *In vitro* assays can also provide useful information regarding the mechanism of action for specific biological responses identified *in vivo*. However, the closed environment of *in vitro* assays does not allow researchers to determine important questions about the behaviour of cell products within a living organism.

Current thinking within the international regulatory community regarding the body of evidence required to support translation of cellular therapy products into the clinic suggests that animal experimentation is necessary for assessing:

(a) The effectiveness of the product towards safely treating the intended indication.
(b) The time course of either positive or negative (adverse) biological responses to the product. This includes the length of time between product administration and a detectable response and the duration of the response.
(c) The possibility of adverse events due to cell migration and engraftment outside of the organ intended for treatment, tumour formation or differentiation into

unexpected cell types. Animal models will also be useful in determining potential adverse events related to cell fusion, a phenomenon that has been documented to occur between blood stem cells and other cell types, such as muscle and liver.

(d) The effect of increasing and decreasing cell dose on measured biological responses.

Choosing the proper animal models for assessing these questions will depend on several factors including the product, the route of administration and the disease being treated. Certain questions, such as the potential for adverse events due to stem cell migration or increasing dose, may be better assessed through the use of more than one animal model. Immune-deficient or immune-compromised rodent models have been developed that are useful for measuring the capacity of human blood stem cells to repair various organs and tissues including blood (Bhatia et al. 1998), liver (Zhou et al. 2009), leg ischemia (Capoccia et al. 2009), spinal cord (Xu et al. 2010) and heart (Sondergaard et al. 2010). Such animals lack a sufficient immune response, even to xenogeneic cells, thus allowing the engraftment and direct study of human stem cells. In some cases, where interactions with the immune system may affect the biological response of the product, it may be necessary to complete studies using rodent stem cells in immune competent animals. Overall, rodent models can provide evidence for product viability and behaviour following administration, such as differentiation, fusion or production of trophic factors that instigate repair. These models may also provide some insight into potential safety issues regarding blood stem cell products. However, it is important to note that the physical size constraints of rodent models, and their relatively short lifespan, limit the extent that the data obtained from such experiments can be extrapolated to humans. For products where chronic adverse events may be expected, such as more than minimally manipulated blood stem cell products that require long-term engraftment to mediate a biological response, or in cases when it is important to examine biological responses in organs of similar size to that of humans, it will be necessary to utilize large animal models. In general, the more pre-clinical data that can be accumulated regarding the product safety and efficacy using model systems that most closely resemble the conditions that occur in the human, the more likely a clinical trial application will be successful. Overall, safety should be the primary concern when accumulating pre-clinical data, especially for the initiation of first in human trials. It is important to take into account all potential safety concerns and know the limitations of each assay system for addressing these concerns. In general, pre-clinical studies should address (1) stem cell identity; (2) properties of biodistribution; (3) general safety issue such as an estimation of the safe dose to be applied in the first clinical trials, the timing administration relative to the onset of the disease, the dosing scheme and schedule, adverse toxicities such as tumorigenicity and immunogenicity and adverse interactions with other drugs; (4) the appropriate route of delivery and safety of the administration procedure and (5) proof of therapeutic benefit. Ultimately, Health Canada recognizes that the experience and expertise of the investigator or sponsor group is critical in determining the appropriate models to utilize for addressing these issues during pre-clinical studies. Regardless of the

models used, a clear rationale for choosing these models should be presented to Health Canada in the CTA.

When evaluating the sufficiency of pre-clinical data submitted in a clinical trial application, Health Canada utilizes a risk-based approach that takes into account both the product and the patient. For most cell-based therapeutics, the manufacturing process has a large effect on the perceived risk associated with the product and, consequently, plays an important role in determining the type of pre-clinical testing that is required. In the case of blood stem cells, the risk scale can range from stem cells that are genetically manipulated and expanded in culture to freshly isolated cells which may be considered of little risk for causing an adverse event in the patient. The route of administration and any potential adverse events that could arise during administration, or the potential to migrate and engraft outside of the intended organ or tissue, should also be taken into account during product risk assessment. In all, the level of risk associated with a product is defined by all aspects of that product from initial purification to processing, storage and administration.

Risk-based analysis can also take into account the proposed indication for the trial. While ensuring safety is of utmost importance for all products, a higher level of risk may be acceptable for patients suffering from an acute terminal disease that cannot be improved with the current standard of care. On the other hand, the acceptable level of risk may be lower for clinical trials involving patients suffering from non-life-threatening injury, or diseases for which the current standard of care provides some efficacy or increased quality of life.

13.3.5 Safety Issues Pertaining to Clinical Trials Involving Blood Stem Cells

Novel blood stem cell therapies are associated with a certain degree of risk. These risks must be addressed, and sufficient evidence of safety must be submitted to Health Canada prior to distribution of stem cell therapies in Canada. However, the exact safety concerns that must be addressed are specific to both the processing procedure and intended use of the product. The next section of this chapter outlines some of the key safety concerns that should be addressed with specific blood stem cell products that have already entered early phase clinical trial or might be anticipated to enter clinical trials in the near future.

13.3.5.1 Products Derived from *In Vitro* Culture of Blood Stem Cells

For decades, investigators have attempted to identify the appropriate *in vitro* conditions to allow expansion of blood stem cells for transplantation. Such conditions would be particularly useful for cord blood–derived stem cells as the number of stem cells required to replace the hematopoietic system of an average-sized adult

cannot be isolated from a single cord. The culture of any cell for the purpose of administration into humans is associated with certain risks.

(a) Infectious disease risks associated with human-derived starting materials
(b) The potential for the introduction of adventitious agents from reagents, equipment, facility and personnel
(c) Inability to terminally sterilize products
(d) Alteration of the cell's biological characteristics

In general, these risks can be reduced by ensuring the manufacturing process is well developed and controlled, and the products are well characterized. This can be accomplished by implementing various measures including, among others: (1) establishing qualification programmes for starting materials, ancillary reagents and other contact materials to ensure they are free from adventitious agents and appropriate for the intended use; (2) validating the aseptic process and the storage and shipping conditions; (3) implementing controls for critical steps in the culture process and intermediates to monitor product quality and safety; (4) implementing measures to prevent product contamination and cross contamination; (5) establishing and complying with specifications for product safety, identity, purity, viability/potency; (6) assessing comparability after manufacturing changes are made; (7) conducting stability studies to support product shelf life and (8) employing good manufacturing practices. Currently, there are two ICH Quality Guidelines that provide recommendations for cell lines used for the preparation of biotechnology/biological products. The guideline entitled *Viral Safety Evaluation of Biotechnology Products Derived From Cell Lines of Human and Animal Origin (Q5A R1) (1999)* provides information regarding the potential sources of virus contamination in cell culture, as well as guidance on the testing and evaluation of viral safety for cell banks. A second guideline entitled *Derivation and Characterization Cell Substrates Used for Production of Biotechnology/Biological Products (Q5D) (1997)* also provides guidance on the generation, characterization and testing of cell banks. While these guidelines are intended for cell lines used for preparing biotechnology/biological products, the information provided can be adapted for some cell therapy products. Some of the specific risks associated with blood stem cell culture include the following:

