

## Chapter 2

# Normal Stem Cells: Biology, Collection/ Harvesting, and Ex Vivo Manipulations

*The good thing about science is that it's true whether or not you believe in it.*

*Neil deGrasse Tyson*

### Introduction

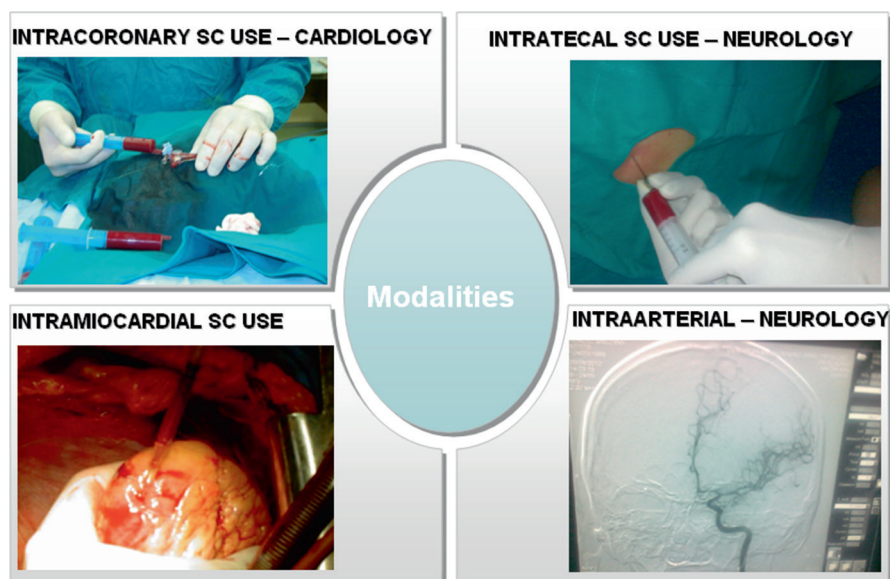
Stem cells (SCs) have an extensive self-renewal capacity and a high potential for proliferation as well as differentiation into pluripotent or committed progenitors and mature blood cells. Hematopoietic events with a complex network of interactive cytokines (grow factors and their inhibitors) are regulated. Different population of SCs expresses CD34 antigen, consequently they are named also as CD34+ cells [1–4]. Thanks to abovementioned characteristics, SCs provide complete and long-term bone marrow (BM) repopulation with subsequent hematopoietic reconstitution after transplantation. A traditional SC transplantation involves myelo (immuno) ablation—the administration of intensive radio-chemotherapy—followed by (re) infusion of harvested cells in order to eliminate of basic disease [5–7]. Similar procedure with reduced-intensity conditioning (RIC) can be offered to patients who are disqualified for high-dose radio-chemotherapy because of their age or comorbidity [8]. Malignant disorders are the most common indication for this therapeutic modality [9–12]. SC transplantation is also used for therapy of benign diseases, such as severe combined immunodeficiency (SCID), metabolic or autoimmune disorders [13–15].

In a few words, in different clinical settings totipotent, pluripotent, and multipotent SCs give rise to repopulation of recipient's BM (engraftment) with subsequent complete, stable, and long-term reconstitution of hematopoiesis. In addition, they are also capable of colonizing different tissues (“homing”). Thus, initial experimental and clinical studies showed that therapeutic use or “implantation” of autologous SCs into damaged and/or ischemic area induces their “homing” and following “transdifferentiation” into the cell lineages of host organ, including collateral vessel formation. Angiogenic growth factors (or genes encoding of these proteins) promote the development of collateral arterioles, and the process is called as “therapeutic angiogenesis” or “neovascularization” [1–3].

In practice, SCs could be collected by multiple aspirations from BM, by mononuclear cell (MNC) harvesting from PB (after mobilizing regimen) or by purification from umbilical cord blood (UCB). Typically, the use of BM or PB derived grafts (allogeneic or autologous) is a standard method in adult setting. UCB transplants have provided hopeful results firstly in pediatric patients—when a matched unrelated BM or PB donor is unavailable [4, 16–25]. In relation to genetic, and particularly HLA relationship between donor and recipient, transplantations can be classified as autologous, allogeneic and syngeneic [1, 4]. The use of autologous transplantation requires both optimized harvesting procedures to get enough SC yield and cryopreservation that guarantees the best possible cell recovery. Despite the fact that cryopreservation are already in routine use, some questions related to optimal freezing method and cryoprotectant (e.g., dimethyl sulfoxide—DMSO, hydroxyethylstarch—HES) type and concentration are not resolved [26–31]. For marrow failure (immunodeficiency, severe aplastic anemia (SAA), BM infiltration) and metabolic disorders, the use of allotransplantation is the therapeutic method of choice if patient has HLA-matched donor. However, allogeneic SC application is not without a risks, such as graft failure, despite intensive myeloablative conditioning regimen applied as well as acute and/or chronic Graft versus Host Disease (GvHD), regardless of immunosuppressive therapy administered [4, 32–34].

