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# Assessment of Differentiation States of Hematopoietic Stem Cells Following in Vitro Culture Using Side and Forward Scatter of Flow Cytometry

**Yongxin Zhang, Farhang Farhangfar, Leonard Giblin, David Wolf, Francesca Vitelli, Yu Zhou, Donnie Rudd**

***Abstract*—Hematopoietic stem cells (HSC) are defined by the International Society of Hematotherapy and Graft Engineering (ISHAGE) as those at low side scatter, positive for CD34 and CD45$^{dim}$ for their numeration with flow cytometry. However, we found that these CD34+ cells increase their granularity and size following in vitro culture, which was exhibited in flow cytometry as more events at higher side scatter and forward scatter. To further determine whether such a change in the cell event distribution is related to HSC differentiation, HSC markers and differentiation markers of in vitro-cultured HSC were detected by flow cytometry at different side scatter and forward scatter levels using modified ISHAGE gating strategies. The results revealed that cultured HSC with higher side scatter have a lower percentage of cells positive for HSC markers and a higher percentage of differentiation makers, while those with higher forward scatter have a higher percentage of differentiation makers but a slightly higher percentage of stem cell markers, suggesting that side scatter and forward scatter levels of cultured HSC correlate with the differentiation level of these cells.**

***Index Terms*—Hematopoietic stem cells, Flow cytometry, Cell culture, Differentiation.**

## I. INTRODUCTION

Pre-cultured hematopoietic stem cells (HSC) from blood and bone marrow are small round, and single-nucleated [1], [2]. When they are examined by flow cytometry, these CD34 positive events demonstrate low side scatter by dot plot analysis [3]-[5], indicating their low granularity. Based on these characteristics, an HSC numeration method made by ISHAGE has been broadly accepted and applied in clinical determination of HSC [3]-[5]. However, following culture with certain cytokines, these cells become larger and more irregular.

In flow cytometry, these cells are shown as the events at higher side scatter for their higher granularity and higher forward scatter for their increased size. In order to determine if these events represent more differentiated HSC and if side and forward scatters of flow cytometry can be used to evaluate differentiation states of HSC following in vitro culture, the events at different levels of side scatter and forward scatter were analyzed by examining the expression of stem cell markers and differentiation markers in this study.

During the development, almost all HSC differentiate into either myeloid linage or lymphoid linage. In the very early stage of differentiation, the pluripotent HSC gains CD33 marker to mainly become a myeloid stem cell or gains CD38 to lymphoid stem cell. CD33 exists at different levels on myeloid cells leucocytes at most maturation stages. The myeloid commitment of hematopoietic progenitors is characterized by the progressive loss of CD34 expression accompanied by the acquisition of CD33 expression.[6]-[8] CD33 expression continues along the myelomonocytic pathway of differentiation, even though its expression is highly down regulated at the ending stage of some cells [6]-[8]. CD38 is expressed on lymphoid stem cells and most mature lymphocytes though the expression on T-lymphocytes could also be down-regulated at various levels [9]-[11]. Therefore, CD33 and CD38 could be the ideal markers to evaluate early HSC differentiation.

**Yongxin Zhang**, Corresponding author, Zyxell Inc. Carrollton, Texas, USA.
**Yongxin Zhang, Farhang Farhangfar, Leonard Giblin, Francesca Vitelli, and Donnie Rudd,** Regenetech, Inc., Houston, Texas, USA
**David Wolf,** NASA, Houston, Texas, USA
**Yu Zhou,** Methrogen Inc., Houston, Texas, USA

## II. MATERIALS AND METHODS

### *A. Cell culture:*

G-CSF mobilized peripheral HSC (AllCells, LLC. Emeryville, CA) were cultured with CellGro®Stem Cell Expansion Media (CellGenix, Inc., Catalog number: 2001) containing 10ng/ml TPO (Peprotech, Inc. Rocky Hill, NJ. Catalog number: 300-18), 50ng/ml SCF (Peprotech, Inc. Rocky Hill, NJ. Catalog number: 300-07), 50ng/ml Flt3 L (Peprotech, Inc. Rocky Hill, NJ. Catalog number: 300-19), 10ng/ml IL-3 (StemCell Technologies Inc. Vancouver, BC, Canada. Catalog number: 02603) and 1X PSA (Life Technologies corporation, Carlsbad, CA) in 5% CO2 incubator at 37°C for 3 days, then diluted by 1:4 with the same media for further 3 days' culture.

### *B. Flow Cytometry*

On the day 0, day 3 and day 6 following the culture, cells for each sample were washed twice and re-suspended in 100µL staining buffer (1x PBS / 0.5% BSA). 20µL Fc receptor blocking antibody (Miltenyi Biotec Inc. Auburn, CA) was added into each reaction and incubated for 10 minutes at room temperature. Then 1ml of staining buffer was added to each reaction and after mixing, cells were aliquoted into different tubes for staining with different antibodies, including CD45, CD34 and CD38 triple staining, CD45, CD34 and CD33 triple staining, CD45, CD34, CD38 and CD33 single staining and unstained and isotype controls. Fluorescence-labeled CD45 and CD38 antibodies and their isotype controls used for the staining were purchased from BD Biosciences Inc.(San Jose, CA). Antibody to CD34 and its isotype control were purchased from Miltenyi Biotec. Antibody to CD33 and its isotype control were purchased from ebioscience, Inc. (San Diego, CA). ISHAGE stem cell numeration protocol [12] was slightly modified for three color flow cytometry and employed for the event gating

in this study. In brief, 2 more dot plots for the third antigen detection (color) and 1 more dot plot for the double positive population of CD34 and CD33 or CD34 and CD38 were added as shown in Figure 1.

*C. Statistics:*

For the analysis of FS levels (levels 1-4) and SS levels (levels 1-4) on the FS/SS dot plot, events were gated into 4 areas (Fig.2a for pre-culture and 2b for post culture), in which the main population was equally divided into 3 areas and the remaining higher part forms the 4$^{th}$ area. For side scatter level analysis and forward scatter level analysis, side scatter and forward scatter versus FL1-FITC or FL2-PE were respectively used (Figures 3 and 4). The CD33 and CD38 positive rates of the events in each area was used to determine the relationship of the cell differentiation and the cell size on Forward scatter or cell granularity on side scatter and the relationship was analyzed by correlation. The correlation coefficient (r) and P values were obtained from z test. The CD33 and CD38 positive rates at different levels of side scatter or forward scatter were compared by ANOVA. SAS-Statview was used for the statistical analysis.

