Efficient Expansion of SALL4–Transduced Umbilical Cord Blood Derived CD133+Hematopoietic Stem Cells

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Abstract- Hematopoietic stem cells (HSCs) were characterized by self-renewal and multilineage potential. Umbilical cord blood-derived (UCB) as an alternative source of HSCs is widely used especially in children for stem cells transplant (SCT). The main limitation in using UCB for transplantation especially in adults is low cell dose. To overcome this limitation besides using double dose UCB, ex vivo expansion is the most important way to increase cell number for transplantation. HSCs are mainly isolated using CD133 or CD34. CD133, as the most primitive marker, shows important physiological role in maintenance and expansion of HSCs. SALL4 plays crucial role in the development and maintaining the pluripotency and self-renewal ability of embryonic stem cells (ESCs) as well as HSCs. Moreover, SALL4 act as a regulator of HSCs expansion, normal hematopoiesis, and hematological malignancies. In the present study, $CD133^+$ cells positively selected and ex vivo expanded in SALL-4 and GFP-transduced group. CD133 expression assessed using flow cytometry at day 0, 7 and 10. Moreover, multilineage differentiation and proliferation potential of expanded cells in both groups evaluated using colony forming unit (CFU) assay, and cells count assay. Karyotyping analysis was performed to assess any chromosomal instability after 7 days of expansion. Obtained results demonstrated that SALL-4 transduced cells showed significant increase in cell number compared to control group. Moreover, immunophenotyping results showed higher expression level of CD133 at day 7 and 10 following expansion in SALL-4 transduced (62 % and 42%) compared to control group (51% and 20.6%). Our results illustrated that SALL4 could act as a positive factor for the expansion of $CD133^+$ derived UCB cells besides maintaining self-renewal and differentiation ability of expanded cell without any numerical and structural chromosomal aberrations .

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Keywords: Hematopoietic stem cells (HSCs); Ex vivo expansion; SALL4; Karyotype; Umbilical cord blood

Introduction

From the first time of umbilical cord blood (UCB) stem cells transplantation in 1988 (1), UCB taken into account as an alternative source to Bone Marrow (BM) and mobilized peripheral blood (mPB) hematopoietic stem/progenitor cells (HSPCs) for treatment of hematological malignancies such as leukemia, lymphoma and non-hematological disorders (2-5). Despite advantages introduced for UCB including low incidence of graft-versus-host disease (GVHD), availability and suitability, the main obstacle in using UCB especially in adults is the low cells dose available for transplantation. One approach to overcome this restriction is ex vivo expansion of isolated cells besides maintaining their self-renewal ability and differentiation potential (6-8). HSCs mostly isolated based on cell surface markers including CD34 or CD133. CD133 (AC133), which is a 5-transmembrane glycoprotein, was first described as a marker of primitive hematopoietic progenitors and neural stem cells. In addition, it is a marker of stem cells pluripotency, and CD133+cells have the ability of multilineage differentiation including endothelial cells, hepatocyte, neural cells, and cardiomyocytes. CD133 also known as a marker of tumor-initiating cells in various types of cancer (9, 10). Various methods have introduced for ex vivo expansion of hematopoietic stem cells (HSCs) such as cytokine cocktails, stromal cells co-culture, three-dimensional (3D) culture as well as engineered overexpression of Notch ligand and HOXB4 (7,11). HSCs niche in bone marrow included non-stem cells, which maintained stem

