Modalities to Improve Cord Blood Engraftment
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Abstract
Umbilical cord blood (UCB) is one of the major sources of Hematopoietic Stem Cell Transplantation (HSCT) with increasing use in clinical practice. UCB can be a life savior for patients who do not have a matched unrelated adult donor or for patients who are in need of an urgent transplantation. Various factors make UCB a significant source of Hematopoietic Stem Cells (HSCs), including ease of procurement and lack of donor attrition, with the ability to process long term storage of donor cells. Importantly, UCB donations can be used right away without the need for a "perfect" HLA match, thereby increasing donor access to HSCT, particularly for minority and mixed ethnicity patients, for whom a suitable matched related or unrelated donor may be difficult to locate. The major limitation of UCB is the quantity of cells to be infused. Although high proliferative potential allows one log less use of HSCs (HSC<10^5/kg) compared to bone marrow (BM) or peripheral blood mononuclear cells (PBSC), even this amount cannot be reached in the majority of patients under donor search. When total nucleated cell (TNC) and CD34+ cell doses in UCB grafts are analyzed, a high correlation is observed with the rate of neutrophil and platelet engraftment, the incidence of graft failure and early transplant-related complications. It has been shown that UCB grafts with more than 3/6 HLA mismatches and with cell doses under the defined minimum threshold lead to higher transplantation related mortality (TRM). Especially when adult cord blood transplantation (UCBT) is the subject, providing units with enough cells remains the major drawback.

Despite certain efforts, there is still an unmet need for increasing HSC cell dose and/or stimulating engraftment without loss of their long term repopulating (LTR) potential and reducing graft versus host disease (GVHD). Intrinsic and extrinsic cellular factors have been proven to act roles in HSC expansion thus justifying their role in in vitro and ex vivo culture conditions. Attempts to regulate these factors through ex vivo expansion methods aim to overcome insufficient cell numbers while methods promoting HSC homing is in favor of the latter. Combination of both appear to work synergistically. Induction or adoptive transfer of UCB derived immune cells particularly Natural Killer (NK) cells and regulatory T cells (T reg) with or without cytokines are also effective approaches for gaining better engraftment levels after UCBT. All of these approaches have been denoted as "successful" in pre-clinical in vitro and animal studies. Many of them have also been tested in early/later phase clinical trials resulting in encouraging results. We aim to review the current knowledge on UCB expansion and engraftment enhancer methods, a very rapidly improving field particularly in the last decade.

Keywords: Cord blood; Hematopoietic stem cells; CD34; Ex vivo expansion; Engraftment enhancer

Ex vivo Expansion
Cytokines/Growth Factors (GFs)

Classical hematopoietic cytokines: The very first attempts of expanding UCB ex vivo were through core hematopoietic cytokines [IL-6, IL-11, IL-3, Flt-3 ligand, stem cell factor (SCF), thrombopoietin (TPO), GM-CSF] that would support survival and expansion of HSC. The challenge of scarcity of stem cells in UCB units was initially thought to be manageable by addition of extrinsic cellular factors such as cytokines. However addition of cytokine cocktails as well as GFs led to a moderate expansion which did not reach the expected cell doses in general. As a first attempt cytokine (SCF, TPO, GM-CSF) rich medium was used by Shpall et al. [1] who were able to achieve only 2-5 fold increase in cell counts. Despite the fact that the safety of the cytokine/ GF cocktails have been proven, either modest or no change in platelet and neutrophil engraftment was achieved.

TPO receptor agonists were also used to regulate HSC fates. TPO is a regulator of thrombopoiesis, supporting megakaryocytogenesis as well as early hematopoiesis of HSC. TPO has also been identified to participate in the production of platelets. C-MPL is the receptor (Rc) of TPO. Non-peptide TPO receptor agonists such as Eltrombopag, an oral thrombopoietic Rc agonist which selectively induces c-MPL in humans and chimpanzees, was investigated for its effect on human UCB HSC and hematopoietic progenitor cell (HPC) expansion [2]. Eltrombopag enhanced expansion of HSC/HPC of human UCB in vivo and in vitro, and promoted multi-lineage hematopoiesis through the expansion of BM HSC/HPC of human UCB in vivo. A novel c-MPL agonist, a small molecule, NR-101 has been found to act as an HSC expansion inducer [3].

Safety and efficiency of Aastrom Replicell system in a clinical setting was initially demonstrated by a phase I clinical trial at Duke University by Jaroscak and colleagues [4] which was followed by many others afterwards. Their results did not reach the expected expansion levels. Cytokines alone were insufficient to improve self-renewal and although triggering HSC differentiation. Progressive loss of in vivo repopulating cells after exposure has remained as the major challenge. As a result, cytokine mediated in vitro expanded HSC characteristics were not predictive of their clinical behavior.

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Novel cytokines/Growth factors: Addition of novel cytokines/GFs to culture systems such as wingless-type (Wnt) proteins, Notch and recently identified novel factors e.g. Angiopoietin (Angptl) like 5, IGFBP2 and pleitrophin among others, result in superior effects on ex vivo expansion of UCB HSC [3]. Among many molecules tested, members of the (Angptl) family of GFs were shown to expand HSC. In particular, Angptl5 has been shown to induce expansion of human NOD/SCID-repopulating cells ex vivo. In a recent study by Blaink [5] human CD34+ cells from UCB were cultured in vitro in the presence or absence of Angptls. The reconstitution capacity of expanded cells was subsequently measured in vivo by transplantation into NOD/SCID or NSG mice and compared with that of uncultured cells. Angptl-4 and Angptl-5 was shown to lead to increased engraftment capacity of SRC but with inter-experimental variation. As a result authors recommend these factors to be used with the aim to maintain SRC activity under ex vivo conditions e.g. for gene transfection. Insulin like GF (IGF) 2 and IGF binding protein (IGFBP) 2 are two other molecules with an expansion capacity. Culturing of CD 133+ UCB cells with Angptl-5 and/or IGFBP 2 improved immune reconstitution in NOD/SCID mice after transplantation [6,7].

