Many novel approaches currently under preclinical and clinical investigation are aimed at enhancing engraftment in CBT recipients. These can be broadly categorized as methods for increasing the absolute numbers of hematopoietic stem and progenitor cells (HSPCs) available at the time of infusion (ranging from increasing the number of cells obtained at collection to infusion of more than one unit of CB or infusion of third-party donor cells and ex-vivo expansion of CB HSPCs) and methods for enhancing the homing capacity of the limited number of HSPCs available in a single or double CB graft. This chapter focuses on ex-vivo expansion of CB HSPCs to overcome delayed engraftment, including initial attempts with cytokine-based expansion systems as well as the more recent approaches of stromal-based culture systems and novel methodologies focused on the ex-vivo manipulation of signaling pathways that may influence HSC self-renewal, namely the Notch signaling pathway. The latter two approaches result in clinically relevant expansions, providing significantly higher numbers of cells for infusion with very promising preliminary data demonstrating that infusion of ex-vivo-expanded CB grafts can result in rapid myeloid (and improved platelet) recovery after CBT.

**Cytokine-Mediated Ex-Vivo Expansion**

As discussed in more detail in other chapters of this book, understanding of the hierarchical development of the hematopoietic system has evolved significantly over the last few decades. This is particularly true with respect to advances in the understanding of regulation of hematopoiesis by the bone marrow microenvironment, as well as better
understanding of the hematopoietic growth factors that support the progressive maturation of the various hematopoietic cell lineages. Less, however, is known about factors that govern the self-renewal of HSPCs, which until recently significantly affected the ability to successfully expand HSCs ex vivo for clinical application. Initial attempts at ex-vivo expansion of HSCs focused on the use of soluble cytokines known to support lineage-committed cells, with the expectation that some of these factors also supported HSC proliferation.3 These studies were based on the belief that cell lineage determination was a stochastic process combined with positive and negative cytokine-mediated regulatory responses controlling survival and expansion of the stem cell population.4 However, these early attempts to expand adult HSCs were clinically ineffective because of the inability to generate sufficient cell numbers and differentiation of the starting HSPC population, resulting in significant expansions of committed myeloid progenitor cells but only a two- to fivefold net increase in long-term repopulating cells after 4 to 21 days in culture.5-8 Despite these results, clinical trials have been conducted using cytokine-based expansion methods and, significantly, have demonstrated the safety of this approach, achieving either modest or no effect on the time to neutrophil recovery.9-12 In a trial of CBT in individuals with hematologic malignancies undergoing high-dose preparative regimens, Shpall and colleagues used a three-cytokine cocktail consisting of stem cell factor (SCF), thrombopoietin (TPO), and granulocyte colony-stimulating factor (G-CSF) to expand a portion of single CB units for a total of 10 days. Patients either received both the nonmanipulated fraction and the ex-vivo-expanded fraction on day 0 or (in the cases where the unit was cryopreserved in a single fraction) received the nonmanipulated fraction on day 0 and the remaining fraction on day 10 of expansion.13 Although modest progenitor cell expansion (fourfold median) was achieved and no toxicities were observed, the time to neutrophil engraftment was not reduced compared to historical controls, with the median time to ANC >500/µL being 28 days in the 37 patients that were enrolled. Based on the favorable safety and feasibility data demonstrated in this trial, a dCBT randomized trial is now under way in which patients are randomized to receive either two nonmanipulated CB units or one nonmanipulated unit and one unit expanded ex vivo.14,15 Although interim analysis shows no significant difference in time to neutrophil engraftment overall, an effect was seen in a subset of patients who received reduced-intensity conditioning (median time to neutrophil engraftment of 7 days vs 14 days). Similarly, Kurtzberg and colleagues reported preliminary data from a Phase I trial in which a portion of a single CB unit was cultured for 12 days using the Aastrom Replicell (Aastrom Biosciences, Ann Arbor, MI) bioreactor in the presence of cytokines (erythropoietin, PIXY321, and Flt-3 ligand) and supplemented with both fetal bovine serum and horse serum.16 The expanded cells were then infused on day +12 to augment the transplant. The average fold expansion of TNCs was 2.4, and no increase in CD34+ fold expansion was achieved (average of 0.5-fold expansion). Clinically, improvement in time to neutrophil or platelet engraftment was not observed.