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cells in undifferentiated state (4). So, engineered overexpression of signaling pathways in this microenvironment such as Notch, HOX-B4 and Wnt have shown efficient results for ex vivo expansion (12). Recently, it has been reported that overexpression of genes including SALL4 has efficient results on the expansion of HSCs (13). SALL4 is a C2H2-type zinc-finger transcription factor (TF) and a member of the Spalt-like (SALL) gene family, which is located within 20q13.2. It was originally cloned based on their DNA sequence homologous to the homeotic Drosophila gene spalt (sal) (14, 15). It plays pivotal roles in normal development and maintaining the pluripotency and self-renewal ability of embryonic stem cells (ESCs) as well as HSCs. In humans, SALL4 gene heterogeneous mutations are associated with Duane-radial ray syndrome (DRRS), acro-renal-ocular syndrome (AROS) and IVIC syndrome (16). SALL4 expression decreased after birth in adult tissues and organs. However, SALL4 is overexpressed in various hematological malignancies and solid tumors including acute myeloid leukemia (AML), chronic myeloid leukemia (CML) blastic crisis, Myelodysplastic Syndrome (MDS) (17), breast, gastric, colorectal and lung cancer (18). It has been reported that overexpression of SALL4 in mobilized peripheral blood CD34+ cells increased ex vivo expansion efficiency by more than 10000 fold in the presence of specific supplementation for CD34+/CD38- and CD34+/ CD38+ (13, 19). In the present study, we aimed to assessed the ex vivo expansion potential of UCB-derived CD133+ as the most primitive marker of HSCs compared to CD34 cells following transduction with SALL4 gene. Also, it has been reported SALL4 was elevated in some hematological malignancies which are associated with chromosomal abnormalities. Thus, karyotype analysis performed to check any chromosomal aberrations following expansion using SALL4 Zinc finger transcription factor.

Materials and Methods

Sample collection

Umbilical cord blood (UCB) samples (n=4) were collected after obtaining written informed consent from full-term patients scheduled to undergo C-section from Iranian Blood Transfusion Organization. Cord Blood (CB) samples were collected into standard bags containing citrate-phosphate-dextrose anticoagulant. Mononuclear cells (MNCs) were isolated from UCB by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) within 24 hours.

Immunomagnetic purification of CD133+cells

CD133 cells were enriched through positive selection of MNCs using human CD133 Microbead Kit and MidiMACS separation LS columns (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Following isolation purity of the CD133 cells were analyzed using BD FACS Calibur flow cytometry (Becton Dickinson, USA).

Flowcytometry

Flow cytometry was performed for CD133 cell surface marker using PE-conjugated anti-human CD133 and PE-conjugated mouse IgG2a isotype control (eBioscience, USA) to evaluate expression percentage of CD133+cells in both SALL4 and cytokine cocktail expanded cells at day 7 and 10 after expansion.

SALL4 transduction of CD133 positive isolated cells

Human SALL4 (Applied Biological Materials (Abm) Cat No. LV295054 Inc, Canada) and PLenti-GFP blank (Abm, Cat No. LV590 Inc, Canada) Lentiviral Vectors were purchased from ABM incorporation. Bacterial Colonies were cultured in Luria broth (LB) medium containing Kanamycin for 16-18 hours in 37° c shaker incubator. Then plasmids were extracted using Exprep Plasmid SV DNA Purification kit according to manufacturer's protocol (GeneAll Biotechnology, South Korea). CD133+isolated cells were nucleofected with SALL4 plasmid using Human CD34 Cell Nucleofector Kit (Lonza, program number U-008) according to manufacturer's instruction.

SALL4-transduced CD133+cells were cultured in 24-well plated under Stemline II Hematopoietic Stem Cell Expansion Medium (Sigma-Aldrich, St.Louis, MO, USA) supplemented with 50 ng/ml Thrombopoietin (TPO), 50 ng/ml Stem cell factor (SCF) and 50 ng/ml Fms-like tyrosine kinase 3 ligand (FLT-3 ligand). All growth factors purchased from Peprotech (Peprotech, USA). The medium was changed every 48 hours.

In vitro clonogenic assay

Clonogenic assay performed before and after expansion in order to assess the colony forming capacity of transduced CD133+cells. Expanded SALL4-transduced cells and freshly isolated cells were plated at concentration of 1×10^3 cells/ml in Methocult™ H4435 enriched containing cytokine cocktails (Stem Cell Technologies, Vancouver, Canada). Each 35-mm prepared petri dish was incubated at 37° c in 5 % Co_2 for 14 days. Hematopoietic colonies including erythroid

burst-forming unit (BFU-E), granulocyte–monocyte colony forming unit (CFU-GM), and granulocyte-erythrocyte-macrophage-megakaryocyte colony forming unit (CFU-GEMM) counted and scored under the inverted microscope after 14 days. Each cluster consisting of 50 cells scored as a colony.

