# Improved *Ex Vivo* Expansion of Hematopoietic Progenitor Cells and their Differentiation into Megakaryocytes and Erythroid Cells in a Novel Serum-Free Medium, StemSpan SFEM II

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## Abstract

We developed a new serum-free medium, StemSpan SFEM II, for *ex vivo* expansion and lineage-specific differentiation of human hematopoietic progenitor cells (HPCs). SFEM II is an enhanced version of the original SFEM medium, which has been supplemented with a chemically-defined lipid formulation. To compare the performance of SFEM II to other media formulated for HPC expansion, cord blood (CB) CD34$^+$ cells ($10^4$/mL) were cultured in SFEM II and, as controls, in SFEM I, H3000, and animal component-free (ACF) medium. The cultures were supplemented with Flt-3 Ligand (FL), stem cell factor (SCF), interleukin (IL)-3, and IL-6. After 7 days, cells were counted, analyzed for CD34 expression and in some experiments assayed to measure colony-forming units (CFUs). Cell viability was high in all cultures (range 93 to 99%; n=10). Total nucleated cell (TNC) expansion in H3000, SFEM I, and ACF media averaged 40-fold (range 14 to 76-fold) and was not significantly different between the three media (P>0.1; paired t-test). TNC expansion in SFEM II medium was significantly higher (on average 60-fold expansion; range 23 to 101-fold; p<0.01; n=10). CD34$^+$ cell output was also highest in SFEM II, but only the differences with H3000 and SFEM I were significant (p<0.01). CFC expansion showed similar differences, with highest expansion of BFU-E, CFU-GM, and CFU-GEMM numbers in SFEM II, and lowest in H3000. The capacity of SFEM II and SFEM I to support megakaryocytic (Mk) and erythroid progenitor expansion and differentiation of CD34$^+$ cells was tested in cultures that were refed on days 4, 7, and/or 10 to maximize expansion and prevent overgrowth, and analysed on day 14. Using a Mk-selective cytokine cocktail [thrombopoietin (TPO), SCF, IL-6, and IL-9], expansion of CD45$^+$CD41$^+$ cells ranged from 13 to 124-fold in eight different CB samples cultured in SFEM I and from 18 to 326-fold in SFEM II, with on average 2-fold higher expansion in SFEM II (p<0.01; n=8). In erythroid-selective cytokines (SCF, IL-3, erythropoietin) and with a glucocorticoid receptor agonist (dexamethasone or hydrocortisone), both media strongly supported production of erythroid cells with 200 to 6,500-fold and 900 to 7,000-fold expansion of cells expressing glycophorin-A (GpA) and/or CD71 in SFEM I and SFEM II, respectively (p<0.05; n=7). These data indicate that StemSpan SFEM II is a superior medium to support the expansion of CB CD34$^+$ progenitors and to promote their differentiation into the Mk and erythroid lineages. SFEM II should prove useful for the continued development of new cellular therapy applications of culture-expanded HSPCs and mature blood cells.

## Materials and Methods

- Cord blood CD34$^+$ cells isolated by EasySep HPC Enrichment Kit (STEMCELL, Cat. # 19356) or EasySep Human Cord Blood CD34$^+$ Selection Kit (STEMCELL, Cat. # 18096) were plated at 10,000 CD34$^+$ cells per mL.
- Culture conditions for TNC and CD34$^+$ cell expansion: FL, SCF, IL-3, and IL-6 (CC100 cytokine cocktail); 7 days of culture.
- Culture conditions for Mk expansion and differentiation: TPO, SCF, IL-6, and IL-9 (CC220 cytokine cocktail); 14 days of culture with addition of fresh medium on day 7.
- Culture conditions for erythroid expansion and differentiation: SCF, IL-3, and EPO and either 1 µM dexamethasone or hydrocortisone; 14 days of culture with medium changes or replating on days 4, 7, and 10.
- Analysis after culture: Total and viable cell counting and immunophenotyping by flow cytometry; CFU assay by replating in semisolid medium (MethoCult H4034).