Generally, SCs can be divided into embryonic and “tissue-specific” (adult) cell compartment [2]. Current researches have recognized that some adult SCs have similar “unlimited” biological potential than embryonic cells. Consequently, adult SCs are able to develop into a variety of somatic cells by “transdifferentiation” or “SC plasticity” [35–37]. Although the term “cell plasticity” became very popular, some studies have suggested that BM might contain different types of SCs that can produce non-hematopoietic (somatic) cells. For example, mesenchymal SCs in BM give rise to osteocytes, chondrocytes, adipocytes, and skeletal muscle. Consequently, adult BM or peripheral blood (PB) derived SCs are clinically applicable for the cell therapy in the field of regenerative medicine, that is cell/organ replacement and/or regeneration—precisely for the treatment of patients with myocardial, liver, vascular, neurological, or other tissue damages [38–43]. The SC applications used in the Institute of Transfusiology and Hemobiology of MMA are presented in Fig. 2.1.

*Autologous SC transplants.* When is BM appropriate to use as source of SCs for autologous resuscitation following myeloablative radio-chemotherapy or RIC depends on the marrow general state and/or infiltration with malignant cells. Fibrosis makes marrow not possible for SC collection by aspirations. Tumor cell infiltrates eliminate marrow as a transplant source as well. Prior pelvic irradiation, poor anesthesia risk, obesity, or patient refusal of marrow collection can limit marrow as an option. Mobilized autologous SCs from PB are commonly used in the above situations and in heavily treated patients. This procedure is nowadays in routine clinical practice and provides more progenitor cell yield than conventional marrow harvest and therefore earlier engraftment, which is a faster hematopoietic recovery. Primarily for this reason, transplant of PB derived SCs has practically replaced BM



**Fig. 2.1** Stem cell application approaches in our center for regenerative medicine

transplant in an autologous setting. As mentioned, autologous PB harvests involve mobilizing the SCs from the patient's BM compartment into the circulation using different growth factors, typically in combination with chemotherapy prior to collection. Once in the circulation, the SCs are collected by apheresis—conventional or large-volume leukapheresis [1–3, 6, 44].

*Allogeneic SC transplants.* Transplant of allogeneic SCs are indicated in the treatment of patients with malignant disease—if they have HLA-matched donors. For patients with immunodeficiency, marrow failure, metabolism disorders, etc., the use of allogeneic SCs is imperative. However, there are also some “atypical data” related to treatment of SAA using autologous SCs [10]. Allogeneic transplant is associated with a risk that immunocompetent donor cells will react against recipient tissues (GvHD), despite immunosuppressive therapy administered. In adult “related allogeneic setting,” the best results are obtained using completely HLA-matched (HLA-identical, i.e., six-antigen-matched donor/recipient pairs) transplants. There is a 25 % chance of a sibling being a complete match, a 50 % chance of a haplotype match, and a 25 % chance of a complete mismatch. Pediatric patients are more tolerant of partially mismatched graft [1–4, 44, 45].

Data obtained up till now has shown that the use of SC donor registers can successfully recruit unrelated donors for collection of BM or PB derived SCs. Thus, matched unrelated donor searches can be initiated for approximately 70 % of candidates without sibling donor. These protocols have possible benefit since higher engraftment potential of allogeneic vs. autologous SCs and following earlier hema-

topoietic reconstitution, as well as occurrence of GvL effect [1, 3, 44]. For definitive choice, additional experimental and clinical trials for comparison of efficacy and outcome of autologous vs. allogeneic (related or unrelated) BM vs. PB derived SC transplant are required.

*Syngeneic and haploidentical SC transplants.* Occasionally recipient has an identical twin—a syngeneic transplant is optimal because the donor and recipient cells are genotypically identical (the first transplants performed in humans) [39]. On the other hand, syngeneic grafts do not induce graft vs. tumor that is GvL effect in recipient. Our knowledge of the immunobiology of SC transplant across major histocompatibility complex (MHC) barriers—haploidentical transplants—has increased significantly over the past decades. The key reason (or limitation) for realization potential haploidentical SC transplant is the absence of a HLA-matched related donor in the majority of families. On the other hand, the conversion of a new hypothetical therapeutic option into the routine haploidentical SC transplant clinical practice is accepted and developed more slowly. The most critical complications of SC transplants across HLA barriers are the graft rejection and/or occurrence of GvHD. However, these adverse events maybe could be successfully prevented and treated using current pharmacologic approaches or manipulations during the haploidentical hematopoietic grafting process [1].

