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Enhancing the Efficacy of Engraftment of Cord Blood for Hematopoietic Cell Transplantation

Hal E. Broxmeyer

Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, Indiana, USA

Abstract

Clinical Cord Blood (CB) Hematopoietic Cell Transplantation (HCT) has progressed well since the initial successful CB HCT that saved the life of a young boy with Fanconi anemia. The recipient is alive and well now 28 years out since that first transplant with CB cells from his HLA-matched sister. CB HCT has now been used to treat over 35,000 patients with various malignant and non-malignant disorders mainly using HLA-matched or partially HLA-disparate allogeneic CB cells. There are advantages and disadvantages to using CB for HCT compared to other sources of transplantable hematopoietic stem (HSC) and progenitor (HPC) cells. One disadvantage of the use of CB as a source of transplantable HSC and HPC is the limited number of these cells in a single CB collected, and slower time to neutrophil, platelet and immune cell recovery. This review describes current attempts to: increase the collection of HSC/HPC from CB, enhance the homing of the infused cells, *ex-vivo* expand numbers of collected HSC/HPC and increase production of the infused CB cells that reach the marrow. The ultimate goal is to manipulate efficiency and efficacy for safe and economical use of single unit CB HCT.

Keywords

Cord blood; Hematopoietic cell transplantation; Hematopoietic stem and progenitor cells; Cord blood collection; Homing of stem and progenitor cells; *Ex-vivo* expansion; Cytokines; Oxygen Tension; Dipeptidylpeptidase 4

1. Introduction

Hematopoietic cell transplantation (HCT) is a life-saving procedure for treatment of malignant and non-malignant disorders, and is usually a last resort for those whom there is no other treatment available [1,2]. The life-saving cells necessary to establish a new hematopoietic system to replace the damaged or malignant cells are hematopoietic stem

Correspondence to: Hal E. Broxmeyer, Ph.D., Distinguished Professor, Mary Margaret Walther Professor Emeritus, Professor of Microbiology/Immunology, Program Leader, NCI-Designated Indiana University Simon Cancer Center Program on Hematopoiesis, Heme Malignancies, and Immunology, Indiana University School of Medicine, Department of Microbiology and Immunology, 950 West Walnut Street, R2-302, Indianapolis, IN 46202, 317-274-7510 (Tel), 317-274-7592 (Fax), hbroxmey@iupui.edu.

(HSC) and progenitor (HPC) cells [3–5]. These cells give rise to all the blood forming elements. Their production is regulated by various proteins, such as cytokines and chemokines, other growth regulatory molecules, the in vivo microenvironmental niche composed of various stromal cells and the extracellular matrix, and the hypoxic atmosphere within the niche [6,7].

HSC and HPC are found in various tissues, including bone marrow (BM) which is the major site of production of blood cells in the adult. HSC/HPC are also found circulating in the blood but their numbers in blood under normal steady state conditions are low, unless these cells are mobilized from the BM with chemotherapy, growth modulating proteins such as Granulocyte Colony Stimulating Factor (G-CSF), or smaller molecules (Macrophage Inflammatory Protein (MIP)-1α or GRO-β), including synthetic ones (AMD3100/Plerixafor) [3,6]. HSC and HPC can also be found in umbilical cord blood (CB), at the birth of a baby [1,2]. Currently the three main clinical sources of HSC and HPC for HCT are BM, mobilized peripheral blood (mPB), and CB. Each have been used successfully and have advantages and disadvantages.

