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EFFECT OF STEMREGENIN1 AND SB431542 SMALL MOLECULES ON EX VIVO EXPANSION OF UMBILICAL CORD BLOOD HEMATOPOIETIC STEM CELLS ON BIOCOMPATIBLE POLYETHERSULFONE NANOFIBER SCAFFOLDS

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Abstract:

Cord blood Hematopoietic stem cells (HSCs) with several advantages including low chance of viral contamination and low rate of Graft versus host disease (GVHD) are appropriate candidate for vast medical applications such as transplantation. The main obstacle of cord blood HSCs is the low number cells. To improve ex-vivo expansion of umbilical cord HSCs we introduced a new culture system. Isolated HSCs were seeded in Three-dimensional(3D) on Polyethersulfone(PES) scaffolds and Two-dimensional(2D) culture conditions and treated with SB431542 and Stemregenin1(SR1) small molecules. On the fifth and tenth days the expanded cells in different groups were investigated for expression of specific markers by flow cytometry, expression of some stemness genes by qRT-PCR and colony formation by methocult medium. SR1 molecule significantly increased expansion of $CD34^+$ cells while SB431542 induced more $CD34^+/38^+$ cells. Also SB431542 treated cells showed higher colony formation capacity. SR1 increased the expression of *c-Myc*, *HOXB4* and *SALL4* while SB431542 seemed to inhibit *HOXB4* expression and increase *SALL4*.Together all, this study introduced a new ex vivo culture setting for further medical application of HSCs. Our data showed simultaneous use of these two small molecules can provide appropriate outcome for HSCs transplantation includes both of engraftment and repopulation.

Keywords: Cord blood; 3D culture; Polyether sulfone; Hematopoietic stem cells; SB431542; Stemregenin1; *c-Myc*; *HOXB4*; *CXCR4*; *SALL4*

Abbreviations: CB –cord blood; HSC –hematopoietic stem cells; GVHD –graft versus host disease; *c-Myc* –avian myelocytomatosis virus oncogene cellular homolog; *HOXB4* –homeobox protein Hox-B4; *CXCR4* –chemokine receptor type 4; *SALL4* –sal-like protein 4; SR1 –stemregenin1; SCF –stem cell factor; FLT3L –FMS-like tyrosine kinase 3 ligand; TPO –thrombopoietin; 3D –Three-dimensional; 2D –Two-dimensional; PES –Polyethersulfone

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Introduction:

Hematopoietic stem cells (HSCs) are defined as multipotent stem cells with ability to differentiate in to variety of progenitor and adult blood cell lineages and also self-renewal. These cells could be isolated from several resources such as peripheral blood , bone marrow and cord blood [1, 2]. Currently a wide-demanding resource of HSCs for stem cell transplantation approaches is cord blood regarding to its noninvasive sampling, availably, low chance of viral contamination, low number of T cell population and low rate of GVHD occurrence after transplantation [3, 4]. mentioned characteristics along with the ability of these cells to differentiate in to non-hematopoietic cells, introduce cord blood HSCs as a potential mean for vast medical approaches [5]. Upon the first cord blood transplantation in 1988 to treat congenital

fanconi anemia, lots of efforts have been done to use cord blood HSCs for treatment of other congenital disorders as well as different malignancies and even autoimmune disorders [6-8]. But in spite of advantages of cord blood HSCs, the main problem with this resource of stem cell is low amount of isolated HSCs from each cord blood which limits cord blood HSCs transplantations in generally high weight recipients and adults [8, 9]. For overcome this limitation several solutions such as using pooled cord blood and in vitro expansion of HSCs have been suggested [9] . So it seems providing efficient in vitro or ex vivo expansion methods for cord blood HSCs could be an advantageous way to obviate this shortage

Generally hematopoietic stem cells are tended to perch in specified regions of tissues which provide structural, mechanical and biochemical circumstances for optimal function and expansion of these cells. These regions are so called "niche". In fact main duties of these 3D porous structures is to build a balance between differentiation and regeneration of hematopoietic stem cells and providing cell to cell connection to enhance stem cell like functions. [10]. recently 3D scaffolds have been produced as ex vivo medium with the ability to mimic in vivo niches [11, 12]. These scaffolds should possess certain properties including bio-compatibility and bio-degeneration and also should provide appropriate attachment for stem cells. in this 3D conditions, other factors such as specific culture media , growth factors and chemical drugs could be added to optimize stem cell function and proliferation [13]. Nano-fiber scaffolds such as PES scaffolds have been widely used in medical approaches regarding to their beneficial properties [14]. Although few studies investigated PES function in enhancement of hematopoietic cells function but it seems that some modifications could increase its efficacy. For example it has been shown that amino acid treated PES efficiently increase hematopoietic cell expansion [15]. Also we previously used PES scaffolds along with a layer of mesenchymal stem cells as a 3D structure for culturing HSCs and showed an increased expansion with minimum differentiation of HSCs in this system [16].

As mentioned above bio-chemical agents could also be used to optimize HSCs functions and self-renewal ability. SB431542 a TGFβ blocker and stemregenin1 (SR1) an aryl hydrocarbon receptor antagonist are two synthetic bio-active small molecules which thought to induce expansion several cells including in stem cell population and minimizing differentiation capacity [17].

Regarding to mentioned data on applications of HSCs and feasibility of in vitro and ex vivo expansion of cord blood derived HSCs, we launched a comparative study to investigate the expansion and differentiation of cord blood HSCs in 3D and 2D cultures in presence of SB431542 and SR1 small molecules and also growth factors such as SCF ،Flt-3 and TPO to open up a new way to improve self-renewal capacity.