In a few words, the intensifying of myelo (immuno) ablative therapy combined with SC transplantation and the introduction of cell-mediated restorative/regenerative methods (“cell therapy”) resulted in increased needs for both SCs conceptual and practical operating procedures inducing minimized cell damages during their harvesting and cryopreservation. In this article, data in the field of practical aspects of an optimized SC harvesting, purification, and cryopreservation will be briefly reviewed. In addition, our results of the investigation of SC harvesting and different graft ex vivo “graft engineering” (cell processing, selection and cryopreservation) protocols will be summarized.

## Bone Marrow Derived Stem Cell Collection

Historically, BM was the first source of SCs for transplant in experimental and clinical setting [4–7]. A marrow harvest is the same for an allogeneic donor as for an autologous patient. SCs are collected by multiple aspirations from the posterior and anterior iliac crest and (seldom) from sternum. The posterior iliac crest provides the richest site of marrow. The procedure is performed under sterile conditions in the operation room, while the donor is generally anesthetized (Fig. 2.2).

In order to provide required number of nucleated cells (TNCs), that is  $\geq 3 \times 10^8$ /kg of body mass (kgbm), around 200 aspirations are required, where single aspirate volume is 2–5 mL. Immediately after the collection, cell aspirate should be filtered in order to remove bone and lipid tissue particles and/or cell aggregates. Anticoagulation is provided using solution containing citrate and by heparin diluted



**Fig. 2.2** BM derived SC collection by multiple aspirations

in saline (5000 IU/500 mL), using autologous plasma or one of the cell culture medium for resuspension of collected cells [1–7, 44, 45].

The target dose of collected marrow is 10–15 mL per kgbm. Thus, the volume of aspirate is relatively large (800–1000 mL) and it contains a high count of red blood cells. Accordingly, in order to prevent anemia in donors, blood for autologous transfusion (to carry out during SC collection) should be collected around 1 week before SC collection and transplant [1–3, 44].

On the other hand, of aspirate volume, precisely red blood cell number and/or plasma quantity reduction is required (by processing—Fig. 2.3), especially for ABO incompatible (major and/or minor) transplants or when cryopreservation is intended (autologous setting).

A commonly used minimum target (after processing) of TNC count—for both autologous and allogeneic transplants—is  $2 \times 10^8/\text{kgbm}$  [1–4, 44]. The concentration of the  $\text{CD34}^+$  cells and/or depletion of T-cells (positive/negative cell selection) in final cell unit is achieved by the *ex vivo* purging procedure using immunomagnetic device for cell selection [2, 44].

These SC purification procedures (processing and purging or selection) enable reduction of the aspirate volume, i.e., reduction of red blood cell for around 80–90 %, or even more precisely, the depletion of mentioned unwanted (malignant or T-cells) cells with efficacy  $\geq 3\text{--}4 \text{ Log}_{10}$  [1–4, 45]. Development of the ability to isolate selected SCs and/or *ex vivo* expand them into a large number is expected to broaden their beneficial therapeutic effects, since the limitation to many of SC applications has been the absolute number of defined target cells.





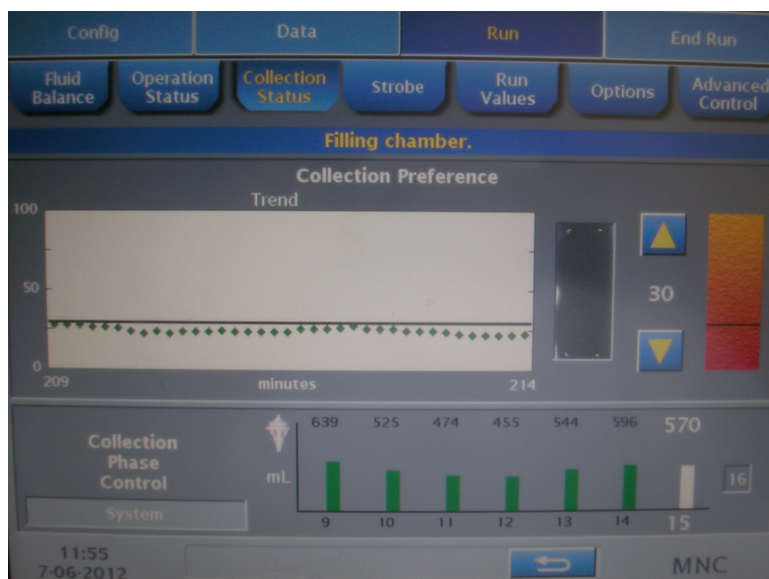
**Fig. 2.3** BM aspirate processing after SC collection using Spectra-Optia device

### ***Peripheral Blood Derived Stem Cell Harvesting/Purification***

CD34 is the cluster designation given to a transmembrane glycoprotein present on SC surface and some stromal cells. Cells expressing the CD34 antigen (obtained from BM or PB) are capable of complete reconstitution of hematopoiesis. The first SC harvests from PB were accomplished in “steady state hematopoiesis”—but using numerous [6–9] collections and following cryopreservation was needed [1–3]. Currently, SCs are harvested after mobilization by the use of chemotherapy and/or recombinant colony-stimulating factors (rHuG-CSF). The typical number of apheresis required is not more than 1–3.

