In vitro expansion of fetal liver hematopoietic stem cells

Rashmi Bhardwaj1, Lalit Kumar1, Deepika Chhabra2*, N. K. Mehra1, Atul Sharma1, Sujata Mohanty1 & Vinod Kochupillai2

Fetal liver hematopoietic stem and progenitor cells (HSPCs) have been considered appropriate for the management of aplastic anemia owing to their proliferative potential. Bone marrow recovery was possible in some cases; the engraftment potential of these cells, however, was unsatisfactory, possibly due to the availability of a smaller number of these cells from a single fetus. The present study explores how we can expand fetal liver hematopoietic stem cells under in vitro conditions. We isolated mononuclear cells from fetal liver and hematopoietic stem cells were identified and analyzed by cell surface marker CD34. CD34+ fetal liver HSPCs cells were separated by magnetic cell sorting positive selection method. HSPCs (CD34+) were cultured by using 5 cytokines, stem cell factor (SCF), granulocyte macrophages-colony stimulating factor (GM-CSF), interleukin-6 (IL-6), Fms-related tyrosine kinase 3 (FLT-3) and erythropoietin (EPO), in 4 different combinations along with supplements, in serum-free culture media for 21 days. Cell viability continued to be greater than 90% throughout 21 days of culture. The cells expanded best in a combination of media, supplements and 5 cytokines, namely SCF, FLT-3, IL6, EPO and GM-CSF to yield a large number of total (CD34+ & CD34-) cells. Even though the total number of nucleated cells increased in culture significantly, levels of CD34 antigen expression declined steadily over this period.

Hematopoiesis appears in the fetal liver (FL) at approximately 5 weeks of gestation and remains the primary site of hematopoiesis until mid-gestation. The HSPCs rapidly proliferate in the liver; undergo maturation and differentiation leading to erythropoiesis, myelopoiesis, B and T lymphopoiesis, and production of megakaryocytes. Thereafter, the bone marrow replaces the fetal liver as the primary site of hematopoiesis. HSPCs have been used to treat a number of nonmalignant conditions such as Aplastic Anemia (AA), Severe Combined Immunodeficiency Disease (SCID), Congenital Metabolic Disorders, and also malignant conditions such as Acute Myeloid Leukemia (AML), Acute Lymphoblastic Leukemia (ALL), Myelodysplastic Syndrome (MDS), Chronic Myeloid Leukemia (CML), and Multiple Myeloma (MM). Fetal liver cells have the advantage of better growth and differentiation because of their higher telomere content. Also, the proportion of HSPCs (marked by surface antigen CD 34) in the fetal liver are greater than those found in cord blood and bone marrow; and are more or less equal to those found in mobilized peripheral blood. Fetal liver hematopoietic stem and progenitor cells (FL-HSPCs) have been found to exhibit proliferative potential compared to the adult bone marrow and cord blood. Increased capacity in serial transfer, greater tritiated thymidine uptake index, evidenced this along with early appearance of the peak number of colonies in semi-solid assays and greater plating efficiency. It was also found that these cells lack proper Human Leukocyte Antigen (HLA) expression. With having all the above-mentioned properties, fetal liver infusion has been utilized for the management of aplastic anemia. A limited quantity of fetal liver cells obtained from a single abortus however, creates problems in carrying out different experiments in the laboratory. A regular supply of the FL-HSPCs, probably can be obtained from expanded cells. Expanded fetal liver CD34+ HSPCs produced under “in vitro” conditions may provide large homogenous population of cells from a single fetal liver source.

Material and methods

Ethical approval. The Human Ethics Committee (Institutional Review Board) of All India Institute of Medical Sciences, New Delhi, India approved the current study. The Patient Information sheet was shared and we obtained Informed consent from patients who underwent Medical Termination of Pregnancy (MTP). We confirm that we carried all methods as per the relevant guidelines and regulations.

1Institute Rotary Cancer Hospital (IRCH), All India Institute of Medical Sciences (AIIMS), New Delhi, India. 2Sri Sri Institute For Advanced Research (SSSAR), Ved Vignan Maha Vidyha Peeth (VVMVP), F003 Soudhamini Apartment, 21st Kanakpura Road, Art of Living International Center, Udaipura, Bengaluru 560082, India. *email: chhabra.deepika@gmail.com
Preparation of cell suspension and cell viability. We obtained human fetuses with gestation periods ranging from 8 to 20 weeks with no known chromosomal abnormality following medical termination of pregnancy (MTP) with Prostaglandin E or Emcradyl injection from the Department of Obstetrics and Gynecology at AIIMS. Immediately after expulsion, we collected fetuses on ice and brought to the Laboratory. We dissected the fetuses under aseptic conditions to separate the liver. The liver was placed into Iscove's Modified Dulbecco Medium (IMDM) containing 10% fetal bovine serum (FBS) and 10 IU/ml heparin. Further, the liver was mashed into small pieces and allowed to pass through sterile muslin cloth repeatedly to obtain single-cell suspension. 10 µl of the fetal liver cell suspension was mixed with 10 µl of Trypan blue dye and loaded onto a hemocytometer and the number of dye excluding cells (viable) were counted. Only those fetus samples which had 80% or more cell viability were taken up for further analysis.