## Results

*Total and CD34$^+$ cell expansion in 14-day cultures in four StemSpan media supplemented with FL, SCF, IL-3, and IL-6 (CC100 cytokine cocktail).*

Approximately 10-11% of cells in SFEM I, SFEM II, and ACF media retained CD34$^+$ expression, while the %CD34$^+$ cells in StemSpan H3000 was 2-fold lower (5.6%; p<0.01, n=10; **Figure 1**). TNC expansion in H3000, SFEM I, and ACF averaged 40-fold (range 14 to 76-fold; p>0.1, paired t-test) and was significantly higher in SFEM II (average 60-fold; range 23 to 101-fold; p<0.01; n=10) (**Figure 2A**). CD34$^+$ cell expansions in SFEM II and ACF were similar but higher than in SFEM I and H3000 (p<0.01; **Figure 2B**). Expansion of CFUs showed similar differences and similar results were obtained in cultures of bone marrow CD34$^+$ cells and after stimulation with SCF, FLT3-L, and TPO (CC110 cytokine cocktail; data not shown).

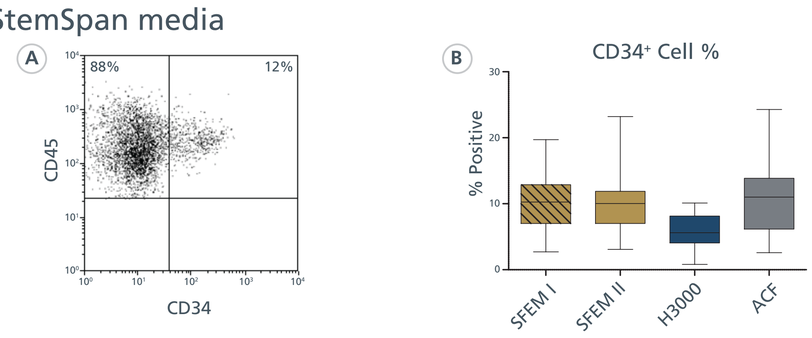
*Megakaryocyte cultures in SFEM I and SFEM II supplemented with TPO, SCF, IL-6, and IL-9 (CC220 cytokine cocktail).*

Both media yielded Mk lineage cells with similarly high purity, i.e., %CD45$^+$CD41a$^+$ cells = 90 ±6 and 88 ±6 in SFEM I and SFEM II, respectively (p=0.2; n=8; **Figure 3A,B**). Average Mk cell output in SFEM II (98 CD45$^+$CD41a$^+$ cells per input CD34$^+$ cell) was two-fold higher than in SFEM I (48 Mk per input CD34$^+$ cell; p<0.01; n=8; **Figure 3C**). Mk expansion in SFEM I was promoted by supplementation with low-density lipoproteins (LDL, 20 ug/mL), but Mk expansion in SFEM II was 1.6 ±0.2 fold higher than in SFEM I + LDL (range 1.3 to 1.8-fold; p<0.05; n=4; **Table 1**). Approximately 90% of the CD41a$^+$ cells expressed the late Mk marker CD42. CD41a$^+$CD42$^+$ platelet-like particles with similar light scatter properties as fresh blood platelets were also detectable, indicating that the media supported terminal Mk differentiation (data not shown).

*Erythroid cultures in SFEM I and SFEM II supplemented with SCF, IL-3, EPO, and glucocorticoid receptor agonist (dexamethasone or hydrocortisone at 1 µM).*