## III. RESULTS

*A. Characterization of pre-cultured HSC.*

Pre-culture cells were triple-stained and analyzed with the modified ISHAGE protocol. The results showed that 89% of the cells are positive for CD45$^{dim}$, 81.10% for CD133, 87.58% for CD34, 76.34% double positive for CD34 and CD133, 44.36% for CD38, 44.18% double positive for CD34 and CD38, 0.86% for CD33 and 0.48% double positive for CD34 and CD33. Based on the area compartmentalization shown in Fig. 3 and Fig.4, CD33 and CD38 positive percentages at the different levels of side scatter and forward scatter are shown in Table 1.

From Table 1, following characters of our primary sample can be seen: (1) Very few of the cells were CD33$^{+}$. Only the events in area 4 of both side and forward scatter were slightly over our positive threshold (1.00%), all remaining numbers fell in the negative territory. (2) In the areas 4 of both side and forward scatter, the percentages of total CD34 positive cells, CD34 positive but CD33 negative cells and CD34 positive but CD38 negative cells are consistently lower than those in other corresponding areas (areas 1-3), indicating the majority of primitive HSC were located in the main population (areas 1-3 for both side and forward scatters) and non-HSC cells in our sample were mainly in areas 4. (3) CD38 positive percentages are negatively

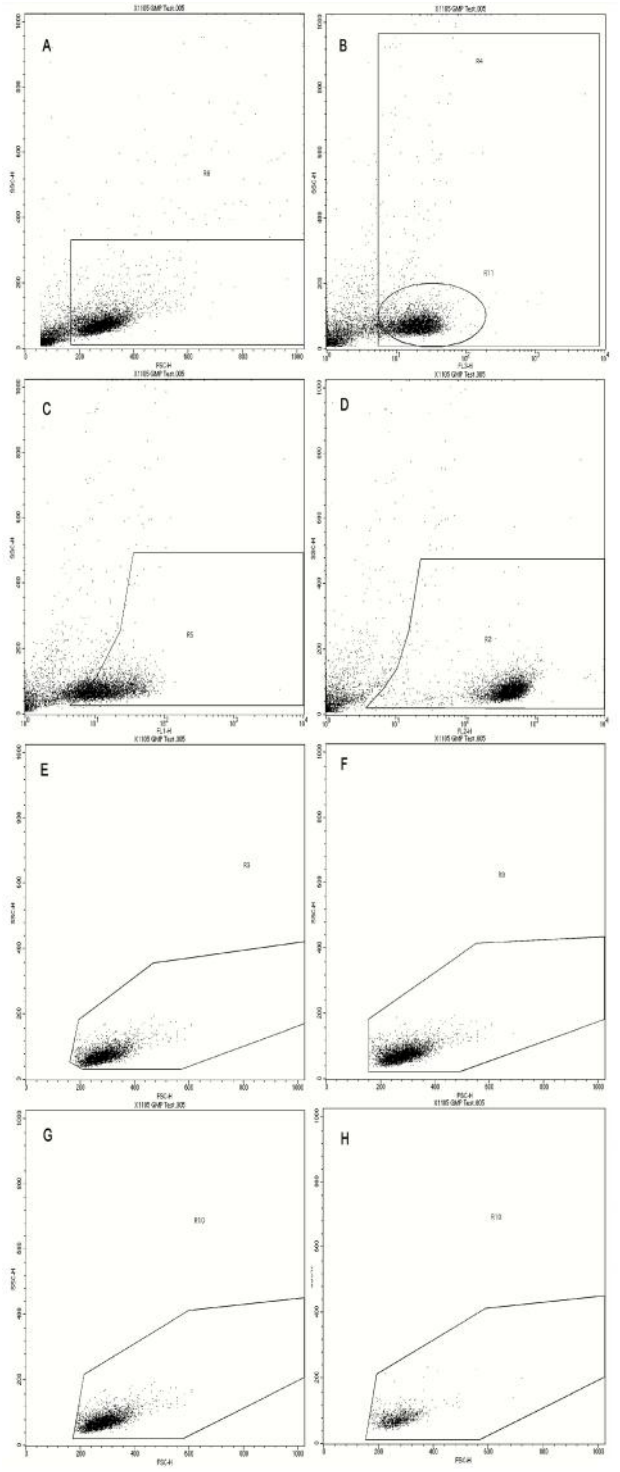


Fig 1. Flow Cytometry of pre-expansion CD34$^{+}$ magnetically selected mobilized peripheral blood cells (HSCs) using a modified ISHAGE gating strategy. Samples were triple stained using anti CD45PerCP, CD34PE, and CD33 or CD38FITC. Thresholds were set with positive events in the negative control set to less than 1% using unstained cells for CD45 and CD45PerCP/CD34PE isotype control/CD33 or CD38FITC isotype control for the other markers. Single stained cells were used for compensation. (A) Mononuclear Cells Gated based on Forward and Side Scatter (FSC and SSC). Panels B-H were based on this gate and showed (B) CD45PerCP staining, (C) CD33 or CD38FITC$^{+}$ Cells, (D) CD34PE$^{+}$ Cells, (E)CD45$^{+}$CD33 or CD38$^{+}$, (F)CD45$^{+}$CD34$^{+}$, (G)CD45$^{+}$CD34$^{+}$CD33 or CD38$^{+}$ (Double positive), (H)CD45$^{+}$CD34$^{+}$CD33 or 38$^{-}$ (Single positive).

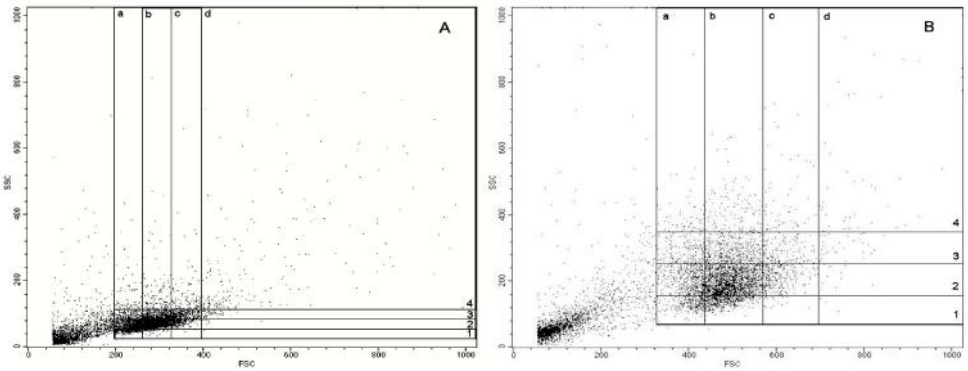


Fig 2. Comparison of Light Scattering Properties of Pre and Post Expansion Cells. The change in Forward and Side Scatter of HSCs from day-0 (A) today 6 (B) was analyzed. To analyze the events at different side scatter levels (1-4) and forward scatter levels (1-4), the main cell population was equally divided into 3 subpopulations respectively on Forward Scatter (gates a - c) and Side Scatter (gates 1 – 3), and the events above main population form gate d on forward scatter and gate 4 on side scatter.