The collection PB-SCs is an aphaeretic procedure with respect to the standardized protocol and cell yield (Fig. 2.4).

Characteristically, for anticoagulation an acid-citrate-dextrose formula B (ACD-B with 1.8 % citrate concentration) or ACD-A (2.2 % citrate concentration) solutions are used alone (seldom in combination with heparin). For allogeneic transplantations, venous access is most frequently realized through antecubital veins. In autologous setting, collection should be performed across central-venous, jugular, or femoral vascular access. Short-term use of femoral catheters appears safe and effective, improving patient comfort and reducing cost. These catheters have simplified cell harvesting, but may be associated with thrombosis of the instrumented vessels. In addition, there is approximately one percent central-venous catheter-related hazard of the local infection, pneumothorax or bleeding [1–3, 18–22, 44].



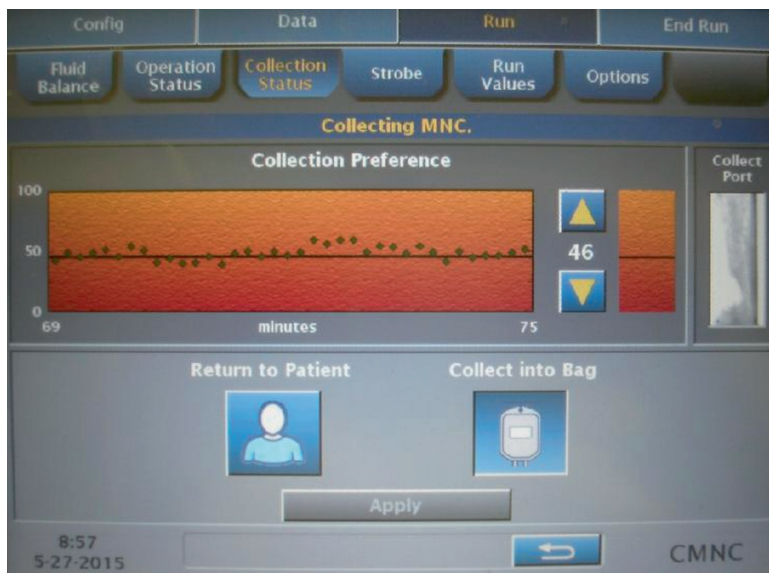
**Fig. 2.4** Peripheral blood SC harvesting and monitoring by Spectra-Optia device

PB-SC transplantation could be described by: (a) absence of general anesthesia and work in surgical division; (b) smaller harvest quantity and higher cell yield; (c) improved engraftment rate and lower transplantation-related morbidity; (d) earlier/faster hematopoietic and immune reconstitution. Due to the mentioned reasons, the number of patients treated by PB-SCs is ever increasing worldwide, especially in autologous SC transplantation setting [1, 44].

For obtaining acceptable SC or CD34<sup>+</sup> yield, efficient mobilization protocol is required. Allogeneic donors are given rHuG-CSF 5–10 µg/kgbm daily subcutaneously. The CD34<sup>+</sup> cell count in the circulation begins to rise after 2–3 days of rHuG-CSF administration and peaks is on the fifth day. When donor mobilization with rHuG-CSF is poor, the only ways to improve yields are to increase the blood volume processed or the number of collections. In autologous setting typical rHuG-CSF doses are higher—patients are given rHuG-CSF 12–16 µg/kgbm daily combined with mono-chemotherapy (cyclophosphamide 4–7 g/m<sup>2</sup>) or by poly-chemotherapy in corresponding doses [1, 18–20].

In the course of cell harvesting, the determination of the optimized collection system (Fig. 2.5) and optimal timing for apheresis are the most critical event.

For allogeneic donors the first apheresis is on the fifth day of rHuG-CSF application. However, the definition of best possible timing of autologous collection from patients who primed by chemotherapy plus rHuG-CSF is more complex and controversial. The optimal timing can be determined based on the specific cell values in the hemogram. Leukocyte and/or MNC counts, as well as the number of circulating CD34<sup>+</sup> cells, have all been used as markers to determine when to initiate harvesting.