The advantages of CB for HCT include the ease of collection of the CB at the birth of the baby, with no problems for the mother or baby, the ability to store CB collections immediately after cryopreservation in either a public CB bank for use by others after HLA-typing, or in a family bank for future use by the baby donor or perhaps for a family member. At present, CB has been used to transplant over 35,000 recipients with success rates equivalent to those done with BM or mPB [1,2]. One outstanding advantage of CB, besides the almost immediate availability of the cells for transplant, is the documented lower graft vs. host disease (GVHD) associated with the use of CB, in comparison to that of BM or mPB [1,2]. This lowered level of resultant GVHD associated with CB as the donor cell population of HSC and HPC has allowed CB to be used in situations of increased HLA-disparity compared to that of BM or mPB, opening up the opportunity for transplants that cannot be performed safely with equivalent partially HLA-mismatched BM or mPB. Thus, there is great optimism for use of CB as a source of HSC and HPC for HCT. However, there are disadvantages to using CB compared to BM and mPB, including the more limited numbers of cells collected at the birth of the baby, which is a one-time only collection, and the slower time to engraftment for neutrophils, platelets, and immune cell reconstitution [1,2]. Being able to successfully address these two concerns would make CB an even more desirable source of transplantable HSC and HPC, and would likely greatly enhance the clinical use of these cells for HCT. Moreover, in addition to use of CB, BM or mPB for transplantation, another treatment has more recently emerged, that of haploidentical HCT, which seems to also have the advantage of increased use in an HLA-disparate setting, lowered GVHD, and with enhanced time to engraftment [8]. However, haplo-identical transplantation is not without its own inherent problems, including enhanced relapse rates over time. Which source of cells will be best for which situation will “play-out” in time. In the meantime, efforts are on-going by numerous research and transplant investigators to find ways to enhance the numbers of HSC/HPC from CB, and to accelerate the time to engraftment with CB. Results are promising, and hopefully efforts in this important endeavor will continue to move forward.

2. Background to the Field

The first CB HCT was performed in October 1988 at the Hopital St. Louis, in Paris under the direction of Eliane Gluckman, M.D., with an HLA-matched sibling CB collection that was processed, frozen and then hand-delivered to Dr. Gluckman by my laboratory [9]. The initial scientific studies suggesting CB as a source of transplantable HSC and HPC [10–14], as well as this first [9] and a number of subsequent HLA-matched sibling CB transplants that started the field of CB HCT came from my laboratory and from our first proof-of-principle CB bank [15–19]. These first CB HCT efforts have been described [9,20–22]. Many of the first HCT advantages and disadvantages first noticed by us and our clinical collaborations still persist to this day, 28 years after the first transplant. While better clinical procedures have enhanced HCT outcome with HLA-matched and-partially matched allogeneic transplants, there is much room for improvement. Efforts towards this outcome by our group and others are described below.

3. Ongoing Experimental Laboratory and Clinical Efforts to Enhance CB HCT

Clinical efforts for, and the status of, CB HCT have been described in detail in several of our recent review articles [1,2]. Present efforts to enhance the efficacy of CB HCT include: A) more effective means to manage high quality and quantity collections of CB that maximize numbers of functional HSC; B) efforts to increase the homing capacity of HSC, since only a small portion of the HSC infused intravenously (i.v.) during HCT actually reach and/or engraft in the BM, a necessary site of eventual lodgement for HSC in order for their maintenance, expansion and differentiation to mature blood cells; C) the capacity to expand numbers of collected HSC and HPC outside the body (*ex-vivo*); and D) determine how best to enhance the production of the cells that eventually reach (home to) the BM, as part of the actual engraftment procedure.

3A.) Enhancing Cell Collections

Our original cell collection procedures have been reported [9,10,23–25]. There is nothing magical about these collections, and different collection sites have different ways to manage the collections at the birth of the baby [26–28]. In fact, there are now a number of companies that have managed these collections with enhanced efficiency [27,28]. However, the numbers of HSC/HPC and other cells collected by the best and most efficient means are still sub-par with regards to optimal collections. Efforts to procure more cells have included perfusing the placenta [29]. While one can collect more cells this way, this procedure is too cumbersome for routine use, and would have to be done at very select birthing centers. We have recently focused on a better appreciation of HSC biology to uncover a means to enhance collection of HSC in single CB collections [30,31]. We initially evaluated collection of HSC from mouse BM before proceeding to collection of CB. Our efforts were based on the increasing knowledge that HSC *in vivo* reside in microenvironmental niches that are grossly hypoxic (1–5% O_2 tension) compared to that of the hyperoxic atmospheric conditions (~20% O_2 tension). Thus, the BM environment needed to nurture, maintain, and expand HSC is extremely hypoxic compared to air. It has been known since the late 1970's,