Pleitrophin, an environmental factor, has recently been shown to trigger ex vivo human UCB HPC expansion (approximately 40-fold expansion of CD34+38− cells after 7 days in culture compared with input cell number). In addition to the in vitro expansion capacity, enhanced in vivo reconstitution of immunodeficient mice with transplanted cells was reported in the same study. The mechanism was elucidated as an interplay between Pleitrophin, phosphoinositide 3-kinase (PI3K) and Notch signaling pathways, regulating HSC renewal and expansion [3,8].

Heparin is a member of glycosaminoglycans (GAGs) family. GAGs and proteoglycans (PGs) are components of extracellular matrix, acting directly/indirectly on proliferation and differentiation of hematopoietic cells. Maurer and colleagues [9] have incubated UCB derived CD34 cells either alone with TPO, SCF, Flt3-Ligand, IL-6, and IL-11 cocktail (5-cytokine) supplemented with autologous serum or in presence of heparin. After 14 days of incubation, heparin induced significantly higher numbers of colony forming units (CFUs) and CD 41+ cells, compared to 5-cytokine controls in expanded cells. As a result, in the presence of the 5-cytokine combination, heparin significantly induced the expansion of CFU-MKs and CD41+ cells accelerating platelet recovery.

Notch signaling pathway

Previous efforts to sustain the necessary cell dose for rapid engraftment using extrinsic cytokine mediated expansion methodologies to generate increased numbers of cells have not shown significant clinical effects. Particularly, the Notch signaling pathway in regulating ex vivo expansion of HPCs has gained attention with the goal of generating increased numbers of progenitor cells capable of rapid repopulation in vivo to improve the kinetics of hematopoietic recovery after UCBT [3,10]. There are four Notch receptors identified as cell surface molecules and specific Notch receptor-ligand binding activates a series of events, inducing differentiation of gene expression. Recent work have pointed out the impact of Notch signaling pathway in hematopoiesis [3,10]. For example, activation of Notch1 by Delta ligands 1 and 4 is required for inducing T-cell and inhibiting B-cell differentiation, whereas Notch2 activation by Jagged1 and possibly Delta1 is sufficient to induce effects on HPC, enhancing generation of HPC during marrow reconstitution [10,11]. The Notch biological functions have led researchers use immobilized exogenous ligands to trigger Notch signaling and self-renewal/maintenance of HSC therein.

Delaney et al. [12] incubated CD34+ UCB HPC with Delta1 ligand combined with fibronectin fragments and cytokines (SCF, TPO, Flt-3 ligand, IL-3, IL-6). This approach has led to a 220-fold increase in the number of CD34+ cells after 17 days incubation, compared with input cell number. Furthermore, nearly 16-fold increase was demonstrated in NOD/SCID mouse repopulating cell frequency.

Based on these experimental results a phase 1 clinical trial was introduced: patients undergoing a myeloablative UCBT received one unmanipulated UCB unit along with a second UCB unit that has undergone Notch-mediated ex vivo expansion. Initial results were encouraging and in favor of expansion: following an average of 6×10^6 CD34+ cells/kg (an average 164-fold expansion of isolated CD34+ cells) and co-infusion of an average 2.4×10^6 CD34+ cells/kg unit, a significant shortening of neutrophil engraftment was observed. Engraftment occurred 50% of the time from the manipulated UCB unit. However in 80% of the patients expanded unit disappeared and the unmanipulated unit dominated the hematopoiesis. Results of phase 2/3 trials are needed before this approach can be widely applicable [13]. Phase II trial from Fred Hutchinson Cancer center is recruiting participants at the moment, comparing double UCBT (dUCBT) with or without Notch mediated ex vivo expanded UCB cells (Clinical Trial Identifier: NCT01690520).

The mechanism of action of Notch mediated expansion is being investigated by different groups. A novel way of Notch ligand Delta 1 mediated expansion via IL-6 induction was recently described by Csaszar E and colleagues [14]. They describe the way Delta 1 alters cytokine receptor distributions on hematopoietic cells, altering feedback networks and their impact on stem cell fate. Latest findings support the fact that Notch signals maintain human HSCs in vitro such that they preserve their hematopoietic-reconstituting ability in vivo delaying the appearance of two newly described early progenitor cells. Interestingly, Notch signaling was shown not to be required for self-renewal of human HSC in vivo by Benveniste et al. [15]. Clinically relevant effects in terms of reduction in TRM, graft failure rates and increase in overall survival (OS) rates are encouraging. The data received so far suggest a significant role of Notch signaling in HSC expansion to be used for UCBT [14,15].

Chemical compounds (Small molecules)

Copper chelators: Copper is an essential element for basic cell functions, such as survival, proliferation, and differentiation. Reduction of cellular copper in ex vivo culture conditions have been shown to enable preferential proliferation of early progenitors leading to increased engraftment [16-18]. Peled and collaborates [16] demonstrated 17 fold and 159 fold increase in HSC proliferation when cultured with tetahydrochloropamine (TEPA) and cytokine cocktail for 3 and 7 weeks, respectively. The results of a phase I study conducted by Petropoulos D [17], carlecortemcel-l, a product derived from ex vivo expansion of UCB cells (Clinical Trial Identifier: NCT01690520).