Chromosomal analysis

Expanded stem cells karyotyping analyses carried out after 7 days of ex vivo expansion. The method used is essentially the same as previously described with some modifications (20). Briefly, hematopoietic cells were incubated with 0.1 μg/ml of Colcemid KaryoMAX (Invitrogen, USA) for 2:30-3 hours. Then cells were incubated in 75 mM KCL for 45 minutes at 37°C and finally fixed in a solution of methanol/acetic acid (3:1). After G-Banding, 20 metaphases characterized for studying structural and numerical chromosomal abnormalities.

Statistical analysis

All statistical analysis performed by GraphPad prism 5.0 (GraphPad software, Inc.). $P<0.05$ considered being statistically significant.

Results

Ex vivo expansion of CD133 isolated cells

Isolated cells were transduced with SALL4 and pLenti-GFP lentiviral vectors using nucleofector kit. The efficiency of nucleofection was evaluated under Inverted microscope (Nikon Corporation, Tokyo, Japan) and was around 40 % in CD133+ HSCs.

To show the purity and expression level of CD133 following expansion in SALL4-transduced and GFP-transduced expanded cells flow cytometry was conducted at day 0, 7 and 10.

The purity of immunomagnetic isolated CD133+ HSCs was approximately 89% (Figure 1A). Flow cytometry results demonstrated that expression level of CD133 was higher in SALL4 transduced cells (62 % and 42%) compared to cytokine-based expanded cells (51% and 20.6%) at day 7 and 10, respectively (Figure 1B). It has been shown that SALL4 helps to maintain stem cell immunophenotype of HSCs at higher level compared to control group. In addition, cells were counted every 24 hours for 7 days to evaluate the rate of expansion in SALL-4 transduced and GFP group with the initial count of 1×10^5 in 24 well plates. As shown in Figure 1C, obtained results demonstrated that expansion rate of SALL-4 transduced cells was greatly higher than the control group after 7 days of expansion in liquid culture medium. A significant increase in the total CD133+ count was observed in SALL4 lentiviral transduced group after 7 days of expansion (24 fold, $P<0.0001$). Cell count in control group showed a 4-fold increase in total CD133+count. Total cell count was shown a significant increase in SALL4 expanded cells compared to control group (6 fold, $P<0.05$). These data showed that SALL4 could significantly increase the expansion ratio in infected group compared to control group following 7 days of expansion in defined condition.

Karyotyping of ex vivo expanded CD133 cells

Ex vivo expanded CD133+cells in both groups were analyzed for chromosomal stability in all expanded UCB samples. All analyzed metaphases demonstrated no numerical and structural changes in chromosomes after 7 days of expansion in SALL4-transduced and GFP-labeled expanded cells in cytokine cocktail. (Figure 3).

Hematopoietic colony assay

To study the proliferation and differentiation potential of expanded UCB- derived HSCs in both sall4-transduced and control group, colony forming unit (CFU) assay was performed using MethoCult semi-solid medium in the presence of various cytokine cocktails. The results revealed that SALL4 infected cells are able to produce multiple colony types including CFU-GEMM, CFU-GM and BFU-E similar to freshly isolated $CD133^+$ cells (Figure 3 A-C). Furthermore, number of colonies was counted 14 days after culturing in the semi-solid medium in both SALL4 and freshly isolated group. There is not a significant difference in colony numbers in both groups.