Isolation of fetal liver mononuclear cells (MNC). Mononuclear cells from fetal liver nucleated cell suspension were separated by density gradient centrifugation for their subsequent use in culture studies. After 5 min of treatment with 1X Red Cell lysis buffer (Sigma) to remove mature erythrocytes and PBS washing, nucleated cells from fetal liver suspension were diluted with IMDM containing 10% FBS in the ratio of 1:3 (fetal liver suspension: IMDM with FBS) and 30 ml of this mix was gently layered on 10 ml of Histopaque-1077 (Sigma Aldrich) in 50 ml sterile centrifuge tubes. The mononuclear cells were isolated by centrifugation (30 min, 1500 rpm, at room temperature) in a swinging-bucket rotor without brake. The white cell ring appearing at the interface of the medium and consisting of mononuclear cells (MNCs) was carefully withdrawn with a fine pasteur pipette. MNCs so obtained were washed thrice in phosphate buffer saline (PBS) to remove any traces of the interface of the medium and consisting of MNCs was recovered into small pieces and allowed to pass through sterile muslin cloth repeatedly to obtain a single-cell suspension.

Expansion of CD34+ FL-HSPCs under 'in vitro' conditions. We followed Stromal-free long-term culture technique to expand the FL-HSPCs. freshly isolated 1X10⁴ FL CD 34+ cells were cultured in a flat bottomed 24 well plates in 1 ml of serum-deprived culture medium STEM PRO-34-SFM (Gibco, Thermo Fisher) supplemented with 100 µg/ml insulin (Sigma), 200 µg/ml transferrin (Sigma), 40 µg/ml low density lipoprotein (LDL) (Sigma), 2 mM Glutamine (Gibco), 10⁻⁴ M Mercaptoethanol (Sigma). Different cytokine combinations (Table 1) comprising SCF (100 ng/ml), FLT-3 (100 ng/ml), IL-6 (20 ng/ml), EPO (2U/ml) and GM-CSF (200U/ml), (Peprotech) were used to expand CD34+ HSPCs for a period of 21 days. Fetal liver CD34+ HSPCs cultures were grown at 37 °C in humidified 5% CO2 incubator. Wells were semi- depopulated once a week. Harvested cells were counted and suitable aliquots were assayed every seven days for the presence of specific surface antigen CD34 by flow cytomtery (BD FACS Calibur).

Colony forming unit assay. Colony Forming Unit (CFU assay) was performed from the in vitro expanded CD34+ HSPCs. 1x10⁴ cells were taken from the expanded culture of Fetal liver CD34+ HSPCs at an interval of 7 days and mixed with methylcellulose semisolid medium (Metbocult H4434). Colony-forming unit-granulocyte macrophage (CFU-GM), Colony-forming unit-granulocyte magakaryocyte macrophage (CFU-GEMM), and Blast-forming unit-erythroid (BFU-E) were scored with an inverted microscope at day 14 of the culture.

Statistical analysis. All data about fetuses and culture characteristics were entered and analyzed using Microsoft excel and SPSS software. Statistical analysis was performed to determine the significance between two
culture populations with minimum of three replicates. Multivariate analysis ANOVA was done between groups to determine statistical significance (**P ≤ 0.01 and ***P ≤ 0.001).

Results
Cell number and viability range of human fetal liver. The age of the fetuses varied from 8–20 weeks (Median = 12 weeks). The median number of total fetal cells was $1.77 \times 10^9$ (Range; $0.02–5.2 \times 10^9$) with mean viability of 93.81 ± 5%. Total viable mononuclear cells obtained from fetal liver nucleated (Non Hepatocytic) cell suspension using density gradient centrifugation were $2.27 \pm 1.81 \times 10^8$ (Range; $0.01–6.78 \times 10^8$) (Table 2).

