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REVIEW ARTICLE

Ex vivo expansion of umbilical cord blood: where are we?

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Abstract Since the first successful clinical use of umbilical cord blood (UCB) in 1988, UCB grafts have been used for over 20,000 patients with both malignant and non-malignant diseases. UCB has several practical advantages over other transplantable graft sources. For example, the ease of procurement, the absence of donor risks, the reduced risk of transmissible infections, and the availability for immediate use make UCB an appealing graft choice. However, UCB grafts suffer from a few limitations related to the limited cell dose available for transplantation in each UCB unit and to defects in UCB stem cell homing. These limitations lead to increased post-transplant complications. In this review, we focus on the issue of limited cell dose in UCB units and discuss the possible approaches to overcome this limitation. We also summarize the various cellular pathways that have been explored to expand UCB units.

Keywords Umbilical cord blood · Stem cells · Expansion · Mechanisms

Introduction

Umbilical cord blood (UCB) is a rich source of transplantable hematopoietic stem cells (HSCs), a fact that inspired the first successful UCB transplant in 1988 [1]. Since then, UCB has been used as a graft source for over 20,000 patients with both malignant and non-malignant diseases [2]. More than 2,800 UCB units were used for adult and pediatric transplants in 2008 alone [3]. Biologically, UCB cells seem to be superior to bone marrow (BM) or peripheral blood mobilized cells (Table 1). For example, when stimulated, UCB cells can generate a larger number of progenitor cells than BM [4]. In addition, CD34(+) UCB cells have higher proliferative potential than BM or peripheral blood mobilized CD34(+) cells, which is thought to be secondary to differences in telomerase activity and telomere lengths [5].

As a graft for transplantation, UCB has several practical advantages over other sources (Table 2). These advantages are related to the ease of procurement, the absence of donor risks, the reduced risk of transmissible infections, and the availability for immediate use [6]. To add, UCB is associated with a lower incidence of graft-versus-host disease (GVHD) despite HLA disparity [7]. In accordance with that, and since UCB allows for a greater HLA disparity in comparison to peripheral blood or BM grafts, UCB extends the application of allogeneic transplant to minority populations who are under-represented in donor registries [8]. In one study, 10/10 matched unrelated donors were identified in 53 % of those with European ancestry compared to only 21 % of patients not of European origin [9]. These advantages make UCB an attractive alternative to unrelated donor transplant. However, this graft source has a few major limitations due to: the limited cell dose available for transplantation in each unit, defects in UCB stem cell homing, delayed immunoreconstitution post-transplant, and an inability to utilize donor lymphocyte infusion to treat relapse. As a result, UCB transplant is associated with delayed neutrophil and platelet engraftment and higher rates of engraftment failure [10]. Though this is most true for adults, even children receiving satisfactory cell doses have a delay in UCB engraftment, in comparison to other graft sources [11]. In myeloablative double UCB

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Table 1 Biological advantages of UCB

Biology of UCB versus BM
UCB cells have increased proliferative potential
UCB cells produce a greater number of progeny
UCB cells have longer telomeres

Table 2 Summary of the advantages and disadvantages of umbilical cord blood (UCB) used for transplantation

Advantages of UCB	Limitations of UCB
Ease of procurement	Limited cell dose in each unit and defects in bone marrow homing:
Availability for immediate use	a. Delayed blood count recovery
Absence of donor risks	b. Delayed engraftment
Reduced risk of transmissible infections	c. Higher rates of graft failure post-transplant
Lower incidence of graft versus host disease	d. Delayed immunoreconstitution post-transplant
Extends transplant to minority populations	Limited options to treat post-transplant relapse

transplants a graft failure rate of 20 % has been reported consistently [12]. Even defects in immune reconstitution post-cord blood transplantation are thought to be a direct result of delayed engraftment post-UCB transplant [13].

In recent years, major advances in the field have helped address these limitations. If these limitations were fully addressed, UCB transplantation could potentially replace unrelated donor transplantation as the transplant of choice in those who do not have a matched sibling donor transplant option.