based on the work of others, and ourselves, that HPC proliferate better *in vitro* under hypoxia, than in ambient air (normoxia) [32–38]. In fact, we showed that cells expand better in long-term *in vitro* culture when they are grown under conditions of hypoxia (e.g. 5% O_2) compared to normoxia [35]. The HPC are also more sensitive to stimulation by growth modulating cytokines in hypoxia. However, virtually all studies of HSC/HPC numbers and function have been performed after their collection and processing in ambient air (normoxia), regardless of their subsequent culture in hypoxia or normoxia. Moreover, the cells have always been injected in recipients (mice or man) under normoxic conditions. Thus, it was possible that the routine collection of BM or CB cells in ambient air, as everyone including ourselves have done, was influencing the HSC/HPC content of the collected cells. We hypothesized that upon collection of BM and CB cells, the immediate exposure of the cells to the high O_2 content of ambient air would grossly alter numbers and function of HSC and HPC, by a phenomenon we termed: "Extra Physiologic Oxygen Shock/ Stress" (EPHOSS) [30,31]. This in fact, turned out to be true. For mouse BM collections, we used two methods. First, the cells were collected and processed from one femur under constant hypoxia (3% O_2), and the contralateral femur was collected and processed in ambient air. Alternatively, two femurs were collected under hypoxia, the cells pooled, and then split such that half remained for processing under hypoxia, and the other half processed in air. For CB the cells were first collected at the birth in air tight arterial, hypoxicequilibrated blood-gas syringes to minimize exposure to air, prior to rapidly placing the collected cells in the hypoxic chamber for pooling and splitting. Half were left in hypoxia, and the other half then removed for replacement in ambient air. It is important to emphasize that the cells collected and processed in hypoxia never left the hypoxic environment, and everything in the hypoxic chamber including glassware, plastic ware, syringes, pipettes, culture and other media had to be equilibrated at 3% O_2 in the hypoxic chamber for at least 18 hours prior to cell collections. The collected cells were assessed for HSC and HPC by phenotype (using rigorous definition for Long-Term (LT)-HSC, Short-Term (ST)-HSC, Multi-Potential Progenitors (MPP), Common Myeloid Progenitors (CMP), Granulocyte Macrophage Progenitors (GMP), etc.) and by function for granulocyte macrophage (CFU-GM), erythroid (BFU-E), and multipotential (CFU-GEMM) progenitors by colony assay *in vitro*, and for *in vivo* infusion in lethally irradiated mice for mouse BM donor cell engraftment, and in sublethally-irradiated immune deficient (non-obese diabetic, severe combined immunodeficient, IL-2 Receptor null: NSG) mice for human $CD34^+$ CB cell engraftment. Even the injections of the hypoxia collected and processed cells were injected i.v. in the hypoxic chamber, where the mice were rapidly placed into a holder where they could breathe 20% O_2 while their tails where the cells were injected were in the hypoxic atmosphere. Collection of cells in hypoxia resulted in 3–5 fold increases in HSC numbers by rigorous phenotyping of mouse BM or human CB cells, and in 3–5 fold increases in numbers of functional HSC, as assayed by limiting dilution analysis of donor cells in lethally-irradiated congenic mouse recipients. This hypoxic collection and processing was concomitantly associated with decreased numbers of phenotypically- and functionally-defined HPC (MPP, CMP, GMP, CFU-GM, BFU-E, and CFU-GEMM). Moreover, the CFU-GM, BFU-E, and CFU-GEMM that were collected and processed in hypoxia were in a slow- or non-cycling-state, compared to the rapid cycling status (~50% in S-phase) of these HPC that were collected and processed in air. Thus, there are many more

HSC and fewer HPC when cells are collected in the more physiologic hypoxic atmosphere than those cells collected in ambient air, and this process of decreased HSC and increased cycling HPC occurs within 15 minutes, or possibly sooner when the collected cells hit normoxic atmospheric conditions. Hence, all investigators have previously greatly underestimated numbers of HSC, and overestimated numbers of HPC when cells are collected and processed in normoxia [30,31]. This difference in numbers of HSC collected could make a major difference in context of CB HCT, where numbers of HSC from single CB collections have thus far been limited.