(a) *The potential for induction of abnormal growth.* Any culture process can have unexpected adverse effects on stem cells, including the loss of normal growth control mechanisms or apoptotic resistance. Such changes in stem cell properties can lead to transformation into a cancerous phenotype. The probability of adverse effects occurring usually increases with increasing duration of culture. Appropriate studies should be completed to compare the growth and differentiation properties of blood stem cells both prior to and following expansion. Tumorigenicity testing in animal models may also be warranted, especially in cases where stem cells have undergone a significant degree of expansion.
(b) *The presence of residual culture products.* Many mammalian cultures utilize products derived from animals or microorganisms, such as bovine serum or recombinant cytokines, for stem cell propagation. Such products can lead to adverse reactions in humans due to development of an immune response,

especially if present at sufficiently high doses. When developing a culture system for the purpose of translating into a product for human use, animal-derived products should be avoided whenever possible as they may cause immune or allergic reactions. Antibiotics should also be avoided as they are associated with hypersensitivity reactions in patients. It is critical to know all the constituents of the media and other reagents, including any proprietary products. Health Canada requires a list of all constituents with the source from which they are derived and proof of their sterility. Assay methods should be established to detect residual amounts of animal- or microbial-derived contaminants in the final product and ensure that they do not exceed levels set out by regulatory agencies.

(c) *The introduction of adventitious agents.* According to *Division 5* of the *Food and Drug Regulations*, clinical trial sponsors must ensure that drugs are manufactured, handled and stored in accordance with the applicable GMP conditions referred to in *Division 2*. GMP ensures that products are manufactured under conditions that reduce the risk of introducing adventitious agents and must be followed when generating cellular products intended for human use. Since product development is an ongoing process that evolves throughout the clinical trial period based on the data obtained throughout each phase, Health Canada recognizes that full GMP compliance may not be achieved in early phase clinical trials. However, adequate measures must be in place to ensure that the manufacturing process employed in early phase clinical trials will consistently result in safe products. Pharmaceutical/GMP grade reagents should be used whenever available to ensure their safety, purity and potency. Pharmaceutical/GMP grade reagents should be used whenever available. If suitable GMP grade reagents are not available, or if a review of the Certificate of Analysis indicates that certain risks have not be adequately addressed, additional testing must be completed to ensure their suitability in a clinical setting. If the use of reagents derived from animal sources (e.g. as bovine serum), cannot be avoided, a transmissible spongiform encephalopathy (TSE) risk assessment must be performed to assess the risk associated with the animal species and determine the TSE risk category of the animal tissue and country of origin. If a TSE risk is identified, information must be provided on risk reduction measures such as the age of cattle, feed controls and pre- and post-mortem veterinary inspections. Tests for animal-specific viruses, such as bovine viruses for bovine-derived reagents, should also be performed where applicable. Human-sourced excipients such as human serum albumin must be approved for use in Canada. Alternatively, sufficient information should be provided to support their identity, purity, potency, stability and safety.

13.3.5.2 Products Involving Gene Transfer into Blood Stem Cells

The introduction of genes into blood stem cells may provide a viable therapy for diseases that are known to result from the mutation of a single gene. However, as the introduction of genes into cells has been associated with oncogenic transformation

and eliciting strong immune reactions (Neschadim et al. 2007; Mingozzi and High 2007), gene therapy is currently perceived to be associated with high safety risks by both the scientific community and the general public. As a consequence, Health Canada may apply a higher degree of scrutiny when reviewing clinical trial applications involving genetically modified blood stem cells. There are several safety concerns that investigators should plan to address when developing blood stem cell–based gene therapy products. Specific safety concerns will vary according to the methods utilized for introducing genes into stem cells. For example, a major concern regarding the use of retroviral-based gene transfer systems is the possibility of cancerous transformation. Concerns regarding adenoviral-based systems, on the other hand, revolve around the generation of an immune response in patients. To avoid the use of viruses for gene therapy, several new methods have been developed that allow either the permanent or temporary introduction of genes into human cells. These include, but are not limited to, meganucleases (Casco-Robles et al. 2011), nanoparticles (Ruan et al. 2011), microporation (Madeira et al. 2011) and piggyback transposons (Woltjen et al. 2009). It is important to note that the safety of these new methods has yet to be rigorously tested, and thus these have not yet been demonstrated as low-risk procedures. When characterizing the safety of any gene transfer method, the following should be taken into account whenever possible:

(a) *Transformation of stem cells into a cancerous phenotype*. Testing of cancerous transformation of stem cells should be completed using the assay systems that are well accepted at the time. Currently, these include karyotyping assays, *in vitro* proliferation assays and the monitoring of tumour formation in immune-deficient animals. When using products with short shelf lives, it may be difficult to obtain such data prior to administration. Under such circumstances, gene transfer methods must be well characterized in pre-clinical experiments using many different samples in order to determine the frequency over which transformation events are expected to occur. Furthermore, when genes are inserted directly into the stem cell genome, it may be important to identify genes upstream and downstream of insertion site. Insertion of a gene sequence can alter surrounding sequences or promoter regions that govern gene expression. As such, the effect of gene insertion on the expression or function of neighbouring genes should be well characterized. This may especially apply for known proto-oncogenes.

(b) *The presence of virus with the ability to replicate and cause infection must be monitored when virus-based systems are utilized to introduce genes.* Virus-based methods for introducing genes into cells utilize a modified virus that is considered ‘replication incompetent’ since it can no longer reproduce inside the cell. These viruses are engineered such that they can enter the cell and deposit the gene of interest but do not carry the machinery necessary to produce new virus for subsequent infection. However, recombination events can occur that may allow an incompetent virus to become ‘replication competent’. This represents a major safety concern for clinical trials in this area as it may allow the

introduction of an infectious agent into study participants. The US FDA has developed two guidance documents that provide useful recommendations for monitoring the absence of replication competent virus in retrovirus-based gene therapy products (http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/ucm078723.pdf.; http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Xenotransplantation/ucm092705.pdf).

Two potential areas where competent virus can arise are from the cells used to produce incompetent virus and from the cells where the gene is being introduced. Thus, testing for competent virus in both virus-producing cell lines and the end product being developed is highly recommended. Also, when creating and utilizing cell lines for the production of replication incompetent virus, Health Canada recommends the development of a cell banking system. This involves the creation of a master cell bank (MCB) which is frozen in liquid nitrogen and stored in several aliquots for seeding and development of a working cell bank (WCB). It is recommended that both MCB and WCB be tested for the presence of replication competent virus.

