Transduction of Human Fetal Liver Hematopoietic CD34+ Stem and Progenitor Cells into a Cell Line by Enhancing Telomerase Activity

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ABSTRACT
Background: Human fetal liver hematopoietic stem cells have proven potential as therapeutics but lack extensive research due to their limited supply. Even *in vitro* expanded fetal liver hematopoietic stem cells enter senescence or lose their self-renewal capacity after a few days in culture. The present study aimed to obtain a homogeneous and persistent supply of hematopoietic stem cells from the fetal liver by establishing a cell line through immortalization of cells by enhancing telomerase activity.
Materials and Methods: Human fetal liver hematopoietic CD34+ stem and progenitor cells were transformed and immortalized using retroviruses carrying the human telomerase (hTERT) gene. Following transduction, telomerase activity was assessed using the TRAP assay and telomere length was examined by Southern blotting in transduced cells. Their characterization was conducted using flowcytometry to analyze the CD34+ population of hematopoietic stem cells and their colony forming potential using colony forming unit (CFU) assay.
Results: After transduction with hTERT, the life span of human fetal liver hematopoietic CD34+ stem and progenitor cells were extended to 80 population doublings, without any change in cell morphology or population doubling times. Constitutive hTERT expression enhanced the replicative capacity and prevented terminal differentiation of CD34+ fetal liver hematopoietic stem and progenitor cells (FLHSPCs). Moreover, hTERT-transduced stem cells maintained their telomere length and telomerase activity.
Conclusion: By introducing telomerase activity into hematopoietic stem and progenitor cells, their lifespan can be extended while maintaining stemness. These modified cells hold promise for *in vitro* research focused on studying hematopoietic stem cells derived from fetal liver.

Keywords: Fetal liver; Hematopoietic stem cells; Immortalization; Telomerase activity, Human telomerase reverse transcriptase (hTERT)

INTRODUCTION

In humans, the fetal liver serves as a predominant site of hematopoiesis before the bone marrow takes over this role and demonstrates a high potential for hematopoietic stem cell (HSC) therapeutics[1]. The advantageous characteristics of fetal liver cells, including a paucity of lymphoid cells, weak expression of human leukocyte antigens (HLA), higher proliferative potential, and superior long-term repopulating ability, have led to their use in the treatment of human immunodeficiency diseases,

acute leukemia, aplastic anemia, inborn errors of metabolism and severe combined immunodeficiency[2–8].

Our previous studies have shown that human fetal liver-conditioned medium contains numerous growth factors and cytokines, suggesting that fetal liver infusion could be an excellent alternative to enhance the recovery of patients with aplastic anemia[2,4]. However, the poor availability of aborted fetuses and the limited number of recovered cells per fetus restricts the therapeutic applications of fetal liver hematopoietic stem cells and research aimed at gaining a better understanding of their biology. Therefore, we demonstrated the high proliferative capacity of human fetal liver hematopoietic stem and progenitor cells (FLHSPCs) and developed a cytokine cocktail to expand these cells under in vitro conditions. Despite the successful expansion of these cells by upto 137-folds, the expression of CD34, a marker of human hematopoietic stem cells and an indicator of stemness, steadily declined over time[9]. Experimental techniques exist to induce cell proliferation at significantly higher densities by utilizing tumor-inducing viruses[10] or chemical agents[11]. Thus, there is an urgent need to transform or immortalize these cells to obtain a homogeneous and persistent supply of FLHSPCs for in-depth in vitro studies.

Cell immortalization can be accomplished through various experimental methods, including the use of tumor-inducing viruses[10] or chemical, physical, and telomere-related techniques[12]. When provided with suitable culture media, the resulting cells (referred to as cell lines) proliferate indefinitely in a culture dish. A cell line serves as an abundant source of immortalized cells with uniform characteristics[13]. Among the available methods for generating immortalized cells, the use of retroviral vectors offers an advantage over chemical and physical approaches because it can lead to stable gene integration within the target genome. However, viral vectors, such as those carrying the SV40-T antigen, pose a risk of tumorigenicity[14]. Toouli *et al.* (2002) compared the efficacy of viral vectors (e.g., SV40) with telomere-based approaches utilizing hTERT. The

hTERT gene encodes the catalytic subunit of telomerase, which adds telomere sequences to chromosome ends, thereby maintaining the replicative capacity of self-renewing stem cells by stabilizing telomere length, which can lead to stem cell immortalization. This study revealed that SV40 induced immortalization was associated with aberrant differentiation, loss of DNA damage response, and karyotypic instability, which in some cases resulted in tumorigenicity. In contrast, hTERT- immortalized cells exhibited characteristics similar to those like normal cells, including fewer karyotypic anomalies, intact DNA damage response, and normal differentiation[15].

Therefore, induction of telomerase activity is a new and effective strategy for cell immortalization. In humans, the ends of chromosomes contain approximately 1,000 - 2,000 telomeric repeats[16]. Beyond the double-stranded region of the telomere, the 3'end of the G-rich strand exhibits a single-stranded extension known as the 3' overhang[11]. This single-strand overhangs folds back onto the duplex telomeric DNA to form a t-loop, which protects the telomere from conventional DNA repair mechanisms[17]. Most somatic human cells lose 50-100 bp of telomeric DNA during each cycle of cell division[16,18]. Activation of telomerase is essential for stabilizing telomere lengths, thereby maintaining the replicative capacity of self-renewing cells such as germ line cells, tumor cells, and potentially stem cells from various tissues[19,20].

