Enhanced Ex Vivo Expansion of Hematopoietic Stem Cells by a Novel Compound Cocktail

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INTRODUCTION

Hematopoietic stem cells (HSCs) are essential for curative therapies targeting hematologic and immune disorders. However, a major barrier to wider clinical and gene therapy applications is the inability to expand functional, long-term (LT) repopulating HSCs ex vivo. Conventional cytokine-based culture systems often promote progenitor proliferation at the expense of self-renewal, resulting in diminished engraftment potential. Despite progress with small molecule modulators and niche-mimetic systems, most ex vivo culture strategies fail to sustain bona fide HSCs. To address this, we developed StemSpan™ HSC Plus Supplement, a small molecule-based cocktail that, when used with cytokine-enriched supplements and serum-free media, enables robust expansion of phenotypically defined primitive or LT-HSCs (CD34+CD90+CD45RA–CD133+EPCR+) in ex vivo cultures. StemSpan™ HSC Plus Supplement significantly increases both the frequency and fold expansion of primitive HSC subsets sourced from cord blood (CB), bone marrow (BM), and mobilized peripheral blood (mPB). Importantly, cells expanded under these conditions retain long-term repopulating capacity in NSG mouse xenograft models, demonstrating functional preservation beyond phenotypic enrichment. Moreover, the supplement supports efficient genome editing workflows. In CRISPR-Cas9–mediated knockout studies, mPB-derived CD34+ cells cultured with HSC Plus supplement yielded higher gene-edited HSCs (CD34+CD45RA-CD90+EPCR+) without compromising viability or editing efficiency. This optimized culture system offers a robust and scalable solution for improving HSC numbers and potency following ex vivo manipulation, empowering advances in transplantation, gene-editing, and cell-based therapies.

METHODS

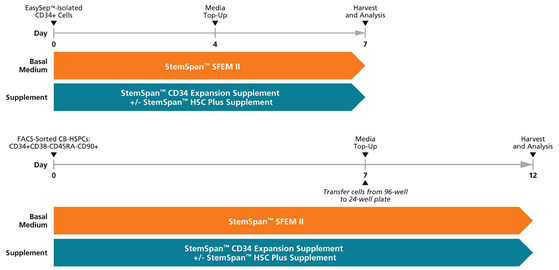


FIGURE 1. Protocol for Ex Vivo Expansion of HSCs

CD34+ cells were isolated from cord blood (CB), mobilized peripheral blood (mPB), or bone marrow (BM) using the applicable EasySep™ Human CD34 Positive Selection Kit II (CB, Catalog #17896; mPB, #100-1569; BM, Catalog #17856). For optimal HSC expansion, cells were cultured in complete medium consisting of a StemSpan™ Medium (SFEM, SFEM II, XF, or AOF) supplemented with a cytokine-based supplement (StemSpan™ CC100, CC110, or CD34+ Expansion Supplement), and StemSpan™ HSC Plus Supplement (omitted in control samples), as specified for each experimental group. Isolated CD34+ cells were plated at a density of 4×10^5 cells per well in 0.4 mL of complete medium in 24-well plates. On day 4, an equal volume of fresh complete medium was added. On day 7, cells were harvested for analysis.
For purified HSC cultures, CD34+CD38-CD45RA+CD90+ cells were FACS sorted and plated at a density of 200 cells per well of a 96-well plate in complete medium. After 7 days, cells were transferred from the 96-well plate into a 24-well plate, and fresh complete medium was added. Cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2 throughout culture duration. On the day of harvest, viable cell were counted using either a Guava® flow cytometer or an automated cell counter. For phenotypic analysis, cells were stained and analyzed by flow cytometry for expression of HSC-specific surface markers, including CD34, CD45RA, CD90, CD133, and EPCR.