**Fig. 2.5** The most recent peripheral blood SC harvesting approach—the use of the Intermediate density layer (IDL) system

It is suggested that optimal time to begin cell collection is when the leukocyte count  $\geq 5\text{--}10 \times 10^9/\text{L}$ . However, the leukocytes do not correlate strongly with the number of SC in the graft. Contrary, circulating  $\text{CD}34^+$  number evidently correlates with collection timing and the SC quantity in harvest (as a function of the volume of blood processed also). Namely, it is presented that for a  $\text{CD}34^+ \geq 20\text{--}40/\mu\text{L}$  of patient's blood the possibility of the  $\text{CD}34^+$  yield  $\geq 2.5 \times 10^6$  cells/kgbm is around 15 % after performance of one “standard” collection or 60 % or more after one LVL. Of course, higher  $\text{CD}34^+$  number in circulation results in superior yield [1, 17–19]. Our results also confirmed high-level efficacy of the LVL. Namely, for the 89.5 % patients using one LVL, the mean  $\text{CD}34^+$  yield was  $12.1 \times 10^6/\text{kgbm}$  (allogeneic) and  $6.5 \times 10^6/\text{kgbm}$  (autologous), respectively. In our group of patients, the circulating  $\text{CD}34^+$  count was also relatively high  $40\text{--}60/\mu\text{L}$  following mobilizing regimen [1, 44, 45].

The efficiency and standardization of PB-SC harvesting can be estimated by MNC (now seldom) and  $\text{CD}34^+$  (typically) quantifications. It is generally considered that the count of MNC should be about  $300 \times 10^8$  per unit, that is  $\geq 2\text{--}4 \times 10^8/\text{kgbm}$ , and  $\text{CD}34^+$  around  $330 \times 10^6$  per unit, that is  $\geq 2\text{--}4 \times 10^6/\text{kgbm}$  in harvest (contamination: no more than  $470 \times 10^9$  platelets and 7.6 mL red blood cell total volume per unit) in order to expect successful transplant. However, recent data support a clinical benefit associated with greater  $\text{CD}34^+$  yield ( $\geq 5.0 \times 10^6/\text{kgbm}$ ) compared to the minimum cell quantity needed ( $\geq 1.0 \times 10^6/\text{kgbm}$ ) in autologous setting [1, 27, 28]. Although commonly accepted, the stated cell yields cannot guarantee



stable and long-term reconstruction of hematopoiesis following transplant. In order to achieve them, the next details are needed: (a) the volume of processed blood in one apheresis  $\geq 2-3$  or more (intensive or LVL) patient's circulating volume, that is around 16–25 L for person with around 70–80 kg of body mass and (b) apheretic procedures should be performed 1–2 (occasionally more) times [1, 20, 27, 44].

However, patients who have been earlier treated with high-dose radio-chemotherapy may be “poor responders” for chemotherapy plus rHuG-CSF induced mobilization. The most efficient approach to obtain adequate SCs from “poor mobilizers” is not resolute still. Simultaneously collection of SCs from BM and PB has not improved engraftment rate significantly. Mobilization with rHuG-CSF alone is perhaps more efficient than rHuG-CSF in addition to chemotherapy. Increased doses of rHuG-CSF or use of rHuG-CSF together with rHuGM-CSF has also effectively mobilized some autologous donors [1, 45].

When PB-SCs are collected, additional harvest ex vivo manipulation, that is, processing (to obtain a red blood cell count and/or plasma volume reduction), is not required because the final hematocrit is small (between 0.05 and 0.10) and the final volume of cell suspension is only around 200–250 mL [44]. On the other hand, the main disadvantage of the use of PB-SCs is high-level T-lymphocyte and occasionally tumor cell “contamination” and subsequent risk of GvHD or disease relapse. However, efficient graft purification methods, that is, depletion of tumor cells (autologous) or T-lymphocytes (allogeneic), were developed using immunomagnetic technique (positive and/or negative selection) by cell sorters [2, 11, 12]. Namely, earlier studies reported an elevated risk of GvHD in allogeneic PB-SC vs. BM-SC recipients, but recent prospective randomized studies found no differences in the incidence of this complication [1, 44]. The use of stated graft purification by immunomagnetic system has been shown to be the most effective method to achieve a 3–4  $\text{Log}_{10}$  T-lymphocyte depletion while retaining around 60–80 % of the  $\text{CD34}^+$  cells in the graft [1, 11, 12].

Our results (Fig. 2.6) also verified that  $\text{CD34}^+$  cell recovery was 70–80 %, when post-selection  $\text{CD34}^+$  purity ( $\text{CD34}^+$  cell percentage in final cell suspension) was around 80–90 % [1–3, 36, 44].

In addition, the objective of our preclinical researches during last years was to optimize SC collection and processing protocols, as well as mobilization/harvesting timing in order to obtain high  $\text{CD34}^+$  and especially a more primitive  $\text{CD34}^+/\text{CD90}^+$  cell yield and recovery (using original controlled-rate cryopreservation), with ultimate goal of improving conditions for complete and long-term hematopoietic reconstitution after autologous SC transplants or therapy in the field of regenerative medicine (Table 2.1).