Egorov *et al.* demonstrated that the introduction of the hTERT gene into normal fibroblasts of adult human skin led to the activation of telomerase activity, elongation of telomeres, and subsequent immortalization[21]. Similarly, Wege *et al.* reported that the restoration of telomerase activity induces indefinite replication in human fetal hepatocytes[22]. Certain studies have shown additional benefits, such as the knockdown of p16 (INK4a) and p53, which can mitigate cellular crises or growth arrest, although these methods occasionally result in aberrant differentiation[23,24]. Despite the availability of various fetal liver cell lines, to our knowledge, a human FLHSC line has yet to be developed. Establishing an FLHSC line is

crucial for studying the biology of hematopoietic stem cells. Furthermore, transformed and immortalized cells can be utilized for gene expression studies of various cytokines and for investigations into the intricacies of hematopoiesis, cell growth, regulation, carcinogenesis, and immune responses. Given its safety and efficacy, we employed the telomere method to immortalize human fetal liver hematopoietic CD34+ stem and progenitor cells. We acknowledge that although hTERT overexpression enhances FLHSPC self-renewal, it may also lead to potential adverse effects, which should be monitored meticulously [25]. In this study, we used a safe hTERT overexpression technique to restore telomere length and stem cell characteristics in human fetal liver hematopoietic stem and progenitor cells, thereby establishing a new transformed cell line.

Materials and Methods
Cell isolation and culture cell source
Human fetuses (n=10, male:6, female:4) with gestation periods ranging from 8 to 22 weeks and no known chromosomal abnormalities were collected after MTP from the Department of Obstetrics and Gynecology, All India Institute of Medical Sciences, India. The gestational age of each fetus was calculated from the first day of the mother's last menstrual period[26]. Fetuses were dissected under aseptic conditions to separate and place the liver into Iscove's modified dulbecco medium (IMDM), supplemented with 10% fetal bovine serum (FBS) and 10 IU/ml heparin[7]. Cell viability was assessed using the trypan blue dye exclusion assay.

Mononuclear cells (MNC) were isolated by density gradient centrifugation using Ficoll. Nucleated cells from fetal liver suspension were diluted with IMDM containing 10% FBS at 1:3 ratio. A total of 30ml of the suspension was gently layered onto 10 ml of Histopaque (Ficoll-Hypaque) in 50 ml sterile centrifuge tubes. Mononuclear cells were isolated by centrifugation (30 min, 1500 rpm, at room temperature) in a swinging-bucket rotor without brakes. The white cell ring at the interface between the medium and Histopaque was carefully collected using a fine Pasteur pipette. The obtained MNC were

washed three times with phosphate-buffer saline (PBS) to remove any traces of Histopaque and subsequently counted using a hemocytometer[27]. The CD34 Direct Isolation Kit (Miltenyi Biotec) was used along with MS$^+$ MiniMACS columns to enrich the CD34$^+$ fraction of fetal liver cells, following the protocol suggested by Fathi et al (2022). The isolation was conducted according to the manufacturer's recommendations and the CD34+ fraction was isolated immunomagnetically using MS+ MiniMACS columns and the CD34 Direct Isolation Kit (Miltenyi Biotec). Mononuclear cells (MNC) from the fetal liver were counted before labeling with magnetic beads or fluorochrome-conjugated antibodies. CD34$^+$ cell purification was performed using the positive selection method[28]. CD 34 + cells were characterized by flow cytometry and cultured as non-adherent cells as described in our recently published study[9].

Cell culture of HEK293T and NIH 3T3 Cell Line
HEK293T (human embryonic kidney cells containing SV40 large T antigen) and NIH3T3 (mouse fibroblasts) cell lines were maintained for transfection and transduction purposes. Both cell lines were cultured in Dulbecco's modified eagle medium (DMEM)supplemented with 10% FBS and 1% of a mixture of antibiotics (penicillin 100 U/mL, streptomycin 100 μg/mL) and an antifungal agent (amphotericin B, 25 μg/mL). Cells were routinely cultured in 75cm^2 (T75) tissue culture flasks at 37°C in a humidified atmosphere of 95% and 5%. CO_2 content. The cells were passaged by trypsinization once they reached approximately 80% confluency[29,30].

Transduction procedure
Plasmids for transfection and retroviral development
Retroviral expression vectors pBABE-puro and Plasmid pBABE-puro-hTERT were obtained from Addgene (Robert Weinberg, MIT, Cambridge, MA, USA) and the Packaging plasmid pCL-Ampho was sourced from Imgenex. All plasmids contained antibiotic-resistance genes (ampicillin) and were amplified using DH5α competent cells[31]. The cells were cultured on Luria–Bertani (LB) plates followed by LB Broth. Plasmid DNA was purified using a

Qiagen purification kit according to the manufacturer's instructions, and quantification was performed using spectrometric analysis[32].

Production of replicative incompetent retroviruses

A total of $0.5\text{-}1 \times 10^6$ 293T cells were plated on 6 cm dishes in 5 ml DMEM/10%FBS medium one day prior to co-transfection. The first transfection was performed using a green fluorescent protein (GFP) reporter to assess the transfection efficiency in the laboratory[33]. The transfection cocktail was prepared with the following components: 1ug of packaging plasmid, one ug expression plasmid (either pBABE-puro-hTERT or pBABE- puro as control), 6 µL of FuGene6 transfection reagent (Promega), and OptiMEM (Invitrogen Cat. No 31985) resulting in a total volume of 100µL. The mixture was gently swirled and incubated for 15 minutes at room temperature. Subsequently, the transfection cocktail was added dropwiseto 293T cells and incubated overnight at 37°C. To reduce cytostatic factors, the medium was replaced the day after transfection. The viral supernatant was harvested at 48 and 72h post transfection[34].