Materials and Methods:

Sampling and HSCs isolation:

Cord blood samples were provided by Iranian National Cord Blood Bank (Tehran. Iran) according to their protocols and awareness consent. After determining the volume of each blood samples, mononuclear cells were isolated using Ficoll-Hypaque density gradient (Sigma, USA). Briefly 50 ml of cord blood sample was mixed with 50 ml PBS (Sigma, USA) and the resultant diluted solution was mixed with Hydroxyethyl starch (HES) (Sigma-Aldrich, Germany) with 1:5 ratio and incubated for 2 hours at room temperature to sediment red blood cells. Then, the supernatant was collected and centrifuged at 300 g for 10 min. The cell pellet was resuspended in PBS-EDTA 0.03% and was gently added to ficoll with 7:3 ratio and centrifuged for 30 min at 300g. All were done at room temperature. At the end the ring like layer which contained mononuclear cells was isolated and washed with PBS twice.

In order to harvest $CD34^+$ HSCs from isolated mononuclear cells, human CD34 Micro Bead Kit and MACS LS separation column (Miltenybiotec, Germany) was used according to manufacturer's instructions. Briefly recommended volume of both Fcγ receptor blocking agent and magnet-labeled anti CD34 antibody were added to cell suspension and incubated in dark at 4°C for 1 hour, then free beads were washed and discarded by PBS-EDTA. Cell suspension was allowed to pass the magnet-column where the $CD34^+$ cells were trapped. At the end HSCs were isolated by mechanical pressure. The whole procedure was done under sterile condition. The purity of isolated cells was evaluated by anti CD34-PE and anti CD38-FITC antibody and flow cytometry method.

PES scaffold preparation and modification

PES nanofiber scaffolds as the 3D culture structure were designated by electro spinning method. To this end PES (Mw: 8000Da) (Sigma Aldrich,

Germany) was diluted in N,N-dimethyl formaldehyde (DMF) (Sigma Aldrich, Germany) and stirred for 4 hours to achieve a 24% (w/v) homogenized solution. Then the solution was vacuumed in to a syringe and fixed on the Electro spinning power supplier. The nanofibers were fabricated using 20 kV voltage. Fibers were collected onto an aluminum coated collector. Before any further experiments, nanofibers were preserved in vacuum oven for solvent removal.

To enhance nanofiber properties plasma-surface modification were performed on the fibers. For this purpose, NanoUHP (Diener Electronics, Germany) was utilized according to machine instruction. The modified fibers were then sterilized using 70% ethanol and UV irradiation.

In order to investigating nanofiber 3D structure and the spatial orientation of cultured cells on nanofibers scanning electron microscopy (SEM) (KYKY EM3200, China) study was done. Briefly the cell-cultured PES scaffolds were washed with PBS and cells were fixed by gelutaraldehyde 2.5% for 1 h. For dehydration, a series of gradient alcohol concentration were used. Nanofibers were also coated with thin gold nano particles to increase conductivity using Sputter coater (KYKY SBC12, China) and then visualized by SEM.

Culture situations

In order to investigate the comparative effects of 2D and 3D culture conditions in presence of different components and growth factors on HSCs, 8 different culture conditions were designed. Culture characterizations are summarized in table 1. MACS isolated $CD34^+$ HSCs were diluted in stempro-34 SFM culture medium (Invitrogen, NY, USA) supplemented with 50ng/ml human SCF, 25ng/ml human Flt3 ligand, 25ng/ml Human TPO (all from stem cell technologies, Canada). 2×10^4 cells were seeded in each well of 2D and 3D (PES coated) 24 well plates (Nunc, Denmark) SR1 (Biovision, USA) and SB431542 (Axon Medchem, Netherlands) were diluted in DMSO (Gibco, USA) and added to desired wells to achieve final concentrations of 1µM and 10µM respectively[18]. Plates were incubated at 37° C and 5% CO2 atmosphere. Viability and number of cells were checked using trypan blue 0.4% (thermofisher, USA) and hemocytometer (weberscientific, USA). Cells were harvested at day5 and day 10 for further assays.

Table1. Culture conditions. Different conditions which HSCs were cultured in are summarized here

2D culture	3D culture (PES coated plate)
SR1+ , SB-	SR1+ , SB-
SR1- , SB+	SR1- , SB+
SR1+ , SB+	SR1+ , SB+
SR1- , SB-	SR1- , SB-

Flow cytometry

HSCs were reaped at day 0 (before expansion), day 5 and day 10 for flow cytometric analysis. For this, 1×10^4 cells were added to each tube and were washed twice with PBS. The cell pellets were resuspended in 100 µl PBS and with predetermined concentration fluorochrome conjugated antibodies including CD34 for HSCs, CD38 as lineage Commitment marker, CD13 for myeloid lineage, CD71and CD235 for erythroid lineage,CD14 as monocytic lineage,CD61 for thrombocyte/megakaryocyte lineage, CD10 and CD3 as lymphoid markers (all from Dako,Denmark) were added to intended tubes. FITC or PE conjugated isotype antibodies were also used appropriately as isotype controls. The whole procedure was done at 4°C. Stained cell were then read by flow cytometer (BD LSRII, USA).

Colony-Forming Assay

For measure the colony forming potency of HSCs, 10^3 cells were harvested from different culture conditions at day0, day5 and day10. Cells were then suspended in Methocult H4435 Enriched medium (Stem cell technologies, Canada) and seeded in $35mm^2$ plates (Nunc, Denmark) and incubated for 14 days at 37°C and humidified 5%CO2 atmosphere.at the end the resultant colonies were detected and counted under light microscope (Nikon, japan).