(c) *The potential for delayed adverse events.* Such events may be due to the expression of the gene itself, changes generated at the insertion site of a gene or the vector used for gene transfer. The potential risk of delayed adverse events varies considerably between different products. As such, the amount of pre-clinical and clinical data that must be accumulated to ensure safety will also vary. For blood stem cell–based products, regulatory authorities will take into account factors such as gene transfer methods utilized, whether the gene is directly integrated into the stem cell genome, duration of gene expression within the stem cells, the disease being treated and the duration of stem cell engraftment in the patient. For high-risk products, pre-clinical evidence of safety can include data from animal studies investigating the bodily distribution of blood stem cells expressing the gene, the persistence of blood stem cell engraftment at sites where they are initially detected and tumour-forming potential assays.

(d) *Immunogenicity of the product.* Immune reactions to blood stem cell–based gene therapy products can potentially develop against components derived from the method used for gene transfer or from the gene product introduced into the cells. Possible sources for initiating an immune reaction in patients are the co-administration of residual proteins or virus particles, expression of virus proteins in the infected cells and the transgene product itself. It is important to note that occurrences of immune reactions are not exclusive to virus-based gene transfer methods. Indeed, cells transduced with liposome-based methods have also been shown to initiate an immune response in animal models (Sakurai et al. 2008). The optimal model systems for determining the immunogenic properties of any cell therapy product are currently being debated, but can range from *in vitro* T-cell activation assays to *in vivo* animal models.

13.3.5.3 Non-homologous Use of Autologous Blood Stem Cells

Pre-clinical studies have identified potential therapeutic benefits for using blood stem cells for treating myocardial infarction, stroke, spinal cord injury, liver damage and skeletal muscle damage. Some of these studies have already led to the initiation of clinical trials. As mentioned above, historical evidence regarding the safety of using blood stem cells for treatment of hematopoietic diseases provides some measure of assurance for the therapeutic use of these cells; however, they do not negate the necessity for clinical trials for other indications. Safety concerns regarding non-homologous use of blood stem cells will vary depending on the tissue being treated and the route of administration. The following safety concerns may be associated with direct administration of blood stem cells into an organ or tissue:

(a) *Potential for organ and tissue damage due to injection.* Such an outcome could be affected by the dose of cells administered, total volume of injection or excipient materials used to resuspend the final product. These issues may need to be addressed pre-clinically using animal models that have organ sizes similar to that of humans.
(b) *Induction of bleeding events and haemorrhage.* Most organs are highly vascularized, and direct injection may result in bleeding that can be difficult to control or may add to any pre-existing organ damage.
(c) *Cell death and the duration of blood stem cell engraftment both inside and outside the damaged organ.* A large amount of cell death, either to the stem cells or surrounding tissue, following administration may instigate an unwanted immune response. In such cases where activated immune cells are already present in the damaged organ, immune responses to the product may exaggerate existing inflammatory reactions. Stem cell migration outside of the damaged organ may also contribute to a reduction in the proportion of injected stem cells. The ability of blood stem cells to migrate through the blood stream and seed various tissues has been well documented. In situations where minimally manipulated stem cells are utilized, migration out of the injection site may have very little consequence. However, the migration and engraftment of modified or culture expanded blood stem cells may be associated with certain safety risks. Thus, for high-risk blood stem cell products, migration and engraftment properties should be thoroughly assessed in pre-clinical models and monitored during the clinical trial when possible.

The majority of non-homologous use regenerative therapies involving blood stem cells are expected to utilize autologous bone marrow–derived cells. It is important to note that autologous cell therapies still qualify as a biologic drug, regulated by Health Canada under the authority of the Food and Drugs Act. For the most part, these products are regulated in the same manner as allogeneic cell products, with the exception of the fact that donor samples procured for the development of autologous products are exempt from infectious disease testing. It is also important to carefully design product specifications for autologous blood stem cells. For clinical trials, sponsors normally develop these specifications to provide an indication of product safety,

quality and efficacy prior to clinical use. These specifications are designed as a screening mechanism to allow rejection of any product the does not meet quality safety and efficacy standards. Thus, the development of stringent specifications for autologous based cell products is not recommended as this may result in a product that is unusable for the clinical trial. On the other hand, some degree of stringency must be applied in order to avoid a large degree of variation in the clinical trial data.

13.3.5.4 Summary

Canadian federal regulations require that health products and therapies meet the highest levels of safety, quality and efficacy prior to distribution in Canada. Blood stem cells used for regenerative therapies are considered biologic drugs under the Food and Drugs Act and, thus, are subject to the *Food and Drug Regulations*. Clinical trials are regulated under *Part C, Division 5* of these regulations and are an important part of ensuring the safety, quality and effectiveness of novel blood stem cell–based products. Successful clinical trials should be developed based on well-designed pre-clinical research that supports all aspects of safety and efficacy. Safety concerns pertaining to the therapeutic use of blood stem cells are specific to the process used to manufacture and administer the therapeutic product as well as the patient population being treated. Understanding the potential safety issues surrounding the product through pre-clinical testing and the development of manufacturing controls to ensure product safety and quality will be critical to successful use of blood stem cells as a novel regenerative medicine.

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Chapter 14
Cell Therapy Regulations from a European Perspective

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Abstract This chapter provides an overview of the current status of cell therapy regulation in Europe. This includes the description of GMP standards and other significant regulations for quality assurance. Accreditation principles and measures will be discussed, and recent regulations on advanced therapy medicinal products which are more than minimally manipulated will be explained.

14.1 General Rules for the Production of Cellular Therapeutics: Good Manufacturing Practice (GMP)

14.1.1 Development and Application of GMP

The manufacture of phamaceutical products has been regulated at a highly defined level (see table 14.1). This is required to safeguard the quality and safety of any medicinal product on the market. These regulations guarantee the efficacy and safety of our therapeutics as an essential part of risk management in order to protect the patient. There are also some legal implications, e.g., defining liabilities for damages caused by the administration of medical products. GMP has been established world-wide through the Pharmaceutical Industry Convention consortium (PIC) which has defined rules and technical standards for the development, production, and distribution of medicinal products. The PIC GMP guidelines have, for more than 20 years, also been the gatekeeper standard for the production of cellular products,

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Table 14.1 European and International regulations for advanced therapies mentioned in the present review