As mentioned, the most common hematological malignancies treated by SCs are different leukemias (nowadays mainly acute myeloid leukemia and Acute lymphoblastic leukemia), Hodgkin's disease or non-Hodgkin's lymphoma, multiple myeloma, and myelodysplastic or myeloproliferative disorders. In addition, a number of neoplasms and nonmalignant diseases were treated with SC transplants such as breast, ovarian, and testicular cancer, Wilm's tumor, neuroblastoma, as well as SAA, SCID, thalassemia, and various congenital or autoimmune disorders.



Fig. 2.6 Positive/negative immunomagnetic cell selection

BONE MARROW vs. PERIPHERAL BLOOD			
CD34+ SUBTYPE INVESTIGATION			
	PB-SCs 1	PB-SCs 2	BM-SCs
CD34 <sup>+</sup> pe / CD90 <sup>+</sup> fitc	1.72±1.47	1.25±0.82	2.72±2.06
CD34 <sup>+</sup> pe / CD38 <sup>+</sup> fitc	2.02±1.18	2.02±1.18	2.3±1.16
CD34 <sup>+</sup> pe / HLA-DR <sup>+</sup> fitc	2.01±0.92	2.0±0.92	2.0±0.88
CD34 <sup>+</sup> pe / CD33 <sup>+</sup> fitc	1.90±1.23	1.9±1.23	2.75±1.12
Apoptotic/necrotic 7-AAD positive cells before and after freezing: 2.58±1.23% i 4.58±2.97%.			

Table 2.1 Flow cytometry of SCs—CD34 subset quantity and ratio  
PB-SCs 1=chemotherapy + rHuG-CSF; PB-SCs 2= rHuG-CSF alone; BM-SCs=BM derived cells

However, specific clinical aspects—such as optimal transplantation timing, therapeutic efficacy, complications—of the treatment of these disorders will not be discussed in this book.

Finally yet importantly, the clinical use of PB-SCs has a specific immune-mediated beneficial antitumor effect, particularly in hematocology. Namely, it has long been known that the administration of the donor-specific lymphocytes (DSLs) resulted with an obvious immunomodulating effect. However, only recently have systems been developed to “separate” the Graft-versus-Leukemia (GvL) effect from GvHD—the best results were obtained in treatment of CML [1, 46]. Our investigations of the use of DSLs also confirmed efficacy of this treatment in patients with Philadelphia-positive CML relapsed after BMT. At the same time, our original *in vitro* test (named as “Test of Mixed Progenitors”) was introduced to predict the clinical outcome of DSL treatment. These patients did not develop GvHD and currently they remain well in complete remission (direct evidence of the GvL effect) [47].

## **Umbilical Cord Blood Derived Stem Cell Usage**

Patient’s requests for SCs have only in  $\leq 30\%$  (related) and  $\leq 70\%$  (unrelated) possibility of finding an adult allogeneic donor. Because of the limited availability of donors, attention has turned to alternative sources of HLA-typed SCs. In recent years, UCB has emerged as a feasible alternative source of transplantable CD34<sup>+</sup> cells for allogeneic transplant, mainly in patients who lack HLA-matched donors of BM or PB derived SCs [1–3, 23–25]. UCB is relatively rich in “more primitive” SCs that can be used not only to reconstitute the hematopoietic system, but have the potential to give rise to non-hematopoietic cells (myocardial, neural, and endothelial cells, etc.) by transdifferentiation (Fig. 2.7).

The “naive” nature of UCB lymphocytes also permits the use of HLA-mismatched grafts at 1–2 loci without higher risk for severe GvHD relative to BM transplant from a full matched unrelated donor. On the other hand, UCB is rich in primitive NK cells, which possess impressive proliferative and cytotoxic capacities and can be induced Graft versus Leukemia (GvL) effect. The use of UCB is an accepted cell source for pediatric patients for whom smaller cell count is enough for engraftment, and for whom a matched unrelated allogeneic BM or peripheral blood SC donor is unavailable. However, a higher risk of graft failure was noticed in children weighing  $\geq 45$  kgmb. Since the number of SCs in UCB is limited and the collection can occur only in a single occasion—its use in adult patients can be more problematic [23–25].

UCB volume is typically 100 mL (range 40–240 mL) with a TNC count around  $1 \times 10^9$  and CD34<sup>+</sup> approximately  $3 \times 10^6$  per unit. UCB can easily be cryopreserved, thus allowing for the establishment of HLA-typed SC banks. Because UCB derived SC banking requires high financial investment and organizational efforts, banking efficiency should be optimized. An important determinant of banking efficiency is the ratio of collections that can be cryopreserved and supplied for transplant.