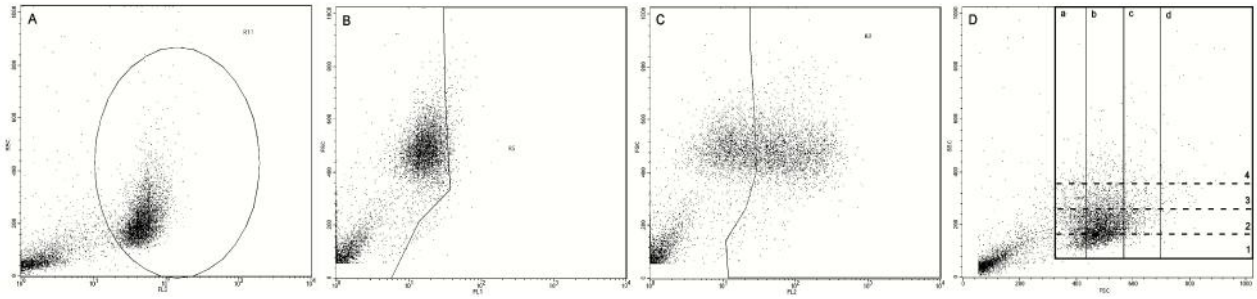


Fig 3. CD33 Distribution by Forward Scatter. (A) CD45PerCP$^{+}$ (gate 11) cells were analyzed in panels B and C. Distribution of CD33FITC (B) and CD34PE (C) stained cells by Forward Scatter. (D) The distribution of CD33 and CD34 expressing cells as determined by panels B and C based on Forward and Side Scatter.

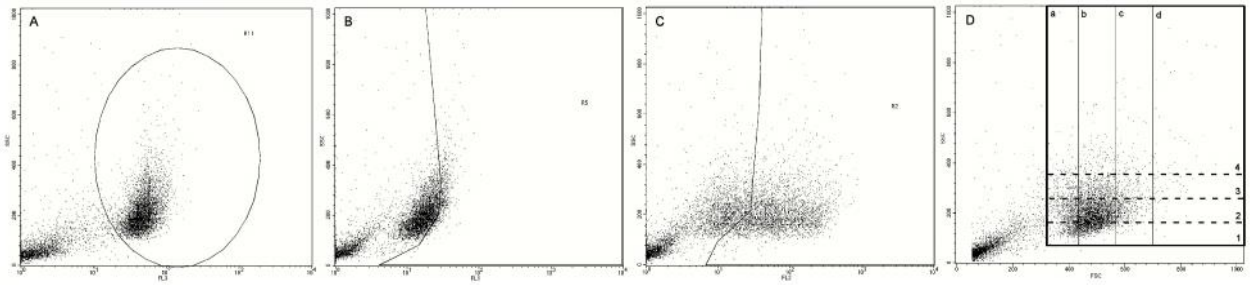


Fig 4. CD33 Distribution by Side Scatter (A) CD45PerCP$^{+}$ (gate 11) cells were analyzed in panels B and C. Distribution of CD33FITC (B) and CD34PE (C) stained cells by Side Scatter. (D) The distribution of CD33 and CD34 expressing cells as determined by panels B and C based on Forward and Side Scatter.

Table 1. CD33 and CD38 positive percentages of uncultured HSC at the different levels of side and forward scatter

| Scatter | Areas | CD33$^{+}$(%) | CD34$^{+}$CD33$^{-}$(%) | CD34$^{+}$(%) | CD38$^{+}$(%) | CD34$^{+}$CD38$^{-}$(%) |
|---|---|---|---|---|---|---|
| Side | 1 | 0.48 | 90.24 | 90.71 | 58.37 | 33.68 |
| | 2 | 0.30 | 95.40 | 95.44 | 49.36 | 46.37 |
| | 3 | 0.32 | 91.75 | 92.07 | 44.91 | 45.34 |
| | 4 | 1.32 | 44.13 | 45.45 | 29.46 | 16.46 |
| Forward | 1 | 0.12 | 87.23 | 87.35 | 41.88 | 46.62 |
| | 2 | 0.21 | 93.25 | 93.46 | 50.13 | 43.35 |
| | 3 | 0.50 | 90.69 | 91.19 | 54.58 | 35.54 |
| | 4 | 1.34 | 47.16 | 48.49 | 31.67 | 12.46 |

correlated to side scatter levels (r=-0.68, P<0.05) as shown in Figure 5, but the percentages for other markers are not significantly correlated to either side scatter levels or forward scatter levels. The negative correlation between CD38 expression and side scatter levels reflects that most of

lymphoid stem cells in our uncultured samples have low level of granularity.

*B. The correlations between CD34, CD33 or CD38 expression and side scatter on HSC in vitro cultured for 6 days (Figure 6).*

Different from non-cultured HSC (not shown), CD33 expressions in total cells (Figure 6A) and in CD34 positive cells (Figure 6B) following 6 days' culture were remarkably at quite high levels on side scatter, and both CD33 positive percentage and CD33 and CD34 double positive ($CD34^+CD33^+$) percentage significantly increased as the side scatter level increased, the positive correlations are shown in Figure 6A and 6B (r=0.787 and 0.719, P<0.01, respectively). In contrast, the percentages of $CD34^+CD33^-$ events and total CD34 positive events were negatively correlated to the side scatter levels (Figure 6C and 6D, r=-0.951 and -0.688, P<0.01, respectively). When the positive percentages on different levels of side scatter were compared, $CD33^+$, $CD34^+CD33^-$ and total $CD34^+$ did not show significant differences between level 1 and level 2 while all marker positive percentages exhibited significant differences between all other two levels. These results suggested that following the in vitro culture some HSC differentiated to granular cells exhibiting on higher side scatter and with the expression of CD33 in flow cytometry, and the population at lower side scatter contains more primitive HSC.

