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Large generation of megakaryocytes from serum-free expanded human CD34$^+$ cells

Te-Wei Chen [a,b,1], Chao-Ling Yao [a,1], I-Ming Chu [b], Tzu-Lin Chuang [a], Tzu-Bou Hsieh [a], Shiaw-Min Hwang [a,*]

[a] Bioresource Collection and Research Center, Food Industry Research and Development Institute, 331 Shih-Pin Road, Hsinchu 30062, Taiwan
[b] Department of Chemical Engineering, National Tsing-Hua University, Hsinchu 30013, Taiwan

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ABSTRACT

Ex vivo generation of megakaryocytes from hematopoietic stem cells (HSCs) is crucial to HSC research and has important clinical potential for thrombocytopenia patients to rapid platelet reconstruction. In this study, factorial design and steepest ascent method were used to screen and optimize the effective cytokines (10.2 ng/ml TPO, 4.3 ng/ml IL-3, 15.0 ng/ml SCF, 5.6 ng/ml IL-6, 2.8 ng/ml FL, 2.8 ng/ml IL-9, and 2.8 ng/ml GM-CSF) in megakaryocyte induction medium that facilitate *ex vivo* megakaryopoiesis from CD34$^+$ cells. After induction, the maximum fold expansion for accumulated megakaryocytes was almost 5000-fold, and the induced megakaryocytes were characterized by analysis of gene expression, polyploidy and platelet activation ability. Furthermore, the combination of megakaryocyte induction medium and HSC expansion medium can induce and expand a large amount of functional megakaryocytes efficiently, and might be a promising source of megakaryocytes and platelets for cell therapy in the future.

Megakaryocytes (Mks) expressing CD41a and CD61 antigens are the progenitors of platelets and play important roles with platelets in thrombosis and haemostasis. Mks are an extremely rare cell population in myeloid cells (<1%), and are generated from HSCs through complex development processes. Mks are mainly located in bone marrow (BM), and also appear in lung, spleen, liver, cord blood (CB), and mobilized peripheral blood (MPB) [1–4]. An insufficient or abnormal amount of Mks or platelets, for example in thrombocytopenia, causes petechia and bleeding diseases [5].

CB collected from the placenta and cord is a rich source of HSCs and is proven to be an alternative to BM or MPB for hematopoietic reconstitution after chemotherapy [6]. However, CB contains lower numbers of HSCs, and CB transplantation requires a longer period for platelet recovery than BM or MPB transplantation [7]. Clinical trials have demonstrated that HSC transplantation combined with the administration of Mks is a safe strategy to accelerate platelet recovery [8,9]. Consequently, *ex vivo* expansion of Mks from HSCs is a promising strategy to accelerate Mk and platelet recovery after CB transplantation.

Cytokines have different effects on cells, depending on target cells, concentrations, and the presence of the other cytokines. The effect of single cytokine on Mk generation was previously studied, such as TPO, SCF, Flt-3 ligand, PDGF-BB, GM-CSF, IL-1β, IL-3, IL-6, IL-9, and IL-11 [10–16]. However, cytokine cocktail, cytokine interaction, and optimal cytokine concentration for *ex vivo* megakaryocytopoiesis has not been well studied.

In previous work, we developed a stromal-free, serum-free and cytokine-optimized medium for *ex vivo* expansion of HSCs (SF-HSC medium) by systematic methodologies [17]. Not only CD34$^+$CD38$^-$ cells, but also colony-forming cells, and long-term culture initiating cells were highly expanded in the SF-HSC medium. Importantly, expanded CD34$^+$ cells that were transplanted into NOD/SCID mice could reconstitute hematopoiesis [18] and maintain the ability to differentiate into natural killer cells *in vitro* [19]. Here, we further explored the application of serum-free expanded CD34$^+$ cells into Mks. In this study, factorial design and steepest ascent (SA) method were combined to develop an expansion and induction medium to generate Mks (Mk medium) from CD34$^+$ cells. We analyzed the individual and interaction effects of cytokines on Mk generation and showed that Mks could be induced from serum-free expanded CD34$^+$ cells efficiently by assessing the biological characteristics, such as surface antigens, mRNA expressions, ploidy levels, and *ex vivo* ability to activate platelets. Our results provided information on the influence and interaction of cytokines in megakaryocytopoiesis and demonstrated that serum-free expanded CD34$^+$ cells might subsequently serve as an alternative source of Mk or platelet cells in cell therapy in the future.