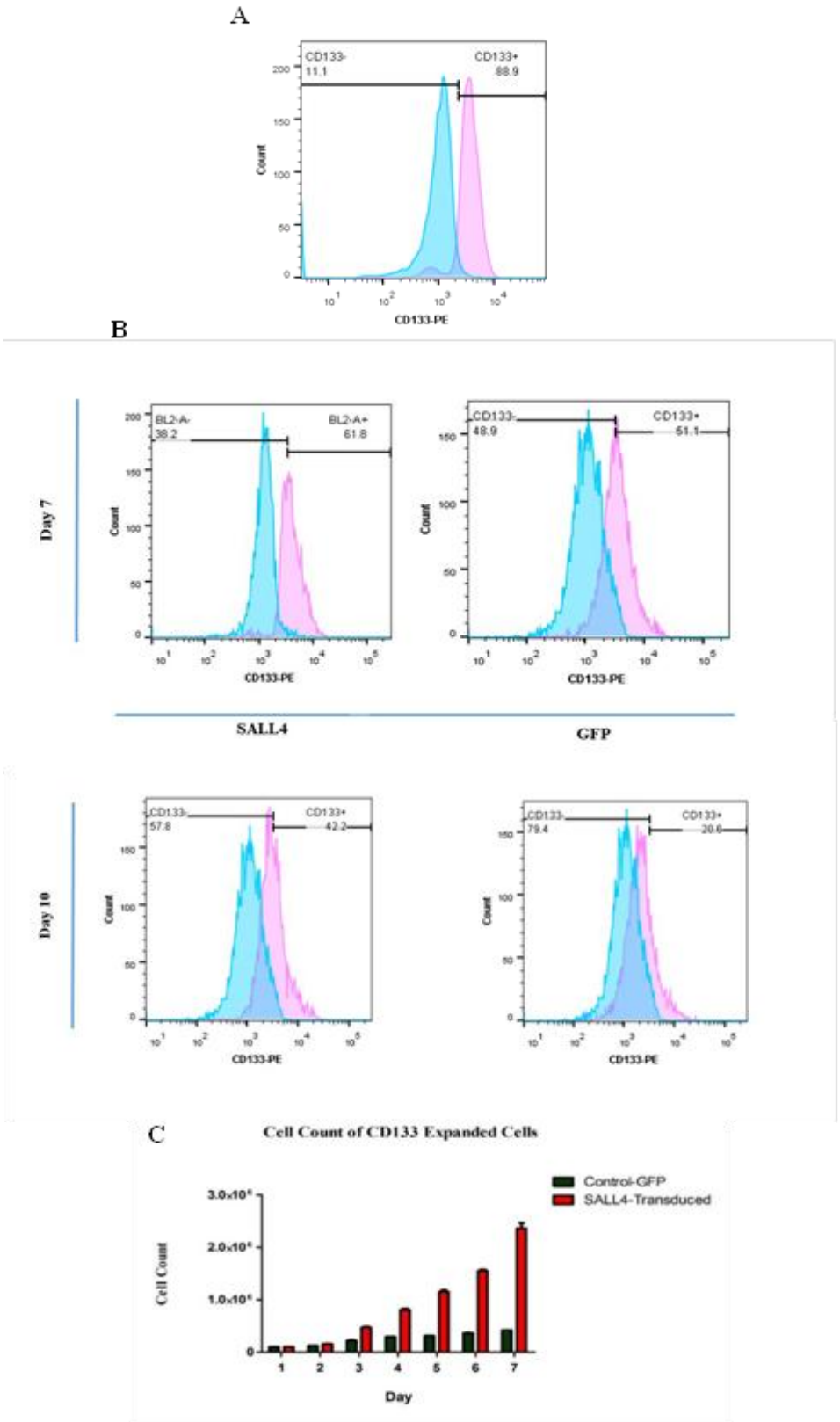

Figure 1. CD133 expression and purity of immunomagnetic isolated HSCs at day 0 (A). CD133 expression was evaluated in SALL-transduced cells and GFP control group at day 7 and 10 following ex vivo expansion. CD 133 expression was higher in SALL4 treated group (62 % and 42%) compared to control group (51% and 20.6%) respectively at day 7 and 10 of ex vivo expansion (B). expanded cells were counted every 24hour for 1 week. Total count of SALL4 infected cells showed significant increase compared to control group at day 7 (6-fold, $P<0.05$) (C).