High CD34 expression on human fetal liver mononuclear cells. Fetal liver MNCs through flow-cytometry showed that the total CD34+ HSPCs population ranged from 1.2% to 12.8% with a median value of 5.5%. Supplementary Table 1 displays the percentage of CD34+ cells among FLMNCs according to gestational age. The peak values of CD34+ HSPCs were observed during 8–16 weeks of gestational age (Fig. 1a). From 8 to 10th week, CD34+ percentage remained high ($8.50 \pm 2.4\%$ to $8.70 \pm 1.8\%$); subsequently it started declining. We performed multivariate linear regression analysis on our results which shows that increase in gestational age of 1 week changes CD34 percentage by 0.8463 units. The overall regression model was significant and depicted 1% level of significance with a coefficient of determination (R²) value at 0.9429.

CD34+ HSPCs purification. The total no. of CD34+ HSPCs separated by MACS positive selection method ranged from $5.20 \times 10^5$–$4.34 \times 10^6$ to (median $2.79 \times 10^6$) with a median recovery of 62.3% (range 31.8–75.3%). The median purity of CD34+ HSPCs isolated from MiniMACS was 95% (Range 89–98%). In all samples, the percentage cell viability of CD34+ isolated HSPCs were greater than 95% as measured by Trypan blue dye exclusion methods. CD34+ cell fraction is nonadherent while CD34- cells adhere to the flask in culture media and this fraction of cells may contain fetal liver stromal cells12.

137 fold expansion of CD34+ fetal liver HSPCs. Estimated cell counts at 7 days intervals are shown in Fig. 2. Cell viability throughout 21 days of culture was greater than 90%. Expansion of total cells was expressed as an increase over initial cell input. Total nucleated cells expanded up to 137.24 ± 8.1 fold (M + S + M4) by day 21 (p < 0.01). A multivariate approach was adapted by performing ANOVA to assess the difference in cell expansion between different culture conditions. Difference between average number of cells in M and M + S + M4 was highly significant (-64.355) as p value (0.000) < 0.01 with 95% confidence level of interval. Highest average number of cells was found in combination M + S + M4 followed by M + S + M3 with mean difference significant at the 0.05 level. Analysis was also performed on different timepoints and it was found that even at earlier timepoints of Day 7 and Day 14, difference between expanded average numbers was highly significant with p-value (0.000) < 0.01 with 95% confidence level of interval.

Declining CD34+ expression on expanded FL-HSPCs. Expanded cells observed from day 0 to day 21, the percentage of CD34+ cells declined quickly after day seven and continued to decline steadily over the

| Characteristics | Number |
|-----------------|--------|
| Total Fetuses collected | 70 |
| Sex Male: Female | 39 (55.7%): 31 (44.2%) |
| Age (Gestation Period) | |
| Mean ± SD | 13 ± 2.91 weeks |
| Median | 12 weeks |
| Range | 8–20 weeks |
| Time between expulsion and processing of Fetus | |
| Mean | 1.00 ± 0.76 h |
| Range | 0.25–3.5 h |
| Non hepatocytic cell viability of liver cells | |
| Mean | 93.81 ± 5.0% |
| Range | 85–100% |
| Total viable Nucleated (Non Hepatocytic) Cells | |
| Mean ± SD | $1.91 \pm 1.31 \times 10^9$ |
| Median | $1.77 \times 10^9$ |
| Range | $5.2 \times 10^7$ |
| Total viable fetal liver mononuclear cells | |
| Mean ± SD | $2.27 \pm 1.81 \times 10^8$ |
| Median | $2.041 \times 10^8$ |
| Range | $0.01–6.78 \times 10^8$ |

Table 2. Fetal liver characteristics at the time of collection.
twenty-one days of culture. FACS analysis of CD34+ expression of five representative experiments at different time points is shown in Fig. 3a. The Percentage of CD34+ among the total nucleated cell count at day 21 was 15.42 ± 4.9 in combination M + S + M1, 14.68 ± 5.1% in M + S + M2, 18.24 ± 4.2% in M + S + M3, and 13.08 ± 2.3% in M + S + M4, however, in media (M) alone it was 1.7 ± 2.0%. ANOVA analysis was adapted to assess the significance levels of difference in decline of CD34 expression between different culture conditions. The decline in CD34+ HSPCs percentage was found to be highly significant among different combination groups with p-value (0.000) < 0.01 with 95% confidence level of interval. Absolute number of total cells and CD34% cells at day 21 are given in Supplementary Table 2.