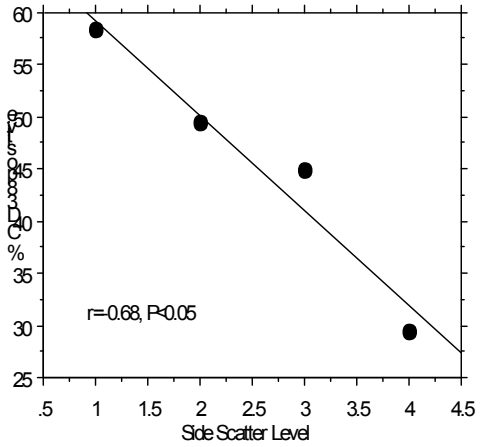


Figure 5. Correlation between CD38 positive percentages and side scatter levels in pre-culture $CD34^+$ HSC isolated from G-CSF mobilized peripheral blood. Cells were triple-stained for CD45, CD34 and CD38 prior to detection with flowcytometer. A flowcytomtric dot plot (SS/FS) was divided into 4 areas and scaled from 1 to 4 to show the scatter levels. A correlation chart was plotted by CD38 positive percentage versus side scatter levels.

Compared to uncultured HSC, CD38 positive ($CD38^+$) event distribution in 6 days' cultured cells is completely changed. The $CD38^+$ percentage is positively correlated to side scatter level (Figure 6E, r=0.887, P<0.01) instead of as the negative correlation shown in uncultured HSC (Figure 5). The reduction of $CD38^+$ cells from day 0 to day 6 following the culture mainly occurred in areas 1 and 2 of side scatter grids, suggesting that CD38 positive lymphoid stem cells might not expand very much in our culture system but become more irregular or more granular. This could lead to an increase in more primitive HSC at lower side scatter.

$CD38^+$, CD34 and CD38 double positive ($CD34^+CD38^+$) and CD34 positive but CD38 negative ($CD34^+CD38^-$) percentages were correlated to side scatter (Figure 6E-6G, r=0.887, 0.884 and 0.805, P<0.01, respectively) in the same pattern as were $CD33^+$, $CD34^+CD33^+$ and $CD34^+CD33^-$, respectively. The results showed that unlike with CD33, no significant difference could be found between side scatter level 1 and side scatter level 2 for all $CD38^+$, $CD34^+CD38^+$ and $CD34^+CD38^-$ percentages. This further confirms that the cultured HSC at the lower side scatter are less differentiated.

Similar to their correlation with side scatter, $CD33^+$, $CD38^+$, $CD34^+CD33^+$ and $CD34^+CD38^+$ percentages were positively correlated to forward scatter levels (Figure 7A, 7B, 7E and 7F, r=0.873, 0.855, 0.718 and 0.718, P<0.01, respectively), while the percentages of $CD34^+CD33^-$ and $CD34^+CD38^-$ events were negatively correlated to the side scatter levels (Figure 6C and 6D, r=-0.89 and -0.74, P<0.01, respectively). However, the total CD34 positive percentages were not correlated to forward scatter levels and no significant differences of CD34 positive percentages among different forward scatter levels were found, suggesting that the size of $CD34^+$ cells is variable but the larger ones are more likely to be differentiated. $CD33^+$, $CD34^+CD33^-$, CD38 and $CD34^+CD38^-$ percentages did not show significant differences between side scatter levels 1 and 2. But all these cell types were at their highest percentages in side scatter level 4. This is further support that the cells at higher side scatter are more differentiated.

Only 4 samples from culture on day 3 had enough cells for all flow cytometric examinations. All correlations (r=0.624-0.832, P<0.05 or 0.01) from the samples on day 3 were consistent with those on day 6 except that no significant correlation was found between $CD38^+$ percentages and side scatter levels.

## III. DISCUSSION

Flow cytometry, as a standard HSC numeration method, has been broadly applied in clinics [13]-[15] . ISHAGE defined the HSC as being $CD34^+$, $CD45^{dim}$ and at low side scatter according to the performance of HSC events on flow cytometric dot plots [16]. In our modified ISHAGE protocol, cells were triple-stained, two more dot plots for the third stain and the dot plot for triple positive event detection were added. Multiple gates in plots of side scatter versus forward scatter formed grids to be used in further analysis. When this system was used to examine the uncultured HSC, it was found that $CD33^+$