Delayed engraftment and graft failure post-UCB transplantation are principally driven by the low cell dose in each UCB unit and defects in UCB stem cell homing. To overcome the cell dose limitations, efforts to expand UCB stem cells have been ongoing. In parallel, using two UCB units instead of one, known as double umbilical cord blood (dUCB) transplantation, has significantly improved the chances of adult patients receiving an UCB transplant. Other efforts are directed at improving cord harvesting techniques [14] and modification of post-thaw procedures [15]. In addition, strategies to improve UCB stem cell homing and others directed at investigating novel preparative regimens are ongoing, too. In this review article, we focus on the efforts to expand UCB ex vivo.

Rationale and methodology of ex vivo expansion of UCB units

The purpose of expanding UCB stem cells ex vivo is to expand HSCs with long-term in vivo marrow repopulating capacity. This is logical since engraftment is inversely related to the cell dose given during transplantation [16]. HSCs are expected to repopulate the BM after high-dose therapy. In pre-clinical settings they are expected to save lethally irradiated mice, which remains the gold standard test to evaluate the human HSC long-term BM repopulating capacity [17]. Several methodologies have been used to expand UCB cells ex vivo. These methodologies fall within the categories of either liquid suspension culture or stromal-cell co-culture. In addition, the introduction of continuous perfusion culture systems has led to more efficient expansion systems.

Liquid cultures

In liquid cultures, isolated UCB stem cells are exposed to a cocktail of cytokines, growth factors, and other factors for a specific time. These cytokines and growth factors are naturally provided in vivo by the marrow stroma in the HSC microenvironment [18]. Prior to culturing, however, the hematopoietic progenitors in the UCB must be isolated. In terms of cytokines, different combinations have been studied. Though the proper mix of growth factors and cytokines for ex vivo expansion remains unknown, a study by Levac et al. [19] concluded that minimal cytokine stimulation using SCF+FLT-3+TPO can successfully expand Lin(−)CD34(+)CD38(−) HSCs without the need for cytokines such as IL-3, IL-6 or the growth factor G-CSF. Overall, it is felt that thrombopoietin (TPO) and stem cell factor (SCF) are the most important up-regulators of HSCs [20]. Additionally, Bordeaux-Rego demonstrated that IL-3 and IL-6 enhance ex vivo expansion of CD-133(+) cells isolated from UCB when added to SCF, fms-like tyrosine kinase 3 (FLT-3), and TPO [4]. One major concern is telomere degradation with continued expansion [5]. However, the use of TPO seems to prevent telomere degradation [5].

In animal models, stroma-free, stroma-noncontact cultures were able to maintain long-term BM repopulating cells with the capacity to engraft primary, secondary, and tertiary xenogeneic hosts [21]. However, in clinical experience, using liquid suspension cultures to expand cord blood stem cells ex vivo has resulted in variable outcomes. The general feeling is that these attempts were mostly unsuccessful because a supporting stromal layer has a stabilizing influence on the UCB stem cells, causing them to self-renew instead of differentiating into more mature progenitors [22]. Another possible explanation for the failure of cytokine expanded HSCs to engraft is related to the downregulation of α4 integrin in the cytokine treated UCB CD34(+) cells in comparison to unmanipulated cells [23]. Since engraftment is mediated by α4 integrin and C-X-C chemokine receptor type 4 (CXCR-4) interaction,

this downregulation of α4 integrin results in downregulation of engraftment. In contrast, a study by Foguenne et al. [23] demonstrated that the repopulation by ex vivo-expanded CD34(+) cells is mediated by α5, though the repopulation by uncultured CD34(+) cells is dependent on α4. Interestingly, the study showed that expanded CD34(+) cells were able to engraft NOD/SCID β_2m-null mice independently of α4 integrin, which indicates a possible cooperative role for α5 that might be uncovered once α4 is inactivated. Another explanation has to do with the transit of expanded HSC population through different phases of the cell cycle, which might result in impairment of engraftment [24].

A main concern with most ex vivo expansion studies is that they are successful in expanding largely the lower quality subset of HSCs, which improves short-term reconstitution with early hematopoietic recovery but ultimately leads to graft failure [25]. One potential explanation considers the fact that HSCs in ex vivo cultures tend to cycle rapidly and differentiate. In contrast, HSCs in vivo tend to remain quiescent and divide at a very slow rate [26]. In a recent study using notch-mediated expanded human UCB progenitor cells, Delaney et al. [27] showed early engraftment mediated by the expanded cells, which occurred at median time of 16 days. However, the expanded cells were lost in virtually all of the recipients over time. Though combining an ex vivo-expanded unit, for early hematologic recovery, with an unmanipulated unit, for long-term sustained hematopoiesis, might seem like an optimal strategy, this was not found to be successful in other trials [28].