Materials and methods

Cytokines and chemicals. Human recombinant TPO, SCF, IL-1β, IL-3, IL-6, IL-9, IL-11, FL, PDGF-BB, G-CSF, and GM-CSF were pur-

* Corresponding author. Fax: +886 3 5214016.
E-mail address: hsm@firdi.org.tw (S.-M. Hwang).
[1] These authors contributed equally to this work.

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chased from PeproTech (EC Ltd., London, UK). Bovine serum albumin (BSA), human insulin, human transferrin, and 2-mercaptoethanol (2-ME) were purchased from Sigma (St. Louis, MO).

Isolation and expansion of HSCs. In this study, CB was collected and processed according to governmental regulation—"Guidelines for collection and use of human specimens for research", Department of Health, Taiwan, and after approval from the scientific committees of Food Industry Research and Development Institute, Taiwan. After obtaining the mother's consent, CB was harvested and processed within 24 h. Mononuclear cells (MNCs) were isolated by Ficoll–Paque (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation. Subsequently, fresh $CD34^+$ cells were purified with CD34 microbeads by a Miltenyi VarioMACS device (Miltenyi Biotec, Bergisch Gladbach, Germany). To serum-free expand $CD34^+$ cells, fresh $CD34^+$ cells were seeded at 5×10^4 cells/ml in the SF-HSC medium that was Iscove's modified Dulbecco's medium (IMDM, HyClone, Logan, UT) containing cytokine cocktail (8.5 ng/ml TPO, 4.1 ng/ml IL-3, 15 ng/ml SCF, 6.7 ng/ml FL, 0.8 ng/ml IL-6, 3.2 ng/ml G-CSF, and 1.3 ng/ml GM-CSF), and serum substitutes (1.5 g/L BSA, 4.4 μg/ml insulin, 60 μg/ml transferrin, and 25.9 μM 2-ME) [17]. After 1-week culture, expanded $CD34^+$ cells were collected for Mk induction.

Fractional factorial design and SA method. Two-level factorial design and SA method were combined to determine the optimal concentration of cytokines for Mk induction from expanded $CD34^+$ cells. Factorial design data were regressed by Design Expert statistical software (Stat-Ease Inc., Minneapolis, MN) to obtain the polynomial. Statistical significance was determined by an *F*-test, and the significance of the regression coefficients was analyzed by a *t*-test. The polynomial takes the form of

$$\begin{aligned} &CD41a^+CD61^+ \text{ cells (cells/ml)} \\ &= \alpha_0 + \sum \alpha_i x_i + \sum \alpha_i j x_i x_j + \sum \alpha_{ijk} x_i x_j x_k \end{aligned} \quad (1)$$

where α's are the fitted constants and x's are coded variables for the tested cytokines. The constants α_i, α_{ij}, and α_{ijk} correspond to the main effect, second-order interaction, and third-order interaction terms. Eq. (1) is a simplified equation. We considered statistically significant main or interaction terms (p-value <0.05) and neglected insignificant higher-order terms. Each positive constant in the equation can screen the effective factors and provide the information to construct the SA path to optimize cytokine concentration for Mk generation. The strategies for development of Mk medium were as follows: (a) screen the effective cytokines in the IMDM plus 10% fetal bovine serum (FBS, HyClone); (b) optimize the concentration of each effective cytokine in the IMDM plus 10% FBS. In all experiments, expanded $CD34^+$ cells were seeded at a concentration of 5×10^4 cells/ml with IMDM plus 10% FBS and variable combination of cytokines according to experimental design. After 1-week induction, cells were analyzed with following assays.

Two culture strategies of ex vivo Mk generation. Two culture strategies were designed to measure the amount of Mks generated from a given amount of fresh $CD34^+$ cells (5×10^4 cells/ml, see Supplementary Fig. 1). In strategy I, fresh $CD34^+$ cells were cultured in the SF-HSC medium for one week and then in the Mk medium for two weeks. In strategy II, fresh $CD34^+$ cells were directly induced in the Mk medium for three weeks. At weekly intervals, the medium was changed and cell density was re-adjusted to 5×10^4 cells/ml.