Another group of investigators pursued the same question using a different copper chelator TEPA StemEx which was used in a phase I/II trial. The study was performed to test the feasibility and safety of transplantation of CD133+ UCB hematopoietic progenitors cultured
Furthermore a long term engraftment could be demonstrated with donors. In the presence of cytokine cocktails, incubation of CD34+ hydrocarbon receptor (AhR) antagonist (StemRegenin1 or SR1) capable are expressed on HSC and Boitano et al. [23] identified a novel aryl receptor antagonists. Treatment of CD34+ cells with Eupalinilide E did not change the total number of nucleated cells after 7 days of culture, but induced a 50% increase in the percentage of CD34+ cells and a 2-fold increase in the number hematopoietic progenitors ex vivo. After 45 days of UCB culture, the total number of cells was 45-fold greater in Eupalinilide E-treated cultures than vehicle-treated cultures. Importantly, this effect was also clear in cultures of UCB CD34+ cells, where Eupalinilide treatment (600 nM) led to a 13-fold increase in CFU-GEMM and 15-fold increase in CFU-GM after 14 days, relative to control. Pre-clinical data should be supported with clinical work and the clinical potential of SR1 and Eupalinilide E are yet to be discovered.

**Histone deacetylase (HDAC) inhibitors and demethylating agents:** Epigenetic changes are regarded as emerging major players for HSC biology. Of the epigenetic modifiers investigated, histone deacetylase (HDAC) inhibitors, such as valproic acid (VPA), suberoylanilidehydroxamic acid (SAHA) and Trichostatin A (TSA) were shown to induce differentiation and apoptosis in a variety of leukemic cells in vitro. They seem to produce a favorable effect on the expansion of normal HSC however their exact impact is still not clear [25]. In the study by Elizalde et al. [26], the VPA target HDAC3 was identified as a negative regulator of UCB HSC expansion. They demonstrated that knock down of the transcript dramatically improves CD34+ cell expansion, which correlates with a higher potential to generate CFUs in functional assays. HDAC3 inhibition does not block commitment to the monocytic lineage and the maturation of monocyte precursors, which are the main inhibited pathways in the presence of VPA. Therefore, their results identify HDAC3 as a promising target for therapies aiming to expand HSCs. Methyltransferase inhibitors are another group of epigenetic chromatin modifiers used in HSC expansion. Hypomethylating drug 5-aza-2'-deoxycytidine (5azaD) is an example which has been shown to effect stem cell expansion kinetics [3,25].

**Nicotinamide (NAM):** NAM, a form of vitamin B-3, has been shown to delay differentiation and increase engraftment efficacy of UCB-derived human CD34+ cells cultured with cytokines. In the presence of NAM, the fraction of CD34+CD38- cells increased and the fraction of differentiated cells (CD14+, CD11b+, and CD11c+) decreased. CD34+ cells cultured with NA displayed increased migration toward stromal cell derived factor-1 (SDF-1) and homed to the BM with higher efficacy [27]. NAM is a known potent inhibitor of several classes of ribosylase enzymes that require NAD for their activity, as well as SirTuin (SIRT1). The NAM effect is SIRT1-specific. The effects of NAM mediated expansion are currently tested in vivo too. With ongoing/completed clinical trials sponsored by Gamida Cell, the clinical potential of NiCord (CB expanded using NAM) in patients with hematological conditions are being extensively investigated.

A pilot study which evaluated the safety and efficacy of NiCord is completed with favorable results. Among 11 patients with hematological cancers receiving NiCord along with a second unmanipulated unit of CB engraftment occurred in a median of just over ten days. A very significant advantage of NiCord over the other expansion techniques was that a single manipulated CB unit has supported the engraftment of 8 patients (Clinical Trial Identifier: NCT01527838). The results of the trial in which 1 of 2 UCB units has been incubated with 16. 16-dimethyl PGE2 (dmPGE2) before infusion demonstrated clear safety with durable multi-lineage engraftment. Encouraging trends in efficacy were also reported with faster neutrophil recovery (17.5 vs 21 days, P = .045) along with long-term engraftment of the dmPGE2-exposed UCB unit in 10 of 12 treated participants [22].

**Arblyhydrocarbon receptor (AhR) antagonist:** Ah receptors are expressed on HSC and Boitano et al. [23] identified a novel aryl hydrocarbon receptor (AhR) antagonist (StemRegenin1 or SR1) capable of enhancing CD34+ cell generation from the blood of mobilized donors. In the presence of cytokine cocktails, incubation of CD 34+ cells and SR1 resulted in a 669-fold increase in the number of CD34+ cells at 3 weeks and a 17.00-fold increase after 5 weeks of incubation. Furthermore a long term engraftment could be demonstrated with second transplants in animal studies.

De Lichtervelde et al. [24] have identified a new pharmacological agent, Eupalinilide E that inhibits erythropoiesis and promotes the expansion of hematopoietic progenitor cells. The activity of Eupalinilide E, a natural product, was found to have additive effects with AhR antagonists.
In order to improve cord blood engraftment, various modalities have been explored to enhance stem cell expansion and maintenance. These strategies include BM engineering and utilization of biomaterials to achieve higher levels of expansion through directing stem cell fates [33]. BM-mimicry strategy based on 3D fibrin scaffold revealed optimal requirements to be implemented into clinical settings for UCB expansion and transplantation.

In vitro expansion of HSC can be achieved by culturing such cells under conditions that mimic the bone marrow microenvironment, with its supporting cells of osteoblasts and stromal cells, allowing hematopoietic differentiation; and expression of these proteins has a role in HSC expansion. These factors are modulators of hematopoietic differentiation and have been found to be effective in expanding HSCs.

**Intracellular factors:** Many intracellular factors namely; transcription factors, cell cycle regulators, chromatin modifiers and microRNAs do act on HSC fate [3,29]. The interconnection between intracellular signaling pathways and cytokines/GFs have a cumulative effect on self-renewal and amplification of HSCs.