RNA extraction, cDNA synthesis and real time PCR

In order to obtained pure RNA samples, First the supernatant media of all wells were discarded at day0, day5 and 10. Then 0.3ml per 10^5 -10^7 cells trizol (Invitrogen, USA) was directly added to each culture wells and to obtain a homogenized suspension, cells were pipetted 30 times by a 20G syringe and incubated for 5 min at room temperature and then 200 µl chloroform (Merck, Germany) was added to each tube and gently mixed and incubated for 3 min. tubes were centrifuged at 12000g and 4°C for 15 min. The aqua phase which contained RNA was collected and transferred to another tube. RNA was precipitated by adding 500µl of isopropanol (Merck, Germany) and Incubating for 10min then centrifuging at 12000g and 4°C for 10 min. After discarding the supernatant, the pellets were

washed with ethanol 70%. At the end, pellet was dissolved in DEPC treated water. Purity and Concentration of RNA samples were checked by UV spectrophotometer (Nanodrop 2000, thermo fisher, USA) and RNA integrity was evaluated by running an aliquot on 1% denaturing agarose Gel.

CDNA was synthesized by QuantiTect Reverse transcription kit (Qiagen, Germany). All procedure was done according to kit instructions without any modification. After genomic DNA elimination and consequent reverse transcription, cDNA samples were preserved at -20°C for further application.

To gene expression analysis, specific primers for *c-Myc*, *HOXB4*, *SALL4* and *CXCR4* were designed using sequencing mask tool pull up fasta file of each gene. Briefly exon-to-exon spanning regions less than 200bp were selected. To avoid repeat run and increase specificity, all sequence was traced in repeat masker software. Qualified sequences were enrolled in primer express3.0 software. Designated primers were checked and evaluated by blast software in order to ensure that chosen primers are specific for intended genes (table 2). After providing the primers, real Time PCR with SYBR green approach for relative quantification of expressed genes was performed. Briefly 25µl Mastermix consist of 12.5µl quantifast SYBR green PCR kit (Qiagen –Germany) plus 1pmol of each primer and 2µl (1:10 diluted) cDNA were mixed and fixed in Corbett Real time PCR thermocycler . A 2 step thermal profile was proceed under following condition: initial denaturation 5 min;1 cycle , 40 cycle of 95 °C 15s;60°C 35 s . Data acquisition was performed during anneal-extension step. Melting curve analysis was analyzed with Corbett real time PCR software with 0.5°C temperature increment at each step. Relative quantification was calculated by Pfaffl's formula approach[19]. All samples were tested triplicate for checking its reproducibility.

Table2. The sequences and the annealing temperatures of utilized primers. F and R represented forwards and reverse primers respectively

Primers	Sequence	Tm oC	PCR product length
c-Myc-F	5`-AGCGACTCTGAGGAGGAAC-3`	59	183
c-Myc-R	5`-CTGCGTAGTTGTGCTGATG-3`	57	
HOXB4-F	5`-TGCAAAGAGCCCGTCGT-3`	57	69
HOXB4-R	5`-GGCGTAATTGGGGTTTACCG-3`	56.4	
CXCR4-F	5`-CGCCACCAACAGTCAGAG-3`	58	176
CXCR4-R	5`-AACACAACCACCCACAAGTC-3`	58	
SALL4-F	5`-CTCTTCAGATCCACGAGCGG-3`	60	260
SALL4-R	5`-GTTCCACACAACAGGGTCC-3`	60	

Graphs and statistical analysis

Graphs were designed using GraphPad prism5 (GraphPad Software, USA). Data was statistically analyzed . One-way ANOVA and Tukey post hoc tests were run for statistical analysis. Data was reported in mean±SD form. P values less than 0.05 were deemed as significant.

Results:

Purity of isolated HSCs

After isolation of HSCs by CD34 positive selection on magnetic column, the purity of isolated proportion was evaluated using anti-CD34 antibody and flow cytometric approach. An example of flow cytometry graph is presented as figure 1 which shows more than 86% of cell populations were $CD34^+$ and less than 15% of cells were $CD38^+$.

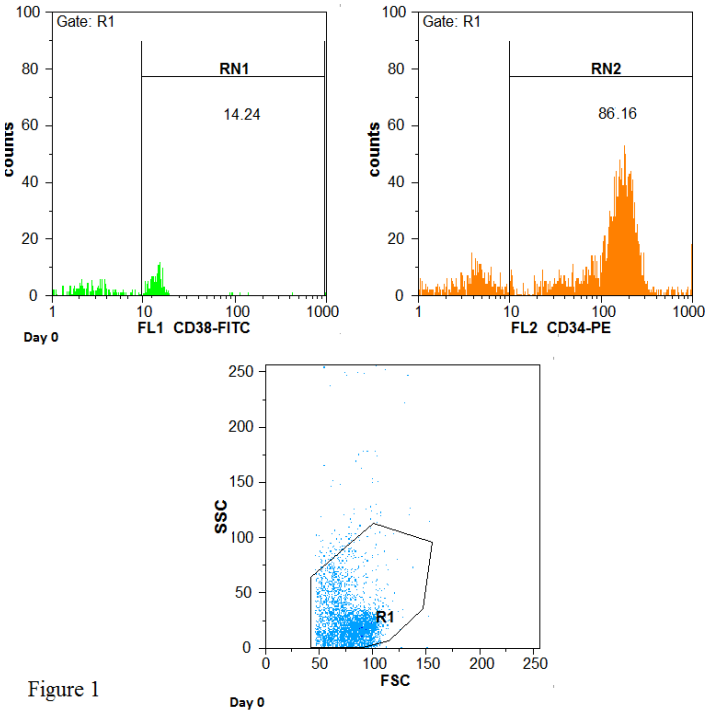

Figure 1

Figure1. Purity of isolated corld blood HSCs. the percentage of CD34⁺ and CD38⁺ cells after isolation by MACS method is presented here.