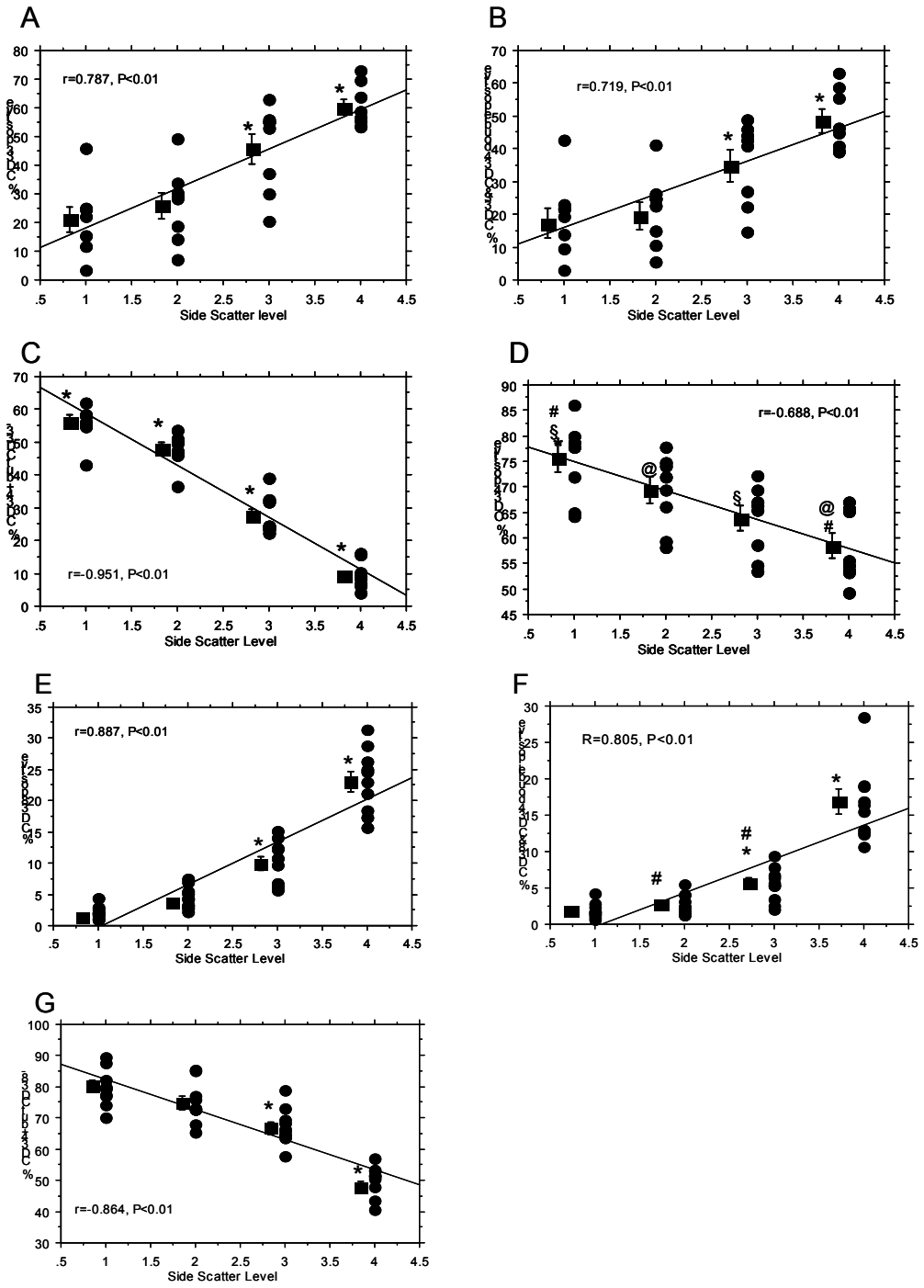


Figure 6. CD34, CD33 and CD38 positive percentages correlate with event levels on side scatter. CD34-positive HSC isolated from G-CSF mobilized peripheral blood were in vitro cultured for 6 days. Cells from 10 cultures were triple-stained for flow cytometric detection of CD45, CD34 and CD33 or CD38. A flow cytomtric dot plot (SS/FS) was divided into 4 areas on the side scatter and scaled from 1 to 4 to show the scatter levels. Correlation charts were plotted by cell marker positive percentages versus side scatter levels. A: Positive correlation between CD33 positive percentages and the event levels on side scatter; B: Positive correlation between CD34 and CD33 double positive percentages and the event levels on side scatter; C: Negative correlation between CD34 positive but CD33 negative percentages and the event levels on side scatter; D: Negative correlation between total CD34 positive percentages and the event levels on side scatter; A: Positive correlation between CD38 positive percentages and the event levels on side scatter; B: Positive correlation between CD34 and CD38 double positive percentages and the event levels on side scatter; C: Negative correlation between CD34 positive but CD38 negative percentages and the event levels on side scatter. Cell marker positive percentages on different side scale levels were also compared with ANOVA. * P<0.01 when compared to all other groups except for the groups marked with #, @ and §, which P values are smaller than 0.01 when compared to the groups with the same symbols in D and smaller
than 0.05 in F.

cells were mainly located at high side scatter though a quite few total positive events were shown. This is

consistent with ISHAGE definition for HSC numerated by Flow Cytometry. However, when $CD38^+$ populations were analyzed, their positive percentages negatively correlated to side scatter levels. Since total $CD34^+$ cell percentages at lower side scatters (levels 1-3) were similar and all above 90%, the $CD38^+$ events at lower side scatter could be confirmed as lymphoid stem cells [17], [18]. Considering the populations with the most $CD34^+CD38^-$ events are at side scatter levels 2 and 3, the uncultured HSC at very low and very high side scatter are less primitive. Compared to the side scatter, the distribution of primitive HSC on forward scatter is much less variable. Only the population at very high level (level 4) consistently exhibited remarkably increased percentages of cells with differentiation markers and decreased percentages of cells with stem cell markers.

To meet the increasing clinical requirements for large amount of HSC, a great deal of effort has been made to expand HSC by in vitro culture [19]-[27]. During the expansion with current technologies, the HSC are unavoidably undergoing to nonspecific differentiation or losing stem cell markers [22], [27], resulting in the less successful engraftment using expanded HSC when compared to the same numbers of uncultured HSC [28], [29]. In the quality and quantity evaluation of expanded HSC, only a few investigators applied ISHAGE protocol in which the side scatter level of HSC was considered. None of studies has shown an analysis of the forward scatter level of expanded stem cells [3], [19], [21]-[29]. The current study demonstrated that the expanded HSC are more differentiated at higher side scatter and higher forward scatter, suggesting that these two scatters can be used as parameters to evaluate the differentiation status of HSC following in vitro culture.

Some differentiated cells could lose the CD33 or CD38 at the ending stage of the HSC development, but this procedure occurs much later than the loss of CD34 [6], [7], [30]-[32]. Therefore, the CD34 and CD33 or CD38 combined analyses in the current study could more significantly demonstrate which cell populations are more primitive, with the $CD34^+CD33^-$ and $CD34^+CD38^-$ cells being more primitive HSC while $CD34^+CD33^+$ and $CD34^+CD38^+$ cells would be at the early stage of the differentiation mainly towards to myeloid linage and lymphoid linage, respectively.

The in vitro expanded HSC usually contains very large portion of nonspecifically differentiated cells [22], [27], [29]. These differentiated cells could potentially affect the efficacy of the stem cell transplantation. It was reported that expanded HSC have less successful engraftment [33], [34]. The purified primitive HSC may be required in some HSC transplantation [35]-[40]. Thus, it would be very important to isolate the less non-specific differentiated HSC from the total expanded HSC. Currently, the most common methods to isolate HSC from the mixed cell population conjugate the cells with magnetic beads or fluorescence-labeled antibodies. The procedure is complicated, expensive, labor-intensive and has an increased chance for contamination (also the antibody itself could potentially affect the cells by cross-linking receptors). Our data showed that the population at lower side scatter and forward scatter (levels 1 and 2) contains the highest percentage of nonspecifically differentiated HSC, which provides a possibility to isolate relatively high primitive HSC using Flow cytometry cell sorter without antibody conjugation.

Event distribution on side scatter and forward scatter could be affected by the culture media and cytokines with which HSC are cultured. TPO, Flt-3L, SCF and IL-3 used in this study were also applied in most other HSC expansion studies [19], [21]-[28]. In addition to CellGro®Stem Cell Expansion Media, IMDM with 10% FBS was also examined. The correlations between side or forward scatters and stem cell or differentiation markers are the same for the cells cultured with IMDM with 10% FBS as those with CellGro®Stem Cell Expansion Media except for significantly increased CD38 expression in former.

The distribution of differentiated HSC on the side versus forward scatter dot plot is also related to culture time. On day 0, the $CD38^+$ percentages were negatively correlated to side scatter levels, but on day 3 following the culture, no significant correlation between them, while on day 6 the correlation became positive. In another study with the same expansion system but different samples, the cells were cultured for 10 days. The correlative relationship between $CD38^+$ percentages and side scatter levels was consistent to this study for the first 6 days' culture. On day 10 following the culture, the $CD38^+$ percentages on all side scatter levels became much lower than before, but they were still positively correlated, suggesting $CD38^+$ cells become more irregular following the culture. This might be resulted from cell senescence.