Stromal co-cultures

In stromal co-cultures, UCB cells are expanded using a stromal layer of supporting cells. The importance of these supporting cells in preventing graft failure can be appreciated by understanding the negative impact of its absence. For example, the chemotherapy given prior to HSC transplantation, called myeloablative preparative regimens, can damage the bone marrow microenvironment [29]. This explains the higher rate of graft failure in transplants using myeloablative preparative regimens compared to those using milder regimens [30]. To understand the role of the HSC "niche," we should review the "niche hypothesis" by Schofield. In this hypothesis, HSCs are fixed cells surrounded by and associated with supporting cells that create a "niche" allowing the HSCs to self renew at the expense of maturation and differentiation [31]. The niche cells provide a unique environment by secreting cytokines and producing extracellular matrix proteins and adhesion molecules [32]. The importance of these cell adhesion and matrix proteins in HSC proliferation and differentiation has been lately revealed in a series of elegant experiments by Zhang et al. [32]. In these experiments, the authors concluded that spindle-shaped N-cadherin(+) CD45(−) osteoblastic cells (SON) are responsible, through physical contact, for the maintenance of long-term HSCs. These SON cells are distributed in a pattern similar to long-term HSCs, which includes the surface of cancellous/trabecular bone and along the endosteal surface of long bone. In addition, this physical contact seems to be mediated by adhesive molecules, including B-catenin and N-cadherin. The importance of BM osteoblasts in expansion of HSCs was further studied in a series of experiments by Mishima et al. [33]. In these experiments, osteogenic-differentiated MSCs were used as a feeder layer to expand human CD34(+)CD38(−) HSCs, in comparison to a feeder layer of undifferentiated MSCs. It was clear that the osteogenic-differentiated MSCs were more potent than the undifferentiated MSCs in expanding the HSCs. These stromal cells were found to express the genes involved in hematopoiesis regulation such as SCF, FLT-3, and TPO, among others. However, the expansion of HSCs was not accomplished without the use of exogenous cytokines, which means that an optimal ex vivo culture system should include both exogenous cytokines in addition to stromal-cell contact. Butler et al. [34] was able to culture HSCs with BM endothelial cells in cytokine-free media, which offers further evidence of the importance of the bone marrow niche. However, the best HSC expansion method likely includes cytokines. In a study by Andrade et al. [35], the optimal cytokine mix for expansion of HSCs/hematopoietic progenitor cells (HPCs) co-cultured with MSCs was found to be SCF 60 ng/mL, FLT 3 55 ng/mL, and TPO 50 mg/mL. Additionally, it was felt that the upregulation of chemokine CXCL12 at the time of osteogenic differentiation was essential for HSC proliferation in the osteogenic-differentiated MSCs. This is probably related to the various effects of CXCL12 on HPC homing and proliferation, as well as counteraction of myelosuppressive chemokines [36]. Though the stromal co-culture system did not traditionally require initial CD-34(+) selection [37], it was found later that other cord blood constituents actually inhibit HSC expansion. In a study by Yang et al. [38], CD3(+) and/or CD14(+) depletion resulted in improved ex vivo expansion of CD34(+) cells obtained with CB mononuclear cell and MSC co-culture.

Continuous perfusion culture systems

Two main developments lead to the introduction of continuous perfusion culture systems, or 'bioreactors' [39]. The first was the development of culture techniques by Dexter et al. [40], which allowed for hematopoiesis support over extended periods of time and identified the importance