HOX factors are modulators of hematopoietic differentiation; and expression of these proteins has a role in HSC expansion. There are 4 members identified so far, namely HOX A4, B4, C4, and D4, encoding proteins with highly conserved homeodomains [30,31]. HOXB4 was one of the first genes identified to have a role in HSC re-programming. Over expression and/or translocation of HOXB4 and HOXC4 in particular, enable efficient expansion of HSC, latter inducing even higher levels of expansion [30,31]. Retroviral over expression of homeobox gene HOX 4 provoked extensive increase in HSC numbers. Amsellem et al. [32]. Sought to evaluate the expansion capacity of HOXB4 without retroviral transduction of the gene to bypass the likely safety issues related to genetic modification of HSC. Hence, passive delivery of a stromal cell layer expressing a HOXB4 fusion protein by human UCB CD34+ cells in culture conditions were used as the experimental approach. HOXB4 fusion protein which passively entered CD34+ cells resulted in a 2.5-fold increase in LTR cells of a NOD/SCID mouse and a 20-fold increase in long-term culture-initiating cells, compared with uncultured control. In the paper by Auvray and collaborators [30], co-culturing HOXC4- producing stromal cells with human CD34+ hematopoietic cells led to 3-6 times ex vivo expansion. Both homeoproteins regulate cell growth pathways and are thought to be eligible to be used in clinical settings for HSC expansion purposes.

Various molecules involved in cellular activities such as Polycomb group of proteins (PcG), DEK, cyclin dependent kinase inhibitors (CDKIs), microRNAs [3] among other intracellular signaling pathways and signaling molecules participate in HSC kinetics which are extensively detailed in the recent review by Bromeyer [29]. Many cytokines and GFs in the immune regulation pathways operate via transcription factors and cell cycle molecules and investigation of the intrinsic cellular factors will help find the interconnection between HSC regulators and the way they act through.

The fact that very high and efficient amounts of expansion is guaranteed by ectopic expression of intrinsic factors, the potential of unwanted stem cell activation as well as malignant transformation possibility are considered as major drawbacks. Even the non-genetic alterations to increase over expression of intrinsic factors have not yet been found reliable enough to bypass the unwanted effects [3]. However, once the more biological functions of intrinsic-extrinsic modulations are disclosed successful manipulations will provide safe and feasible clinical benefits [29].

**Scaffolds**

Mimicking in vivo physical environment conditions has a potential of achieving the capacity of ex vivo expansion through directing stem cell fates [33]. BM engineering in vitro and utilization of biomaterials were the first attempts of providing an artificial niche. Biomaterials such as olyvinlyalcohol-co-itaconic acid (PVA) do play a significant role in maintaining a platform-niche. Higher levels of expansion and increasing number of CFU-GM/GEMM have been detected, when HSC were cultured on PVL coated dishes in the presence of oligonucleotide fragments or fibronectin [34].

Ferreira et al. [35] have tested four different 3D biomaterial scaffolds (PCL, PLGA, fibrin and collagen) for freshly isolated UCB-CD34+ cell expansion either in presence of exogenous cytokine supply and MSC-UCB combination. Cell morphology, growth and proliferation were analyzed in vitro as well as multi-organ engraftment and multi-lineage differentiation in a murine transplantation model. All scaffolds, except 3D PLGA meshes, supported UCB-CD34+ cell expansion, which was additionally stimulated by UC-MSC support. UCB-CD34+ cells cultured on human-derived 3 fibrin scaffolds with UC-MSC support reached 5×10⁵-fold expansion of TNC after 14 days of incubation and 3×10⁶-fold expansion of CD34+ cells after 7 days (p<0.001). The mentioned conditions maintained superior immune-phenotypes with better migratory and adhesive properties and showed the significantly highest numbers of engraftment and multi-lineage differentiation in BM, spleen and peripheral blood in long-term transplanted NSG mice compared to the other 3D biomaterial scaffolds. They concluded that BM-mimicry strategy based on 3D fibrin scaffold revealed optimal requirements to be implemented into clinical settings for UCB expansion and transplantation.

Single or multi walled novel carbon based nanomaterials are being used in many different applications. A carbon nanotube (f-SWCNT-COOH) has been introduced with the ability to support the viability and ex vivo expansion of UCB derived HSC. In vitro phenotypic and functional data identified a role for f-SWCNT-COOH for inducing and supporting ex vivo expansion of HSC. Immunodeficient mice models also proved that f-SWCNT-COOH expanded HSC could repopulate without exhibiting any detectable considerable safety [36]. Researchers from Kansas University have completed a clinical observational study proposing a higher expansion of cord blood stem cells ex vivo in a 3D microenvironment, with its supporting cells of osteoblasts and stromal cells (Clinical Trial Identifier: NCT 01061879). The results shall indicate the clinical utility.

**Co-culturing with Mesenchymal Stem Cells (MSC)/stromal cells**

Expansion of progenitor cells can be achieved by culturing such cells in the presence of different combinations of recombinant stimulatory cytokines; in contrast, expansion of actual HSCs has proved to be more difficult because, in addition to needing recombinant cytokines, HSCs seem to deeply depend on the presence of stromal cells and/or elements that promote the activation of particular self-renewal signaling pathways [37].