We also noticed that the width of the grid (gate) could affect the result. When the very narrow grids such as the gates for uncultured HSC levels 1 to 3, were used to analyze post-cultured HSC, the result for CD38 on forward scatter could be different, clearly because area 4 on forward scatter includes a great portion of main population, which leads the shift of positive percentages at different forward scatter levels. Although the $CD38^+$ percentage at forward scatter level 1 could be higher than other levels in this case, the differences between different levels are less than 2%, which does not make any substantial effect on the primitive stem cell isolation. Moreover, $CD34^+CD38^-$ percentages are still negatively correlated to forward scatter levels ($r=-0.554$, $P<0.01$). Therefore, it can still be concluded that more primitive HSC following in vitro culture are located at lower side scatter and lower forward scatter, even though the smaller gates were used for the analysis.

## IV. CONCLUSION

*The in vitro* expanded HSC have tendency to increase size and granularity and to spontaneously differentiate into myeloid ($CD33^+$) and lymphoid ($CD38^+$) lineages.

The changes of post-expaded HSC in morphology, exhibited on flow cytometry plot as higher forward scatter and higher side scatter, are positively correlated to the increased CD33 and CD38 expression on these cells, suggesting that the non-specific differentiation of *in vitro* expanded HSC could be evaluated using flow cytometry by examining the distribution of cell events on side scatter and forward scatter.

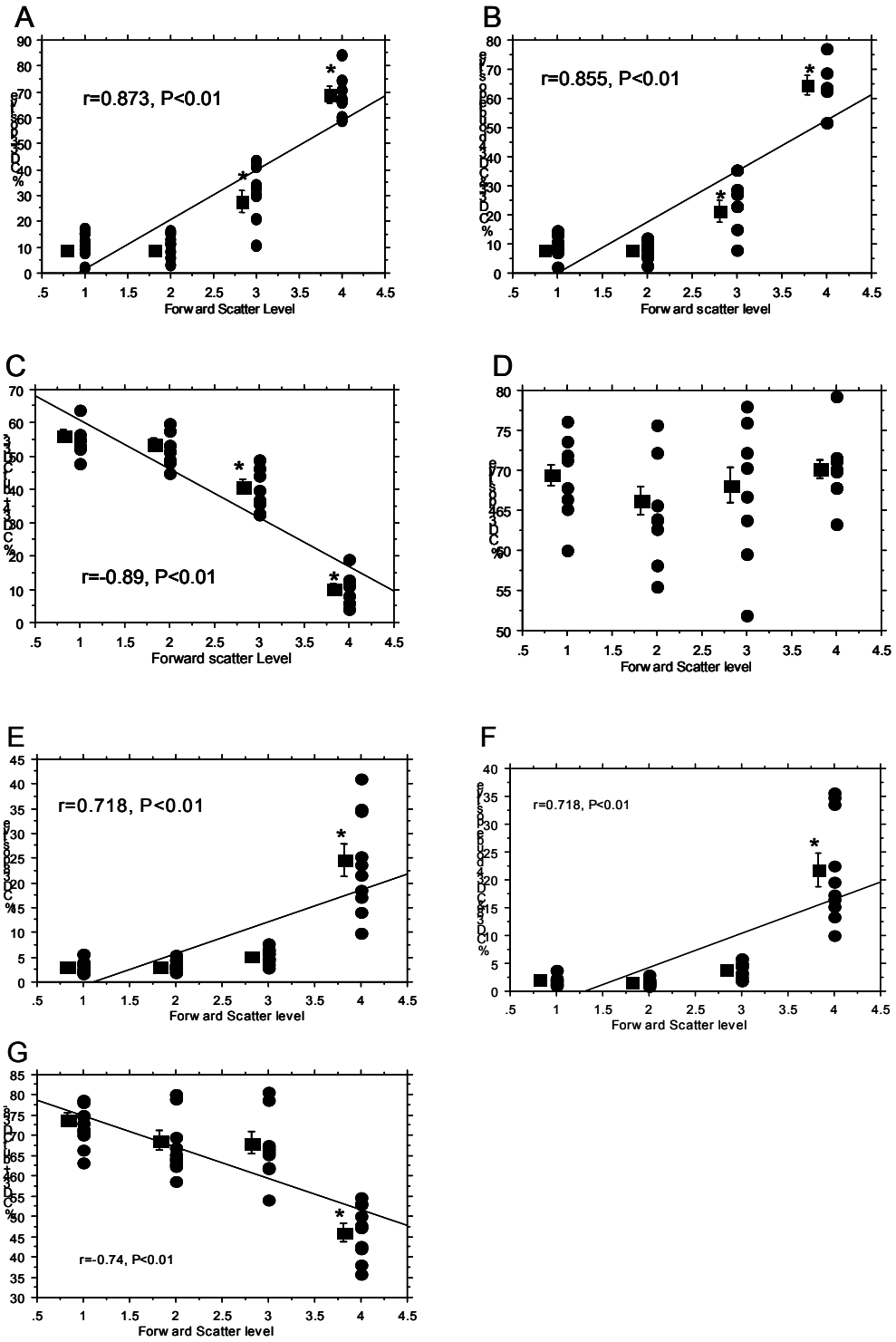


Figure 7. CD34, CD33 and CD38 positive percentages correlate with event levels on forward scatter. CD34-positive HSC isolated from G-CSF mobilized peripheral blood were in vitro cultured for 6 days. Cells from 10 cultures were triple-stained for flow cytometric detection of CD45, CD34 and CD33 or CD38. A flow cytomtric dot plot (SS/FS) was divided into 4 areas on the side scatter and scaled from 1 to 4 to show the scatter levels. Correlation charts were plotted by cell marker positive percentages versus forward scatter levels. A: Positive correlation between CD33 positive percentages and the event levels on forward scatter; B: Positive correlation between CD34

and CD33 double positive percentages and the event levels on forward scatter; C: Negative correlation between CD34 positive but CD33 negative percentages and the event levels on forward scatter; D: Negative correlation between total CD34 positive percentages and the event levels on forward scatter; A: Positive correlation between CD38 positive percentages and the event levels. On forward scatter; B: Positive correlation between CD34 and CD38 double positive percentages and the event levels on forward scatter; C: Negative correlation between CD34 positive but CD38 negative percentages and the event levels on forward scatter. Cell marker positive percentages on different forward scale levels were also compared with ANOVA. * P<0.01 when compared to all other groups.

## REFERENCES


[1] SAIGO, K., HASHIMOTO, M., KUMAGAI, S., SUGIMOTO, T.: [Detection of hematopoietic stem cells by flow cytometry, hematology analyzer or in vitro culture method]. Rinsho Byori Suppl 110, 124-130, 1999.