of stroma in that process. This culture system was made of adherent and non-adherent populations of cells. The adherent population contained what was described as phagocytic mononuclear cells, "epithelial" cells, and "giant fat" cells. The second development was the finding that media change is of major importance in maintaining optimal conditions to support ex vivo expansion [41]. Continuous medium perfusion seems to stimulate the production of growth factors by stromal cells [42] and prevents nutrient depletion and build up of metabolic waste products [43]. Finally, primitive long-term culture-initiating cell (LTC-IC) numbers have been shown to decline in conventional static human culture conditions, even with exogenous cytokine combinations [43]. The ability of perfusion bioreactor systems to expand non-enriched cells from mobilized peripheral blood or BM extended the use of the same system to expand UCB to a clinical-scale level enough to enable its use in transplantation [44]. Using a clinical-scale automated cell production system (CPS) by Aastrom Biosciences, Koller et al. showed that small inoculums of $1.5 \times 10^5/cm^2$ of viable nucleated cells can generate on average 4.4×10^8 cells and 1.7×10^7 colony-forming units granulocyte macrophages (CFU-GM). Interestingly, the cord blood cultures were insensitive to variations in the medium perfusion rates, contrary to BM cultures. This was felt to be related to the lack of stromal support in cord blood cultures. Cord blood cells expanded using a continuous perfusion system, the Aastrom-Replicell System, were used in a phase I clinical trial reported by Jaroscak et al. [39]. In this study, 28 patients were enrolled and received expanded UCB cells on day 12 as a boost to a conventional graft. While the device increased nucleated cells 2.4-fold and CFU-GM 82-fold, the time to myeloid, erythroid, or platelet engraftment was not altered. This study confirmed the ability to expand UCB cells for clinical use and that the administration of the expanded cells was well tolerated.

Cellular mechanisms explored to expand UCB ex vivo

Notch-mediated ex vivo expansion

Because primitive hematopoietic cells express Notch homolog, a role for Notch in HSC biology has been suggested [45]. Indeed, several groups have shown that Notch ligand family members can expand human HPCs in vitro [46–49]. Butler et al. [34] found that BM endothelial cells in cytokine-free media stimulate Notch signaling on hematopoietic cells. In addition to HSCs, this Jagged/Notch signaling pathway seems to be expressed by the bone osteoblasts, which provides another explanation for their role in regulating hematopoiesis [50]. For example, the use of parathyroid hormone, which results in expansion of bone osteoblasts, was found to contribute to increasing the self-renewal of LTC-ICs. In a recent study using Notch-mediated expanded human UCB progenitor cells, Delaney et al. [27] applied these observations into clinical practice and showed early engraftment mediated by the expanded cells, which occurred at median time of 16 days.

Wnt signaling pathway

The ability of Wnt to expand human CD34(+)Lin(−) cells in vitro was demonstrated in a series of experiments by Berg et al. [51]. The Wnt gene family (Wnt-5A, Wnt-2B, and Wnt-10B) was cloned from human fetal bone stromal cells and to a variable extent was expressed in hematopoietic cell lines derived from T cells, B cells, myeloid cells, and erythroid cells. Interestingly, only Wnt-5A was expressed in CD34(+)Lin(−) primitive progenitor cells. The exposure of hematopoietic progenitor cells to stromal-cell layers expressing Wnt genes resulted in higher numbers of mixed CFUs, CFU-GMs, and burst-forming units-erythroid, in comparison to controls. Finally, the presence of Wnt genes resulted in higher numbers of less differentiated hematopoietic cells and fewer mature cells than controls. However, the mechanism by which Wnts exercise their effect on HSCs remains inconclusive, and there is a feeling that the stronger role of Wnt in in vitro expansion of HSCs might not be duplicated in vivo [20]. Heinonen et al. [52] found a non-canonical pathway for Wnt signaling that activated Jnk kinases and suggest that Wnt4 might assist in niche restoration post-transplant.

Combined notch and Wnt

Recent data suggest that these two pathways actually act together to maintain the HSC pool, though the mechanisms underlying this interaction remain unclear [53]. Several theories have been proposed to explain the synergy between Notch and Wnt [54]. In one, it is speculated that the Wnt signal exerts its influence by activating the Notch pathway and that the Notch signaling is required for Wnt's effect on HSCs. This is supported by the observation that Wnt-3A upregulates Notch target genes. The other possibility is that the Wnt and the Notch pathways are actually parallel pathways in HSCs, in which Wnt enhances proliferation and survival whereas Notch prevents differentiation [53].