Analysis of surface antigens and ploidy levels. To analyze surface antigens, CD34-FITC, CD34-PE, CD61-PE (Miltenyi Biotec), and

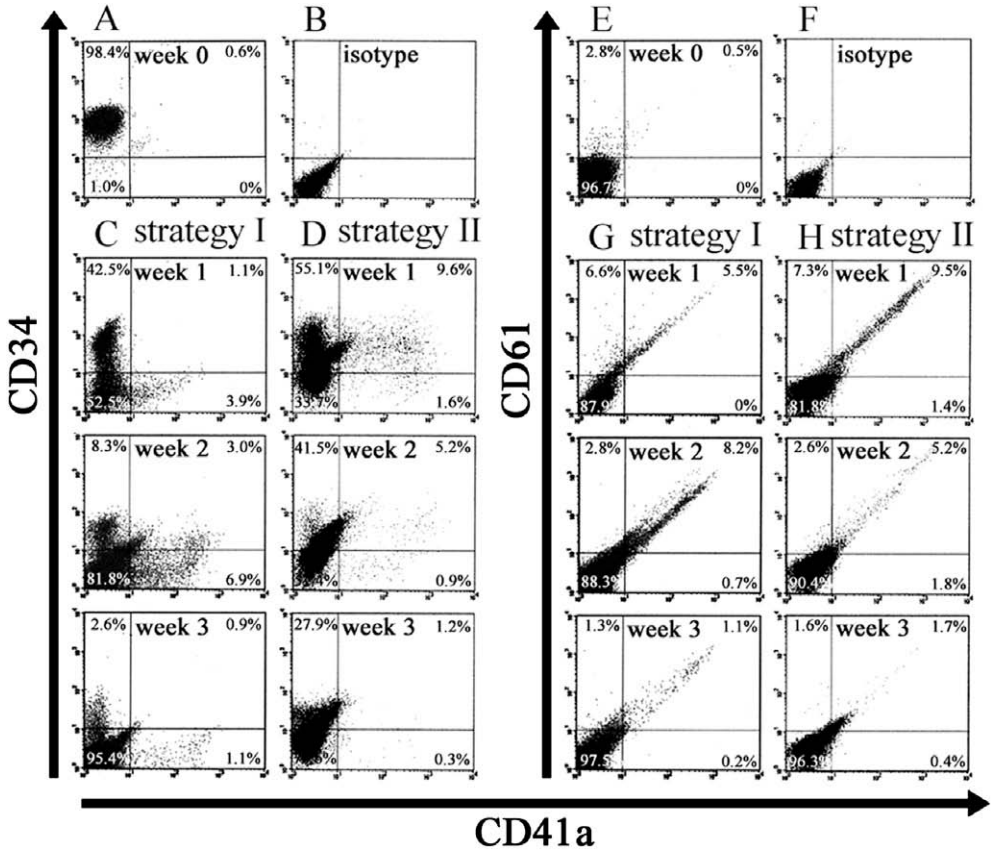

Fig. 1. Surface marker analysis ($n = 4$) of cells generated *via* two culture strategies. Fresh $CD34^+$ cells (A,E) were double stained with CD41a/CD34 and CD41a/CD61 and their isotype control (B,F). CD41a/CD34 and CD41a/CD61 expression of generated cells were cultured *via* strategy I (C,G) and strategy II (D,H) at weekly points. The numbers within dot plots indicate the percentages of marker expressions.

CD41a-FITC (eBioscience, San Diego, CA) were used. A replicate sample was stained with FITC- or PE-conjugated mouse IgG_1 as an isotype control. The labeled cells were analyzed on FACSCalibur flow cytometry with CellQuest software (Becton–Dickinson, San Jose, CA). Mks were defined as $CD41a^+CD61^+$ cells [4]. After induction, induced cells were purified with anti-CD41a magnetic microbeads to measure ploidy. Isolated $CD41a^+$ cells were perforated by FACS™ permeabilizing solution 2 (Becton–Dickinson) and then stained with the staining solution (50 μg/ml PI, 0.1% Triton-X, and 0.1 mg/ml RNase in D-PBS, Sigma) according to the methods described previously [3].