However, while it is logistically possible to do CB collections and processing in hypoxia, this would be a complicated procedure, and would have to be done in very selected CB collection centers that maintain good manufacturing procedures. Thus, it was necessary to understand the mechanisms involved in the phenomenon of EPHOSS, to see if there was a simpler means of preserving the cells, as they would be in their residing *in vivo* hypoxic environment. We found that collection of HSC in ambient air was associated with increased generation of mitochondrial reactive oxygen species (ROS), increased mitochondrial mass, and higher membrane potential [30,31]. We linked EPHOSS directly to a mitochondrial permeability transition pore (MPTP), -cyclophilin D-P53 axis, in which increased O_2 present in ambient air opened the MPTP causing increased release of mitochondrial ROS. ROS is known to be an inducer of cell differentiation. We also linked EPHOSS to an hypoxia-inducing factor-1alpha, and hypoxamir micro RNA (miR)210 activity [30,31]. We were able to use the GVHD antagonizing drug cyclosporine A (CsA), to keep the MPTP closed under normoxic conditions, which ablated the EPHOSS effects. Thus, the immediate collection and processing of mouse BM or human CB cells in ambient air but in the presence of CsA mimicked the effects of hypoxia with collection of increased numbers of phenotypically- and functionally-defined HSC, and with an associated concomitant decrease in HPC. It now remains to be determined if CsA can be used in a CB banking scenario to enhance numbers of collected HSC. More work is necessary in this area as CsA is not an easy compound to work with. It is difficult to get into solution and the exact concentration to use for best advantage may need to be worked out for different collection scenarios.

3B.) Enhancing the Homing Capabilities of HSC for More Efficient Engraftment

There have been a number of attempts to enhance the capacity of HSC to home to the BM after i.v. injection/infusion for enhanced engrafting capability. Some current efforts include: inhibition of the enzymatic activities of Dipeptidylpeptidase (DPP)4 [39–43], use of prostaglandin E (PGE) [44–46], short term treatment of cells with hyperthermia [47], and enforced fucosylation of the cells [48,49].

3Bi.) Inhibition of DPP4—DPP4 has the capacity to truncate certain proteins at a penultimate N-terminal amino acid that is a proline or alanine, and to a lesser extent when that amino acid is a serine and perhaps a threonine [50,51]. There are an increasing number of cytokines and chemokines with putative and documented real DPP4 truncation sites [41,50,51]. One regulatory protein that we originally focused on was the CXC chemokine, stromal cell derived factor-1 (SDF-1; also known as CXCL12) which binds and signals through the CXCR4 receptor [52]. SDF-1/CXCL12 is a potent chemotactic (directed cell