Viral titer determination

Retroviral supernatants with high titers were harvested within 36-72 hours post-transfection. Both control (pBABE-puro) and test (pBABE-puro-hTERT) viral supernatants were collected after 48h and 72h of transfection, filtered using a 0.45 µM syringe filter, and subsequently stored at -70°C. The viral titer was determined using the NIH 3T3 cell line to evaluate puromycin resistance.

A standard gene transducing unit assay was conducted to estimate the concentration of viral supernatants. For this assay, 5×10^4 cells per well were plated in 6 well culture plates. Various dilutions of the viral supernatant were used to determine the viral titer. After reaching approximately 40% confluency, transduction was performed using Hexadimethrine Bromide (polybrene) at a concentration of 8 gm/ml[35].

Transduction of human fetal liver HSPCs

Enriched CD34+ fetal liver cells were isolated through Mini MACS using CD34 microbeads as described by Fathi et al[28]. 2×10^6/ml cells were pre-stimulated for 48 hours in Iscoves modified Dulbecco medium (IMDM) containing a serum substitute (BIT, Stem Cell Technologies, Vancouver, BC, Canada). 10^{-4}M mercaptoethanol (Sigma), and 40 µg/ml low-density lipoproteins (Sigma) supplemented with recombinant human cytokines: 100 ng/ml Flt-3 ligand, 100 ng/ml SCF and 20 ng/ml IL-6 (Pepro Technologies) were used[9]. After 48 hours, the cells were resuspended in 0.2 µm filtered virus-containing supernatant supplemented with the same cytokines and Hexadimethrine Bromide (Polybrene) at a concentration of 8 µg/ml and incubated in tissue culture dishes for 1 hour. This procedure lasted for two consecutive days for a total of three infections. Cell cultures were maintained at 5% CO2 and 37ºC. Transduction was conducted with pBABE-puro and pBABE-puro-hTERT containing supernatants of retroviral vectors[36]. Mock transduction was performed in parallel using the same medium, growth factors and Hexadimethrine Bromide but without the addition of viral vectors. Puromycin resistance was evaluated after 15 days of culture. Retrovirally transfected puromycin-resistant FLHPSC were reselected using mini MACS and cultured for up to 8 months.

Characterization Assays
Characterization of transduced human fetal liver HSPCs

At various time points during culture, cells were harvested, counted, and used for additional analysis and continuation of the culture. Characterization included the assessment of morphology, cell surface antigen, colony-forming potential, telomerase activity, and telomere length at different time intervals.

Cell morphology and surface antigen

Cell morphology was examined using an inverted microscope (Nikon) for both untransduced and transduced fetal liver CD34+ cells. Cell surface antigen of untransduced, control vector-transduced, and hTERT-transduced fetal liver cells were analyzed by flow cytometry (BD FACS Calibur) using CD34 APC and CD38 PECy7 antibodies (BD Biosciences).

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Colony-forming assay (CFU) of transduced FL HSPCs

Colony-Forming Unit (CFU assay) were performed using untransduced, control vector transduced, and hTERT transduced fetal liver CD34+ cells. A total of 1X 10^4 cells from each type of culture were collected at day 30 and day 50 post-transduction, mixed with methylcellulose semisolid medium (MethoCult H4434), and plated in triplicate in 35-mm petri dishes. The dishes were incubated for 14 days at 37°C in a 5% CO2 incubator. Committed progenitors in the form of colony-forming unit-granulocyte megakaryocyte macrophage (CFU-GEMM), colony-forming unit-granulocyte macrophage (CFU-GM), and blast-forming unit-erythroid (BFU-E) were counted with an inverted microscope on day 14 of culture[9,37,38].

Analysis of transgene expression and telomerase reconstitution: TRAP (Telomeric Repeat Amplification) assay

Telomerase activity of transduced cells was evaluated using the *TeloTAGGG* PCR ELISA PLUS kit (Roche) following the manufacturer's protocol (Supplementary Table 1)[39–41]. Telomerase adds telomeric repeats (TTAGG) to the 3'-end of the biotinylated primers. The elongation products were amplified using polymerase chain reaction (PCR). Following denaturation, the PCR products were hybridized to digoxigenin-labelled telomeric probes. Subsequently, horseradish peroxidase conjugated antibodies to were added, and the amplicons were detected using the peroxidase-sensitive substrate TMB (3,3- 5,5- tetramethyl benzidine), resulting in visible color development. The ELISA signal of the sample was compared with that of a control template containing a known number of telomeric repeats. The relative telomerase activity (RTA) values were calculated as follows:

$$RTA = \frac{\frac{Absorbance\ of\ sample - Absorb\quad of\ Rnase\ treated\ sample}{Absorbance\ of\ internal\ standard\ of\ the\ sample}}{\frac{Absorbacne\ of\ control\ template - Absorbance\ of\ lysis\ buffer}{Absorbance\ of\ internal\ standard\ of\ the\ control\ template}} X100$$

These values were translated in the percentage of telomerase activity of the transformed cells taken along with the test. Telomerase activity was considered positive if the difference in absorbance (absorbance of sample - absorbance of RNAse treated samples) exceeded the background activity (absorbance of RNAse treated samples).