Topic	Reference number	Effective from	Remarks
E 6 (R1) Guideline for Good Clinical Practice	CPMP/ICH/135/95	July 1996	The objective of this ICH GCP is to provide a unified standard for the European Union (EU), Japan and United States to facilitate the mutual acceptance of clinical data by the regulatory authorities in these jurisdictions
EudraLex-Volume 4 Good Manufacturing Practice (GMP) Guidelines	EudraLex-Volume 4 The Rules Governing Medicinal Products in the European Union	continuously adjusted	Contains guidance for the interpretation of the principles and guidelines of good manufacturing practices for medicinal products for human and veterinary use laid down in Commission Directives 91/356/EEC, as amended by Directive 2003/94/EC, and 91/412/EEC respectively
Directive 2001/20/EC of the European Parliament and of the Council	Directive 2001/20/EC	April 2001	This Directive is focused on the approximation of the laws, regulations and administrative provisions of the Member States relating to the implementation of good clinical practice in the conduct of clinical trials on medicinal products for human use
Commission Directive 2003/94/EC	Directive 2003/94/EC	October 2003	This Directive lays down the principles of good manufacturing practice in respect of medicinal products for and investigational medicinal products for human use
Community E Directive 2001: Directive 2001/82/EC of the European Parliament and of the Council of November 6, 2001, on the Community Code Relating to Veterinary Medicinal Products	Directive 2001/82/EC	April 2004	This Directive is only one stage in the achievement of the aim of freedom of movement of veterinary medicinal products [Art (9)]
EU Directive 2004/23/EC: Towards Standards of Quality and Safety for Human Tissues and Cells in Europe	Directive 2004/23/EC	April 2004	This Directive represents a milestone in the establishment of a regulatory frame
Directive 2001/83/EC of the European Parliament and of the Council of November 6, 2001, on the Community Code Relating to Medicinal Products for Human Use	Directive 2001/83/EC	November 2004	This Directive represents an important step toward achievement of the objective of the free movement of medicinal products [Art (14)]

Committee for Human Medicinal Product (CHMP) Guideline on Human Cell-Based Medicinal Products	EMEA/CHMP/ 410869/2006	January 2007	This guideline is focused on the development, manufacturing and quality control as well as non-clinical and clinical development of cell-based medicinal products
Regulation (EC) 1394/2007 of the European Parliament and of the Council	Regulation 1394/2007	November 2007	This *lex specialis* regulates advanced therapy medicinal products which are intended to be placed on the market in Member States and either prepared industrially or manufactured by a method involving an industrial process [Art (6)]
FACT-JACIE International Standards for Cellular Therapy Product Collection, Processing and Administration, Accreditation Manual 4th Edition	FACT-JACIE 4th Edition	November 2008	These Standards are designed to provide minimum guidelines for programs, facilities, and individuals performing cell transplantation and therapy or providing support services for such procedures
21 C.F.R. Part 16 - Regulatory Hearing Before the Food and Drug Administration	44 FR 22367	April, 1979	This regulation provides a person with an opportunity for a hearing on a regulatory action, including proposed action
21 C.F.R. Part 1270 - Human Tissue Intended for Transplantation 62 FR 40444 July, 1997 This regulation applies to human tissue and to establishments or persons engaged in the recovery, screening, testing, processing, storage or distribution of human tissue 21 C.F.R. Part 1271 - Regulations Under Certain Other Acts Administered by the Food and Drug Administration 66 FR 5466 Jan. 19, 2001 This regulation applies to human cells, tissues, and cellular and tissue-based products (HCT/P's) and establishes donor-eligibility, current good tissue practice, and other procedures to prevent the introduction, transmission, and spread of communicable diseases by HCT/P's.			These Standards apply to cellular therapy programs that collect, process, or administer cells isolated from bone marrow or peripheral blood, including organizations that provide support for such services
NetCord-FACT International Standards for Cord Blood Collection, Banking, and Release for Administration	NetCord-FACT 4th Edition	January 2010	

including stem cells for transplantation. The standards can be recognized as generally accepted throughout Europe and beyond, since they are used by almost every country and have also become standards for inspections by competent authorities.

The GMP regulation has been transformed into national legislation in individual European countries with small differences which are beyond of the scope of this chapter. With the foundation of a European initiative, experiences in drug licensing and regulation of the production of therapeutic agents have merged into the establishment of The European Medical Agency (EMA). The EMA has been endorsed to grant licenses for pharmaceutical products first, parallel to the nationally established licensing pathways, and, in the meantime, centrally to provide licenses and, in consequence, medicinal products of the appropriate quality in Europe. The recent years have increasingly driven the regulation of the production of cellular therapeutics toward EMA-controlled principles.

The production of cellular therapeutics, regardless of whether they consist of minimally manipulated cells or engineered products such as culture-differentiated or genetically altered cells therefore have to comply with GMP standards throughout Europe and worldwide. Subsequently, the relevance of GMP to the manufacture of cellular therapeutics will be discussed.

14.1.2 Main Characteristics and Definitions of the GMP Process

GMP covers a part of the quality assurance system for the manufacturing and testing of medicinal products with the goal to meet preset specifications. This requires not only the demand for the traceability of raw materials, but also a production according to validated standard operating procedures (SOPs). This ensures a consistent manufacturing process yielding products meeting preset criteria, thus providing for their clinical safety (Alici and Blomberg 2010). Next, the main parts of the manufacturing process are outlined including recent additions by new EU regulations to the basic requirements.

14.1.2.1 Process Description

In a GMP process, at first, a hierarchical and detailed production strategy has to be set, considering the acceptance criteria of all steps involved in the production. This includes a clear and well-understood definition of the expectations and requirements of the project's goals and objectives, preparing the user requirements specifications, operational flow diagrams and the facility conceptual design (Alici and Blomberg 2010).

14.1.2.2 Personnel, Procedures and Documentation

The establishment and maintenance of a satisfactory system of good manufacture of medicinal products relies on people. Therefore there must be sufficient qualified personnel to carry out all the tasks which are the responsibility of the manufacturer.

Good documentation comprises an essential part of the quality assurance system and is critical to compliance with GMP requirements. Documentation may exist in a variety of forms, including paper-based, electronic, or photographic media. The main objective of the documentation system must be to establish, control, monitor, and record all activities that are involved in all aspects of the quality of medicinal products. Production operations must follow clearly defined procedures; they must comply with the principles of good manufacturing practice in order to obtain products of the requisite and appropriate quality in accordance with the relevant manufacturing and marketing authorizations. The independence of quality control from production is considered fundamental to the satisfactory operation of GMP and quality assurance activities: Before release, each product has to be released by a qualified person who could be, for example the head of production or the head of quality control. Like all other steps of the manufacture, the product release is a strictly defined procedure ensuring that each batch of the medicinal product is manufactured and tested in accordance with the regulations applicable to the trade in medicinal products.