RT-PCR analysis. Total cellular RNA was extracted with Tri-Reagent (Molecular Research Center, Cincinnati, OH). mRNA was reverse transcribed into cDNA using the reverse transcriptase set (Fermentas Inc., Glen Burnie, MD). Mk gene-specific primer sets were as follows: GATA-1 (forward: CTCCCTGTCCCCAATAGTGC, reverse: GTCCTTCGGCTGCTCCTGTG), NF-E2 (forward: CTACTCACTC ATGCCCAA, reverse: GGTGCTGGAAAATGTCA) and GAPHD (forward: AGCCTCAAGATCATCAGCAATG, reverse: TTTTCTAGACGGCA GGTCAGG). Specific cDNA was amplified by PCR using Taq DNA polymerase kits (Invitrogen, Carlsbad, CA), and the amplification program was as described previously [20].

Analysis of platelet activation. After induction *via* two strategies, the isolated $CD41a^+$ cells were treated with the platelet activation reagent (Sigma) containing adenosine diphosphate, collagen, and epinephrine. After stimulation, cells that expressed CD41a and CD62P (eBioscience) were defined as activated platelets.

Statistical analysis. The experimental results from multiple independent experiments were shown as means ± SD. *P*-value <0.05 was considered as statistical significance, using the paired samples *t*-test and *, **, and *** indicated that *p*-value of the comparison was <0.05, <0.01, and <0.001, respectively.

Results

Cytokine screening

Based on extensive review, 11 cytokines (TPO, SCF, IL-3, IL-6, FL, IL-11, IL-1β, PDGF-BB, IL-9, G-CSF, and GM-CSF) were selected for *ex vivo* induction of Mks from serum-free expanded $CD34^+$ cells. In our preliminary experiments, TPO, SCF, FL, IL-3, IL-6, IL-9, and GM-CSF had significantly positive effects, and IL-11 had a significantly negative effect on the Mk generation, while IL-1β, PDGF-BB, and G-CSF had no effect (data not shown). Therefore, a 2^{8-3} fractional factorial matrix was adopted to identify the effect of these eight significant cytokines on total nuclear cell (TNC) and $CD41a^+CD61^+$ cell growths (Supplementary Table 1) and the first-order model was regressed according to Supplementary Table 1.

$$\begin{aligned}&CD41a^+\ CD61^+\ \text{cells/ml}\ (\times 10^4)\\&= 5.63 + 2.28x_1 + 0.96x_2 + 3.35x_3 - 0.23x_4 + 1.24x_5\\&\quad + 0.63x_6 + 0.61x_7 + 0.62x_8 + 0.35x_1x_2 + 1.84x_1x_3\\&\quad - 0.39x_1x_4 - 0.37x_1x_5 + 0.49x_3x_5 + 0.41x_3x_7 + 0.17x_5x_6\\&\quad + 0.26x_5x_8 - 0.43x_1x_3x_4 \qquad (2)\end{aligned}$$

In Eq. (2), x_1 to x_8 are coded variables for TPO, IL-3, SCF, IL-11, IL-6, FL, IL-9, and GM-CSF, respectively and only listed significant terms (p-value <0.05). Eq. (2) specifies that all cytokines (with positive coefficients) can promote $CD41a^+CD61^+$ cell generation, except IL-11 (with negative coefficient) inhibited $CD41a^+CD61^+$ cell growth. The individual cytokine effects on Mk generation rank as follows: SCF > TPO > IL-6 > IL-3 > FL = IL-9 = GM-CSF.

Optimize cytokine concentration

SA path was determined by the coefficients in Eq. (2) to optimize the concentration of TPO, IL-3, SCF, IL-6, FL, IL-9, and GM-CSF for maximal $CD41a^+CD61^+$ cell generation (Supplementary Table 2). TNC and $CD41a^+CD61^+$ cell growths increased along SA path initially, reaching 2.24×10^6 and 3.76×10^5/ml at step 4, respectively. After step 4, there were no additional increases in TNC and $CD41a^+CD61^+$ cell growths. Consequently, the optimal cytokine concentration in Mk induction medium was IMDM containing 10% FBS, 10.2 ng/ml TPO, 4.3 ng/ml IL-3, 15.0 ng/ml SCF, 5.6 ng/ml IL-6, 2.8 ng/ml FL, 2.8 ng/ml IL-9, and 2.8 ng/ml GM-CSF.