Verifying the structure of PES scaffolds

To ensure the appropriate spatial orientation of 3D scaffold, Scanning electron microscopy was utilized. The resultant figures revealed that the scaffolds are well- oriented and porous (figure 2A). Also Cells were attached appropriately to 3D scaffold appropriately (figure 2B). Light microscopy of cultured cells in 2D condition by inverted microscope is also shown in figure 2C

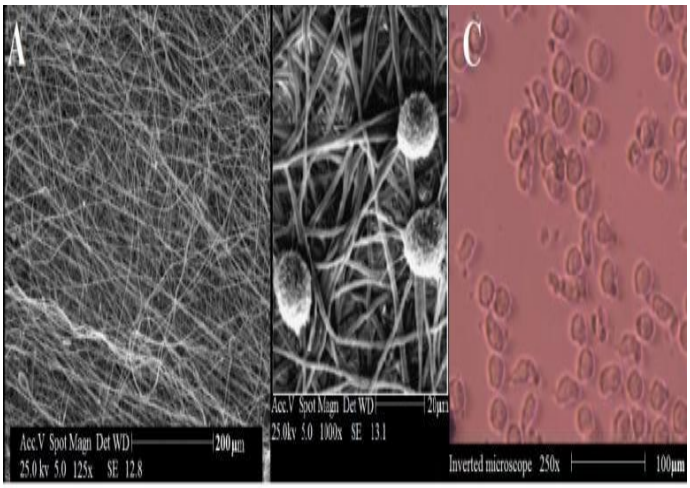

Figure 2

Figure2. Structure of 3D and 2D culture settings. The structure and form of PES scaffolds without cells (A) and along attached cells (B) by scanning electron microscopy and the form of 2D culture (C) are presented by inverted microscope in figure.

Overall expansion of cultured HSCs

After culturing and counting the number of HSCs in different conditions and different days, data showed that in all conditions the number of cultured cells were significantly more than day0 (all p-values were less than 0.01) . Overall in both 3D and 2D conditions the numbers of cells in day 10 were significantly higher than day5. It should be noted that the mean total number of cells in day 10 in 2D culture were 7233 , 123333 , 113333, 146666 cells in no treatment, SR1 treatment, SB treatment and SR1+SB treatment conditions respectively while in same conditions in day 10 the mean number of cells were 113333, 186666, 18000, 24333. As mentioned these differences were also seen in 3D culture settings. The mean total number of cells at day 5 in 3D culture settings were 61000, 113333, 103333, 143333 in no treatment, SR1 treatment, SB treatment and SR1+SB treatment conditions respectively whilst with same treatments the mean total number of cells at day 10 were 96666, 156666, 146666 and 236666. This data indicated the positive effects of time on expansion. In case of SB and SR1 effects, data also revealed that in in both 3D and 2D cultures SR1 showed better effects on cells expansion rather than SB molecule (mean total numbers of cells in all conditions are mentioned above) . These data in more details information are presented in figure 3

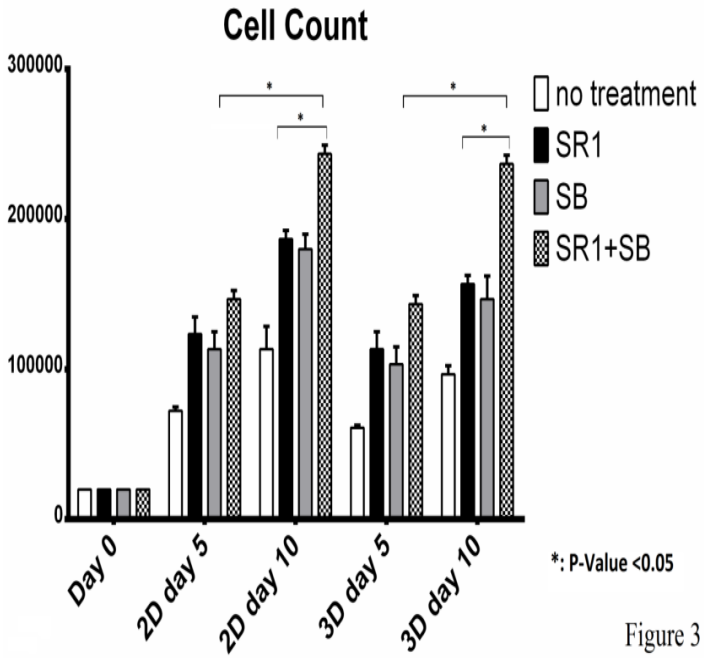

Figure 3

Figure3. The count of HSCs in each culture condition at day5 and day10. The numbers of harvested cells from different culture conditions at day 5 and 10 is presented in form of mean±SD.