movement) and homing molecule for HSC and HPC [6,7,52]. DPP4 truncation of SDF-1/CXCL12 results in a molecule that lacks chemotactic activity, but the truncated SDF-1/CXCL12 can block the chemotactic activity of full-length SDF-1/CXCL12 [39]. Inhibition of DPP4 or deletion of the *dpp4* gene in mice results in enhanced chemotaxis of HPC, and probably HSC, and enhanced homing and engrafting capability of mouse BM HSC into lethally-irradiated mice [40,41] and of human $CD34^+$ CB cells (enriched for HSC and HPC) into sublethally-irradiated immune-deficient mice [53]. Inhibition of DPP4 also accelerates hematopoietic recovery in mice after radiation or chemotherapeutic drugs. Based in part on this information, a clinical trial was set into motion in which human recipients with end-stage leukemia and lymphoma were conditioned as usual, but also given oral administration of sitagliptin [42,43], an FDA approved DPP4 inhibitor that had previously been used by others to treat patients with Type 2 diabetes. The conditioned patients were then infused i.v. with a single CB unit. The results were encouraging in that the time to neutrophil engraftment with sitagliptin was 21 days, compared to the greater than 24 days associated with single unit CB transplantation [42]. However, the sitagliptin was only given to recipients once a day for 4 days, and the DPP4 enzymatic levels, while immediately being decreased after the first of each sitagliptin administration, quickly rebounded, and sometime over-bounded within 4–6 hours [42,43]. Thus, the patients did not respond as well as normal donors to the sitagliptin, since normal donors that received sitagliptin demonstrated reduced DPP4 levels for at least 24 hours after administration of a single dose of oral sitagliptin. A follow-up trial using sitagliptin twice a day for 4 days in the same context of single CB transplantation has resulted in a much reduced time to neutrophil engraftment of 16–17 days (Farag and Broxmeyer, unpublished data). This simple procedure of adding a relatively inexpensive orally-active reagent such as sitagliptin to the transplant procedure bodes well for a simple and inexpensive means to enhance single CB transplantation. More efforts are needed to be sure about the efficacy of this treatment, and if it also allows enhanced engraftment of platelets and immune cells, as these types of cells are also delayed in context of CB transplantation. It may be that DPP4 inhibition in context of CB transplantation is not only enhancing the activity of a chemotactic/homing molecule such as SDF-1/CXCL12 by blocking its truncation [39] in the setting of conditioning and transplantation of the patient, but inhibition of DPP4 also enhances the activity of other hematopoietically active molecules, such as granulocyte (G), macrophage (M) colony stimulating factor (CSF), G-CSF, interleukin (IL)-3, erythropoietin (EPO), and probably thrombopoietin (TPO), as well as many other cytokines/chemokines with DPP4 truncation sites [41,50,51]. Moreover, SDF-1/CXCL12 as well as other cytokines also have cell survival activities [54,55], so inhibiting DPP4 activity *in vivo* is also likely enhancing the survival and other functional activities of cytokines and chemokines [41], allowing for enhanced HSC/HPC self-renewal and proliferation that also figure into the engrafting process.

3Bii) PGE—PGE has long been known to have functional activities on HSC/HPC populations, both as a negative and positive regulator of these cells [56–58]. PGE has now been shown to enhance the homing and engraftment of HSC in a mouse BM recipient context [44,45]. Use of PGE has been instituted in context of a double CB HCT situation with encouraging results [46]. However, it is not yet clear whether or not PGE will be advantageous in context of a single CB HCT. This is important, because it is necessary to

enhance single CB HCT, rather than that of double CB HCT, for economic reasons [59]. Of interest, is that in a mouse BM HCT context, the combined use of a DPP4 inhibitor and of PGE resulted in better engraftment than that of either a DPP4 inhibitor or PGE, each alone [60]. Thus, there are still ways for even greater enhancement in reducing the time to blood cell recovery.

3Biii) Treatment of Donor Cells with Short-Term Hyperthermia—CXCR4 is a potent receptor for SDF-1/CXCL12 activities on hematopoiesis [52]. For CXCR4 to be most potent, it is necessary for it to be incorporated into cell membrane lipid rafts. In this context, hyperthermia pre-treatment of cells was assessed to see if it enhanced the potency of CXCR4 for response to the chemotactic activity of SDF-1/CXCL12 [47]. Heating $CD34^+$ human CB cells, and the CXCR4-expressing factor-dependent cell line MO7e for 4 hours at 39.5°C greatly enhanced the chemotactic response of these cells to SDF-1/CXCL12, and also the sensitivity of the cells to SDF-1/CXCL12. The hyperthermia-pre-treated cells responded to much lower concentrations of SDF-1/CXCL12 than did the cells treated at 37°C. The enhanced chemotactic response to hyperthermia-treated $CD34^+$ cells was equally apparent for the immature $CD34^+$ $CD38^-$ containing HSCs and the more mature $CD34^+$ $CD38^+$ (containing HPC) subsets of $CD34^+$ cells. Of relevance for HCT, pretreating human $CD34^+$ CB cells for 4 hours at 39.5°C prior to transplantation into sublethally irradiated NSG immune-deficient mice significantly enhanced the engraftment of the human CB cells by at least 2 fold as assessed by human $CD45^+$ CB chimerism in the NSG mice [47]. Hence, this is another inexpensive, and easily performed method to *ex-vivo* prime CB HSC/HPC for better engraftment. Heat treatment may be useful alone or as an adjuvant with other therapies to enhance the efficacy of CB HCT. This has yet to be tried in context of clinical CB HCT.