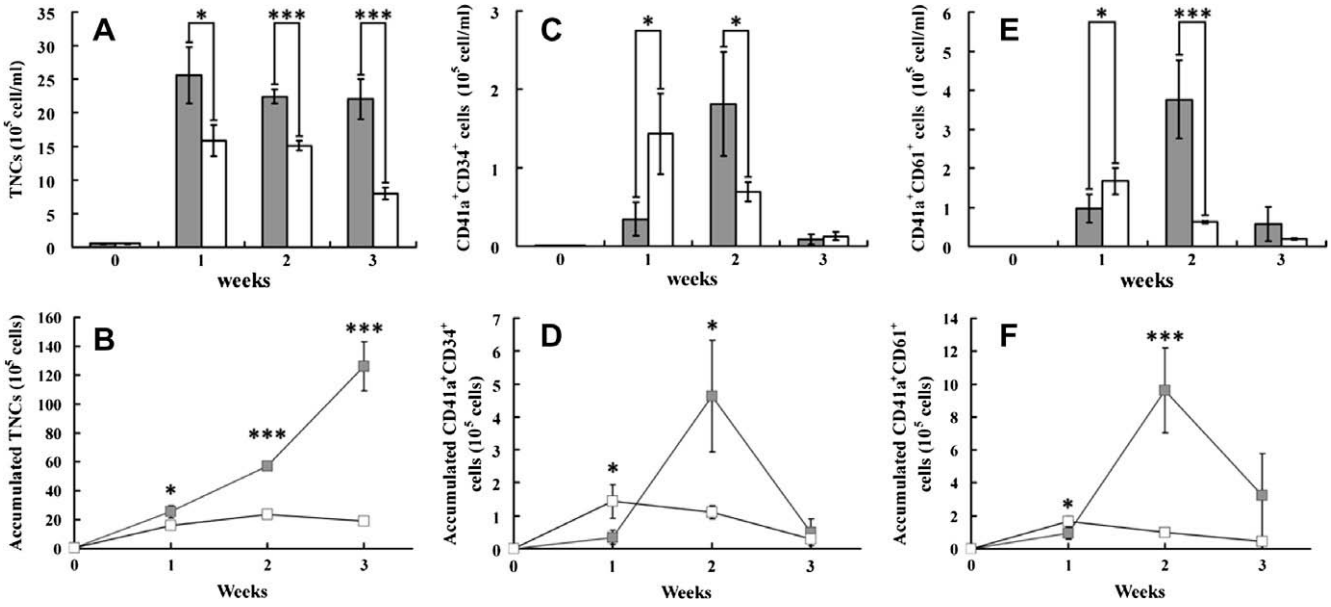

Fig. 2. Growth kinetic of cells generated from fresh $CD34^+$ cells *via* two culture strategies (strategy I: black bar and square and strategy II: white bar and square, $n = 4$). (A) Total nuclear cell density. (B) Accumulated total nuclear cell number. (C) $CD41a^+/CD34^+$ cell density. (D) Accumulated $CD41a^+/CD34^+$ cell number. (E) $CD41a^+/CD61^+$ cell density. (F) Accumulated $CD41a^+/CD61^+$ cell number.

Mk generation and analysis of cell surface antigens

Two strategies were designed to compare the megakaryocytopoietic potential of freshly isolated $CD34^+$ cells and serum-free expanded $CD34^+$ cells (see Supplementary Fig. 1). After CD34 microbead isolation, the purity of the fresh $CD34^+$ cell fraction was over 98.0% (Fig. 1A), and $CD41a^+CD61^+$ cells were almost undetectable (Fig. 1E). After 1-week culture in the SF-HSC medium, there was a 27-fold increase in $CD34^+$ cell number [17]. In strategy I, when serum-free expanded $CD34^+$ cells were cultured in the Mk medium, CD34 expression decreased gradually, and CD41 and CD61 expressions began to be detectable (Fig. 1C and G). The highest percentages of $CD41a^+CD34^+$ cells and $CD41a^+CD61^+$ cells in the total cultured cells were 3.0% and 8.2% at week 2, respectively. In strategy II, when fresh $CD34^+$ cells were cultured directly in the Mk medium, CD34 expression decreased continuously (Fig. 1D). The highest percentages of $CD41a^+CD34^+$ cells and $CD41a^+CD61^+$ cells in the total cultured cells were 9.6% and 9.5% at week 1, respectively (Fig. 1D and H). Expression of CD41a and CD61 declined in the cultured cells when prolonged culture in Mk medium (strategies I and II, week 3).