Telomere length assay

Telomere lengths were analyzed using the TeloTAGGG Telomere Length Assay (Roche). Terminal restriction fragments were analyzed after digestion of the genomic DNA with *Hinf*I and *Rsa*I restriction enzymes[42,43]. Southern blot analysis was conducted on DNA extracted from both untransfected and transfected cells to confirm the integration of the transfected plasmid DNA. Genomic DNA was extracted, digested with HinfI and RsaI, electrophoresed, blotted, and transferred to positively charged Magnacharge membranes using alkaline blotting. The membrane was hybridized with ^{32}P-(TTAGGG)$_3$ as a telomeric probe using Hybrisol II and subsequently washed. The mean terminal restriction fragment (TRF) length was calculated by integrating the signal intensity above the background over the entire TRF distribution[44].

Supplementary Table 1: TRAP-PCR reaction cycle

	Time	Temperature	Cycle
Primer elongation	10-30 min	25°C	1
Telomerase inactivation	5 min	94°C	1
Denaturation	30 s	94°C	1-30
Annealing	30 s	50°C	
Polymerization	90 s	4°C	
	10 min	72°C	1
Hold		4°C	

Statistical analysis

Statistical analyses were conducted using SPSS 11.3(SPSS Inc, Chicago, IL, USA). Data regarding fetuses and culture characteristics were entered and analyzed to determine the significance of the differences between the two culture populations. Measures of central tendency are presented as mean ± standard error of the mean. Two-tailed Student's t-

test and ANOVA for repeated measures were used to analyze the differences between groups, with p-value ≤ 0.05 considered statistically significant.

RESULTS
Viral titer determination

HEK293T and NIH3T3 cell lines were used for transfection and transduction, as depicted in Figure 1. The Plasmid quality of pBABE-puro and pBABE-puro-hTERT was confirmed by amplification and purification (Figure 2). Retroviral titers containing the pBABE- puro and pBABE-puro-hTERT genes were calculated based on the number of colonies present at the highest dilution, multiplied by the dilution factor. Colonies are represented as colony-forming units (cfu)/ml. The viral titer range for the control (pBABE-puro) was $2X10^3$ - $1X10^5$, whereas that for the test vector (pBABE-puro-hTERT), it ranged from $1X10^4$ - $1X10^5$.
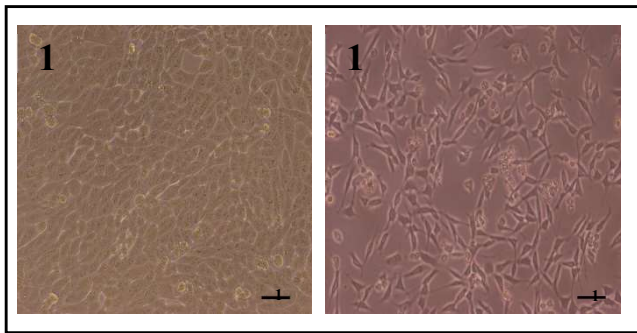

Figure 1: HEK 293T (human embryonic kidney cell line) (10X) (1A). NIH 3T3 cell line (mouse embryonic fibroblast cell line) (10X) (1B)
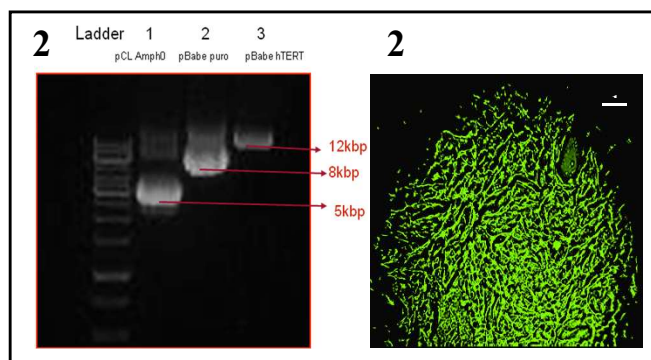

Figure 2: Plasmid quality after amplification and purification from Bacterial Cultures. Lane 1: pCL Ampho, Lane2: pBABE-puro, Lane 3: pBABE-puro-hTERT(2A). Fluorescence Microscopy of HEK293 T cells transfected with FuGene HD: Cells were transfected with GFP using FuGene HD reagent to produce green fluorescent protein. Image taken 48hrs post-transfection (2B).