3Biv.) Fucosylation—The ability of cells to home to the BM involves many steps, including the capture, rolling and arrest of the cells on the BM endothelium. This is mediated in part by endothelial E- and P- selectin interactions with the HSC and HPC. For this interaction to occur involves specific fucosylations on the cells interacting with and traversing the endothelium. Since there are apparently reduced levels of fucosylation of E- and P- selectin ligands on CB cells, it was postulated that increasing the levels of fucosylation on the surface of cell populations containing HSC and HPC would improve the engraftment capabilities of these donor cells. This was first addressed in human-mouse chimera studies [48], and then in a human clinical trial [49], both which demonstrated positive results. In the clinical study, the median time to neutrophil engraftment was 17 days compared to historical control values of 26 days, platelet recovery was reduced to 35 compared to controls of 45 days [49]. This clinical study was done in context of a double CB HCT, and will have to be verified in a single CB unit HCT setting before its full worth is determined. The time to neutrophil engraftment seen in this double CB HCT scenario was similar to that seen above in a single CB HCT setting using sitagliptin twice a day for 4 days. Whether or not combining fucosylation with DPP4 inhibition will further accelerate the time to donor engraftment in the setting of single unit CB HCT remains to be seen.

3Bv.) Future Efforts to Enhance Homing of CB for More Efficacious HCT— There are now encouraging reports that the homing and then subsequent engraftment of CB cells can be enhanced. Above, we noted DPP4 inhibition [39–43], PGE treatment [44–46], hyperthermia [47], and fucosylation [48,49] in this context. There are likely other simple methods to accomplish this end-point, but combined treatments, such as already demonstrated with DPP4 inhibition plus PGE [60], may be advantageous.

3C. Ex-vivo Expansion of HSC and HPC to Increase the Numbers of these Cells, and Enhance CB HCT

One limiting factor, believed in part to be due to the slower time to engraftment with CB compared to BM or mPB, is the limited numbers of cells, including HSC and HPC, that can be collected at the birth of a baby from a single collection. The numbers of HSC/HPC decline rapidly within hours after the birth of a baby, and what one collects is all one can get in this situation [11]. To make up for this relative paucity of cells in a single CB collection, investigators went to use of double CB HCT [1,2]. This did help move the field of CB HCT forward, but at this time there is no definitive proof that double CB HCT is any more effective than single CB HCT [1,2], although most centers still use two CB units for HCT. Double CB HCT certainly served its purpose for increased numbers of CB HCT, but there is no apparent difference in times to engraftment with double compared to single CB HCT, both of which still lag behind the accelerated times to engraftment noted with BM- or mPB-HCT. The expense for two vs. one CB unit is usually twice the cost, and one way to decrease the cost, is to find a relatively inexpensive means to enhance single CB HCT [59]. As a means to find a way to increase the numbers of CB HSC/HPC for CB HCT, different groups have experimented with means to expand the numbers of these cells outside the body (*ex-vivo*). Towards this ultimate aim to increase numbers of HSC and HPC, the cytokines stem cell factor (SCF), Flt3-ligand (FL), and TPO have been used to good advantage in an experimental situation. There have been a number of other means to ex-vivo expand HSC, and some of these have been assessed in a clinical setting, but it is of interest that many of the *ex-vivo* expansion procedures, regardless of their efforts have in some way had to incorporate some cytokine combination such as SCF, FL and TPO in the experimental design. The additional maneuver enhances *ex-vivo* expansion of HSC beyond that of the cytokine combination. However, without the cytokine combination these procedures have little or no effect by themselves.