Stromal elements along with non-hematopoietic cells are considered to provide the "niche" and molecular signaling needed for efficient ex vivo expansion of stem UCB-derived stem cells, mimicking in vivo conditions [10]. Hence, another approach for expansion has been co-culturing UCB stem/progenitor cells with stromal or mesenchymal cells. MSC were proven to be the most effective supporters of ex vivo expansion so far. An unmanipulated UCB unit with a 14 day expanded unit in the presence of cytokines on either family member donor-derived MSC or "off-the-shelf" MSC using dUCBT was evaluated derived MSC or "off-the-shelf" MSC using dUCBT was evaluated[10]. Hence, another approach for expansion has been co-culturing UCB stem/progenitor cells with stromal or mesenchymal cells. MSC were proven to be the most effective supporters of ex vivo expansion so far. An unmanipulated UCB unit with a 14 day expanded unit in the presence of cytokines on either family member donor-derived MSC or "off-the-shelf" MSC using dUCBT was evaluated [10]. Hence, another approach for expansion has been co-culturing UCB stem/progenitor cells with stromal or mesenchymal cells. MSC were proven to be the most effective supporters of ex vivo expansion so far. An unmanipulated UCB unit with a 14 day expanded unit in the presence of cytokines on either family member donor-derived MSC or "off-the-shelf" MSC using dUCBT was evaluated[10]. Hence, another approach for expansion has been co-culturing UCB stem/progenitor cells with stromal or mesenchymal cells. MSC were proven to be the most effective supporters of ex vivo expansion so far. An unmanipulated UCB unit with a 14 day expanded unit in the presence of cytokines on either family member donor-derived MSC or "off-the-shelf" MSC using dUCBT was evaluated [10]. Hence, another approach for expansion has been co-culturing UCB stem/progenitor cells with stromal or mesenchymal cells. MSC were proven to be the most effective supporters of ex vivo expansion so far. An unmanipulated UCB unit with a 14 day expanded unit in the presence of cytokines on either family member donor-derived MSC or "off-the-shelf" MSC using dUCBT was evaluated[10]. Hence, another approach for expansion has been co-culturing UCB stem/progenitor cells with stromal or mesenchymal cells. MSC were proven to be the most effective supporters of ex vivo expansion so far. An unmanipulated UCB unit with a 14 day expanded unit in the presence of cytokines on either family member donor-derived MSC or "off-the-shelf" MSC using dUCBT was evaluated [10]. Hence, another approach for expansion has been co-culturing UCB stem/progenitor cells with stromal or mesenchymal cells. MSC were proven to be the most effective supporters of ex vivo expansion so far. An unmanipulated UCB unit with a 14 day expanded unit in the presence of cytokines on either family member donor-derived MSC or "off-the-shelf" MSC using dUCBT was evaluated [10]. Hence, another approach for expansion has been co-culturing UCB stem/progenitor cells with stromal or mesenchymal cells. MSC were proven to be the most effective supporters of ex vivo expansion so far. An unmanipulated UCB unit with a 14 day expanded unit in the presence of cytokines on either family member donor-derived MSC or "off-the-shelf" MSC using dUCBT was evaluated [10]. Hence, another approach for expansion has been co-culturing UCB stem/progenitor cells with stromal or mesenchymal cells. MSC were proven to be the most effective supporters of ex vivo expansion so far. An unmanipulated UCB unit with a 14 day expanded unit in the presence of cytokines on either family member donor-derived MSC or "off-the-shelf" MSC using dUCBT was evaluated [10]. Hence, another approach for expansion has been co-culturing UCB stem/progenitor cells with stromal or mesenchymal cells. MSC were proven to be the most effective supporters of ex vivo expansion so far. An unmanipulated UCB unit with a 14 day expanded unit in the presence of cytokines on either family member donor-derived MSC or “off-the-shell” MSC using dUCBT was evaluated (Angioblast Ltd). In this study, the authors demonstrated a median 40-fold expansion of CD34+ cells and a median time to neutrophil and platelet engraftment of 15 (range 9-42) and 40 (range 13-62) days, respectively [10]. Some results of the clinical trial led by MD Anderson
Cancer Center (Clinical Trial Identifier: NCT 00498316) for testing UCB expansion on MSC was reviewed recently by De lima et al. [38]. Co-cultures with mesenchymal stromal cells led to an expanded number of total nucleated cells by a median factor of 12.2 and of CD34+ cells by a median factor of 30.1.

Xiong et al. [39] has conducted a prospective randomized trial comparing the MSC combined UCBT with only MSC transplantation, concluding that although 2 strategies were effective for EF and do not result in GVHD or increase the risk of tumor relapse, the MSC plus UCB regimen had a superior effect on neutrophil reconstruction. Impact of UCB co-infused with MSC was studied by McMillan and co-workers [40] in which ex-vivo culture-expanded MSCs from haploidentical parental donors were infused at the time of UCBT. The results of the phase II clinical trial has shown increase in engraftment among patients receiving UCB-MSC co-infusions. The results point out a safe clinical translation of ‘off the shelf’ allogeneic HLA-mismatched MSC products.

There are many other ongoing/completed clinical trials at the moment for the clinical utility assessment of co-culturing and co-infusion with MSC/stromal cells (http://www.clinicaltrials.gov). The value of BM derived MSC have been shown by Robinson et al. [41] in earlier work and MSC sources other than bone marrow (such as placental tissues) were suggested to reach better supporting conditions lately [42].

Engraftment and Homing Enhancers

UCB-derived specific immune cells

Alloimmune responses consisting of UCB derived immune cells as well as cytokine and growth factor milieu have a strong impact on engraftment, GVHD or graft versus leukemia (GVL) status after HSCT [43]. The immature and naive nature of immune cells in UCB certainly have an impact on the lower GVHD rates observed after UCBT compared to bone marrow transplantation (BMT) or peripheral blood stem cell transplantation (PBSC) [43-45]. UCB immune cells are characterized by lower expression of GFs as well as lower levels of effector cytokines (e.g.IL-12, TNF alpha, IFN gamma) compared to adult peripheral blood T cells. Low cell doses achieved in addition to the inexperienced nature lead to slower engraftment with delayed immune reconstitution and increased risk of opportunistic infections after UCBT [44,45]. Adoptive transfer of viral specific (CMV, EBV, Adenovirus or BK virus specific) T cells have been shown to circumvent the high infection rates seen after HSCT [45]. This strategy is not as feasible in UCBT as in HSCT, due to the low quantity and naiveté of cells in UCB grafts. Thus, ex-vivo polyclonal expansion of UCB derived T cells, primed with CMV or EBV transformed cells in the presence of cytokines such as IL-7, IL-12 and IL-15 have been used to produce cytotoxic T cells against CMV, EBV of ADV [46,47]. Recently, exogenous Rcs on ex vivo expanded peripheral T cells (chimeric antigen receptors, CARs) have been targeted against malignancies [44]. Micklewaite et al. [48] have transduced UCB derived multi virus specific cytotoxic T lymphocytes to express CAR CD19 (B cell malignancy marker). A Phase I/II trial is recruiting at the moment to utilize pathogen specific expanded T cells (Clinical Trial Identifier: NCT 00880789).