Characterization of FL CD34+ cells transduced with hTERT

Fetal liver CD34^{+} HSPCs were transduced with the pBABE-puro-hTERT plasmid and the control plasmid pBABE-puro, followed by puromycin selection after 15 days. No morphological changes were observed in the transduced cells (Figures 3A and 3B). Puromycin-resistant CD34^{+} cells were sorted using MiniMACS and maintained under serum-free conditions in the presence of SCF (100 ng/ml), Flt-3(100 ng/ml), and IL-6 (20 ng/ml). Untransduced CD34^{+} FL (FLUT) cells were used as controls. Flow cytometric analysis of CD34 and CD38 cell surface antigen revealed no differences before (Figure 3C) or after (Figure 3D) transduction. In all cases (n=3), control CD34^{+} FL cells underwent differentiation and senescence-associated proliferation arrest after approximately three months of culture (Figure 3E). FL CD34^{+} cells transduced with the pBABE-puro vector (FLT3 in Figure 3E) exhibited a pattern similar to that of untransduced cells. In contrast, CD34^{+} FL cells transduced with hTERT prolonged their proliferation. Three FL hematopoietic cell lines were established from fetuses aged 9, 10, and 12 weeks, two of which were continuously propagated in culture for over 8 months. Assessment of the growth factor requirement of these cell lines revealed adequate growth in the presence of SCF (50 ng), Flt-3(50 ng), and IL-6 (10 ng). Consequently, the cells were routinely maintained with all three cytokines. Karyotype analysis could not be performed on transduced cells because of some technical issues.
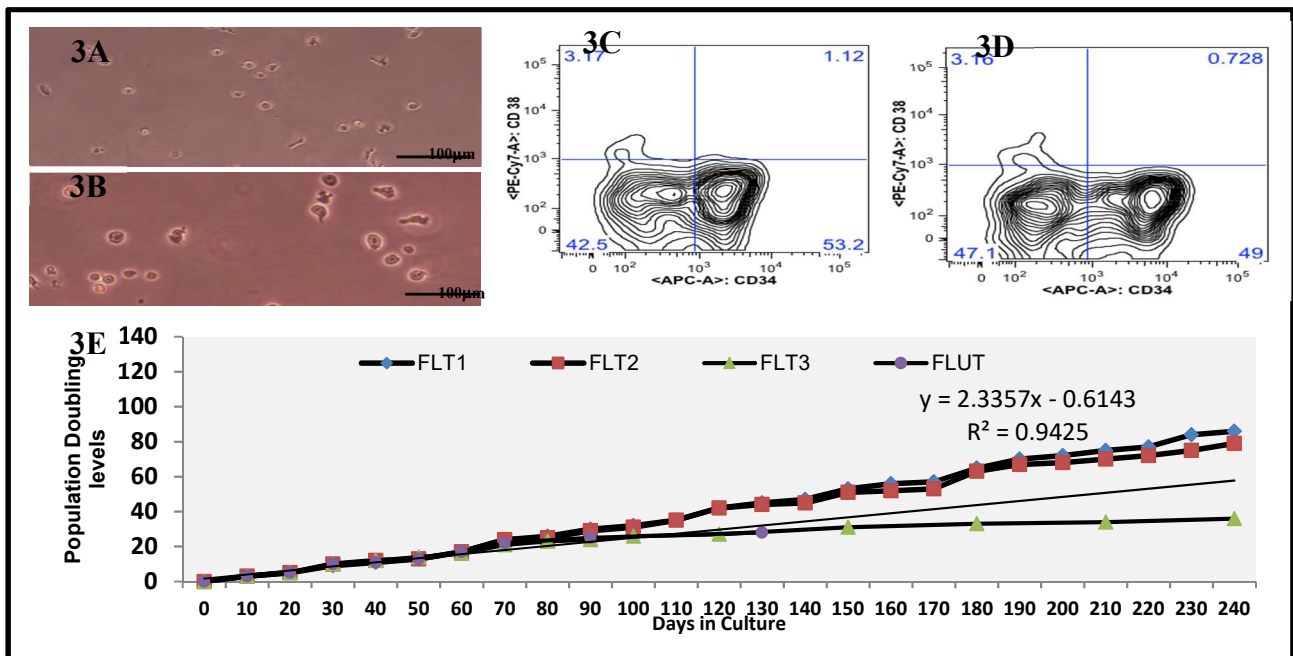
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Figure 3: Extended Lifespan and stemness in FL CD34+ cells transduced with hTERT: Fetal liver CD34^{+} cell morphology before transduction (3A) and after transduction with pBABE-puro-hTERT plasmid after Day 15 of puromycin selection (3B). Characterization of untransduced(3C) and hTERT transduced (3D) Fetal liver cells with CD34 and CD38 marker by flow cytometry. Compared with untransduced (FLUT) and pBABE-puro (Vector without telomerase gene) (FLT3) entering senescence at ~80 population doublings, Fetal liver CD34^{+} cells transduced with pBABE-puro-hTERT (FLT1 and FLT2) bypass senescence (3E).

Colony assay of FL CD34+ hTERT transduced cells

The Colony forming ability (CFU-GM, BFU-E and CFU-GEMM) of $1X\ 10^4$ Fetal liver CD34+ untransduced and transduced cells (both with control vector pBABE-puro and test vector pBABE-puro-hTERT) was evaluated by colony-forming assays after 3 and 50 days of culture in serum-free liquid suspension media. On day 0, the colony-forming potential of CD34^{+} cells were 132± 5.65 CFU-GM colonies, 166.5± 10.60 BFU-E colonies, and 111.5± 3.53 CFU-GEMM colonies. Although a decline in all colony types was observed in untransduced and pBABE-puro cells, a significant increase in colony-forming ability was detected in hTERT-transduced cells on both day 30 (p<0.01) and day 50 (p<0.01) (Figure 4).
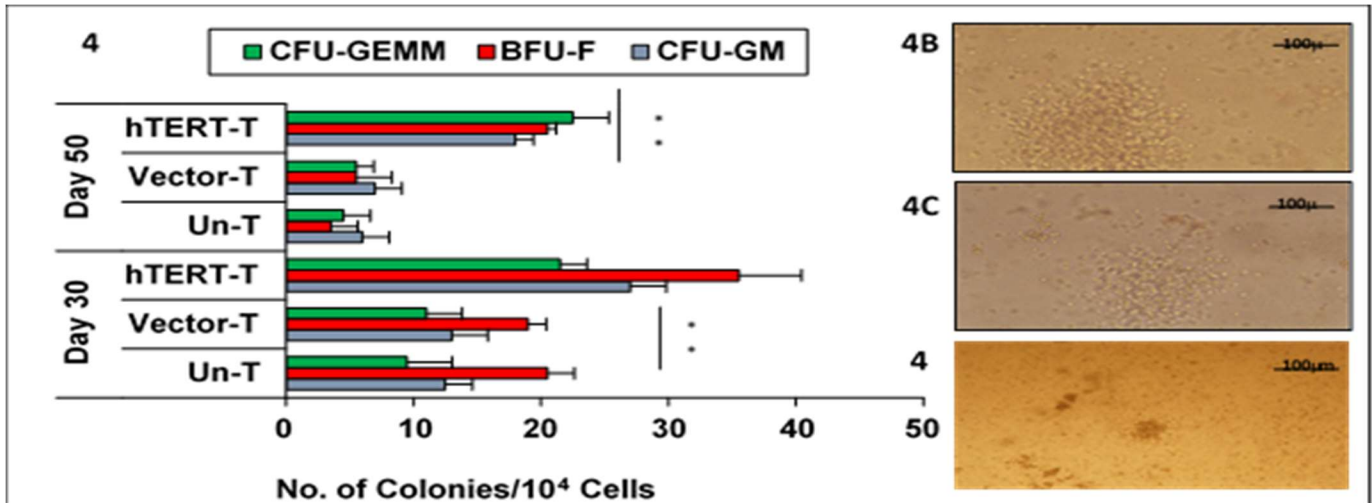
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Figure 4: Colony Assay of Transduced FL CD34+ Cells: Number of committed progenitors (including CFU-GM, BFU-E, and CFU-Mix) derived from 1X 10^4 untransduced, Control vector transduced and hTERT transduced Fetal liver CD34$^+$ cells after 30 days and 50 days of culture in serum-free liquid suspension(4A). Representative Picture of CFU-Mix (4B) CFU-GM (4C) and BFU-E (4D) colonies (10X) at day 14 derived from hTERT transduced Fetal liver CD34$^+$ cells.