14.1.2.3 Qualification of Premises and Equipment

Another main area of GMP regulation is the qualification of the premises and the equipment used. In a GMP process, it is required to monitor the operational suitability of the equipment by predefined specifications. Therefore, reviews of the performance of both equipment and facilities are required, including corrective and preventive actions as needed. Vice versa this means that products that have been manufactured with nonqualified machinery cannot be released.

14.1.2.4 Involvement of Other Partners in the Production Process

Contract manufacture and analysis must be correctly defined, agreed upon, and controlled in order to avoid misunderstandings which could result in a product or task of unsatisfactory quality. There must be a written agreement between the contract giver and the contract acceptor which clearly establishes the duties of each party.

14.1.2.5 Manufacture of Sterile Products

The manufacture of sterile products is subject to special requirements in order to minimize risks of microbial, particulate, and pyrogen contamination. Therefore skills, training, and behavior of the personnel involved in the process play an essential role in product quality. Quality assurance is particularly important, and this type of manufacture must strictly follow carefully established and validated methods of preparation and related procedures (Community E 2003a, EC Guide to Good Manufacturing Practice Revision to Annex 1). Regarding the manufacture of biological medicinal products, certain specific considerations arising from the nature of the products and the processes must be mentioned.

Since most of the products cannot be subjected to sterilization, the manufacture has to be performed in a strictly defined environment. The most common technical solution in this context is a laminar airflow cabinet placed in a cleanroom. According to the above-mentioned Annex 1, extensive particular and microbial environmental monitoring has to be in place.

The sterility testing of the products has to be performed according to the European Pharmacopoeia. These cultural methods take at least one week indicating that faster methods for the detection of microbial contamination are urgently needed because of the short shelf-life of some products (Marshall and Marshall 2003). In addition, other methodical problems remain to be solved such as optimal growth conditions of relevant bacterial strains (Montag et al. 2010).

The ways in which biological medicinal products are produced, controlled, and administered make some particular precautions necessary by taking the variability of the pharmaceutically active compounds and the by-products into account.

14.1.3 Installation of Tools to Recall Defective Products

All complaints and other information concerning potentially defective products must be reviewed carefully according to written procedures. In order to provide for all contingencies, and in accordance with Article 117 of Directive 2001/83/EC and Article 84 of Directive 2001/82/EC (Community E Directive 2001), a system should be designed to, if necessary, promptly and effectively recall products known or suspected to be defective from the market. Self-inspection should be conducted in order to monitor the implementation and compliance with good manufacturing practice principles and to propose necessary corrective measures (European Commission 2008), The Rules Governing Medicinal Products in the European Union, Volume 4 EU Guidelines to Good Manufacturing Practice Medicinal Products for Human and Veterinary Use).

14.1.4 Quality Assurance and Risk Management in the Production of Cellular Therapeutics Through GMP

Good manufacturing practice (GMP) has become a general rule governing the production of essentially all medicinal products in the European Union. GMP is part of quality assurance since it ensures that products are controlled to the quality standards appropriate to their intended use and as required by the marketing authorization and product specification. Good manufacturing practice is therefore concerned with both production and quality control.

Also, the basic concepts of quality assurance, good manufacturing practice, quality control, and quality risk management are interrelated. Quality assurance is a wide-ranging concept which covers all matters that individually or collectively

influence the quality of a product. It is the sum total of the organized arrangements made with the objective of ensuring that medicinal products are of the quality required for their intended use (EudraLex, 2008, The Rules Governing Medicinal Products in the European Union Volume 4 EU Guidelines to Good Manufacturing Practice Medicinal Products for Human and Veterinary Use).

GMP is also an essential prerequisite for good clinical practice, which is a guideline for investigational medicinal products (IMPs) used and tested in clinical trials (CPMP/ICH/135/95) (Community 2003a; Signore and Jacobs 2005; F.D.A. 21 CFR Parts 16, 2005; European Commission 2008; Commission Directive 2003).

As an important part of GMP, quality control focuses on sampling, specifications and testing, as well as on management, documentation, and release procedures. The latter ensure that the necessary and relevant tests are actually carried out and that materials are not released for use, nor are products released for sale or supply until their quality has been judged to be satisfactory (EudraLex, 2008, The Rules Governing Medicinal Products in the European Union Volume 4 EU Guidelines to Good Manufacturing Practice Medicinal Products for Human and Veterinary Use). In addition, the cited EU guidelines include two other important issues such as the product quality review and quality risk management. In order to verify the consistency of the existing process, the appropriateness of current specifications for both starting materials and finished product, regular periodic quality reviews of all licensed medicinal products, including export only products should be conducted. At this hierarchical level, quality risk management is a very important systematic process which ensures that the assessment, control, communication and review of risks to the quality of the medicinal products are performed accordingly (Community E 2003b).

The production of investigational medicinal products involves added complexity in comparison to marketed products by virtue of the lack of fixed routines, variety of clinical trial designs, consequent packaging designs and the need for randomization and blinding, and increased risk of product cross-contamination and mix-up. The increased complexity in manufacturing operations requires the implementation of a highly effective quality system.

14.2 Tools to Supervise GMP and Standards in Practice and Quality

In the last two decades, responsible individuals and groups acting in the field of production of cellular therapeutics have founded committees to harmonize quality assurance and GMP practices in the field of cellular therapy. The promotion of quality in therapies using cellular products is also a major objective of the Foundation for the Accreditation of Cellular Therapy and the Joint Accreditation Committee (FACT-JACIE) – ISCT (International Society of Cell Therapy), EBMT (European Group for Blood and Marrow Transplantation), International Standards for Cellular Therapy Product Collection, Processing, and Administration. They apply to all phases of collection, processing, storage, and administration of these cells that have

been derived from marrow or peripheral blood, including various manipulations such as removal or enrichment of various cell populations, expansion of hematopoietic cell populations, and cryopreservation. For hematopoietic progenitor cells or therapeutic cells derived from umbilical cord and/or placental blood, these standards apply only to the administration of the cellular products, and apply the clinical standards for transplantation of allogeneic or autologous hematopoietic progenitor cells, as is appropriate. These standards do not apply to the collection, processing, or banking of umbilical cord and placental blood cells. Standards for the latter processes are found in the current edition of NetCord-FACT International Standards for cord blood collection, processing, testing, banking, selection, and release.

These standards are compliant with the above-mentioned European regulations. The accreditation of facilities compliant with European standards is offered by JACIE. Accreditation of European laboratories and production sites for stem cell therapeutics has increased enormously over the last decade, and has become routine in many parts of Europe.