Angiopoietin-like (Angptl) proteins

Angptl proteins are a family of proteins structurally similar to the angiogenic regulating factors angiopoietins. They are encoded by seven genes, Angptls 1–7 [55]. Angptl genes

are expressed by the HSC supporting cells in the fetal liver, which comprise 1–2 % of fetal liver cells [56]. These HSC supporting cells were identified by Chou et al. [57] as SCF(+)DLK(+). In addition to Angptl 3, these cells produce IGF2, SCF, and TPO, the last two of which have already been identified as important factors for HSC expansion. In the fetal liver, these cells are also the primary expressers of CXCL12, which is required for HSC homing. These findings clearly implicate these cells in the process of HSC expansion and homing during fetal development. In a study by Zhang et al., the HSC-supportive CD3(+) cells in the mouse fetal liver were found to specifically express the proteins Angptl 2 and Angptl 3. The use of these proteins produced a 24- or 30-fold net expansion of long-term HSCs confirmed by reconstitution analysis. Angptl 5 and Angptl 7 also supported the expansion of HSCs in culture [58]. The combination of SCF, TPO, FGF-1, and IGFBP2 with Angptl 5 in a serum-free culture supported an approximately 20-fold net expansion of repopulating human cord blood HSCs [59].

TAT-HOXB4

Hox transcription factors have been recently recognized as important regulators of hematopoiesis. In a study by Antonchuk [60], HOXB4 was found to be a potent enhancer of primitive hematopoietic cell growth. In these experiments, retroviral vectors were generated with the GFP reporter gene ± HOXB4 and used to transfect HSCs. Stem cell recovery was measured by long-term competitive repopulating cells. The HOXB4-overexpressing cells demonstrated an enhanced growth in vitro, rapid dominance in mixed cultures, and shortened population doubling time. A subsequent study by Huang et al. [61] demonstrated that the use of purified recombinant TAT-HOXB4 resulted in ~7.5-fold increase in CD34(+) progenitor cells from UCB and peripheral blood. The expanded cells retained their repopulating capacity and multipotency, as evidenced by LTC-ICs and NOD/SCID mice repopulating assays. Lee et al. [62] found two potential downstream targets of HOXB4: Gp49a and Laptm4b. Both of these genes are preferentially expressed in long-term HSCs and down-regulated in more mature cells. However, the exact functions of these genes are relatively unknown, especially in regards to hematopoietic development.

Agonist of myeloproliferative leukemia virus protooncogene (c-MPL)

c-MPL is the receptor for TPO, which is the major regulator of megakaryocytic differentiation and platelet production [63]. TPO's binding to c-MPL activates three major pathways: janus kinase (JAK)/signal transducer and activator of transcription (STAT), ras/mitogen-activated protein kinase (MAPK), and phosphatidylinositol-3-kinase (P13K/AKT) [64, 65]. Interestingly, c-MPL is not only expressed in cells of megakaryocytic lineage, it is also expressed in HSCs and HPCs and seems to play a role in maintaining HSCs [66–68]. TPO, generated by the HSC niche, seems to maintain HSC quiescence [69]. Based on that, most ex vivo culture systems use TPO as part of the cytokine cocktail to expand HSCs [70], including UCB stem cells [35]. In a study by Nishino et al. [71], a small molecule agonist of c-MPL, NR-101, was used to expand human UCB stem cells. In comparison to TPO, the study found that NR-101 increased the HSCs and HPCs more efficiently and resulted in 2.3-fold increase in the SCID-repopulating cells over TPO. The effect on megakaryocytopoiesis was comparable. These results are particularly encouraging and suggest NR-101 might be replacing TPO for this indication. The mechanism by which NR-101 activates c-MPL, however, remains unknown.

zVADfmk and zLLYfmk

The ex vivo expansion culture is known to encourage the initiation of apoptosis [22]. Caspase and calpain, both cysteine proteases, have been implicated in graft apoptosis in neuronal cells [72]. Thus, Sangeetha et al. [73] studied the addition of protease inhibitors zVADfmk (caspase inhibitor) and zLLYfmk (calpain inhibitor) to the culture of UCB CD34(+) cells. The addition of these inhibitors preferentially expanded the CD34(+) cell content by 3.5- to 4-fold relative to the control, with no enhancement of differentiation.

ER chaperone protein GRP94

As was previously mentioned, integrins play an important role in homing. Glucose-regulated protein 94 (GRP94) is an endoplasmic reticulum chaperone protein that is required for the expression of many integrins [74]. Luo et al. created a mouse model in which they could knock-out the GRP94 gene. HSCs that did not express GRP94 were also lacking α4 integrin. Not surprisingly, knock-out mice had impaired HSC homing to the niche, with an expanded population of primitive HSCs.