Modulating donor active T cell clones by Tregs [CD4+CD25highCD127lowforkhead box protein 3 (FoxP3+)] as well as intracellular regulatory cytokines such as IL-10 and TGF-beta have been shown to play role in immune tolerance, reducing GVHD while still retaining GVL effect in allo HSCT [43,49-51] and UCB [52,53]. Inhibition of proliferation and function of T, B, NK and NKT cells and antigen presenting cells through production of IL-10 and IL-35, absorbing IL-2 is of the mechanisms proposed [44,45]. Adoptive transfer of T regs with or without conventional T cells in HLA mismatched setting in mice and human allogeneic HSCT have resulted in better GVHD status whereas removal was shown to accelerate rejection [45,51,53]. There are two clinical trials at the moment testing the impact of adoptive transfer of Tregs on prevention of GVHD [54,55]. In UCBT setting, isolation of Tregs have been more troubling, due to low numbers of Tregs in UCB. Not only the quantity but also the quality of T regs have also been found different for UCB [45]. UCB derived Tregs can be highly expanded in vitro using antibody coated beads 200-1000 fold [56] and with universal artificial antigen-presenting cell (aAPC) [53]. There have been pre-clinical attempts to adoptively transfer ex vivo expanded UCB derived T regs and Brunstein et al. [57] have conducted a clinical trial in UCBT demonstrated encouraging results. The IL-2 expanded third party UCB T regs were infused to 23 dUCBT patients on different days. A reduction in grade II-IV GVHD was observed compared to 108 controls in this clinical trial.

NK cell activity is very important for viral clearance and for GVH effect after HSCT. The influence of NK inhibition through killer inhibitory receptors (KIR) on protecting against graft rejection after HSC/UCBT is very well investigated [43-45,58]. NK cells found in UCB show differences to peripheral blood NK cells, with decreased expression of activator Rcs NKG2C and increased levels of inhibitory Rcs such as NKG2A and KIRs [44]. The fact that UCB has low amounts of NK cells due to low volume and low cell dose have led researchers to in vitro expand NK cells to be applied in UCBT for faster and better engraftment with low GVHD [44,45]. Increasing UCB derived NK population through in vitro/vivo manipulation with or without conventional immunosuppressive drugs to stimulate better transplantation outcome is a promising field. Utility of NK cells in UCBT is tested in a clinical trial at the moment (Clinical Trial Identifier: NCT 01619761).

IL-7, IL-2 and IL-15 cytokines, growth hormone and keratinocyte like growth factor among other immune molecules are under investigation for a likely role to enhance immune reconstitution after HSCT [45]. The effect of low dose IL-2 therapy on reducing chronic GVHD via restoring T reg homeostasis have been shown previously by Matsuoka et al. and Koreth J et al. [59,60]. An ongoing clinical trial to test the role of IL-2 in T cell depleted dUCBT for refractory AML is recruiting patients at the moment (Clinical Trial Identifier: NCT 01464359).

Using CB derived immune cells in vivo to be used in cellular therapies in UCBT is promising for enhancing immune tolerance resulting in less graft rejection and better engraftment [43,45].

Intrabone infusion

A large amount of HSC fails to reach BM microenvironment after the infusion of UCB. Intra osseous BMT is one of the primary applications of HSCT. Relative ease of intravenous transplantation has replaced this method totally. Studies in animals irradiated with limb shielding have shown that only a small fraction of cells injected intravenously migrate to haematopoietic sites, and direct intra bone injection of HSC resulted in 10-times more efficient repopulation of the marrow of lethally irradiated mice than compared to intravenously injected HSC.
Frassoni et al. [61] performed a phase I/II study on 32 patients with leukaemia who received a single UCBT injected directly in the iliac crest after standard myeloablative conditioning. No complication occurred during the infusion. Intrapro co-transplantation of MSC with UCB CD34+, has further improved engraftment in NOD/SCID mouse model [62]. There is ongoing work by the same group to test human intrabone transplantation of UCB with MSC. There are several trial records with the purpose of investigating the outcome of intrabone UCBT (http://www.clinicaltrials.gov).

**CXCR4–SDF-1 axis**

SDF-1 is a cytokine secreted from BM stromal cells, involved in fetal and adult life in homing of HSC in the marrow microenvironment. CXCR-4 is the receptor of SDF-1. Responsiveness of CXCR4 on HSCs to an SDF-1 gradient; and an effective level of SDF-1 expression in the BM environment are two main parameters which are of special importance for the engraftment of HSC in BM. Following the conditioning regimen, BM increases SDF-1 secretion which results in increased responsiveness of HSCs to an SDF-1.

SDF-1 possesses the ability to induce motility, chemotactic responses, adhesion and secretion of matrix metalloproteinases (MMPs) and angiopoietic factors (e.g., VEGF) in cells expressing CXCR4. SDF-1 also increases adhesion of early HSC to VCAM-1, ICAM-1, fibronectin and fibrinogen by activating/modulating the function of several cell surface integrins. In addition to regulation of cell trafficking, SDF-1 is claimed to directly or indirectly affect cell proliferation and survival. CXCR4 expressed on UCB CD4+ T cells may play an important role in their retention to BM after transplantation and allogeneic engraftment.