14.3 Principles of Current European Regulations and Legislation and Specifics for Cell Therapy–Related Medicinal Products

The above mentioned FACT-JACIE standards have been a voluntary measure by physicians, pharmacists, and scientists in the field of hematopoietic stem-cell transplantation, and have provided practicable standards for North America and Europe together. At the time of their installation, national legislation has prevailed in the field. Since then, European legislation has introduced into the field a number of new supporting documents. It is relevant to also mention here that European regulations are generally applied in three different forms. The aim is to convert all content into national legislation. This still leaves room for community or governmental laws or regulations to establish additional requirements in a given individual country. This legislation differs from country to country, and will therefore not be discussed in further detail here.

European regulation includes three major classes of documents:

1. **Guidelines** (guidelines on human cell-based medicinal products, EMEA/CHMP/410869/2006) set a scientific standard, but cannot be enacted as law. They instead may be considered as recommendations, directly addressing users.
2. **Directives** are documents aiming for a minimal standard in all European counties. They call upon all individual countries which have to install the contents in their national legislation within a period of two years. Failure to convert can result in the EU commission suing a nation before the European High Court in The Hague. The relevant directive in the field of cellular therapy is the Human Tissues and Cells Directive 2006/17/EC, a directive on setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage, and distribution of human tissues and cells-2004/23/EC). It implements

technical requirements for the donation, procurement and testing of human tissues and cells.

3. **Regulations** are documents which are imposed as binding law directly into the legislation in every EU member state. In the cell therapy field, the most relevant document is the Advanced Therapy Medicinal Products (ATMP) European Regulation 1394/2007, which has implemented regulation on so-called advanced therapy medicinal products (Regulation 1901/2006; Regulation 1394/2007).

14.3.1 Classification of Cellular Therapeutics According to Manipulation Grade or Indication

GMP production includes all therapeutics which are beyond the usual "minimal manipulation" steps that are required for the isolation and preparation of, for example, hematopoietic stem cells, bone marrow, and lymphocytes.

Over the past decade, very clear European legislation initiatives have been created concerning the classification of products as medicinal products (MPs), minimally manipulated MPs, and advanced therapy medicinal products (ATMPs). ATMPs are now defined as either a gene therapy MP, a somatic cell therapy MP, or a tissue-engineered product. A tissue-engineered product is defined as an MP that contains cells or tissues that have either been "substantially manipulated" or that will not be used for the same essential function than in their original state (Alici and Blomberg 2010). According to Article 2(1)(c), the cells or tissues shall be considered "engineered" if they fulfil at least one of the following conditions:

- The cells or tissues have been subject to substantial manipulation, so that biological characteristics, physiological functions, or structural properties relevant for the intended regeneration, repair, or replacement are achieved. Procedures during manufacturing like cutting, grinding, shaping, centrifugation, soaking in antibiotic or antimicrobial solutions, sterilization, irradiation, cell separation, concentration or purification, filtering, lyophilization, freezing, cryopreservation, and vitrification are not considered to substantially manipulate a product. (Annex 1, REGULATION (EC) No 1394/2007),
- The cells or tissues are not intended to be used for the same essential function or functions in the recipient as in the donor.

The Regulation on Advanced Therapies (Regulation (EC) 1394/2007) defines advanced therapy medicinal products (ATMPs) as gene therapy, somatic cell therapy and tissue-engineered products (Article 2). Article 29 of the above-mentioned regulation implies that ATMPs on the community market, in accordance with national or community legislation, will have to comply with the new legislation by December 30, 2011 (ATMPs other than tissue engineered products), or December 30, 2012 (tissue engineered products). These products will have to undergo a marketing authorization procedure, unless they are exempted in accordance with Article 28(2)4 of Regulation (EC) 1394/2007. This exemption applies to ATMPs

prepared on a nonroutine basis according to specific standards, and used within the same member state in a hospital under the exclusive professional responsibility of a medicinal practitioner in order to comply with an individual medical prescription for a custom-made product for an individual patient.

For the purpose of compliance with Article 29 of Regulation (EC) 1394/2007, a marketing authorization application in compliance with Annex I to Directive 2001/83/EC, as amended, and with the Regulation on Medicinal Products for Paediatric use (Regulation (EC) 10901/2006, as amended) will have to be submitted to the EMEA for evaluation in a centralized procedure. The review process will follow the normal procedure for the centralized procedure for ATMPs.

Since January 1, 2009, it has been possible to ask for a scientific recommendation on ATMP classification (Article 17 of Regulation (EC) 1394/2007).

However, neither a manufacturing license nor marketing authorization replaces the requirement for controlled clinical trials. For these different regulations – standards of good clinical practice (GCP) apply. (see DIRECTIVE 2001/20/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of April 4, 2001, on the approximation of the laws, regulations and administrative provisions of the Member States relating to the implementation of good clinical practice in the conduct of clinical trials on medicinal products for human use).

The Advanced Therapy Medicinal Products (ATMPs) have been subject to considerable interest and debate. Following the European regulation on ATMPs, a consolidated regulatory framework for these innovative medicinal products has recently been established. Central to this framework is the Committee for Advanced Therapies (CAT) at the European Medicines Agency (EMA), comprising a multidisciplinary scientific expert committee representing all EU member states and European Free Trade Association countries, as well as patient and medical associations (The Committee for Advanced Therapies (CAT) and the CAT Scientific Secretariat, 2010). Regulatory requirements of ATMPs together with the technical requirements contained in the revised Annex I, part IV of Dir 2001/83/EC introduce specific requirements with which manufactures of ATMPs must comply. These requirements involve specific rules for clinical trials (they have to be designed according to specific good clinical practice (GCP) guidelines and medicinal products have to be manufactured according to good manufacturing practice (GMP), follow up of adverse events, risk management, and traceability (Jekerle et al. 2010). In addition, postauthorization efficacy follow-up has been introduced as a special feature for ATMP in order to address the need for long-term surveillance of efficacy particularly for tissue-engineered products with a regenerative mode of action.

The concept of a risk-based approach has been introduced through the revision of Annex I, part IV of Dir 2001/83/EC. The risk analysis serves to determine the extent of quality, nonclinical, and clinical data. These data have to be included in the marketing authorization application and the amount of data will depend on the level of risk and state of knowledge identified for the product under development and experience of the manufacturer with other ATMPs.

The EU Directive (EUD) 2001/83/EC is concerned with products that are classified as MPs and covers both gene and cell therapy products (Community E Directive 2001).

GMP regulations which have been first installed by the US Food and Drug Administration are contained within the European Commission Directive 2003/94/EC (Commission Directive 2003). One other regulation, the EUD 2004/23/EC, acts as a framework regulation of ATMPs, with the intention of unifying the regulatory frameworks for medical devices, tissue engineering, and medicinal products including gene and cell therapy products (Commission Directive 2004). The latter EUD 2004/23 is applicable to the donation, procurement, and testing of the tissues and cells contained in these products (Community Directive 2004). Regulatory requirements are one of the many aspects to consider when planning and building an ATMP-manufacturing facility, and a considerable amount of foresight is crucial to be able to adapt the suitability of the facility for future regulations.

