Summary & Conclusions
Ex vivo expansion of hematopoietic stem/progenitor cells are attempted worldwide to circumvent the major problem of low stem/progenitor numbers. This strategy is of particular importance pertaining to the cord blood derived stem cells owing to their lower collection volume which limits the use of stem cells for various therapeutic purposes inspite of the inherent attractive features compared to the other adult stem cell sources. Therefore, in the present scenario the adverse effects of pooling of the multiple cord blood samples can be obviated if we can expand the functional HSPCs from a single CB unit. However, the occurrence of differentiation, apoptosis and cellular homing defects involved during culture, even in presence of well optimized cytokines combination have made new strategies mandatory for their in vitro expansion. One of the important physiological mechanisms which control the stem cell compartment is the cellular apoptosis. Their function in asymmetric cell division in drosophila has opened up a new, conserved and previously unexpected role of the apoptosis components in stem cell functionality.

Our present study which involves the expansion of cord blood CD34+ cells with cytokines in the presence of caspase inhibitor; zVADfmk or calpain inhibitor; zLLYfmk is a novel step in this direction. In the present study we made a systematic effort to analyze the role of apoptosis inhibition during the expansion of CB derived HSPCs.

After isolation of CD34+ cells from cord blood samples, we cultured these cells in serum-free media with cytokines alone (as reported in previous studies) and in media with cytokines in the presence of pan caspase inhibitor zVADfmk or calpain 1 inhibitor zLLYfmk for 10 days. Surprisingly we have observed about 3 fold increase in primitive stem cell (CD34+CD38-) population in cells which were cultured in presence of either zVADfmk or zLLYfmk compared to cells grown in cytokines alone, along with significant reduction in apoptosis cascade. Also, the improvised culture method of cord
blood stem cells, helped to increase the total nucleated cell yield about 30 to 40 folds compared to freshly isolated CD34+ cells in 10 days. We observed a preferential expansion of CD34+ cell content which was a result of an increased transcription of the CD34 gene.

The analysis of signaling molecules that participate in HSC renewal and expansion demonstrated that the use of zVADfmk/zLLYfmk up regulated Notch, Wnt5a and fork head transcription factors which are crucial for reducing oxidative stress and for the stem cell pool expansion.

The functional properties of these cultured cells showed a significant increase in total CFU and LTC units. The other important factor in the functional biology of expanded graft was to assess their in vitro and in vivo homing ability. The SDF1-CXCR4 axis was functionally more efficient in the test expanded cultures compared to the cytokine cultured counterpart. Moreover, these cultures were superior in preserving the adhesion molecules and major integrins on their surface. The concerted consequences of these observations resulted in an enhanced migration and adhesion of the zVAFfmk/zLLYfmk cultures in vitro.

The cytoskeletal re arrangements are pertinent for efficient homing. When we analyzed the polymerization kinetics of the expanded test HSPCs, we observed that, these cells respond to SDF1 stimuli within a short time frame. This provided the test HSPCs to an early and higher polarization, facilitating the chemotaxis towards SDF1. The higher migration was not merely due to a higher cell polarization, but the presence of these compounds upregulated functional CXCR4 on the cell surface. However, the cytoplasmic pool of the CXCR4 was unaffected in these cultures.

To explore the reason behind the higher actin polymerization, we analyzed the expression of RhoGTPase members RhoA, Rac1 and Cdc42. The presence of these compounds enhanced the expression of these molecules at both
protein and transcript levels hinting towards a possible activation of RhoGTPases.

The clinical utility of the compounds were assessed in the cryopreservation studies in two steps: first, dealing with the expansion of frozen CD34+ cells and second in the cryopreservation of expanded cells. We observed a potential role of these additives in the cryotechnology, as the functionality of the test cultures were maintained and also further augmented upon additional supplementation of either zVADfmk/zLLYfmk in the freezing medium.

The in vitro functions of the expanded HSPCs, correlated well in improving the in vivo homing (24 hrs), short term engraftment (4 weeks) and long term engraftment (16 weeks) of the cells expanded in the presence of zVADfmk/zLLYfmk as assessed in NOD/SCID mice repopulation experiments.

The important conclusions and novel findings documented in the present work are:

➤ The activation of apoptosis cascade during cytokine mediated expansion negatively affects the cellular functions of hematopoietic stem/progenitor cells (HSPCs).

➤ Transient regulation of apoptosis using cell permeable inhibitors of caspases and calpains are feasible and safe.

➤ The presence of zVADfmk/zLLYfmk upregulated CD34+ cell content which was a result of the higher transcription of the CD34 gene.

➤ The presence of protease inhibitors along with cytokines upregulated the major signaling molecules like Notch-jagged, Wnt5, and Foxo3a which may account for one among the resons for the improved expansion outcome.
zVADfmk/zLLYfmk cultures showed a higher multipotent progenitor content as assessed from CFU assays conducted after the 10th day and 4 weeks of expansion and also after the LTC assay.

The expanded test HSPCs showed a higher expression of functional CXCR4 and several adhesion molecules and integrins resulting in an enhanced migration and adhesion in vitro.

The actin polymerization studies demonstrated a higher and early response to activating stimuli resulting in a polarized distribution of F actin, higher expression of RhoA, Rac1 and Cdc42.

We observed that these two compounds were equally effective in expanding frozen thawed CB samples and also for the cryopreservation of expanded HSPCs underscoring their clinical utility.

Final functional assessment in the NOD/SCID mouse showed an augmented homing and long term multilineage engraftment potential of the test HSPCs compared to the control.

Thus, through this study, we have demonstrated a novel application of these apoptotic protease inhibitors in increasing the CD34+ cell content of the graft during ex vivo expansion. These data may help in resolving some of the persistent problems faced during cytokine expansion of HSCs. Moreover, the study may yield a functionally superior graft which can be effectively cryopreserved for future use. The outcome of this study may also compliment the existing protocols for expansion and transplantation, opening up new avenues in the field of apoptosis and stem cell biology.