RESULTS

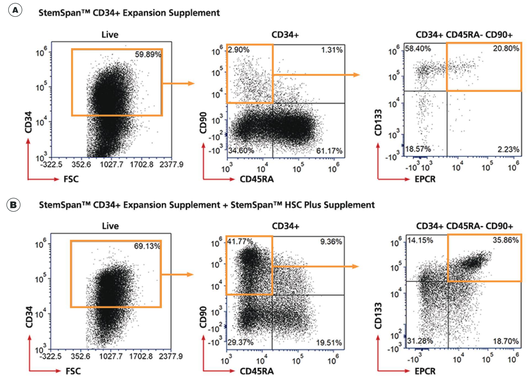


FIGURE 2. Gating Strategy for Immunophenotyping Cord Blood-Derived CD34+ Cells Expanded for Seven Days in StemSpan™ Medium With or Without StemSpan™ HSC Plus Supplement

Purified CD34+ cells derived from cord blood (CB) were cultured for 7 days in StemSpan™ SFEM II medium supplemented with **(A)** StemSpan™ CD34+ Expansion Supplement alone (**B**) or together with StemSpan™ HSC Plus Supplement. After 7 days, the cultured cells were stained with fluorescently labeled antibodies against CD34, CD45RA, CD90, EPCR, and CD133, in addition to viability dye Zombie Yellow™, and analyzed by flow cytometry. Hematopoietic stem and progenitor cell (HSPC) subsets were gated using fluorescence-minus-one controls. Sequential gates (orange boxes) were used to determine the percentages of viable CD34+ cells, CD34+ CD45RA-CD90+ cells and CD34+CD45RA-CD90+CD133+EPCR+ cell subsets.

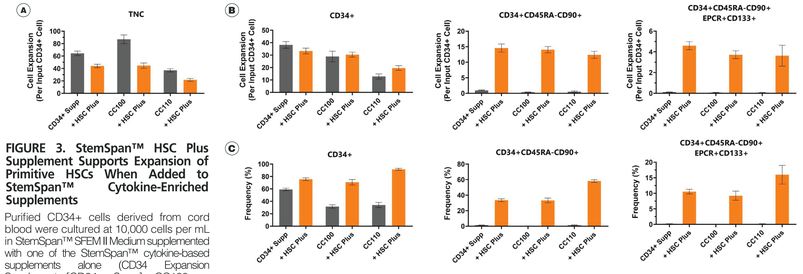


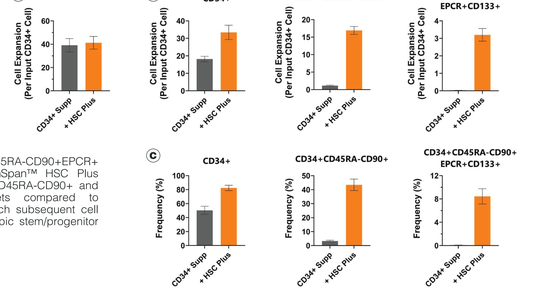
FIGURE 3. StemSpan™ HSC Plus Supplement Supports Expansion of Primitive HSCs When Added to StemSpan™ Cytokine-Enriched Supplements

Purified CD34+ cells derived from cord blood were cultured at 10,000 cells per mL in StemSpan™ SFEM II Medium supplemented with one of the StemSpan™ cytokine-based supplements alone (CD34 Expansion Supplement [CD34+ Supp], CC100, or CC110; grey bars), or together with StemSpan™ HSC Plus supplement (HSC Plus; orange bars). After 7 days of culture, **(A)** total nucleated cell (TNC) expansion, **(B)** expansion, and **(C)** frequency of CD34+ HSPCs and primitive HSC subsets (CD34+CD45RA-CD90+ and CD34+CD45RA-CD90+EPCR+CD133+) were analyzed by flow cytometry. StemSpan™ HSC Plus Supplement promoted greater expansion of CD34+CD45RA-CD90+ and CD34+CD45RA-CD90+CD133+EPCR+ HSC subsets compared to cytokine-based supplements alone. Each subsequent cell subset was progressively more enriched for phenotypic stem/progenitor cells. Data represent mean ± SEM (n = 24 for CD34 Expansion Supplement dataset and n = 9 for CC100 and CC110 datasets).

Primitive HSCs from Mobilized Peripheral Blood

FIGURE 4. StemSpan™ HSC Plus Supplement Supports Expansion of Primitive HSCs Derived from Mobilized Peripheral Blood

Purified CD34+ cells derived from G-CSF mobilized peripheral blood were cultured at 10,000 cells per mL in StemSpan™ SFEM II Medium supplemented with StemSpan™ CD34 Expansion Supplement alone (CD34+ Supp; grey bars) or together with StemSpan™ HSC Plus Supplement (HSC Plus; orange bars). After 7 days of culture, the **(A)** total nucleated cell (TNC) expansion, **(B)** expansion, and **(C)** frequency of CD34+ HSPCs and primitive HSC subsets (CD34+CD45RA-CD90+ and CD34+CD45RA-CD90+EPCR+CD133+) were analyzed by flow cytometry. StemSpan™ HSC Plus Supplement promoted higher expansion of CD34+CD45RA-CD90+ and CD34+CD45RA-CD90+CD133+EPCR+ HSC subsets compared to StemSpan™ CD34 Expansion Supplement alone. Each subsequent cell subset was progressively more enriched for phenotypic stem/progenitor cells. Data represent mean ± SEM (n = 6).