The GMP regulations require manufacturers to ensure that products are safe, pure and effective. To achieve these goals, all aspects of manufacturing should be monitored: quality assurance management, personnel, premises and equipment, documentation, production, quality control, contract manufacture and analysis, complaints, and product recall (Sensebe et al. 2010).

In Europe for example, mesenchymal stem cells (MSCs) are considered advanced therapy medicinal products (ATMPs), as defined by European Regulation (European Commission [EC] 1394/2007) (Commission Directive 2006). Under this regulation, MSCs are considered somatic-cell therapy products or tissue-engineered products depending on the source, manufacturing process, and proposed indications. The regulation contains rules for authorization, supervision, and technical requirements regarding the summary of product characteristics, labeling and package leaflets of ATMPs that are prepared according to industrial methods and in academic institutions. This regulation includes the following chapters:

- Facilities (Part 1271.190a and b)
- Environmental control (Part 1271.195a)
- Equipment (Part 1271.200a)
- Supplies and reagents (part1271.21a and b)
- Recovery (part 1271.215)
- Processing and process controls (Part 1271.220)
- Labeling controls (Part 1271.250a and b)
- Storage (part 1271.260a–d)
- Receipt, predistribution shipment, and distribution of an HCT/P (Part 1271.265a–d)
- Donor eligibility determinations, donor screening, and donor testing (Parts 1271.50, 1271.75, 1271.80, and 1271.85)

In conclusion, the regulation on ATMPs provides a clear regulatory framework for the approval of ATMPs in the European Union. Because major challenges remain for the developers of ATMPs and for the authorities reviewing the marketing authorization application (MAAs) of ATMPs, the European Medicines Agency (EMA) is promoting an open dialogue with developers of ATMPs to discuss scientific challenges, including a close interaction with the newly established specialist committee, the CAT (The Committee for Advanced Therapies (CAT) and the CAT Scientific Secretariat, 2010).

14.4 Quality of Cell Products: Important Guidelines Regarding the Authenticity, Identity, Genomic Stability and Status of Differentiation

Cellular therapies that either use modifications of a patient's own cells or allogenic cell lines are attracting more and more attention from the medical community in that they are becoming a treatment option for a variety of disorders. In contrast to well-established cell-based therapies in transfusion medicine and organ transplantation, the new advanced therapies are based on prolonged cultivation and *ex vivo* manipulation, e.g., differentiation and genetic modification (Slifka and Whitton 2000; Cheng et al. 2008; Hay 2002). However, the extended cultivation time and manipulation enhance the risk for mutation and contamination which can potentially lead to loss of function, infection-associated problems and even tumorigenesis. Special attention has to be paid to minimizing the risks of contamination, especially microbial contamination during the cultivation process and intra- or interspecies contamination with other cells.

Besides the technical issues of optimal isolation, cultivation and modification, quality control of the generated cellular products is increasingly being considered to be more important. Recently, methodology to ensure identity of cell products has been reviewed, taking the case of two contaminated cell lines (Dittmar et al. 2010), including a review of the recent developments to verify cell lines, stem cells and modifications of autologous cells, exemplifying misinterpretations generated from the analysis of cell lines which were not properly tested for authenticity. The first example included a human cell line that was used for the generation of neurons using specific isolation and cultivation protocol. In order to document the human origin of this cell line, mitochondrial DNA (mtDNA) analysis was performed. Astonishing data revealed that the cell line was not of human origin but in fact originated from rat (reviewed by Dittmar et al. 2010). A second example included a Stem-1 cell line that was used as a human mesenchymal stem cell (MSC) line able to produce differentiated cells depending on the differentiation cytokine cocktail. Genetic profiling analysis demonstrated that the Stem-1 cell line had an identical profile with SAOS-2, which was established in 1973 from the primary osteosarcoma tissue of an 11-year-old Caucasian female (reviewed by Dittmar et al. 2010).

Among the causes that can determine the appearance of contamination one can include:

1. Cross-contamination with other cell lines
2. Chromosomal stability
3. Contamination with microorganisms (particularly *Mycoplasma* spp. In 20–30% of cases (Stacey 2000; Stacey 2002)

Several methods used for cell line authentication were successfully tested:

1. Isoenzyme analysis which detects interspecies contamination of at least 10% (Nims et al. 1998)
2. DNA fingerprinting based on restriction fragment polymorphism (RFLP), (Drexler et al. 2000)

3. Cytogenetic analysis (karyotyping), identifying chromosomal alterations (Drexler et al. 2000; South and Blass 2001)
4. Comparative genomic hybridization (Niini et al. 2011)
5. Analysis of telomere length alterations (Kimura et al. 2010; Berardinelli et al. 2010)
6. Species-specific mitochondrial DNA analysis (Bartlett and Davidson 1992; Unseld et al. 1995)
7. Short tandem repeat analysis (STR) which is becoming the principal method for DNA analyses in forensic cases (Brown and Aaron 2001)

It is important to emphasize that for safe and effective cellular therapies, characterization of the final cell products including karyotype, genetic and epigenetic alterations, expression profiles, phenotype and differentiation potential as well as functional characterization depending on their intended use is fundamental.

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Chapter 15
EBMT Registry of Nonhematopoietic Stem Cells and Regenerative Therapy (Cellular and Engineered Tissue Therapies in Europe)

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Abstract Cellular therapy is an increasingly important therapeutic modality for repairing damaged or destroyed cells or tissues. In hematopoietic stem cell transplantation (HSCT), stem cells are introduced to repopulate the destroyed hematopoietic system after lethal chemoradiotherapy. Moreover, donor lymphocyte infusion was successfully introduced in the 1990s to cure patients suffering from disease relapse after HSCT. Extensive progress in the field of hematopoietic and nonhematopoietic stem cell biology has helped to establish cellular therapy as a new therapeutic option for tissue repair, tissue replacement, and immune modulation.

15.1 Introduction

Cellular therapy is an increasingly important therapeutic modality for repairing damaged or destroyed cells or tissues. The most established stem cell therapy is hematopoietic stem cell transplantation (HSCT), which was first performed in 1957 and, following a decade of development, came to play an essential role in curing

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leukemia or otherwise irreversible bone marrow damage (Thomas et al. 1957; Thomas et al. 1975; Appelbaum 2007; Gooley et al. 2010). In HSCT, stem cells are introduced to repopulate the destroyed hematopoietic system after lethal chemoradiotherapy. A further cellular therapy in the form of donor lymphocyte infusion was successfully introduced in the 1990s to cure patients who underwent a relapse after HSCT (Kolb et al. 1990). Extensive progress in the field of hematopoietic and nonhematopoietic stem cell biology and cell culture in recent years has helped to establish cellular therapy as a new therapeutic option for tissue repair, tissue replacement, and immune modulation. Today, both primary and in vitro cultured selected or nonselected stem and progenitor cells of various lineages from autologous as well as allogeneic donors have been infused, locally applied or given subcutaneously to mediate immune modulation, to repair, to replace but also to perform local paracrine healing and protective functions (Martin et al. 2010; Le Blanc et al. 2004).