We compared the growth kinetics of the cells that were cultured *via* two strategies with the same amount of fresh $CD34^+$ cells. TNC densities were maintained approximately at 2.5×10^6 cell/ml and 1.5×10^6 cell/ml in strategy I and strategy II at each week, respectively (Fig. 2A). TNC density increased more rapidly and was a greater extent in cells prepared from strategy I than those from strategy II (Fig. 2B). At week 0, subpopulations of $CD41a^+CD34^+$ cells and $CD41a^+CD61^+$ cells in the fresh $CD34^+$ cells were almost undetectable. In strategy I, the maximum cell densities of $CD41a^+CD34^+$ cells and $CD41a^+CD61^+$ cells reached $1.81 \pm 0.67 \times 10^5$ and $3.76 \pm 1.00 \times 10^5$ cell/ml at week 2, respec-

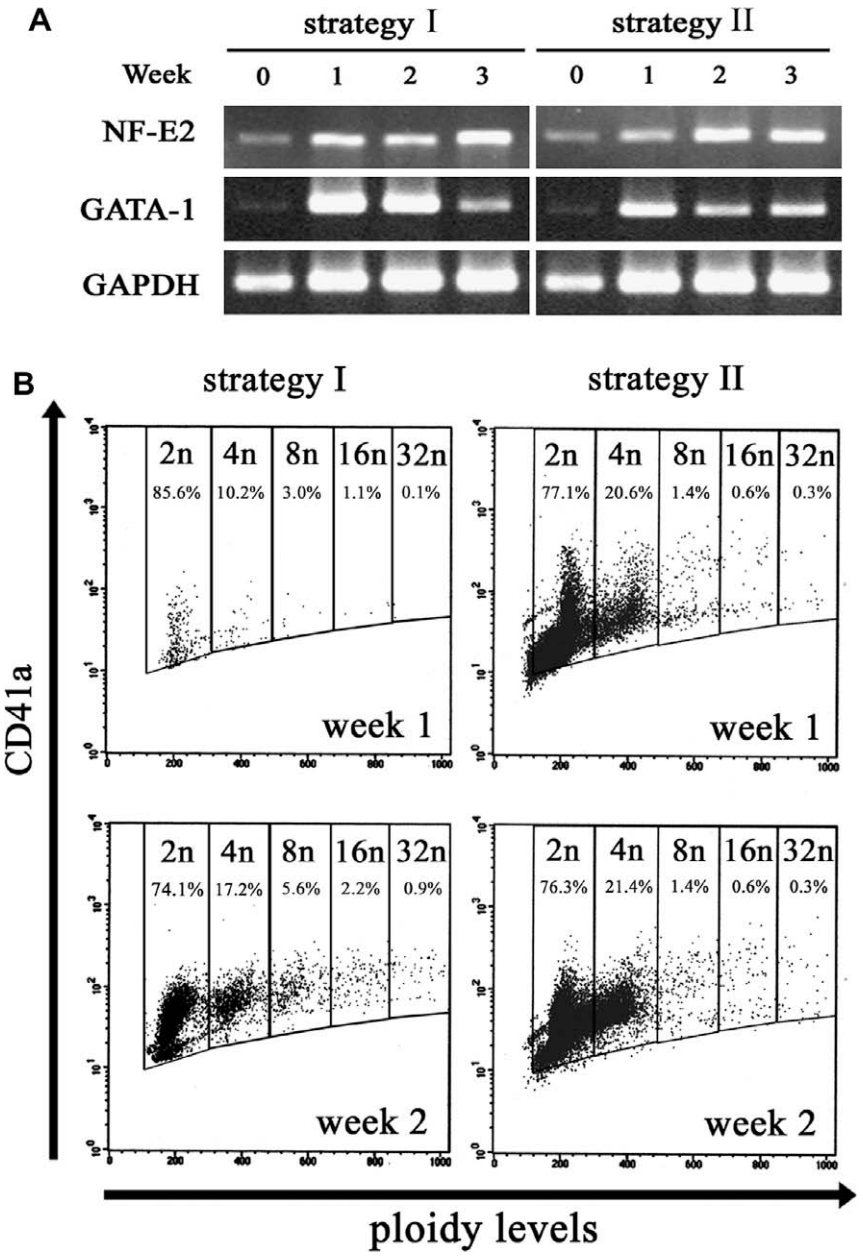

Fig. 3. (A) NF-E2, GATA-1, and GAPDH expressions of generated Mks were induced *via* strategy I or strategy II ($n = 3$). (B) DNA distribution of generated $CD41a^+$ cells induced *via* strategies I and II. The numbers within dot plots indicated the percentages of cells with corresponding ploidy level ($n = 3$).