The SDF-1–CXCR4 axis may be modulated by various molecules related to inflammation (e.g., C3a anaphylatoxin, desArgC3a, fibronectin, and hyaluronic acid), coagulation (e.g., fibrinogen, uPAR, and thrombin) or cell activation (e.g., s-VCAM-1, s-ICAM-1, membrane- derived vesicles). All these molecules are constituents of leukapheresis products collected from G-CSF-mobilized patients [10]. It was recently found that supernatants from leukapheresis products collected from G-CSF-mobilized patients [10].

Homing and retention of UCB stem and progenitor cells into the marrow microenvironment is a highly critical step after transplantation and SDF-1(CXCL-12)–CXCR4 axis plays a significant role in this course by better seeding efficiency as well as faster neutrophil/platelet recovery [64]. SDF-1(CXCL-12) is a chemokine implicated in retention and survival of HSC and HPC [65,66], CD26/DPPIV is an enzyme cleaving CD26–DPPIV in a truncated form, hindering its chemotactic effect. Moving on from this point, it was shown that inhibition of CD26/DPPIV efficiently enhanced engraftment in the mouse models through an increase of homing [65,66].

Sitaglitin is a DPP-IV inhibitor FDA approved for the treatment of type II diabetes mellitus. Sitaglitin is currently investigated in two different clinical phase II studies by Sarah Farag to assess the efficacy of CD26/DPP-IV inhibition for increasing and speeding up the engraftment following transplantation of UCB (Clinical Trial Identifiers: NCT 00862719 and NCT 01720264). The median time to engraftment was observed to be 21 days using single UCB in this very first clinical trial with DPP-4 inhibitors. The simple and cost effective approach of using DPP-IV inhibitor with single UCBT offers potential for clinical use. The optimization of Sitaglitin dose as well as the duration is required to be investigated in further clinically studies [67].

**Fucosylation**

Addition of a fructose (fucosylation) to CD 34+cell surface have been shown to enhance engraftment in vitro and in vivo mouse models [68,29]. The major challenge of delayed engraftment after UCB transplantation was found to be due, at least in part, to low fucosylation of cell surface molecules important for homing to the bone marrow microenvironment. Targeted fucosylation to specific cell surface ligands is required before effective interaction with selectins (P/E Selectins) expressed by the bone marrow endothelium can occur. Altering fucosylation increases the expression of molecules critical for homing properties (such as CXCR4) and PGE2 [69].

Robinson and colleagues [70] have tried a simple 30-minute ex vivo incubation of UCB hematopoietic progenitor cells with fucosyltransferase-VI (α1-3 fucosyltransferase) and its substrate (Guanosine diphosphate –fucose/ GDP) to increase levels of fucosylation. In their study they observed a concomitant improvement in engraftment with increased number of fucosylated CD34 cells in a NSG mouse model. FTVI and FTVII fucosylated CB CD34+ cells in vitro, and both led to enhanced rates and magnitudes of engraftment compared with untreated CB CD34+ cells in vivo. In contrast, only FTVI was able to fucosylate T and B lymphocytes as indicated by Robinson and colleagues [71].

Trummer et al. [72] have used circulating microparticles (MP) with P-Selectin and its ligand (PSGL-1) to improve engraftment and to amplify adhesion of HSC to BM. In this abstract that was presented in American Society of Hematology Conference 2013, synergistic effect of MP s with fucosylation was investigated. They used an incubation protocol with GDP and fucosyltransferase-VII and concluded that the fucosylation was mediated through MP s whereas there was no synergy between the two pathways. A phase II clinical trial by MD Anderson Cancer Center fucosylation of cord blood CD34 cells is recruiting participants at the moment (NCT 01471067) with the expected finding of faster engraftment via fucosylation of UCB derived CD34+ cells.

**Parathyroid Hormone (PTH)**

In mouse models, administration of parathyroid hormone (PTH) is an effective way to enhance the ability of limited numbers of hematopoietic stem cells to support hematopoiesis. When UCB cells were co-cultured with osteoblasts in the presence of human PTH, a 4-fold increase in CD34+ CD38- cells was detected after 7 days of incubation [73]. A significant increase in adhesion molecules (CD44/ VLA4) was also noted in favor of cell expansion which was due to human PTH.

Findings of a phase II trial conducted by Ballen K et al. [74] were reviewed. After either a myeloablative or a reduced-intensity double UCB transplantation, patients received human PTH at 100 µg/day for 28 days. Thirteen patients (median age, 42 years) were enrolled. The median time to neutrophil and platelet engraftment of >20×109 cells/L in patients was 30 days and 61 days, respectively. The occurrence of four deaths before day 100 led to the early closure of the trial. Overall survival was reported to be 62% at 6 months after transplantation with 39% disease-free survival at 2 years. At the dose and schedule studied, there was no evidence that PTH influenced blood count recovery.

Human PTH is approved for clinical use and findings that
administration of PTH can promote recovery of HSC when CBU with low cell numbers are to be infused. Preclinical research has sought to determine the mechanism for which PTH affects the process of supporting hematopoietic recovery. It was indicated that, PTH may induce an in vivo increase of HSC via Notch signaling pathway. Their findings suggest that PTH binding on its receptor on osteoblasts results in an increase in Notch ligand Jagged-1 thus overcoming the negative effect of low adhesion molecule levels of UCB.

Other Approaches

HLA matching in UCBT

Many studies have shown an interaction between TNC dose and HLA mismatch level [75,76]. In two retrospective analyses by Eapen et al. [76,77], it was shown that reduction in TRM along with longer overall survival rates were significantly correlated with less HLA- mismatches. The impact of HLA-C matching in UCBT was also stressed in the latter. It was suggested that TNC dose plays more significant roles with higher number of HLA mismatches [78].