The above studies demonstrate that cytokine-mediated expansion methods are safe but generate only moderate increases in progenitor cell numbers with, at best, modest improvements in clinically relevant outcomes such as time to neutrophil recovery. This lack of clinical success has led to a paradigm shift in current approaches to ex-vivo expansion to target molecular pathways involved in stem cell self-renewal, including those that play fundamental roles in governing cell-fate decisions throughout development. Work at the author's laboratory with the Notch signaling system is an example of one such approach.
Notch-Mediated Expansion and Clinical Translation

A role for Notch in hematopoiesis was initially suggested by detection of the human Notch-1 gene in CD34+ or CD34+Lin- human hematopoietic precursors. Additional studies by the author's group demonstrated that use of a retrovirus to overexpress the intracellular domain of Notch-1 in primary murine HSCs led to emergence of enhanced self-renewal and an immortalized pluripotent cytokine-dependent cell line capable of multilineage in-vivo repopulation, demonstrating a role for Notch in HSPC self-renewal. These findings further suggested that manipulation of the Notch signaling pathway ex vivo in primary HSCs could prove to be a novel approach for expanding HSPCs. To this end and to avoid the potential safety concerns of retroviral transduction, the group engineered a Notch ligand consisting of the extracellular domain of the Notch ligand Delta-1 in order to activate endogenous Notch signaling in HSPCs during ex-vivo expansion.

Initial use of engineered ligand immobilized to the tissue culture surface revealed profound effects on the growth and differentiation of isolated murine marrow precursors with a multilog increase in the number of Sca-1+Gr-1- cells with short-term lymphoid- and myeloid-repopulating ability. With the goal of generating increased numbers of HPCs capable of providing rapid myeloid recovery in vivo for patients undergoing CBT, this ex-vivo expansion approach was extended to human CB HSPCs, where a response of CB HSPCs to Notch ligand was noted. In optimizing this methodology for clinical application, it was determined that there was a ligand dose-dependent effect in culture, whereby relatively lower densities of immobilized ligand substantially enhanced generation of nonobese diabetic/severe combined immunodeficiency (NOD/SCID) repopulating cells. In contrast, culture of CB HSPCs in the presence of higher ligand densities promoted differentiation toward the T-cell lineage at the expense of repopulating cells. Similarly, concurrent murine studies found that the relatively lower amount of Notch signaling induced in cells cultured with lower densities of Delta-1 led to self-renewal of progenitors with primarily B-lymphoid and myeloid potential, whereas higher amounts of Notch signaling inhibited B-cell differentiation and promoted differentiation toward the T-cell lineage. In further preclinical assessments of this methodology, incubation of CB CD34+ cells with immobilized ligand Delta-1 and fibronectin fragments and serum-free media supplemented with cytokines [SCF, TPO, Flt3 ligand, interleukin (IL)-3, IL-6] led to a >2-log increase in the number of CD34+ cells and nearly a 16-fold increase in NOD/SCID mouse repopulating cell frequency when cultured on Delta ligand compared to control.

Moreover, the expanded progenitors were capable of rapid reconstitution of the myeloid compartment in immunodeficient mice, indicating their potential clinical utility. In-vivo persistence of transplanted cells at 9 weeks was observed, and positive reconstitution of secondary transplant recipients also suggested the presence of both long-term and short-term repopulating cells following culture of human CB progenitor cells on Notch ligand. Thus, based on the results of these preclinical studies, this methodology is now under clinical investigation.

An ongoing Phase I trial involves transplantation of a nonmanipulated unit along with CB progenitors from a second CB unit that have undergone Notch-mediated ex-vivo expansion. The primary objective is evaluation of the safety of infusing the ex-vivo-expanded CB progenitors, while secondary objectives include evaluation of the kinetics and durability of hematopoietic reconstitution and the relative contribution to engraftment as provided by the expanded and nonmanipulated CB units. To date, 11 patients with high-risk acute leukemia have been treated, with a median age of 26 years (range = 3 to 43) and a median weight of 57 kg (range = 16 to 79).