Primitive HSCs from Bone Marrow

FIGURE 5. StemSpan™ HSC Plus Supplement Supports Expansion of Primitive HSCs Derived from Bone Marrow

Purified CD34+ cells derived from bone marrow were cultured at 10,000 cells per mL in StemSpan™ SFEM II medium supplemented with StemSpan™ CD34 Expansion Supplement alone (CD34+ Plus; grey bars) or together with StemSpan™ HSC Plus Supplement (HSC Plus; orange bars). After 7 days of culture, the **(A)** total nucleated cell (TNC) expansion, **(B)** expansion, and **(C)** frequency of CD34+ HSPCs and primitive HSC subsets (CD34+CD45RA-CD90+ and CD34+CD45RA-CD90+EPCR+CD133+) were analyzed by flow cytometry. StemSpan™ HSC Plus Supplement promoted higher expansion of CD34+CD45RA-CD90+ and CD34+ CD45RA-CD90+CD133+ EPCR+ HSC subsets compared to StemSpan™ CD34 Expansion Supplement alone. Each subsequent cell subset was progressively more enriched for phenotypic stem/progenitor cells. Data represent mean ± SEM (n = 8).

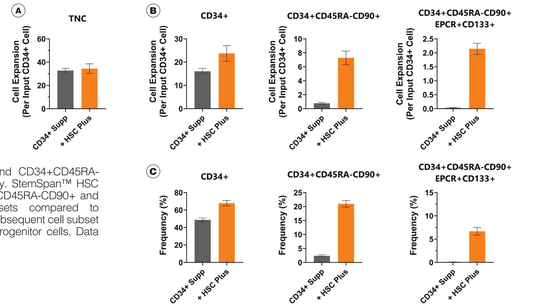
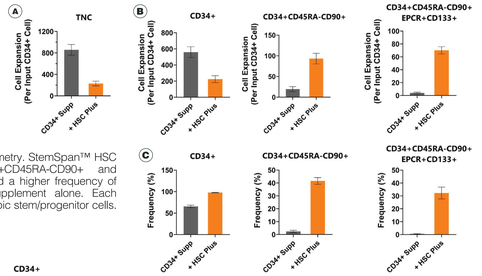


FIGURE 6. StemSpan™ HSC Plus Supplement Supports Expansion of CD34+CD38-CD45RA-CD90+ Purified HSCs

Cord blood-derived CD34+CD38-CD45RA-CD90+ cells were purified by FACS and cultured at a density of 200 cells per well of 96-well plate in StemSpan™ SFEM II medium. Cultures were supplemented either with StemSpan™ CD34 Expansion Supplement alone (CD34+ Supp; grey bars) or together with (HSC Plus; orange bars) StemSpan™ HSC Plus Supplement. After 12 days of culture, the **(A)** total nucleated cell (TNC) expansion, **(B)** expansion, and **(C)** frequency of CD34+ HSPCs and primitive HSC subsets (CD34+CD45RA-CD90+ and CD34+CD45RA-CD90+EPCR+CD133+) were analyzed by flow cytometry. StemSpan™ HSC Plus Supplement promoted greater expansion of CD34+CD45RA-CD90+ and CD34+CD45RA-CD90+CD133+EPCR+ HSC subsets and maintained a higher frequency of primitive HSCs compared to StemSpan™ CD34 Expansion Supplement alone. Each subsequent cell subset was progressively more enriched for phenotypic stem/progenitor cells. Data represent mean ± SEM (n = 3).