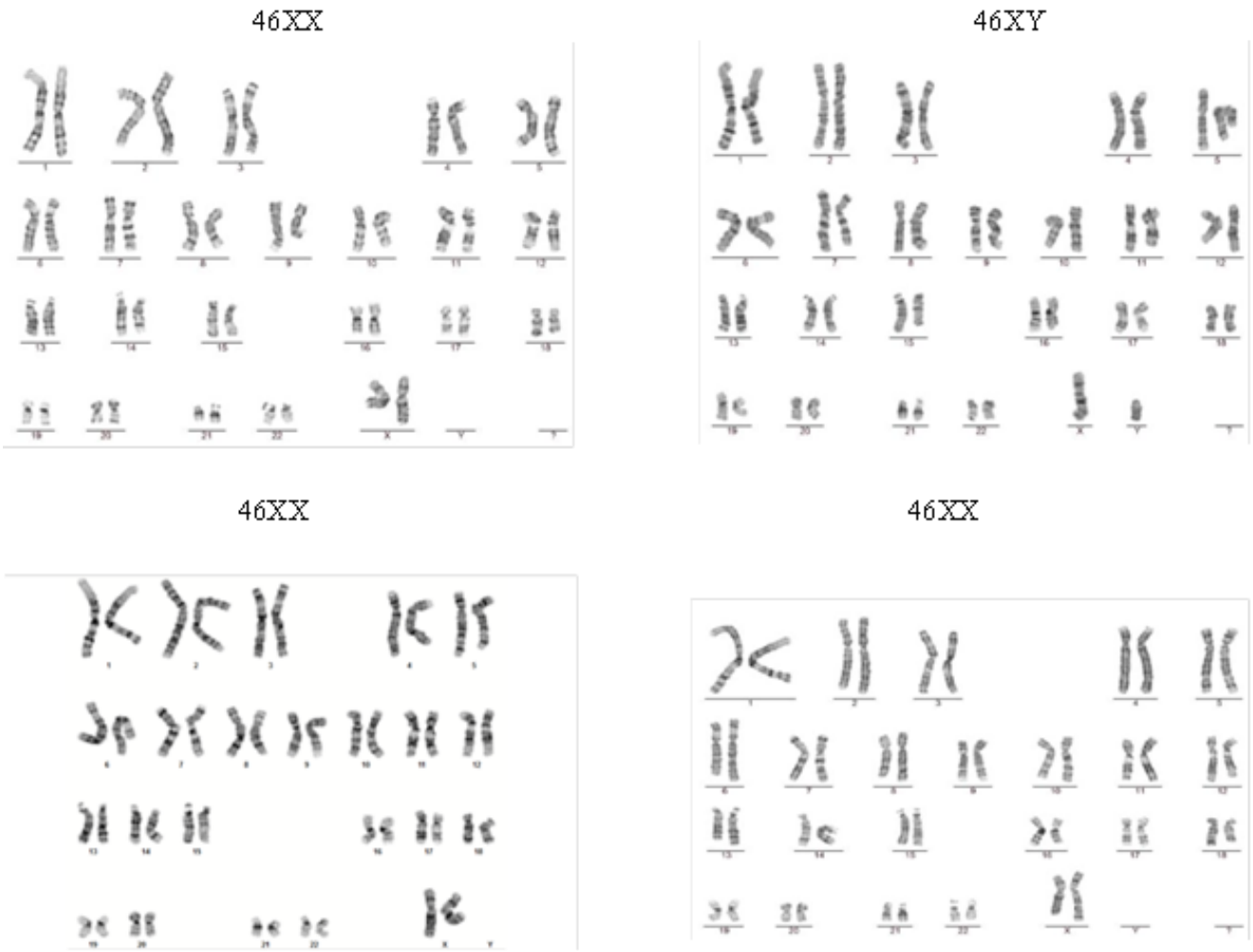

Figure 2. Chromosomal study. G-banding study of 20 metaphases did not show any chromosomal structural and numerical abnormalities in expanded cells 7 days after ex vivo expansion in SALL-4 treated $CD133^+$ HSCs

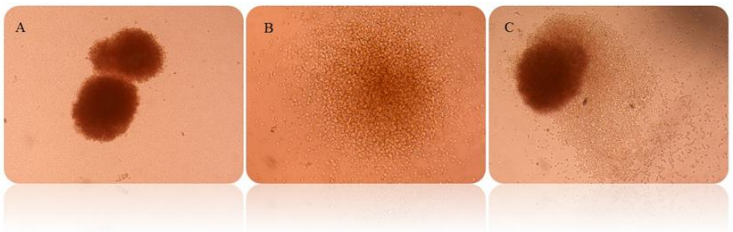

Figure 3. Colony forming assay. Various lineages observed in the Clonogenic assay in SALL4-transduced $CD133^+$ HSCs, including BFU-E, CFU-GM, and CFU-GEMM, respectively (A-C). It has demonstrated that the expanded HSCs have the differentiation potential to different toward various blood cell lineages. A number of CFU colonies formed from SALL4-induced hematopoietic stem cells. The number of CFU colonies was counted 14 days after SALL4 delivery and in freshly isolated cells. It has been represented that there is no significant difference in a number of colonies in SALL-4 and control group. Types of CFU colonies formed at day 18 (D)