Colony forming unit assay from in vitro expanded FL-HSPCs. In vitro expanded cells FL-HSPCs were counted and analyzed every 7 days for their colony formation (CFU-GM, BFU-E and CFU-GEMM) potential (Fig. 4a, b and c). The Colony forming potential of freshly isolated CD34+ HSPCs was 120 ± 17 for CFU-GM, BFU-E 161 ± 15 and 101 ± 17 for CFU-Mix. Figure 5 shows the comparison in different types of colonies gener-
by day 21 is certainly consistent with this possibility. Growth factor dosing is another important issue. At high concentrations of glutamine and mercaptoethanol has been used by us in the current study for a period of 21 days. Expansion of nucleated cells happened by 137.24 ± 8.1 fold. Early acting cytokines supporting the growth of very primitive HSPCs only when synergized with other growth factors, including SCF (100 ng/ml), IL-3 (10 ng/ml), IL-6 (10 ng/ml), granulocyte colony stimulating factor (13 ng/ml) and 0.4 U EPO19 and 10U/ml TPO14,20,21. With these different combinations, expansion of CD34+ cells could be observed by day 7; expansion ranged from 2.44 to as high as 25.11 ± 13.50-fold by day 7. One possibility is that growth factors might positively alter self-renewal versus differentiation decisions of stem cells (25). One possibility is that growth factors might positively alter self-renewal versus differentiation decisions of stem cells (25). One possibility is that growth factors might positively alter self-renewal versus differentiation decisions of stem cells (25). One possibility is that growth factors might positively alter self-renewal versus differentiation decisions of stem cells (25). One possibility is that growth factors might positively alter self-renewal versus differentiation decisions of stem cells (25).

Discussion

HSPCs transplantation therapy has been effectively utilized for the treatment of several life-threatening hematopoietic diseases. Bone marrow (BM) and mobilized peripheral blood are the major sources of HSPCs; however, there is a paucity of human leucocyte antigen (HLA) matched donors. Hence, umbilical cord blood (UCB) has been used as an alternative to obtain HSPCs for transplantation therapy. UCB transplantation therapy has the limitation of availability of low numbers of HSPCs per unit of UCB. HSPCs from Fetal liver can be considered as potential candidates owing to their superiority in self renewal and differentiation potential. However, sample availability, as well as consistency of fetuses in terms of fetus age and HSPC count becomes a hurdle in using this important source. In vitro expansion of HSPCs from fetal liver has the potential to provide large number of cells useful for stem cell transplants as well as experimental studies. Possibility of in vitro expansion of HSPCs has been studied by several researchers to address this issue. Combination of various growth factors have commonly been employed to expand HSPCs in vitro. Mixture of stem cell factor (SCF), thrombopoietin (TPO), and FMS like tyrosine kinase 3 ligand (FLT-3L) was found to support the expansion of HSPCs. This study verified the effect of cytokines IL-6, FLT-3L, along with the role of serum supplementation in a short-term liquid culture. Human recombinant FLT-3 ligand (FL), (> 300 ng/ml)25. According to McNiece et al., SCF synergizes with other cytokines e.g., Epo, IL-3, GM-CSF, and G-CSF to support growth of BFU-E, CFU-GM, and CFU-GEMM cells. SCF alone resulted in 50% increase in colony forming potential as compared to control (p-value < 0.05), levels of CD34+ antigen expression however, declined steadily over this period. It has been the general observation that even when there was clear demonstration of in vitro amplification of HSPCs, there was a net decline over input numbers (6.5 fold after 4 weeks) indicating that the culture conditions were suboptimal for stimulating HSPCs self-renewal and/or their continued survival (25). One possibility is that growth factors might positively alter self-renewal versus differentiation decisions of stem cells (25). Concentrations and combination of different cytokines has proven their role in stem cell expansion studies. In one of the similar studies, although stimulation of HSPCs expansion by IL-11 was observed at low concentration range; yet saturation effects were not achieved even at high concentration with factors such as steel factor (SF) or FLT-3 ligand (FL), (> 300 ng/ml)25. According to McNiece et al., SCF synergizes with other cytokines e.g., Epo, IL-3, GM-CSF, and G-CSF to support growth of BFU-E, CFU-GM, and CFU-GEMM cells. SCF alone resulted...
in no significant colony formation, however, along with other cytokines viz. G-CSF and rhIL-3, SCF stimulated a synergistic increase in colony numbers\textsuperscript{27}. A study by Sui et al., reported that although SCF (100 ng/ml) alone has modest effect on colony growth, but in the presence of other cytokines like of lL-3 (200 U/ml), and EPO (2 U/ml), SCF increases both the size and the number of colonies\textsuperscript{28}. Our study, similar to that of Matsunaga et al., depicts that the combination of SCF (100 ng/ml)), IL-6 (100 ng/ml), and FLT-3 ligand (100 ng/ml) can support the proliferation, differentiation, and terminal maturation of BFU-E in vitro, even in the absence of EPO\textsuperscript{29}.