Enhanced telomerase activity in CD34$^+$ FLHSPCs ectopically expressing hTERT

TRAP assay was conducted to evaluate telomerase activity before and after transduction of the telomerase gene in CD34+ cells. The relative telomerase activity' (RTA) values were determined in untransduced and virus-transduced cells in culture (after puromycin selection and MiniMACS sorting) at various time points from day 10 to day 50. The endogenous telomerase activity of enriched untransduced CD34$^+$ cells was 74.34$\pm$2.6 % (n=5) of the activity in a representative telomerase-positive immortalized cell line (HEK293T). In CD34$^+$ cells transduced with the hTERT gene; telomerase activity was significantly enhanced relative to that in control cells throughout the culture period (Figure 5). Compared with vector-only transduced cells, hTERT transduced cells exhibited a substantial increase in telomerase activity (p<0.01), which was proportional to the levels found in immortalized cells such as HEK293T cells.
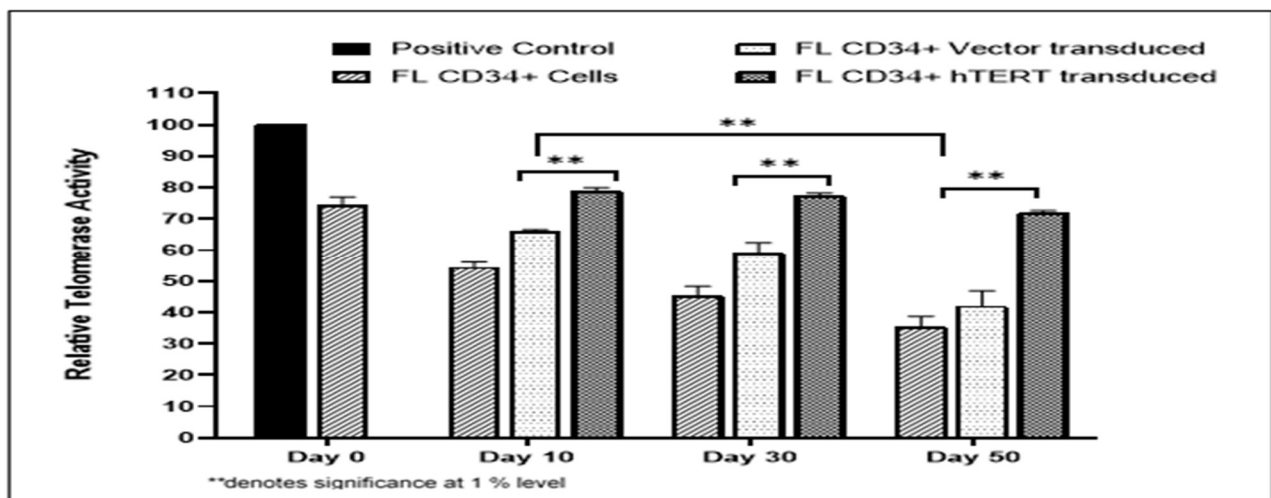

Figure 5. Elevated levels of telomerase activity in CD34$^+$ FL cells ectopically expressing hTERT: TRAP assay for transduced cells: Telomerase activity (n=5) was measured relative to 293T cell line extract positive control set at 100%. Statistical significance was analyzed via ANOVA and is depicted with double (**) asterisks indicates P ≤ 0.01.