Discussion

Hematopoietic stem cells (HSCs) are rare adult stem cells which have the potential of Self-renewing and multilineage differentiation (4). It has been isolated from different sources including Bone marrow (BM), Peripheral blood (PB) and umbilical cord blood (UCB). HSCs from UCB are able to produce in vivo long-term repopulating stem cells (21). The main disadvantage of using this source of stem cells is low cells dose available for therapeutic purpose, especially in adults. Cell dose increased following ex vivo expansion. Currently, several approaches exist for ex vivo expansion of hematopoietic progenitor cells (HPCs) which is especially isolated based on CD34 or CD133 cell surface marker. In the present study, we isolated CD133+subset of UCB-derived HSCs. It has been reported that CD133+cells demonstrated the higher capacity of long-term culture initiating cells (LTC-ICs) and more efficient proliferation potential compared to cells isolated based on CD34 expression (22, 23). There are various methods described expanding CD34+ isolated HSC from various sources (24). Recently, transcription factors such as SALL4, HOXB4, and Notch-Delta1 used to expand HSCs isolated from BM and other sources (25). In 2011, it was reported that SALL4 acts as stimuli of expansion in CD34+ HSCs derived from mobilized peripheral blood cells(13). In another experiment, Shen, Bin *et al*., used SALL4B isoform to expand BM-derived CD34+ nonhuman primate HSCs (25). We demonstrated that SALL4-transduced CD133+UCB derived HSC, as more primitive cells to CD34+HSCs, expanded efficiently in the presence of SCF, FLT-3 ligand and TPO compared to control group. SALL4 as a member of Zinc finger transcription factor plays critical role in hematopoiesis including normal hematopoiesis, stimulation of ex vivo expansion and acting as oncogene in leukemia and hematological malignancies. In a study which performed in murine, it has been demonstrated that overexpression of both sall4 isoforms (SALL4a or SALL4b) leads to blocking normal hematopoiesis and decrease self-renewal potential in HSCs in a dose-dependent manner (26). In contrast to their results, we demonstrated that SALL4 lentiviral transduction causes a 6-fold change in total cell count CD133+HSCs compared to control group (Figure 2C). We also observed that it SALL4 overexpression in isolated HSCs increase the rate of expansion (24-fold, $P<0.0001$). Moreover, flowcytometry analysis demonstrated that the expanded cells presented higher levels of CD133+expression after 7 days of ex vivo culture. In the same study, colony formation assay demonstrated that both isoforms SALL4 overexpression at medium to high levels resulted in decreased in colony size and lowering proliferation potential of transduced cells. In a different pattern, we observed that there is no significant difference in forming CFU-GEMM, BFU-E, and CFU-GM in methylcellulose semi-solid medium compared to freshly isolated CD133 HSCs (Figure 3 which our results are similar to the findings which have reported following the expansion of mobilized CD34+subpopulation of HSCs (13). They demonstrated that 1-month-old SALL-4 transduced cells formed similar numbers of various colony types compared to GFP- labeled control cells. An important aspect of ex vivo expansion of UCB derived stem cells besides maintaining stem cells potential of HSCs is clinical safety. During this processes, some chromosomal abnormalities might be present in expanded cells, which could cause the transformation to cancer stem cells. Chromosomal analysis performed to show chromosomal aberrations in expanded cells. It has been reported that chromosomal abnormalities were observed in all expanded CD34+ UCB derived HSCs after 14 days (27). They observed no genetic instability and transformation findings in soft agar studies. In the current study, all analyzed metaphases didn't show any numerical and structural chromosomal instability, 7 days after expansion of SALL4-tranduced cells that is compatible with the reports presented on the expansion of CD34+expanded cells(20, 28).

Our results demonstrated that SALL4 is an efficient intrinsic factor which leads ex vivo expansion of UCB derived CD133+cells with no chromosomal aberrations as previously it has been reported that it has effect on transformation to hematological malignancies especially AML.

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