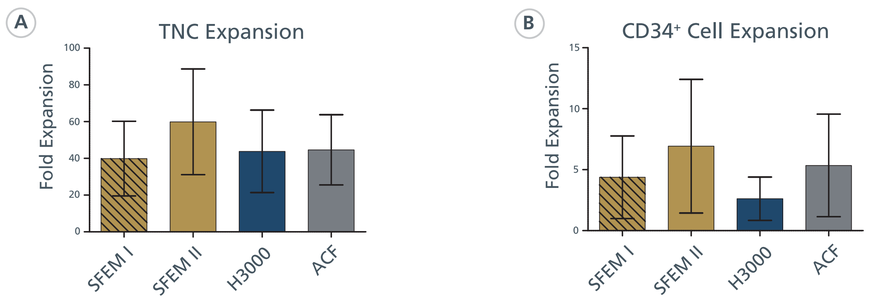
Both media yielded similar erythroid cell frequencies: average ±SD = 76 ±13% and 81 ±6% in SFEM I and II, respectively (p=0.3; n=7; **Figure 4A,B**). Approximately 40-60% of cells were CD71$^+$GpA$^+$ erythroblasts, while CD71$^+$GpA$^-$ pro-erythroblasts and CD71$^-$GpA$^+$ normoblasts were much less frequent (**Figure 4B**). Cell output in SFEM II (average: 2,800 erythroblasts per input CD34$^+$ cell; range 900 to 7,000) was ~20% higher than in SFEM I (average: 2,300; range 200 to 6,500; p<0.05; n=7; **Figure 4C**). Cumulative cell output in three cultures that were maintained for 26 days ranged between $10^5$ and $10^6$ erythroblasts per input CD34$^+$ cell (data not shown).

FIGURE 1: CD34 expression after 7 days of culture of purified CB CD34$^+$ cells in four different StemSpan media



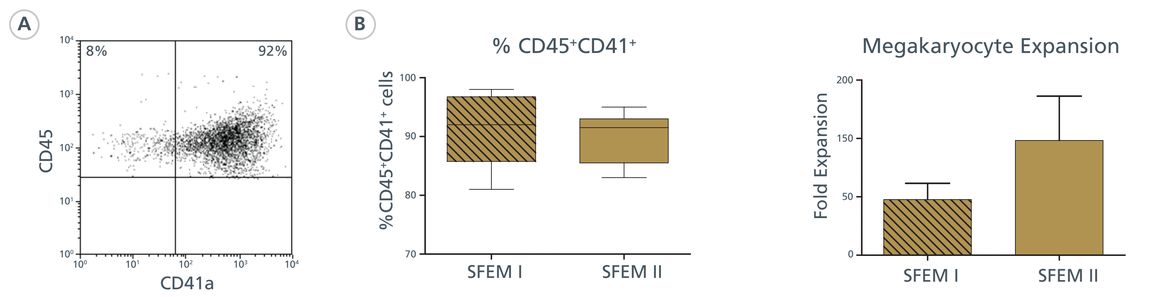

**A)** Flow cytometry dot plot showing CD45 and CD34 expression after 7 days of culture of CB CD34$^+$ cells in medium supplemented with FL, SCF, IL-3, and IL-6 (CC100 cytokine cocktail). **B)** Percentage of CD34$^+$ cells as measured by flow cytometry as depicted in the example plot shown in A. Culture of CB CD34$^+$ cells in SFEM I, SFEM II, and ACF media resulted in similar frequencies of CD34$^+$ cells (p>0.5; paired t-test; n=10), which were ~2-fold higher than in H3000 medium (p<0.01). Box and whisker plot show median, 25-75 percentile and full data range.

FIGURE 2: Total and CD34$^+$ cell expansion after 7 days of culture of purified CB CD34$^+$ cells in four different StemSpan media




Average fold increase of **A)** Total nucleated cells (TNC) and **B)** CD34$^+$ cells after 7 days of culture. Bars represent standard deviation (n=10). SFEM II supported, on average, ~50% higher TNC expansion compared to the other three media (p<0.01). SFEM II and ACF media supported similar ~8-fold CD34$^+$ cell expansion (p>0.5; paired t-test; n=10), which was ~2-fold higher than in H3000 medium (p<0.01).