Although controversies arise due to the differences in clinical findings in retrospective studies, avoiding HLA mismatches particularly in HLA A, B and DRB1 regardless of TNC dose is recommended particularly for single UCBT. Since, TNC dose is still by far the most important determinant when choosing a CBU [3,68], the expansion techniques may allow the usage of an HLA matched but otherwise insufficient single UCB unit, avoiding the application of a mismatched CBU.

Double UCBT

An increased risk of delayed engraftment, graft failure and higher TRM is strongly associated with lower cell doses in UCBT. Especially when the level of HLA mismatch is high, higher TNC doses [78-80] are required in order to compensate the outcome which is very difficult to achieve in older children and adults [79]. Using double UCB units to sustain donor engraftment is another approach effecting transplant outcome and since its first use by Minnesota group in 2000, the method was proven to be safe and feasible.

Currently, dUCBT is performed more than single UCBT in adults with the expected result of faster engraftment. Although decrease in relapse rates are reported, reaching adequate cell doses and the median neutrophil and platelet recovery time is still far from optimal [10,14]. One of the major advantages of dUCBT is that it maintains the platform to test novel expansion techniques. The manipulated-unmanipulated unit combination and investigation of the unit dominance provide a platform for ex vivo expansion and enhancement modalities. A recent prospective study conducted by Kindwall Keller and colleagues [81] comparing single versus dUCBT in adult patients demonstrated higher relaps risk in single unit transplants and similar survival outcomes in those transplanted with one adequate unit or two if one adequate unit is not available. With the advancement in engraftment enhancer modalities, it may be possible to circumvent the need of bystander third party cells provided by a second UCB unit to support expansion. Haploidentical UCBT and RIC are other clinical approaches to improve UCBT outcomes which are beyond the scope of this review.

Conclusion and Final Remarks

UCBT has taken significant place not only in the management of malignant/ nonmalignant diseases but also for gene therapy purposes. Despite the fact that UCB is an increasingly used alternative source of HSC with certain advantages, many different groups have reported UCBT with higher rates of delayed engraftment and higher TRM particularly when low cell doses were infused. Since UCB hematopoietic cells have the longest telomere length among all adult type HSCs, to expand them in vitro has been one of the first attempts of expanding cellular pool. Rapid advancement has been achieved in new methodologies developed for ex vivo expansion and enhancing engraftment of UCB derived HSCs. With above mentioned HSC manipulation techniques in addition to novel approaches that are on the way, UCB derived products will surely have a positive impact on UCBT outcomes provided that optimal culturing and infusion conditions are determined [37].

Most of the expansion methods lead to transient myelopoesis through generation of short term progenitors. It is important to define and optimize culture conditions to maintain LTR cells. Ensuring the presence of clinically relevant HPC/mature cells in the UCB graft is stressed by Czaszar et al. [14]. The authors suggested that, a 15 fold expansion of LTR HSC would enable patients up to 162 kg to receive a recommended cell dose of an unmanipulated unit if the HSC that are important for long term engraftment are present. Thus, the level of expansion and the type of the expected HSC population should also be characterized when modulating HSC fate. If both short term and long term cells are to be expanded, HLA mismatched otherwise small UCB units may be of use. Also, with ongoing scientific research and clinical work, it may be possible to efficiently use only one manipulated UCB unit, avoiding complexity and reducing the cost.

With recent studies suggesting that UCB derived HSCs may as well differentiate into nonhematopoietic cells, such as neural, cardiac, mesenchymal, and muscle cells, treatment of neural, metabolic, orthopedic, cardiac, and neoplastic disorders is also possible. Bioengineering strategies allow reprogramming of UCB cells into different cell types and methods for the formation of induced pluripotent cells (iPSC) in particular are under extensive research. Judicious application of re-programming may lead towards establishing a UCB derived iPSC bank in the future. Some stem cell products are already about to reach clinic such as iPSC for macular regeneration which was discovered by Takahashi and collaborators [82]. In vivo infusion of ex vivo expanded “off the shelf” non HLA matched UCB products are also currently tested under a Phase II clinical trial (NCT 01175785) conducted by Fred Hutchinson Cancer Center. HEMACORD (name changed to “HEMACORD HPC, Cord Blood” in April 2013), the first stem cell therapy product, was FDA licensed in 2011.

To achieve better engraftment with less GVHD in myeloablative or T cell depleted HSCT/UCBT, strong immunosuppressive therapy is critical. The regulatory and suppressive Tregs from UCB can be effectively utilized as a future alternative, provided that adequate expansion and safety is guaranteed for the clinical adoptive transfer of these immune cells. More pre-clinical and clinical data is needed for optimizing the kinetics of CB cellular immune therapies. Transplantation of ex vivo expanded UCB cells is becoming a reality, while the issue of expanded cell banking emerges as an option allowing the conservation of the product for future use [82]. Widespread application can only be achieved via robust expansion. Another fundamental task is to protect the genomic integrity of the modified UCB products and to ensure that they resemble the unmanipulated counterparts [10]. Detrimental safety risks like unwanted cell differentiation or oncogenicity should be assessed thoroughly. Optimization of bioprocesses and large scale production of safe cellular products will lead to wider and more cost-
effective banking strategies for manipulated and re-programmed UCB units. Combination of the current methods with “yet to be developed” approaches will definitely have a strong effect not only on UCBT but also on regenerative cellular therapies. Production of immediate “off the shelf” use of modified UCB is a major goal of all ongoing investigations. However this goal would surely require very serious GMP measures as well as enormous amount of time and money to provide the clinical efficacy and safety [3,14,29,83,84]. Efficient clinical utility can only be tested with randomized clinical trials, prospective if possible, to exclude any bias.

References

37. Flores-Guzman P, Fernandez-Sanchez V, Mayani H (2013) Concise review: ex vivo expansion of cord blood-derived hematopoietic stem and progenitor...
cells: basic principles, experimental approaches, and impact in regenerative medicine. Stem Cells Transl Med 2(11): 830-838.[PubMed]