3Ci.) Clinical Assessment of *Ex-Vivo* Expanded CB Cells—The first successful attempt at clinical use of ex-vivo expanded CB cells involved Notch-mediated culture of $CD34^+$ CB cells [61]. The isolated $CD34^+$ cells were cultured for 16 days in culture vessels pre-coated with $Delta^{ext\text{-}IgG}$ with fibronectin fragment CH-296 overnight, and then in serum-free medium with IL-3, IL-6, TPO, FL, and SCF. The infusion of these cultured cells resulted in an apparent shortening of the time to neutrophil engraftment. CB cells were also cultured by others in the presence of nicotinamide and a non-cultured T-cell fraction which resulted in encouraging results [62]. More recently, a third group took advantage of background studies that demonstrated that a small molecule, SR1 [63], in combination with a cytokine cocktail could expand HSC numbers, in order to bring this procedure to the clinic [64]. This effort also resulted in quite impressive results. However, all these clinical studies

were done in context of a double CB HCT in which the recipients were provided with donor cells from a manipulated and also unmanipulated CB collection. Thus, although the results noted above are impressive, it remains to be seen if in fact the *ex-vivo* cultured cells can by themselves, without the added presence of a second unmanipulated CB unit actually engraft, and if so, can it accelerate the time to neutrophil, platelet and immune cell recovery compared to that noted by either single or even double unmanipulated CB HCT.

3Cii.) Additional Experimental Laboratory Assessment of *Ex-Vivo* Expanded CB Cells—There are a number of small molecule experimental procedures that have been evaluated in a laboratory setting, all of which enhance the *ex-vivo* expansion of CB $CD34^+$ cells and the HSC and HPC population within this phenotyped population of $CD34^+$ cells beyond that of a cocktail of cytokines. This includes SR1 [63,64], an aryl hydrocarbon receptor antagonist, and also pyrimidoindole derivatives [65], which acted as agonists of human HSC self-renewal events.

It was known that enhancing the expression of the homeobox protein HoxB4 resulted in *ex-vivo* expansion of HSCs [66,67], and most recently we reported that activation of the pluripotent transcription factor Oct 4, using an Oct4-activating compound (OAC1) in the presence of SCF, FL and TPO greatly expanded both phenotypically- and functionally-defined human HSC and HPC from a starting population of human CB $CD34^+$ cells [68]. Interestingly, the OAC1-expansion was mediated by enhanced expression of HOXB4, as siRNA that decreased expression of either Oct4 or HoxB4 prevented the OAC1-induced enhancement in *ex-vivo* expansion of HSC and HPC [68]. Since enhanced sustained expression of HoxB4 has been associated with leukemogenesis, we checked if we could detect signs of leukemia with our *ex-vivo* expanded cells. The expanded cells neither caused the formation of teratomas when subcutaneously injected into nude mice, whereas the injection of a human embryonic stem cell line as a control did cause teratomas that contained tissue from all three germ layers [68]. We did not detect any evidence of leukemia in the NSG mice injected with the *ex-vivo* expanded cells for the 4 months of the engraftment period in primary mice or in the secondary recipients after another 4 months [68]. It seems likely that the transient induced expression of Oct4 and HoxB4 by short term expansion of the $CD34^+$ CB cells to OAC-1 may be safe, and that only prolonged activation of HoxB4 results in leukemogenesis. However, caution is needed in evaluating this, as well as any other *ex-vivo* expanded cell population when considering their use in a clinical setting [68].

Epigenetic modification of cells is also being considered in the context of *ex-vivo* expansion [69]. While still quite experimental, and with the understanding that caution is necessary if one were to proceed to clinical analysis, the use of histone deacetylase inhibitors (HDACIs), primarily valproic acid (VPA) in context of cytokines resulted in very large increases in number of engraftable cells in NSG mice [70]. The investigators determined that the frequency of SCID-repopulating cells (SRC; a measure of human HSCs used in many of the above noted reports [53,63–65,68]) after *ex-vivo* culture of CB cells with cytokines and VPA was 1 SRC in every 31 cells while culture with only cytokines produced 1 SRC in 9,225 cells, as compared to the unexpanded input CB cells of 1 SRC in 1,115 cells. This translated

to a phenomenal expansion of 32,258 SRCs compared to the starting unexpanded number of 897 SRCs [70].