FIGURE 3: Megakaryocyte outgrowth of CB CD34$^+$ cells in two StemSpan SFEM media

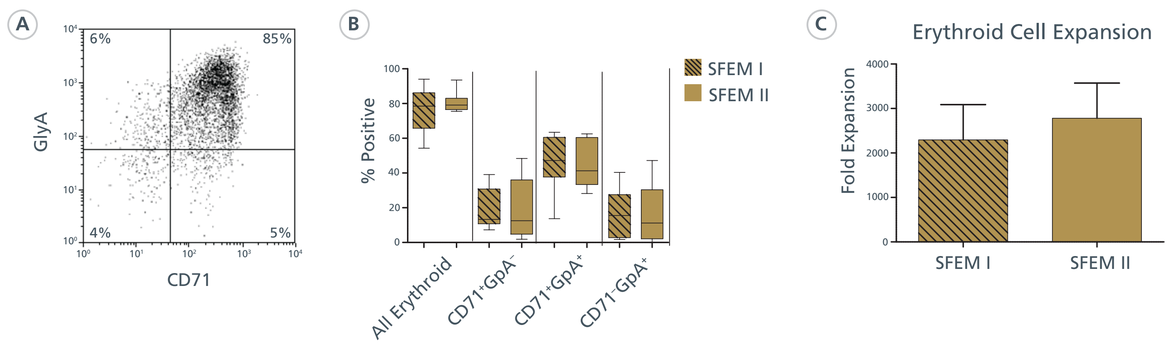



**A)** Example of CD45 and CD41 expression after 14 days of culture of CB CD34$^+$ cells under Mk-specific conditions (TPO, SCF, IL-6, and IL-9, CC220 cytokine cocktail). **B)** Percentage of CD45$^+$CD41$^+$ Mk was measured by flow cytometry as depicted in the sample plot shown in A. Box and whisker plot show median, 25-75 percentile and full data range. **C)** Average fold Mk expansion, expressed as number of CD45$^+$CD41$^+$ cells generated per input CD34$^+$ cell. Bars represent standard deviation (n=8).

TABLE 1: SFEM II supports higher Mk output in cultures of CB CD34$^+$ cells compared to SFEM I supplemented with low-density lipoproteins

| | Fold expansion of CD45$^+$CD41$^+$ cells | | |
|---|---|---|---|
| **Exp** | **SFEM I** | **SFEM I + LDL** | **SFEM II** |
| 1 | 16.0 | 19.7 | 35.7 |
| 2 | 20.7 | 25.8 | 44.3 |
| 3 | 47.0 | 53.2 | 67.6 |
| 4 | 124.1 | 213.6 | 326.4 |
| **Average** | **52.0** | **78.1** | **118.5** |
| **SD** | **50.0** | **91.5** | **139.2** |

FIGURE 4: Erythroid outgrowth of CB CD34$^+$ cells in two StemSpan SFEM media




**A)** Example of CD71 and GpA expression after 14 days of culture of CB CD34$^+$ cells under erythroid-specific culture conditions (SCF, IL-3, EPO, dexamethasone). **B)** Percentage of all erythroid cells and of erythroid subsets identified on the basis of CD71 and GpA expression as shown in A. Box and whisker plot show median, 25-75 percentile and full data range. **C)** Average fold expansion of all erythroid cells (CD71$^+$ and/or GpA$^+$) expressed as number of cells generated per input CD34$^+$ cell. Bars represent standard deviation (n=7).

## Conclusions

- **StemSpan SFEM II supports stronger *ex vivo* expansion of human CB and BM hematopoietic cells, with higher numbers of nucleated cells and CD34$^+$ cells in short-term (7 days) cytokine supplemented cultures than original StemSpan SFEM (SFEM I), a xeno-free medium (StemSpan H3000) and an animal and human serum protein-free medium (StemSpan-ACF).**
- **StemSpan SFEM II strongly supports megakaryocyte (Mk) expansion and differentiation of CB CD34$^+$ cells under Mk-specific culture conditions, resulting in ~100-fold cell expansion and generation of ~90% pure Mk lineage cells after 14 days of culture.**
- **StemSpan SFEM II also strongly supports erythroid expansion and differentiation of CB CD34$^+$ cells under erythroid-specific culture conditions, resulting in large cell expansion (>$10^3$ and >$10^5$ fold after 14 and 26 days, respectively) and generation of highly pure populations of CD71$^+$ and/or Glycophorin-A$^+$ erythroid lineage cells.**