Transforming growth factor beta 1 (TGF beta 1) at concentration of 1–50 pg/ml stimulated colony formation but at higher concentration, it had an inhibitory effect\textsuperscript{30}. Stimulation of selective growth factor pathways, may yet be a crucial determinant of HSPCs self-renewal. For instance, regeneration of normal HSPCs in recipients lacking TPO got impaired by 10–20-fold\textsuperscript{31}. TPO has been implicated as a positive regulator of HOXb4 (part of a family of transcription factors) expression, a potent enhancer of HSPCs expansion. HOXb4 induced self-renewal in vitro in conditions that were sub optimal for untransduced HSPCs\textsuperscript{25}. Over expression of another gene namely SALL4 has also been shown to have the capacity to substantially increase the number of HSPCs in vitro\textsuperscript{32}. More recently, Jing et al.\textsuperscript{33} demonstrated the expansion of both murine BM HSPCs and human UCB HSCs with the

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**Figure 3.** Percentage fetal liver CD34\textsuperscript{+} HSPCs cells during 21 days of expansion: CD34 expression of in vitro expanded cells from day 0–21 done through flowcytometry. Mean cell percentage is indicated (n = 5) with SEM (a). Statistical significance among different combinations as well as days was analyzed via ANOVA and is depicted with asterisks (*). double (***) asterisks indicate $P \leq 0.01$. (b) Representative image of immunophenotyping of CD34 antigen from in vitro expanded cells using M + S + M4 media and cytokines combination at Day 14 (b) and Day 21(c) done through flowcytometry.
combination of a moderate concentration of p38 inhibitor plus a GSK 3 inhibitor. Thus, it appears that despite the availability of various methods to expand HSPCs in vitro, an ideal method to increase the number of HSPCs in vitro is yet to be discovered.

**Conclusion**

Fetal liver, a major site of hematopoiesis during second trimester of pregnancy is an excellent source of hematopoietic stem cells. The higher cell number required for experimental studies is hindered by poor availability of fetuses and a smaller number of cells obtained per fetus. To overcome this problem, magnetically sorted CD34+ hematopoietic stem and progenitors cell populations have been subjected to expansion, using media supplements and different combinations of cytokines. Although the total number of nucleated cells increased in culture, levels of CD34 antigen expression declined steadily with time.

Figure 4. Colony Forming Unit Assay from in vitro expanded FL CD34+ HSPCs. Number of committed progenitors (including CFU-GM, BFU-E, and CFU-Mix) derived from $1 \times 10^6$ Fetal liver CD34+ HSPCs after 7 days (a), 14 days (b) and 21 days (c) of serum-free liquid suspension culture in the presence of different combinations of cytokines M1, M2, M3 and M4 ($n = 3$). Statistical significance among colony forming potential from different media combinations as well as days was analyzed via ANOVA and is depicted with asterisks (*). Double (***) asterisks indicate $P \leq 0.01$. Representative Picture of CFU-GM (d), BFU-E (e) and CFU-Mix (f) colonies derived from Fetal liver CD34+ HSPCs (10×) from combination M + S + M4, day 14 of expansion.
**Figure 5.** Colony Forming Unit Assay from M+S+S4 vs Day 0. Number of committed progenitors (including CFU-GM, BFU-E, and CFU-Mix) derived from $1 \times 10^4$ freshly isolated Fetal liver CD34+ HSPCs, denoted as Day 0 vs. colonies generated from in vitro expanded cells collected at day 7, day 14 and Day 21 from media combination M+S+S4 ($n=10$). Statistical significance among colony forming potential from different media combinations as well as days was analyzed via ANOVA and is depicted with asterisks (*). double (**) asterisks indicate $P \leq 0.01$.

**Data availability**
All data generated or analyzed during this study are included in this “to be published” article.

Received: 27 January 2021; Accepted: 15 April 2021
Published online: 04 June 2021

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Acknowledgements
We gratefully acknowledge the research grant provided by the Department of Biotechnology, Government of India and AIIMS, New Delhi.

Author contributions
Dr. V.K. and Dr. L.K. designed the study. Dr. R.B. collected, assembled, analysed and interpretated the data. Dr. D.C. drafted the manuscript which was discussed amongst Dr. N. K.M., Dr. A.S., Dr. S.M. Dr. L.K. and Dr. V.K. gave the final approval.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-91272-6.

Correspondence and requests for materials should be addressed to D.C.

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