3Ciii.) Thoughts on *Ex-Vivo* Expansion—The above and other efforts to *ex-vivo* expansion of CB cells for use in CB HCT [71–74] appear encouraging, and we and others continue to work in this area. Greater insight into the biology of HSC and HPC will no doubt enhance our capacity to evolve additional ways to *ex-vivo* expand CB and other sources of HSC. However, until the clinical studies are done in context of a single transplantable CB unit, it is not clear how this can change how CB HCT is done. Economically, two CB units cost more than one CB unit, usually double the cost of a single CB unit, although efforts are underway by some public CB banks to reduce the cost of procuring two units. Add the cost of two units to the probably significant cost of the *ex-vivo* culture procedure, which would most likely have to be performed in very selective processing centers with good manufacturing procedure facilities, and the costs continue to add up [59]. The question becomes how impressive the actual clinical results will be and if these are better than other less expensive means for enhancing the efficacy of CB HCT.

D.) *In Vivo* Enhancement of the Engrafted Cells

One potential means to enhance the efficacy of CB HCT is to find a way to accelerate the self-renewal and differentiation capacity of the CB cells that have already homed to and lodged in the BM of the recipients. This would be done by infusing growth factors, which has been done already in context of adding G-CSF (e.g. neupogen). Presently, it is not clear that G-CSF has actually reduced the time to neutrophil engraftment [1]. I believe that TPO may have also been tried in the past to enhance engraftment of platelets, but likely without much success as there are no published reports on this.

Another possibility is to use a DPP4 inhibitor such as sitagliptin for more prolonged periods after the engraftment process has started. Since DPP4 itself is increased in amount and enzymatic activity during stresses such as radiation and chemotherapy [41], likely due to cell death which releases endogenous DPP4, and it is now clear that DPP4 can truncate and change the functional activities of a number of hematopoietically active cytokines such as GM-CSF, IL-3, G-CSF, EPO, and TPO by downregulating their positively acting functions [41,50,51], it may be that DPP4 inhibitors that will prevent this cytokine truncation can accelerate recovery after radiation or drugs such as 5 Flurouracil or cyclophosphamide. Hematopoietic recovery is greatly accelerated in CD26/DPP4 knockout mice, or in mice receiving a DPP4 inhibitor such as oral sitagliptin or i.v. administered Diprotin A (a tripeptide: ILE-PRO-ILE) [41]. When our clinical trials were first designed [42,43], it was with the knowledge that SDF-1/CXCL12 the chemotactic and homing chemokine [52] could be truncated [39]. Thus, we were only considering the effects on the homing process, hence the short-term treatment of patients with sitagliptin. Now that we understand the wide range of hematopoietically-active cytokines and growth factors with DPP4 truncation sites [41,50,51], it might be worthwhile to see if adding orally-active sitagliptin to patients for more than 4 days can enhance time to recovery faster than we have now seen with administering it 2x/day for 4 days, starting one day before the infusion of the CB unit (Farag, Broxmeyer, unpublished data). The safe design of such a further trial needs to be

considered. Another interesting way to enhance engraftment that needs further verification, is the use of hyperbaric oxygen for the recipient [75].

E.) Concluding Remarks

The field of CB HCT has come a long way [1,2] since the initial background laboratory studies that led to the field [10] and the first CB transplants, first in an HLA-matched sibling [9,15–21], and then in an allogeneic HLA-matched and partially HLA-matched situation [1,2]. Many laboratory scientists and clinical investigators continue to work to better understand the biology of HSC and HPC and the mechanisms involved in the regulation of HSC and HPC, and means to enhance CB HCT so that there is more rapid engraftment of neutrophils, platelets, and immune cells, without loss of the lesser amounts of GVHD found in CB HCT in comparison to BM or mPB HCT, and with maintenance of the anti-leukemia, anti-cancer effects of the CB. The newer findings will also have to take into account the economics of the transplant [59]. Without sacrificing the safety and efficiency of the procedure, cost should be taken into account. When all is said and done, simpler is usually better, so if one can find simple less-complicated and- expensive means that are safe and effective in making CB HCT better, than this needs to be seriously considered by the clinical-investigators and- transplanters.

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