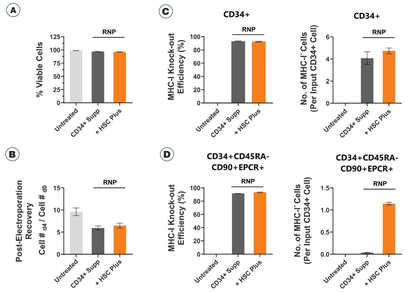


FIGURE 7. StemSpan™ HSC Plus Supplement Supports Efficient Gene Knock-out in Mobilized Peripheral Blood-Derived CD34+ Cells

Purified CD34+ cells derived from mobilized peripheral blood were pre-cultured for 2 days in StemSpan™ SFEM II medium supplemented with StemSpan™ CD34 Expansion Supplement alone (CD34+ Plus; dark grey bars) or together with StemSpan™ HSC Plus Supplement (HSC Plus; orange bars). After two days, ~6 x 10^4 cells were electroporated with ribonucleoprotein (RNP) complexes targeting the *B2M* gene using the Neon® Transfection System. Cells not exposed to the electroporation were cultured with the StemSpan™ CD34 Expansion Supplement and StemSpan™ HSC Plus Supplement (untreated; light grey bars). Four days post-electroporation, all conditions **(A)** maintained >80% viability but **(B)** exhibited reduced cell recovery in RNP-treated conditions. The frequency and yield of MHC-I⁻ cells (i.e. *B2M* knock-out efficiency) in CD34+ cells and primitive CD34+CD45RA-CD90+EPCR+ HSC subset were assessed via flow cytometry. **(C)** The frequency and yield of *B2M* knock-out in CD34+ progenitor populations were similar between cultures with (orange bar) or without (dark grey) StemSpan™ HSC Plus Supplement. **(D)** The yield of primitive CD34+CD45RA-CD90+EPCR+ *B2M* knock-out cells per input CD34+ cell was higher in cultures containing StemSpan™ HSC Plus Supplement. Data represent mean ± SEM (n = 3).

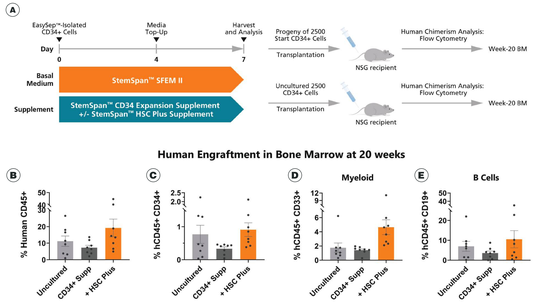


FIGURE 8. Cord-Blood CD34+ Cells Expanded with StemSpan™ HSC Plus Supplement Support Long-term In Vivo Engraftment in Immunodeficient Mice

(A) Illustration of the treatment protocol. Immunodeficient NSG mice received transplants of either uncultured 2,500 cord blood CD34+ cells (Uncultured; light grey bars) or the progeny of 2,500 cord-blood CD34+ cells cultured for 7 days in StemSpan™ SFEM II medium supplemented with StemSpan™ CD34 Expansion Supplement alone (CD34+ Supp; dark grey bars) or together with StemSpan™ HSC Plus Supplement (HSC Plus; orange bars). Long-term multilineage engraftment was assessed in the bone marrow of transplanted NSG mice at 20 weeks post-transplantation. Data represent mean ± SEM (n = 8 mice). Cells expanded with the StemSpan™ HSC Plus Supplement demonstrated similar or higher levels of human engraftment in the bone marrow, as measured by frequency of human **(B)** CD45+ cells, **(C)** CD45+CD34+ progenitor cells, **(D)** CD45+CD33+ myeloid cells, and **(E)** CD45+19+ B cells relative to the other tested conditions. All transplanted cells showed lower or undetectable T cells engraftment (data not shown).

Summary

StemSpan™ HSC Plus Supplement is a novel small molecule-based cocktail that:

- Enhances ex vivo expansion of primitive HSC subsets (CD34+CD45RA-CD90+EPCR+CD133+) from cord blood, bone marrow, and mobilized peripheral blood.
- Can be combined with various cytokine-based media (StemSpan™ CC100, CC110, or CD34 Expansion Supplement).
- Supports high cell viability and efficient CRISPR-Cas9–mediated gene knockout in mPB-derived CD34+ cells.
- Increases the yield of gene-edited primitive HSCs without compromising editing efficiency.
- Retains long-term, multilineage engraftment capacity in NSG mice of culture-expanded HSCs.
- Enables robust and scalable expansion of functional HSCs for gene editing, transplantation, and other therapeutic and non-therapeutic applications.

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