**Fig. 2.7** Intramyocardial SC application in the cardiosurgery



Although there were reasons for removing UCB units that may be less amenable to improvement, such as low volumes and low cell counts, a number of obstetric factors influencing the outcome of collections could be evaluated further, including the time of cord clamping, length of gestation, length of labor, the newborn's body weight, and the weight of the placenta [1–3, 24, 36].

## Long-Term Storage of Stem Cells by Cryopreservation

Cryobiology is a scientific discipline that estimates the effects of ultra-low temperature on cell integrity and functionality in the “refrigerated biological system” and determines data applicable in cryopractice. Cryopreservation is beneficial when cells appear to be biologically, chemically, or thermally unstable after liquid-state storage. Its primary aim is to obtain both better cell recovery and postthaw viability. Thus, cryopreservation includes specific approaches/techniques designed to extend “therapeutic shelf life” of the cells (prolonged storage time) and to obtain minimum thermal damages (cryoinjury) [1–3, 26–30].

The use of cryobiology for living cell preservation began in 1949 with the freezing of bovine sperm cells, using glycerol as a cryoprotectant [48]. Afterwards, glycerol and dimethyl sulfoxide (DMSO) techniques were applied for cryopreservation of different blood-derived (progenitor or mature) cells [44, 49–56]. The basic goal

of these initial cryoinvestigations was to predict the cell response to freeze/thaw processes and cryoprotectant addition/removal. However, evaluation of certain cryobiological variables (biophysical, physicochemical, and other events/parameters responsible for cryoinjury), as well as standardization of conceptual and practical aspects of cryopreservation, is still a question of considerable interest to researchers and practitioners [1–3, 27, 57–60].

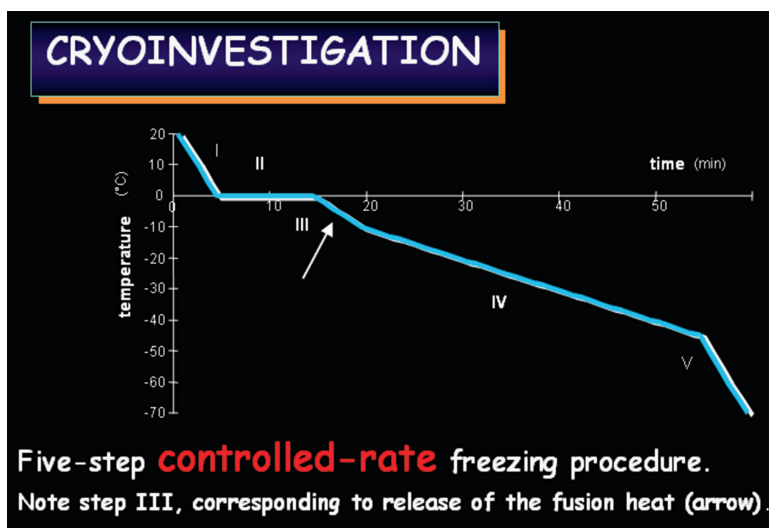
As mentioned, SC cryopreservation is now in routine practice, but recent cryoinvestigations suggest that freezing strategies should be revised to optimize specific cryobiosystems, i.e., to minimize the cryoinjuries and maximize cell recovery. Microprocessor-restricted (controlled-rate) freezing is a time-consuming process, which requires high-level technical expertise. Uncontrolled-rate (“dump-freeze” without programmed cooling rate) technique is less costly because it does not require a programmed freezing device. However, the controlled-rate method is a high-class alternative to the uncontrolled-rate technique due to superior quantitative, morphological, ultrastructural, and functional cell recovery [28–30, 44].

Cryoinjuries can be detected as cell lesions, caused by the decrease of selected functions to the total cell destruction, i.e., cytolysis. At present it is considered that cryoinjuries result from the extensive volume reduction (cellular dehydration or solution effect) and/or massive intracellular ice crystallization (mechanical damage). Although independent, these mechanisms can also act together. The first event is expressed primarily at low-rate ( $\leq 10$  °C/min) freezing, and the second one in high-rate ( $\geq 10$  °C/min) freezing [2, 28, 56].

Therefore, determination of an optimal freezing rate (specific for each cell type and cryobiosystem) should be considered. It is the speed of cooling high enough to prevent cell dehydration and adequately low to make possible efflux of water from the cell. It would be ideal to find a cooling rate just less than the one, which causes intracellular crystallization [10, 27]. Optimal freezing rate is the function of the ratio between cell surface and volume, as well as of cell membrane permeability for water and its corresponding temperature coefficient—but it also depends on what type of cryopreservation strategy is applied. Last but not least, a higher degree of cell destruction has occurred when transition period from liquid to solid phase (fusion heat releasing) is prolonged (Fig. 2.8). The released heat of fusion—if not considered during controlled-rate freezing—could result in additional temperature fluctuation. That is why the period of transformation from liquid to solid phase will be prolonged, and its duration is directly related to the degree of cryoinjury [2, 26, 28].