Evaluation of specific cells expansion

Different antibodies were used in flow cytometry experiments to evaluate the expansion of specific cells with specific markers. Here The results are presented in form of percentage of each markers and absolute count of each in figure 4. The ratio of $CD34^+/CD38^+$ cells is also provided as determinant for self-renewal/differentiation ratio. In case of $CD34^+$ cells, the number of cells in both 3D and 2D conditions was higher at day 10 rather than day5 except in not treatment conditions (the exact number of CD34+ or CD38+ cells are presented in table 3 and table 4). Although SB and SR1 molecules effects in expansion of these cells but it is Worthy to note that SR1 had more effects on $CD34^+$ expansion in comparison to SB. For example

the number of CD34+ cells in in 3D culture setting at day 5 were 95933 and 74400 in treatment with SR1 and SB respectively (p= 0.01). The second note is that the number of CD34⁺ at day 5 in 3D culture with all form of treatments were significantly higher than 2D cultures (all p values were less than 0.01) but this difference at days 10 was seen just when cells were treated with both SR1 and SB (p-value less than 0.001).

Table 3. The percentage and the number of CD34 expressing cells in different culture conditions

CD34				
Condition	Mean of expressing cells (%)	SD of expressing cells (%)	Mean of absolute count	SD of absolute count
Day 5 (2D)	29	1	20986.67	1312.45
Day 5 (SR1.2D)	65	1	80166.67	7617.30
Day 5 (SB.2D)	57.66	2.51	65333.33	7023.76
Day 5 (SB+SR1.2D)	61.33	2.08	89933.33	4045.16
Day 10 (2D)	25	2	28466.67	5746.59
Day 10 (SR1.2D)	57	3	106400	6581.79
Day 10 (SB.2D)	50.33	2.08	90633.33	7074.13
Day 10 (SB+SR1.2D)	53.33	1.52	129766.70	4308.51
Day 5 (3D)	44.33	0.57	27050	1125.83
Day 5 (SR1.3D)	84.66	3.51	95933.33	10358.25
Day 5 (SB.3D)	72	3	74400	8944.83
Day 5 (SB+SR1.3D)	80	3	114566.70	2250.18
Day 10 (3D)	36.33	0.57	35100	1558.84
Day 10 (SR1.3D)	68.33	3.05	107033.30	5692.39
Day 10 (SB.3D)	57.33	2.08	84100	9600.52
Day 10 (SB+SR1.3D)	61.66	3.51	146066.70	11545.27

As data are shown in figure 4 and table 4, CD38⁺ Cells in 2D conditions at day 10 had the highest number in comparison to other conditions. In 3D conditions the number of cells day 10 were significantly more than day 5 (all p-values were less than 0.01). According to corresponding graph and table it is crystal clear that SR1 and SB increased the absolute number of CD38⁺ proportion at day 10 more than day5. For example the absolute numbers of CD38+ cells in 3D culture at day 10 were 45900 and 53333 cells in treatment with SR1 and SB respectively while in same conditions the numbers of these cells at day 5 were 42666 and 42433. It is worthy to note that the percentage of CD38⁺ cells was higher when treated with SB in comparison to SR1 (33% and 29% in 2D culture at day 5 , 40% and 35% in 2D culture at day 10, 41% and 37% in 3D culture at day 10, 37% and 29% in 3D culture at day 10) . At day 5 the effects of SR1 and SB didn't differ with each other but at day10 the positive effects of SB on CD38⁺ cells number was significantly more than SR1 (p- values were less than 0.01 for both 3D and 2D culture settings).

Table4. The percentage and the number of CD38 expressing cells in different culture conditions

CD38				
Condition	Mean of expressing cells (%)	SD of expressing cells (%)	Mean of absolute count	SD of absolute count
Day 5 (2D)	26.33	1.52	19073.33	1776.55
Day 5 (SR1.2D)	29.33	1.15	36266.67	4734.27
Day 5 (SB.2D)	33	1.73	37333.33	3401.96
Day 5 (SB+SR1.2D)	33.33	2.08	48966.67	4878.86
Day 10 (2D)	35.66	1.52	40566.67	7099.53
Day 10 (SR1.2D)	35.66	1.15	66600	3651.02
Day 10 (SB.2D)	40.66	2.08	73233.33	6045.10
Day 10 (SB+SR1.2D)	38	1.73	92433.33	3600.46
Day 5 (3D)	25.66	2.08	15650	1203.12
Day 5 (SR1.3D)	37.66	1.52	42666.67	4424.17
Day 5 (SB.3D)	41	1.73	42433.33	5810.62
Day 5 (SB+SR1.3D)	32.33	2.51	46266.67	2369.24
Day 10 (3D)	33	1.73	31866.67	1858.31
Day 10 (SR1.3D)	29.33	1.52	45900	953.93
Day 10 (SB.3D)	37.66	2.08	55333.33	7400.90
Day 10 (SB+SR1.3D)	35	2.64	82733.33	4215.84

As mentioned above the CD34/CD38 ratio was calculated in all conditions and is presented in figure 5. Data showed that in all conditions SR1 resulted in higher CD34/CD38 ratio indicating the higher effects of SR1 on expansion rather than differentiation.

CD13 as a marker of myeloid progenitor cells was also investigated. As it is shown in figure 4 the highest percentage of CD13⁺ cells were in no

treatment group in either 3D or 2D culture (25% and 41% at days 5 and 10 in 2D culture setting, 25% and 38% at days 5 and 10 in 3D culture setting) and SB and SR1 significantly reduced the $CD13^+$ proportion of HSCs.

In case of $CD71^+$ and $CD235^+$ cells, as markers for erythroid precursors the results showed that this proportion of cells were more abundant at day 10 in 2D cultures, especially in presence of SB (38966 cells). It seems that SR1 did not play any role in increasing $CD71^+/235^+$ cells. Generally the number of $CD71^+/235^+$ was more in 2D cultures in comparison to 3D culture in all conditions. More details are presented in figure 4.