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Telomere length in hTERT transduced CD34+ FL HSCPCs

The mean telomere length of the transduced cells was determined using the Telo TAGGG telomere length assay and southern blot analysis of terminal restriction fragment digestion of genomic DNA with HinfI and RsaI (Figure 6A), which stabilized the telomere length of CD34+ fetal liver cells. The mean terminal restriction fragment (TRF) length of the untransduced FL CD34+ cells, serving as ectopic telomere-negative control cells, was 13.26 kb, which decreased to 4.96 kb after 50 days of culture. In contrast, the mean TRF value of hTERT-transduced cells harvested after 30 days of culture increase to 14.04 kb, which was maintained beyond 50 days in culture. Significant differences (p< 0.01) were observed between the telomere lengths of hTERT-transduced cells, untransduced cells and vector-only transduced cells (Figure 6B).
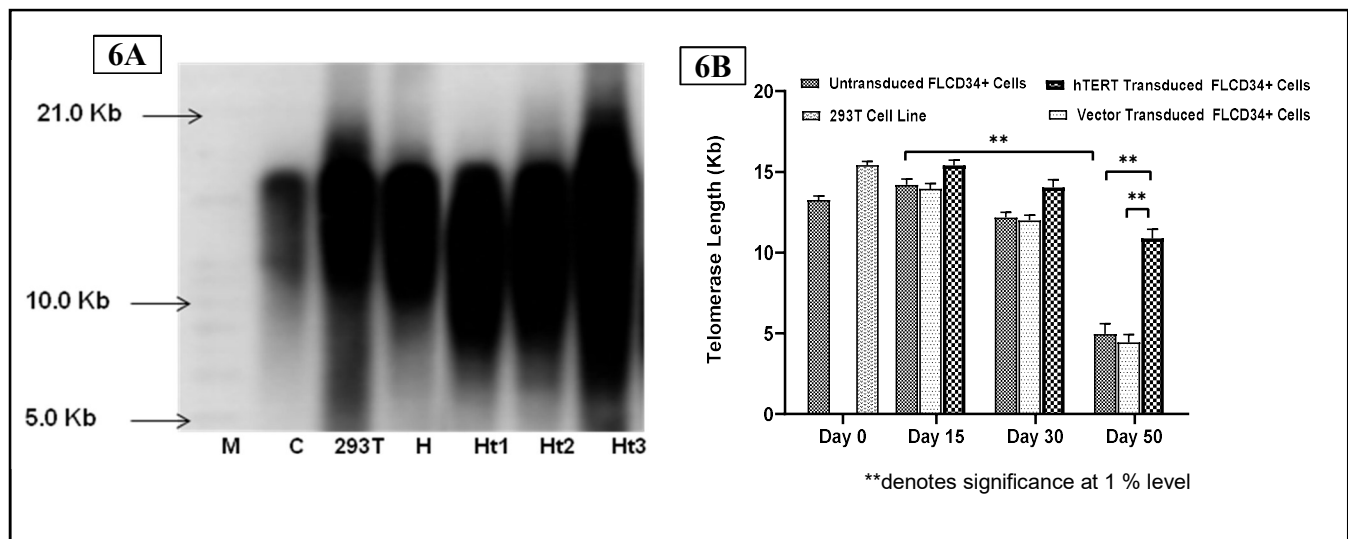

Figure 6. Telomere Length in hTERT transduced CD34+ Fetal liver Cells: Southern Blot for Terminal Restriction Fragment (TRF) length: Expression of hTERT stabilizes telomere length. The positions of size standards (Kb) are indicated on the left. M: Marker, C: control, 293T: 293T cell line, H: Untransduced Cells, Ht1: After 10 days culture, Ht2: After 20 days culture, Ht3: After 30 days culture (6A). Mean TRF (Kb) values (n=5) during 'in vitro' culture for hTERT transduced CD34+ Fetal liver cells, their vector only and non-transduced counterparts(6B). Statistical significance was analyzed via ANOVA and is depicted with double (**) asterisks indicates P ≤ 0.01.

DISCUSSION

To immortalize human hematopoietic stem cells from fetal liver samples, we explored the retrovirally mediated expression of exogenous hTERT. Retroviral vectors are currently the most widely used vehicles for gene transfer into hematopoietic cells, owing to their high gene transfer efficiency, stability following gene transfer, and minimal cellular toxicity[45]. Fetal liver stem cells are particularly suitable for retrovirus transfection, as most cells actively divide and are not in a quiescent state, as observed in adult stem cells[46]. To produce retroviral vectors containing the hTERT gene, we co-transfected HEK293T cells with the amphotropic packaging plasmid pCL-Ampho, the expression plasmid pBABE-puro-hTERT, or the control plasmid pBABE-puro. Transient transfection into HEK293T cells is an effective method to overexpress telomerase and increase its activity. The HEK293T cell line, derived from human kidney epithelial cells, is transformed with the adenovirus E1A gene product and expresses the SV40 large T antigen, enabling episomal replication of plasmids containing the SV40 origin and early promoter region[47]. After verifying the viral supernatant for transgene expression in NIH3T3 cells, fetal liver hematopoietic stem and progenitor cells were

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transduced with the same viral titer. The enrichment of CD34+ cells using immunomagnetic columns allowed us to conduct experiments using a pure population of these cells. Subsequently, the viral supernatants were transduced into fetal liver CD34+ cells, leading to the growth of puromycin-resistant cells in serum-free media containing SCF, IL-6, and Flt-3. The morphology of the transduced fetal liver HSC cultures appeared normal, as depicted by phase-contrast microscopy, and these cells expressed typical stem cell surface antigens (Figure 3). The transduced cells exhibited homogeneous populations of non-adherent cells and continued to proliferate for an extended period. However, karyotype analysis, which would have provided further characterization of the transduced cells, could not be conducted because of technical issues.

In total, three fetal liver HSPC lines were successfully established, two of which were continuously propagated in culture for over eight months. A significant increase in colony-forming ability (CFU-GM, BFU-E, and CFU-GEMM) was observed on day 30 (p<0.01) and day 50 (p<0.05) in hTERT transduced cells. This confirms that the transduction of hematopoietic stem cells does not adversely affect their colony-forming ability in methylcellulose media. Our findings are supported by Goldman et al. who reported minimal differences in cell proliferation and colony formation following viral gene transfer of telomerase activity in HSCs[48]. Additionally, our results are consistent with those of other researchers, who observed that hTERT-transduced adipose tissue-derived CD34+ cells maintained phenotypic characteristics and multi-differentiation potential similar to those of untransduced cells[49,50].