[2] WICKENHAUSER, C., THIELE, J., KUMMEL, T., FISCHER, R.: [Hematopoietic stem cells of the human. Function and morphology]. Pathologe 16, 1-10, 1995.

[3] CHAPPLE, P., PRINCE, H. M., WALL, D., FILSHIE, R., HAYLOCK, D., QUINN, M., BRETELL, M., VENTER, D.: Comparison of three methods of CD34+ cell enumeration in peripheral blood: dual-platform ISHAGE protocol versus single-platform, versus microvolume fluorimetry. Cytotherapy. 2, 371-376, 2000.

[4] SUTHERLAND, D. R., ANDERSON, L., KEENEY, M., NAYAR, R., CHIN-YEE, I.: The ISHAGE guidelines for CD34+ cell determination by flow cytometry. International Society of Hematotherapy and Graft Engineering. J. Hematother. 5, 213-226, 1996.

[5] SUTHERLAND, D. R., NAYYAR, R., ACTON, E., GIFTAKIS, A., DEAN, S., MOSIMAN, V. L.: Comparison of two single-platform ISHAGE-based CD34 enumeration protocols on BD FACSCalibur and FACSCanto flow cytometers. Cytotherapy. 11, 595-605, 2009.

[6] CROCKER, P. R.: Siglecs in innate immunity. Curr. Opin. Pharmacol. 5, 431-437, 2005.

[7] CROCKER, P. R., REDELINGHUYS, P.: Siglecs as positive and negative regulators of the immune system. Biochem. Soc. Trans. 36, 1467-1471, 2008.

[8] FREEMAN, S. D., KELM, S., BARBER, E. K., CROCKER, P. R.: Characterization of CD33 as a new member of the sialoadhesin family of cellular interaction molecules. Blood 85, 2005-2012, 1995.

[9] DORRELL, C., GAN, O. I., PEREIRA, D. S., HAWLEY, R. G., DICK, J. E.: Expansion of human cord blood CD34(+)CD38(-) cells in ex vivo culture during retroviral transduction without a corresponding increase in SCID repopulating cell (SRC) frequency: dissociation of SRC phenotype and function. Blood 95, 102-110, 2000.

[10] HUANG, S., TERSTAPPEN, L. W.: Lymphoid and myeloid differentiation of single human CD34+, HLA-DR+, CD38- hematopoietic stem cells. Blood 83, 1515-1526, 1994.

[11] RAWLINGS, D. J., QUAN, S., HAO, Q. L., THIEMANN, F. T., SMOGORZEWSKA, M., WITTE, O. N., CROOKS, G. M.: Differentiation of human CD34+. Exp. Hematol. 25, 66-72, 1997.

[12] SUTHERLAND, D. R., ANDERSON, L., KEENEY, M., NAYAR, R., CHIN-YEE, I.: The ISHAGE guidelines for CD34+ cell determination by flow cytometry. International Society of Hematotherapy and Graft Engineering. J. Hematother. 5, 213-226, 1996.

[13] KEENEY, M., CHIN-YEE, I., WEIR, K., POPMA, J., NAYAR, R., SUTHERLAND, D. R.: Single platform flow cytometric absolute CD34+ cell counts based on the ISHAGE guidelines. International Society of Hematotherapy and Graft Engineering. Cytometry 34, 61-70, 1998.

[14] SAIGO, K., HASHIMOTO, M., KUMAGAI, S., SUGIMOTO, T.: [Detection of hematopoietic stem cells by flow cytometry, hematology analyzer or in vitro culture method]. Rinsho Byori Suppl 110, 124-130, 1999.

[15] SUTHERLAND, D. R., KEATING, A., NAYAR, R., ANANIA, S., STEWART, A. K.: Sensitive detection and enumeration of CD34+ cells in peripheral and cord blood by flow cytometry. Exp. Hematol. 22, 1003-1010, 1994.

[16] SUTHERLAND, D. R., ANDERSON, L., KEENEY, M., NAYAR, R., CHIN-YEE, I.: The ISHAGE guidelines for CD34+ cell determination by flow cytometry. International Society of Hematotherapy and Graft Engineering. J. Hematother. 5, 213-226, 1996.

[17] RAWLINGS, D. J., QUAN, S., HAO, Q. L., THIEMANN, F. T., SMOGORZEWSKA, M., WITTE, O. N., CROOKS, G. M.: Differentiation of human CD34+. Exp. Hematol. 25, 66-72, 1997.

[18] ROBIN, C., BENNACEUR-GRISCELLI, A., LOUACHE, F., VAINCHENKER, W., COULOMBEL, L.: Identification of human T-lymphoid progenitor cells in CD34+ CD38low and CD34+ CD38+ subsets of human cord blood and bone marrow cells using NOD-SCID fetal thymus organ cultures. Br. J. Haematol. 104, 809-819, 1999.

[19] BOIRON, J. M., DAZEY, B., CAILLIOT, C., LAUNAY, B., ATTAL, M., MAZURIER, F., MCNIECE, I. K., IVANOVIC, Z., CARAUX, J., MARIT, G., REIFFERS, J.: Large-scale expansion and transplantation of CD34(+) hematopoietic cells: in vitro and in vivo confirmation of neutropenia abrogation related to the expansion process without impairment of the long-term engraftment capacity. Transfusion 46, 1934-1942, 2006.

[20] FAN, X., BAO, C., LIU, Y., LIU, T., SUN, X., GE, D., MA, X.: [The investigation of ex-vivo expansion of hematopoietic stem/progenitor cells]. Sheng Wu Yi. Xue. Gong. Cheng Xue. Za Zhi. 24, 870-875, 2007.

[21] GAMMAITONI, L., BRUNO, S., SANAVIO, F., GUNETTI, M., KOLLET, O., CAVALLONI, G., FALDA, M., FAGIOLI, F., LAPIDOT, T., AGLIETTA, M., PIACIBELLO, W.: Ex vivo expansion of human adult stem cells capable of primary and secondary hemopoietic reconstitution. Exp. Hematol. 31, 261-270, 2003.

[22] IVANOVIC, Z., DUCHEZ, P., DAZEY, B., HERMITTE, F., LAMRISSI-GARCIA, I., MAZURIER, F., PRALORAN, V., REIFFERS, J., VEZON, G., BOIRON, J. M.: A clinical-scale expansion of mobilized CD 34+ hematopoietic stem and progenitor cells by use of a new serum-free medium. Transfusion 46, 126-131, 2006.