The absolute amount and the frequency of other lineages showed no notable significant differences. Detailed data on other markers is presented in figure 4.

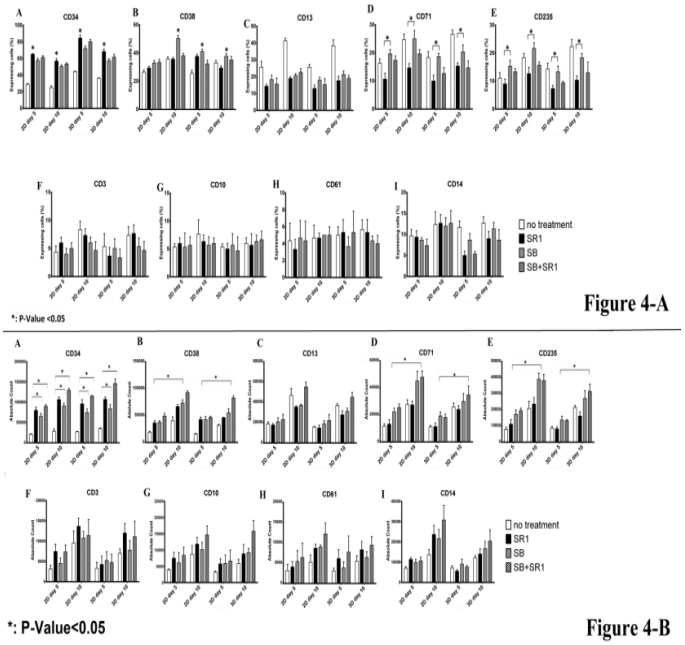

Figure4. The percentage(A) and absolute count(B) of cell lineages in each culture condition at day 5 and day10. The percentage and absolute numbers of cells expressing lineage specific markers including CD34(A) as HSC marker, CD38(B) as lineage Commitment marker, CD13(C) as myeloid marker, CD71(D) and CD235(E) are known as erythroid markers, CD3(F) and CD10(G) are known as lymphoid markers, CD61(H) as thrombocyte/megakaryocyte marker and CD14(I) as monocytic lineage marker harvested from different cell culture conditions are presented in form of mean±SD.

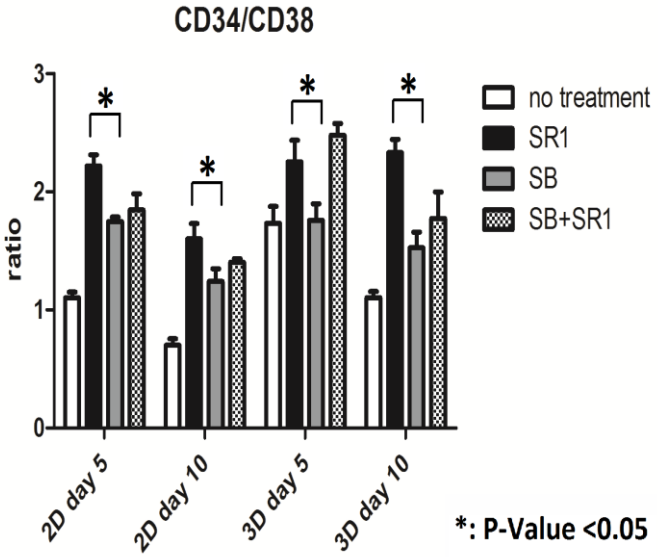

Figure 5

Figure5. The ratio of $CD34^+$ cells/ $CD38^+$ cells. The ratio of CD34/CD38 is presented here as determinant for evaluation of

expansion/differntation. Data is presented in form of mean±SD

Colony formation of isolated cells from different culture conditions

Fresh HSCs or isolated cells from different culture conditions were seeded in methocult media to investigate colony forming capability of isolated cells. The number of colonies was counted and data is presented with more details in figure 6. Briefly data revealed that freshly isolated HSCs from cord blood forms more colony units rather than cultured HSCs (35 colonies) . About cultured cells, the highest ability of colony formation were detected in cells from 3D culture of day5 (17,25,28,29 colonies in no treatment, SR1 treatment, SB treatment and SR1+SB treatment conditions respectively) following by 2D culture of day 5 (13,21,24,26 colonies in no treatment, SR1 treatment, SB treatment and SR1+SB treatment conditions receptively) , 3D culture of day 10 (12,18,22,25 colonies in no treatment, SR1 treatment, SB treatment and SR1+SB treatment conditions receptively) and 2D culture of day 10 (8,15,17,20 colonies in no treatment, SR1 treatment, SB treatment and SR1+SB treatment conditions receptively) in turn. As it could be find out from mentioned colony numbers, in every days and conditions the effects of SB on increasing colony formation was more than SR1. at the end it is worthy to note that if after fresh HSCs, the highest ability of colony formation was detected in cells from 3D culture of day 10 in presence of

both SB and SR1 (25 colonies) and the lowest ability belonged to cells from 2D culture of day 10 with no treatments (8 colonies). Also BFU/CFU-E colonies increased more when the culture systems were treated by SB than other conditions (figure 6).