We quantified telomerase activity in fetal liver hematopoietic stem cells before and after transduction. Upon initial expansion, untransduced CD34+ cells exhibited telomerase activity level of 74.34±0.5%; however, this activity subsequently declined as shown in Figure 5. These results are consistent with those of Holt et al. (1996)[51] and Engelhardt et al. (1997)[52], which demonstrated that telomerase is upregulated in rapidly expanding cells and downregulated upon differentiation[53]. In our study, ectopic transfer of hTERT in CD34+ FL cells

increased telomerase activity, as evidenced by the Telomeric Repeat Amplification Protocol (TRAP). The highest fold increase in telomerase activity in hTERT-expressing cells was recorded at PD10, reaching 78.9±1.0% (p<0.05). Notably, the telomerase activity of hTERT-transduced cells remained consistent even after 30 population doublings, demonstrating that transduction of the hTERT gene successfully maintained telomerase activity in CD34+ fetal liver cells. This finding is further supported by similar studies[48,54,55].

To evaluate the effects of hTERT expression on telomere length and cellular life span, we extracted genomic DNA from both vector control-infected cells and hTERT retrovirus-transduced cells at early passages and after maintaining the cultures for at least ten days post-infection. The telomere restriction fragment (TRF) length of telomerase-negative control cells was significantly longer at early passage than at later passages, reflecting the loss of telomeric DNA during extended culture. These findings are in accordance with earlier research[52,56] indicating that telomere length shortens during cell proliferation and differentiation. In agreement with earlier studies conducted by Vaziri and Benchimol[57], we observed telomere shortening in hematopoietic cells on proliferation, despite the presence of telomerase activity. Telomeres that shorten to such an extent may no longer protect chromosome ends, leading to genomic instability and cell death associated with the crisis[58,59]. Allsopp and Weissman suggested that most primitive hematopoietic cells lose telomeric DNA at a rate comparable to that of other somatic cells (50-100 bp per doubling)[18]. Although control vector-infected cells also enter crisis as their telomeres shorten, hTERT-expressing cells maintain telomere lengths around 12 kb over time and do not enter crisis, thereby continuing to proliferate. Primary fetal liver CD34+ cells exhibit limited growth potential and enter a senescence-like state after 10-15 doublings. In contrast, transfected CD34+ cells, cultured for more than 50 population doublings, showed no evidence of senescence, as assessed by cell morphology and trypan blue dye exclusion. The significant increase in colony-forming ability observed at both day 30 (p<0.01) and day 50 (p<0.05) in hTERT-transduced cells suggests that the

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transfection of the hTERT gene subsequently extends the life span of FL CD34+ cells. Our data indicated that the constitutive hTERT expression enhances the replicative capacity of CD 34+ FLHSPCs and prevented these cells from undergoing terminal differentiation under the examined culture conditions. Importantly, hTERT expression stabilizes telomere length, resulting in an immortalized FL CD34+ hematopoietic stem and progenitor cell line that maintains normal morphology. This observation is consistent with recent findings suggesting that hTERT regulates the DNA damage response pathway and contributes to chromatin maintenance independent of telomere length[59]. As noted by Zhao et al. overexpression of hTERT increases cell proliferation, telomerase activity and telomere length in stem cells[54]. Our findings are also supported by those of Raval et al., who demonstrated that the reactivation of telomerase activity in telomerase knockout hematopoietic stem and progenitor cells restored proliferation, normalized DNA damage, and improved red cell production[55].

These findings establish the feasibility of bypassing senescence in human hematopoietic stem cells through genetic engineering. Our investigation provides novel insights into the use of hTERT for the immortalization of fetal liver HSPCs, demonstrating the enhanced proliferative capacity, telomere length preservation, and functional integrity of transduced cells. These findings pave the way for future research and potential clinical applications of hematopoietic stem cell therapies.

CONCLUSION

Our findings demonstrate that the introduction of telomerase activity effectively extends the lifespan of fetal hematopoietic stem cells without altering the cell morphology or population doubling time. Enhanced telomerase activity is crucial for sustaining the viability and functionality of these cells in culture, thereby increasing their overall lifespans. Moreover, the introduction of hTERT into hematopoietic stem cells allows for the maintenance of telomere length for a longer duration than in untransduced cells. These modified cells hold significant potential for use in various in vitro experiments focused on studying

hematopoietic stem and progenitor cells derived from the fetal liver. Despite the widespread use of retroviral vectors for gene expression, the novelty and significance of our findings are highlighted by the scarcity of literature on retrovirus-mediated hTERT expression in hematopoietic stem cells.

A significant avenue for future research involves karyotype analysis, which has previously been constrained by technical limitations. Subsequent studies will emphasize the genetic integrity, phenotypic expression, and long-term stability of the transduced cells. Further exploration of primitive hematopoietic stem cell biology will enhance our understanding of the mechanisms governing self-renewal and differentiation, as well as the regulatory processes associated with telomerase-related hematological malignancies. These investigations are expected to provide valuable insights into critical biological processes.

CONFLICTS OF INTEREST

The authors have no conflicting financial interest.

Ethical approval and consent to participate

Ethical approval was obtained from the Human Ethics Committee of All India Institute of Medical Sciences, New Delhi, India. To collect human fetuses, we shared patient information sheets and received informed consent from patients who underwent Medical termination of pregnancy (MTP). Therefore, we declare that we have performed all procedures according to the relevant guidelines and regulations.

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