15.2 The Challenges of Cellular Therapy and the Role of Registries

While offering a rapidly evolving new therapeutic field and hope for many patients for whom no other treatment options are available, cellular therapies also present new challenges. In order to facilitate the most efficient development in this field, it is necessary to provide an adequate supportive and regulatory environment while avoiding legislative obstacles. It is particularly important in this respect to prevent misuse or even abuse of novel therapies. Such therapies are still experimental and must be conducted within the boundaries of medical ethics and performed in the context of prospective clinical studies. To this end, the European Medical Agency (EMA) has introduced the committee for advanced therapies in order to respond to the needs of novel cellular therapies which, after chemicals and biologics, are becoming an increasingly important and challenging treatment modality (Schneider et al. 2010).

During phases of rapid development, as was the case decades ago for HSCT, registries play an essential role (Horowitz 2008). With this simple but important instrument, it is possible to assimilate current status and experience without delay and to make wide-ranging retrospective analyses of indications, frequencies, and changes. The European Blood and Marrow Transplantation Group (EBMT) has implemented a very simple annual survey of HSCT activity in Europe. The annual EBMT survey has become an essential instrument for the observation of trends and the monitoring of changes in the use of HSCT for the treatment of hematological disorders primarily in Europe but more recently also worldwide. The sharing and rapid dissemination of information is a major goal of this activity, as is the establishment of a formal basis for patient counseling and health care (Gratwohl et al. 2011).

In 2009, the EBMT activity survey was extended through a collaborative effort involving five societies (EBMT, EULAR, TERMIS-EU, ISCT-EU, ICRS-EU) to include novel cellular therapies performed in the previous year. A total of 1040 patients receiving hematopoietic and nonhematopoietic cellular therapies for different

indications were collected in 2008. The main indications were cardiovascular disorders (29%) (79% of which involved myocardial ischemia, bypass grafts, and cardiomyopathy), musculoskeletal disorders (18%) (61% for cartilage and bone repair), neurological (9%), epithelial/parenchymal (9%) (52% for skin reconstruction or liver insufficiency), autoimmune diseases (12%) (60% multiple sclerosis), and graft-versus-host disease (23%). The majority of the cells were of autologous (64%) and the rest of allogeneic origin (36%), with allogeneic cells being used predominantly in neurological and epithelial/parenchymal disorders. It is not surprising that in the early stages of rapid development, the cell type and source (hematopoietic, nonhematopoietic, marrow, placenta, cord blood, blood, and fat), the cell processing (nonexpanded, expanded, untransduced, transduced, unsorted, or sorted), the indications, and the application (intravenous, intraorgan, membrane/gel, or 3D scaffold) were highly heterogeneous, often even for the same clinical indication. Interestingly, the activities differed between European countries with the highest activities in Belgium, the Netherlands, Slovenia, Switzerland, and Greece (Martin et al. 2010). In 2009, 1142 cellular therapies from 105 teams in 22 countries were reported to the joint cellular therapy and EBMT activity survey. The main indications were cardiovascular (416, 64% autologous), tissue repair (192, 98% autologous), autoimmune disease (103, 84% autologous), epithelial (90, 73% autologous), neurological (34, 50% autologous), and unspecified (307, 7% autologous). An increase of 11% could be seen in the cardiovascular disorders, while a decrease of 6% was seen in the neurological disorders.

The annual EBMT survey is complementary to the EBMT registry, which collects more extensive individual patient and donor reports by MED A-C forms. In 2009, more than 31,000 HSCTs (41% allogeneic and 59% autologous) from 624 centers in 43 countries were reported to the EBMT and more than 50,000 HSCTs were collected by the worldwide network for blood and marrow transplantation (WBMT). The EBMT registry has been assimilating patient-specific information including outcome for more than 3 decades. Detailed data from more than 385,000 HSCTs are now available in the EBMT registry database with similar numbers in the registry of the Center for International Blood and Marrow Transplant Research (CIBMTR), representing an invaluable source of information for retrospective studies. Registries have made and will continue to make an essential contribution to the success of HSCT (Gratwohl et al. 1998; Gratwohl et al. 1993; Goldman and Horowitz 2002).

Starting in 2008, detailed patient-specific data on novel cellular therapies were collected within the EBMT registry in a specially designed MED-A form. It is perhaps not surprising that the activity reported at this level is somewhat less than that apparent from the survey. This may be due to the inclusion of patients in prospective clinical studies, to the intensive paper work required to report and update, or to the interdisciplinarity of the effort involved. The EBMT registry included 149 registrations of mesenchymal cells and 48 of dendritic cells in the first year. These figures increased to 257 mesenchymal cells and 52 dendritic cells applications in 2009. In 2010, 464 patients with procedures were registered, including 408 mesenchymal and 59 dendritic cell applications. Thus, a continuous and significant annual increase of activity has been observed to date.

15.3 Conclusion

In conclusion, the new frontier of novel cellular therapies is expanding and promises further interesting developments in the near future. While large studies on efficacy are still lacking, preliminary reports are promising and exciting. However, many questions remain to be answered, and we are still far from established treatment modalities. Efforts to clarify these issues should be undertaken in the most efficient and coordinated way possible. Particular attention should be paid to the definition of the optimal cell type and source, the cell processing, and delivery routes for each application. Dosing is another open question of fundamental importance, as is the issue of short- and long-term vigilance. In this respect, scientific societies around the world are set to assume an essential role in coordinating activities and providing important tools for registering and analyzing developments. Both the survey and the registry will continue to be fundamental and will assume an increasingly important scientific and regulatory role in the future. Report forms can be requested from Helen Baldomero (EBMT-survey) and from Carmen Ruiz (EBMT-registry) or downloaded from http://www.ebmt.org/4Registry/registry3.html.

EBMT: European Blood and Marrow Transplantation Group
WBMT: Worldwide Network for Blood and Marrow Transplantation
EULAR: EUropean League Against Rheumatism
ISCT-EU: International Society of Cellular Therapy-EUropean section
TERMIS-EU: Tissue Engineering and Regenerative Medicine International Society-EUropean chapter
ICRS-EU: International Cartilage Repair Society
CIBMTR: Center for International Blood and Marrow Transplant Research
MED: minimal essential data

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