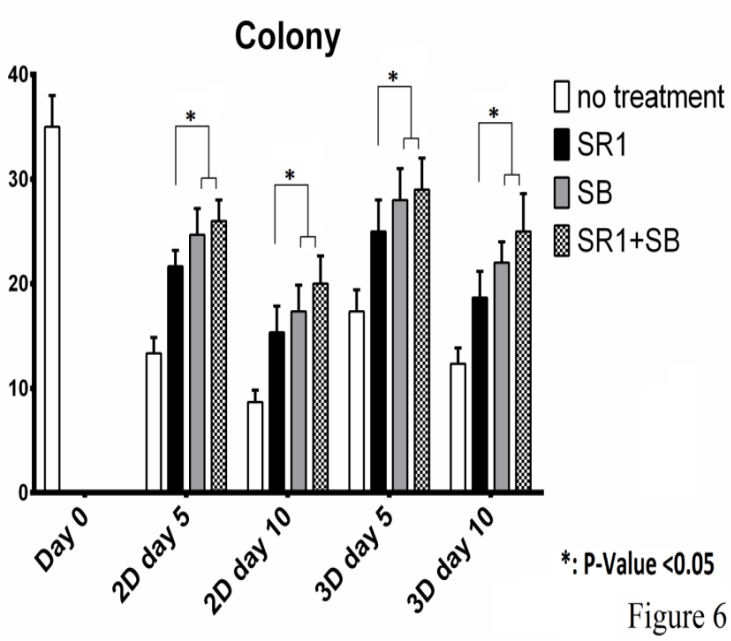

Figure 6

Figure6. The colony assay test of isolated cells from different culture conditions. The numbers of formatted colonies by isolated cells from different culture conditions in methocult medium are presented in form of mean±SD.

c-Myc, _CXCR4_, _HOXB4_ and _SALL4_ Gene expressions

The expressions of various genes in isolated HSCs were estimated before culture or any treatment and after culturing in different conditions and different periods interval. For more precise data presentation, the relative changes in expression of each genes regarding to baseline expression (day0) is presented in figure 7. According to resultant data, the expression of *c-Myc* at day 5 in 3D culture condition and in presence of SR1 was the highest level (1.7 fold change) in comparison to other conditions. Moreover in other forms of treatments, the highest increases were observed at day 5 and in 3D cultures. The expression of *c-Myc* in 2D cultures and at day 10 was less than day 5 so that the lowest level of expression was observed at day 5 in 2D culture and in absence of any treatment.

Relative to day0, the expression of *CXCR4* decreased more obviously in 2D culture conditions. We observed the lowest level of expression in 2D cultures with no treatment at day 5 and day10 (0.3 and 0.17 fold changes respectively) but SR1 and SB treatments at these conditions reduced this decline in expression of *CXCR4*. In general view, the highest level of expression was observed at day 5 in 3D culture in simultaneous presence of SB and SR1 (1.2 fold change) and at day 10 in 3D culture and in presence of both SB and SR1 the expression was preserved like day zero.

The highest level of *HOXB4* was also at day 5 in 3D culture especially in treatment with SR1 (1.5 fold change) and in presence of both SR1 and SB (1.6 fold change). In either 3D or 2D cultures, the lowest expressions were at day 10 in absence of SR1 and SB. Moreover the data revealed that the use of SB alone resulted in a decreased level of *HOXB4*

In case of *SALL4* gene, the expression pattern was somehow similar to *HOXB4*. Generally the expression levels at day 5 were more than day 10 and 3D cultures resulted in higher expression of *SALL4* rather than 2D culture. The highest expression was observed at day 5 in 3Dculture (1.4 folds change) and as it could be expected the lowest level was at day 10 in 2D culture with no treatment. More Detailed data and analysis of all four mentioned genes are presented in figure 7

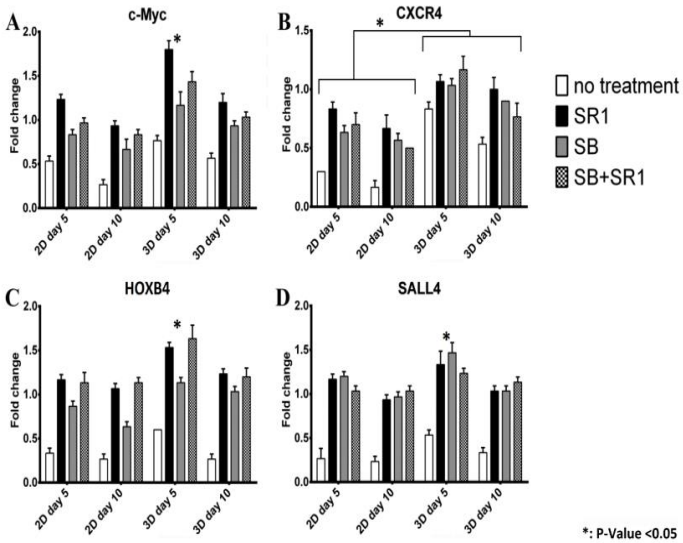

Figure 7

Figure7. Expression of specific genes by isolated cells from different culture conditions. The relative expressions of c-Myc (A), CXCR4 (B), HOXB4 (C) and SALL4 (D) in different culture conditions according to baseline expression of day0 are presented in form of mean±SD.

Discussion:

Considering the vast advantages and applications of cord blood HSCs and regarding to low amount of isolated HSCs from each single cord blood sample, this study aimed to open up a new way for expanding cord blood HSCs to overcome the mentioned shortage [3, 4, 20]. To this end we used 2D and 3D culture setting and 2 different small molecules to enhance HSCs expansion.