Prostaglandin E2 (PGE2)

Based on previous findings that PGE2 increases HSC numbers in vitro, as well as in ex vivo, Goessling et al. [75] investigated the use of PGE2-pretreated HSCs in murine transplant models. They found that UCB HSCs do express PGE2 receptors and respond to ex vivo PGE2 stimulation. The PGE2 treated human UCB cells had lower apoptosis

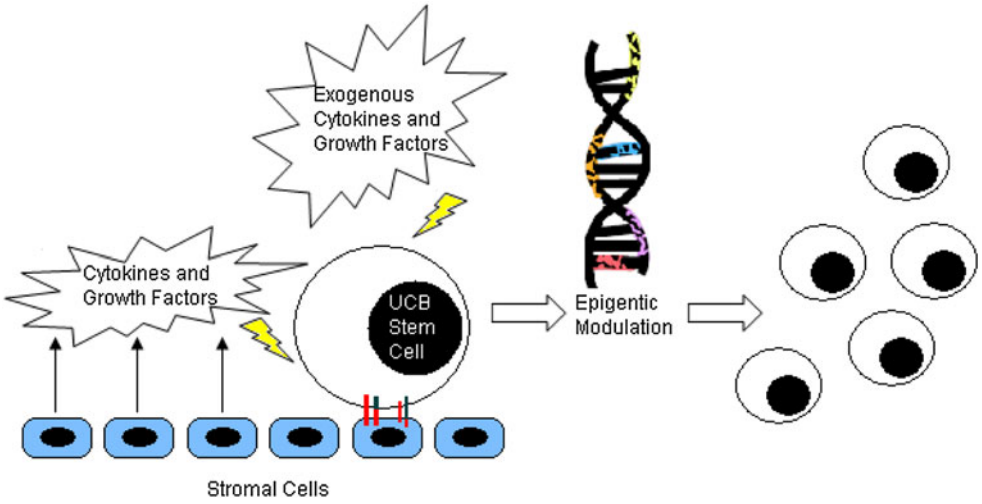

Fig. 1 Various aspects of culture conditions that lead to effective expansion of UCB cells ex vivo

levels and increased proliferation. They were also better able to engraft NOD/SCID mice.

Aryl hydrocarbon receptor antagonist SR1

Boitano et al. [76] found that StemRegenin 1 (SR1), when added to other cytokines in culture media, increased hematopoietic stem and progenitor cell as much as 10-fold compared to cytokines alone. When SR1 was removed, the cells rapidly differentiated. UCB CD34(+) cells expanded with SR1 were better able to engraft NOD/SCID mice, in both the short and long term, than uncultured cells or cells expanded in cytokines alone. Boitano et al. suggest the inhibition of aryl hydrocarbon receptor as a mechanism for these effects.

Impact of epigenetics on cord blood stem cell expansion

Human cord blood stem cells expanded ex vivo, using serum-free medium, seem to lose their ability to repopulate the BM of irradiated NOD/SCID mice [24]. This is felt to be partly secondary to 'reversible silencing' of engraftment potential in the expanded stem cell population [24]. During the expansion process, these stem cells transit through different phases of cell cycle but they fail to enter G_0, which results in defects in BM repopulating capacity. This defect is felt to be related to epigenetic mechanisms and can be potentially reversed with the use of hypomethylating agents [77]. As supportive evidence, hypomethylating agents [78] and histone deacetylases [79] have been shown to improve stem cell self-renewal activity and engraftability, respectively [78, 79]. One specific example of this is Garcinol, a histone acetyltransferase inhibitor that stimulated expansion of CD34(+) HSCs [80].

Conclusions

Though ex vivo expansion of cord blood cells is far from being perfect, a lot has been learned about the process. With its reduced risk of chronic GVHD, UCB has the potential to be the default unrelated donor source for allogeneic transplant in patients with various hematologic and non-hematologic conditions for whom allogeneic transplant is considered a potentially curative procedure. Efforts to expand cord blood stem cells are fundamental to overcoming the delayed engraftment and delayed immunoreconstitution limitations of cord blood transplantation. The ultimate technique to achieve this task should combine growth factors, stromal support, and possibly epigenetic modulation (Fig. 1). Insight into the basic mechanisms that govern HSC fate will help in these efforts that aim at UCB stem cell expansion without significant differentiation.

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Conflict of interest The authors declare that they have no conflict of interest.

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