[23] KAWANO, Y., KOBUNE, M., YAMAGUCHI, M., NAKAMURA, K., ITO, Y., SASAKI, K., TAKAHASHI, S., NAKAMURA, T., CHIBA, H., SATO, T., MATSUNAGA, T., AZUMA, H., IKEBUCHI, K., IKEDA, H., KATO, J., NIITSU, Y., HAMADA, H.: Ex vivo expansion of human umbilical cord hematopoietic progenitor cells using a coculture system with human telomerase catalytic subunit (hTERT)-transfected human stromal cells. Blood 101, 532-540, 2003.

[24] KAWANO, Y., KOBUNE, M., CHIBA, H., NAKAMURA, K., TAKIMOTO, R., TAKADA, K., ITO, Y., KATO, J., HAMADA, H., NIITSU, Y.: Ex vivo expansion of G-CSF-mobilized peripheral blood CD133+ progenitor cells on coculture with human stromal cells. Exp. Hematol. 34, 150-158, 2006.

[25] LAM, A. C., LI, K., ZHANG, X. B., LI, C. K., FOK, T. F., CHANG, A. M., JAMES, A. E., TSANG, K. S., YUEN, P. M.: Preclinical ex vivo expansion of cord blood hematopoietic stem and progenitor cells: duration of culture; the media, serum supplements, and growth factors used; and engraftment in NOD/SCID mice. Transfusion 41, 1567-1576, 2001.

[26] LI, K., LI, C. K., CHUEN, C. K., TSANG, K. S., FOK, T. F., JAMES, A. E., LEE, S. M., SHING, M. M., CHIK, K. W., YUEN, P. M.: Preclinical ex vivo expansion of G-CSF-mobilized peripheral blood stem cells: effects of serum-free media, cytokine combinations and chemotherapy. Eur. J. Haematol. 74, 128-135, 2005.

[27] LIU, Y., LIU, T., FAN, X., MA, X., CUI, Z.: Ex vivo expansion of hematopoietic stem cells derived from

umbilical cord blood in rotating wall vessel. J. Biotechnol. 124, 592-601, 2006.

[28] HOFMEISTER, C. C., ZHANG, J., KNIGHT, K. L., LE, P., STIFF, P. J.: Ex vivo expansion of umbilical cord blood stem cells for transplantation: growing knowledge from the hematopoietic niche. Bone Marrow Transplant. 39, 11-23, 2007.

[29] ROBINSON, S. N., NG, J., NIU, T., YANG, H., MCMANNIS, J. D., KARANDISH, S., KAUR, I., FU, P., DEL ANGEL, M., MESSINGER, R., FLAGGE, F., DE LIMA, M., DECKER, W., XING, D., CHAMPLIN, R., SHPALL, E. J.: Superior ex vivo cord blood expansion following co-culture with bone marrow-derived mesenchymal stem cells. Bone Marrow Transplant. 37, 359-366, 2006.

[30] HUANG, S., TERSTAPPEN, L. W.: Lymphoid and myeloid differentiation of single human CD34+, HLA-DR+, CD38- hematopoietic stem cells. Blood 83, 1515-1526, 1994.

[31] RAWLINGS, D. J., QUAN, S., HAO, Q. L., THIEMANN, F. T., SMOGORZEWSKA, M., WITTE, O. N., CROOKS, G. M.: Differentiation of human CD34+. Exp. Hematol. 25, 66-72, 1997.

[32] ROSS, R. E., JETER, E. K., STUART, R. K., SELF, S. E., LAVIA, M. F.: CD34+ and CD33+ stem cells: predictors of hematologic recovery? Prog. Clin. Biol. Res. 377, 505-512, 1992.

[33] SEET, L. F., TENG, E., LAI, Y. S., LANING, J., KRAUS, M., WNENDT, S., MERCHAV, S., CHAN, S. L.: Valproic acid enhances the engraftability of human umbilical cord blood hematopoietic stem cells expanded under serum-free conditions. Eur. J. Haematol. 82, 124-132, 2009.

[34] KIM, D. H., YOO, K. H., YIM, Y. S., CHOI, J., LEE, S. H., JUNG, H. L., SUNG, K. W., YANG, S. E., OH, W. I., YANG, Y. S., KIM, S. H., CHOI, S. Y., KOO, H. H.: Cotransplanted bone marrow derived mesenchymal stem cells (MSC) enhanced engraftment of hematopoietic stem cells in a MSC-dose dependent manner in NOD/SCID mice. J. Korean Med. Sci. 21, 1000-1004, 2006.

[35] AWAD, O., DEDKOV, E. I., JIAO, C., BLOOMER, S., TOMANEK, R. J., SCHATTEMAN, G. C.: Differential healing activities of CD34+ and CD14+ endothelial cell progenitors. Arterioscler. Thromb. Vasc. Biol. 26, 758-764, 2006.

[36] GIASSI, L. J., PEARSON, T., SHULTZ, L. D., LANING, J., BIBER, K., KRAUS, M., WODA, B. A., SCHMIDT, M. R., WOODLAND, R. T., ROSSINI, A. A., GREINER, D. L.: Expanded CD34+ human umbilical cord blood cells generate multiple lymphohematopoietic lineages in NOD-scid IL2rgamma(null) mice. Exp. Biol. Med. (Maywood. ) 233, 997-1012, 2008.

[37] HOGAN, C. J., SHPALL, E. J., MCNIECE, I., KELLER, G.: Multilineage engraftment in NOD/LtSz-scid/scid mice from mobilized human CD34+ peripheral blood progenitor cells. Biol. Blood Marrow Transplant. 3, 236-246, 1997.

[38] NOVELLI, E. M., CHENG, L., YANG, Y., LEUNG, W., RAMIREZ, M., TANAVDE, V., ENGER, C., CIVIN, C. I.: Ex vivo culture of cord blood CD34+ cells expands progenitor cell numbers, preserves engraftment capacity in nonobese diabetic/severe combined immunodeficient mice, and enhances retroviral transduction efficiency. Hum. Gene Ther. 10, 2927-2940, 1999.

[39] PIACIBELLO, W., SANAVIO, F., SEVERINO, A., DANE, A., GAMMAITONI, L., FAGIOLI, F., PERISSINOTTO, E., CAVALLONI, G., KOLLET, O., LAPIDOT, T., AGLIETTA, M.: Engraftment in nonobese diabetic severe combined immunodeficient mice of human CD34(+) cord blood cells after ex vivo expansion: evidence for the amplification and self-renewal of repopulating stem cells. Blood 93, 3736-3749, 1999.

[40] SIVAN-LOUKIANOVA, E., AWAD, O. A., STEPANOVIC, V., BICKENBACH, J., SCHATTEMAN, G. C.: CD34+ blood cells accelerate vascularization and healing of diabetic mouse skin wounds. J. Vasc. Res. 40, 368-377, 2003.