As expected, first of all our data revealed that the 3D culture conditions are more effective in HSCs expansion in comparison to 2D culture settings. It is worthy to note that the highest number of cells was observed at day10 in 3D culture settings. This finding has been shown by several other studies [21, 22]. Notably modifications on fabricated 3D scaffolds seem to be necessary for maximum efficacy. For example on study showed that using fibronection-conjugated 3D scaffold results in high expansion of HSCs [22]. Another study which used PES scaffolds also suggested same results [23]. This later study showed that although adding carboxylic, hydroxyl or amine groups to PES scaffolds could increase the efficacy of 3D culture but the most efficient one was the amine groups [15, 23]. Given that the CD34/CD8 ratio is a considerable determinant for evaluating the expansion and differentiation of HSCs, our results showed that 3D conditions are more in favor of expansion capacity of HSCs in comparison 2D culture which induced more differentiation as the CD34/CD38 ratio was below 1 in 2D culture at day 10 in absence of any treatment. These finding also have introduced by other studies [24].

We also used two small molecules so called SR1 and SB in 2D and 3D conditions. The resultant data showed that both SR1 and SB431542 could increase expansion of HSCs in comparison to no treatment conditions but SR1 seems to be more efficient in expansion while SB seems to induce the increase in CD38 positive cells , in other words differentiation. Others have also attributed these effects to SR1. Beyond that SR1 seems to have minimum toxicity which makes it an appropriate choice for medical approaches [18, 25]. The mechanisms of SR1 in increasing cell expansion have been investigated so far. It has shown that the SR1 could act as an Aryl Hydrocarbon Receptor (AhR) agonist which is expressed on CD34 positive cells [18, 26]. AhR seems to regulate other transcription factors which are critical in cells expansion and proliferation and increases the expansion of HSCs in this way [27].As far as we know there is no study investigated the effects of SB on expansion and differentiation of HSCs and further studies are required to find out . It has shown that the SR1 could act as and Aryl Hydrocarbon Receptor (AhR) agonist which is expressed on CD34 positive cells. But generally SB supplies its effects by binding to activin receptor-like kinases receptors and inhibits TGF-β signaling pathway. It should be noted that one study suggested that SB inhibits differentiation of mouse embryonic stem cells by inhibiting the differentiation related transcription factors [28]. This finding is somehow in controversy to our findings which SB induced differentiation of HSCs. for sure of find out the reasons more investigations are required.

Furthermore our results showed that SB could induce of differentiation HSCs in to erythroid progenitors as it leads to higher frequency of CD71 and CD235 expressing cells and both SR1 and SB molecules were able to reduce myeloid differentiation as they reduced CD13+ frequency.

About colony formation ability of cultured cells the data showed that , highest ability of colony formation were in cells from 3D culture of day5 which were in accordance with others findings[22, 24].

The expression of several genes including *c-Myc*, *CXCR4*, *HOXB4* and *SALL4* were also investigated in different conditions to find out the mechanisms underline the mentioned effects. As it mentioned in results SR1 and 3D culture setting showed the highest effects in increasing *c-Myc* expression.. A study which exclusively have investigated the roles of *c-Myc* in HSCs provides interesting data on this molecule. They suggested that *c-Myc* has a regulatory role in fine tuning and balancing self-renewal and differentiation of HSCs (29). It has been also evidenced that AhR agonists like SR1 could increase the expression of expansion related transcription factors such as *c-Myc* [30]. So it could be concluded that one of the proper mechanisms of SR1 in inducing cell expansion is through increasing *c-Myc* expression. Other gene which analyzed was *CXCR4* , the expression of this gene was only increased in 3D culture and in presence both SR1 and SB. It well understood that *CXCR4*-SDF-1 axis in bone marrow niches is account for expansion and hemostasis of HSCs [31]. Other studies also have shown that mimicking in vivo niches by in vitro and ex vivo culture conditions could provide *CXCR4*-SDF-1 axis for HSCs which in turn results in enhanced expansion of stem cells [32]. Beside 3D culture SR1 is thought to induce *CXCR4* expression. This

phenomenon has been observed in breast cancer cells in which the AhR signaling results in higher expression of *CXCR4*[33]. In case of *HOXB4*, the results showed increases in 3D culture and treatment with SR1 but decreases in treatment with SB. This findings suggest that SB may be a potent inhibitor of *HOXB4*. It is well defined that *HOXB4* is a strong positive regulator of self-renewal in HSCs [34]. The higher efficacy of SR1 in inducing cell expansion in CD34 positive in comparison to SB molecule could be attributed to mentioned fact. Resultant data about *SALL4* also indicated the higher expression of this gene in 3D culture and in presence of small molecules. It has been evidenced that *SALL4* is a regulator for HSC self-renewal, which increases self-renewal activity and inhibits differentiation [35].

Like any other studies our studies have its own limitations that could be considered for further studies. Using potential inhibitors of subjected genes would provide confirmative data for their roles. Beside that assessing other genes and factors such as tyrosine kinases would open up other mechanisms which are involved in expansion of HSCs in these conditions. It is also suggested to use other scaffolds and 3D culture structure to perform a comparative study.

Conclusions:

This study introduces a new ex-vivo culture system for further biological and clinical aspect of HSCs. According to our study using of SR1 and SB in 3D culture resulted in a demanded state of HSCs expansion..

The hallmark of our study was the simultaneous use of SR1 and SB431542 that caused increase self-renewal through SR1 and on the other hand, some degrees of differentiation due to effect of SB431542.

It seems that simultaneous use of these two small molecules can provide appropriate outcome for hematopoietic stem cell transplantation in recipients, which includes both of engraftment and repopulation, successful engraftment due to desired level of $CD34^+$ cells and good repopulation by the appropriate number of $CD34^+/38^+$ and differentiated cells.

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