Determination of the optimal freezing approach is essential, but it cannot solve all problems related to cell cryoinjury. To be precise, postthaw cell recovery and viability are high only when cryoprotectants are present in the cryobiosystem. They prevent or reduce the degree of cell thermal damages. In brief, cryoprotectants can express protective effect by the reduction of cell dehydration, as well as by decreasing the intensity of intracellular crystallization. However, they cannot protect the cell from an already existing excessive dehydration or from the effect of already formed intracellular ice crystals [26].





**Fig. 2.8** Controlled-rate cryopreservation with compensation of the released fusion heat

Generally, cryoprotectants can be classified into nonpenetrating or extracellular and penetrating or intracellular compounds. Mechanisms of their action are complex and only partially recognized. Due to the differences in its chemical and other properties, it is not possible to discover a cryoprotective mechanism common for all cryoprotectants. In brief, extracellular agents could protect cells during high-rate freezing, reducing the intracellular ice crystal formation. On the other hand, intracellular cryoprotectants could provide protection in the course of low-rate freezing, decreasing the degree of cell dehydration [48–51, 61–64].

In practice, bone marrow SC cryopreservation consists of the following steps: (a) graft purification (if it is needed); (b) equilibration (cell exposure to cryoprotectant) and freezing; (c) cell storage at  $-90 \pm 5$  °C (mechanical freezer), at temperature from  $-120$  to  $-150$  °C (mechanical freezer or steam of nitrogen) or at  $-196$  °C (liquid nitrogen); and (d) cell thawing in a water bath at  $37 \pm 3$  °C. Cryopreservation of PB-SC has to be adapted to conditions which depend on the: (a) higher blood cell count; (b) presence of plasma proteins; (c) absence of lipid and bone particles in HSPC concentrate [2, 28].

Immediately after thawing, cells are transfused through a central vein catheter. Generally, patients tolerate the infusion of unprocessed SCs well, with no side effects. However, the grade of the potential reinfusion-related toxicity is associated with DMSO quantity in the cell concentrate [2]. Alternatively, cryoprotectant can be removed by washing, but this procedure results in substantial cell loss. The integrity of residual granulocytes is compromised within cryopreserved HSPCs and consequential DNA release during the thawing procedure may lead to cell “clumping” with resulting extra cell loss. To avoid this problem, a washing protocol by recombinant human deoxyribonuclease (rHu-DNase) is recommended [2,

65]. The addition of rHuDNase to cell concentrate seemingly proves to be effective in preventing “clumping” and it does not cause decreased expression of adhesion molecules, although it is not free of potential risks for patients. Moreover, the use of specific additives (e.g., membrane stabilizers) could improve postthaw cell recovery and it is probably a more effective approach than the decrease of DMSO concentration [28, 65].

Our results are in agreement with the abovementioned studies. Namely, we have found that the recovery of pluripotent and committed haematopoietic progenitors (CFU-Sd12 and CFU-GM) in the presence of 5 % vs. 10 % DMSO is superior [26]. However, it has also been demonstrated that the recovery of very primitive pluripotent haematopoietic stem cells (Marrow Repopulating Ability—MRA) is better when 10 % DMSO is used. These results imply a different “cryobiological request” of MRA cells in comparison with the nucleated cells and progenitors. Moreover, we have demonstrated that differences in cell recovery are not related to the changes in the total number of frozen/thawed cells, regardless of the use of cryopreservation strategy [26]. As a final point, our clinical studies showed that therapeutic use of the controlled-rate cryopreserved SCs in treatment of leukemias (ALL, ANLL, CML), multiple myeloma, Hodgkin’s and non-Hodgkin’s lymphoma, breast and ovarian cancer, and extragonadal non-seminal germ cell tumor resulted with high cell recovery (91 %) and rapid posttransplantation haematopoietic reconstitution (on the 11th day in average) [1, 9, 10, 44, 45].

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The intensification of myeloablative therapy with SC rescue, as well as increase in the use of allogeneic transplantations and different cell-mediated therapeutic approaches have resulted in higher needs for both SCs and practical operating procedures in minimizing cell damage during collection and cryostorage. SC collection systems have to effectively protect the biological and physical properties of cells that can be altered radically by the harvesting and/or purification processes, and have to include techniques and materials appropriate for human use. Although cryopreservation of SCs represents one complex process and the number of potential questions is higher than the number of possible answers, considering ever increasing needs for SCs as well as a rising use of different cell-mediated curative methods, it should also find its appropriate place in the current medical practice.

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