tively. In strategy II, the maximum cell densities of $CD41a^+CD34^+$ cells and $CD41a^+CD61^+$ cells reached $1.44 \pm 0.51 \times 10^5$ and $1.67 \pm 0.33 \times 10^5$ cell/ml at week 1, respectively (Fig. 2C and E). The maximum numbers of accumulated $CD41a^+CD34^+$ cells and $CD41a^+CD61^+$ cells induced from 5×10^4 fresh $CD34^+$ cells were $4.63 \pm 1.70 \times 10^5$ (728 ± 268-fold versus initial $CD41a^+CD34^+$ cell number at week 0) and $9.62 \pm 2.56 \times 10^5$ cells (4932 ± 132-fold versus initial $CD41a^+CD61^+$ cell number at week 0) at week 2 in the strategy I group, respectively, compared to $1.44 \pm 0.51 \times 10^5$ (226 ± 81-fold versus initial $CD41a^+CD34^+$ cell number at week 0) and $1.67 \pm 0.33 \times 10^5$ cells (856 ± 170-fold versus initial $CD41a^+CD61^+$ cell number at week 0) at week 1 in the strategy II group, respectively (Fig. 2D and F). Therefore, each fresh $CD34^+$ cell could generate an average of 19.2 Mks *via* strategy I (week 2) and 3.3 Mks *via* strategy II (week 1). These results showed that strategy I could generate more Mks than strategy II (5.8-fold).

RT-PCR analysis of Mk-lineage transcription factors

The Mk-lineage mRNA expression of NF-E2 and GATA-1 was analyzed (Fig. 3A). Fresh $CD34^+$ cells showed a low-level of mRNA expression for NF-E2 and GATA-1 (week 0). In strategy I, cells began to prominently express NF-E2 and GATA-1 at week 1 and maintained these high levels of expression until week 2. GATA-1 expression, however, noticeably decreased during week 3. In strategy II, cells maintained almost the same level of expression of NF-E2 and GATA-1 throughout the whole 3-week Mk induction, although NF-E2 expression was slightly weaker at week 1.

Ploidy analysis of induced Mks

After induction, induced $CD41a^+$ cells were purified to analyze DNA distribution (Fig. 3B). In strategy I, expanded $CD34^+$ cells (week 1) were almost diploid (2*n*). Hyperploidy $CD41a^+$ cells (>4*n*) were obtained when fresh $CD34^+$ cells or expanded $CD34^+$ cells were induced in the Mk medium. $CD41a^+$ cells that were induced from serum-free expanded $CD34^+$ cells had higher levels of hyperploidy (8.7 ± 0.3%, week 2 in strategy I) than those induced from fresh $CD34^+$ cells (2.3 ± 0.4%, week 2 in strategy II).

Ability analysis of platelet activation

After induction, induced $CD41a^+$ cells were purified to test their ability to produce activated platelets. CD62P expression was used as the marker for platelet activation (Fig. 4A and B). Before stimulation with the platelet activation reagent, the numbers of accumulated $CD41a^+CD62P^+$ cells were $3.55 \pm 0.54 \times 10^5$ cells and $0.42 \pm 0.08 \times 10^5$ cells at week 2 in strategy I and at week 1 in strategy II, respectively. After stimulation, CD62P expressions were both upregulated in the $CD41a^+$ cells that were induced via strategies I and II, and the numbers of accumulated $CD41a^+CD62P^+$ cells were $6.11 \pm 1.01 \times 10^5$ cells and $0.73 \pm 0.14\ 10^5$ cells at week 2 in strategy I and at week 1 in strategy II, respectively (see Supplementary Fig. 2). These results showed that strategy I could generate more Mks that had the ability to become active platelets than strategy II.

Discussion

Thrombocytopenia is observed in patients after high-dose chemotherapy or hepatitis virus-related cirrhosis [21]. To reduce the period of thrombocytopenia and to accelerate platelet recovery, two strategies were considered to treat: (a) TPO alone/with G-CSF injection [11], or (b) Mk transplantation [8,9]. However, TPO and G-CSF injection may induce side effects. Therefore, *ex vivo*

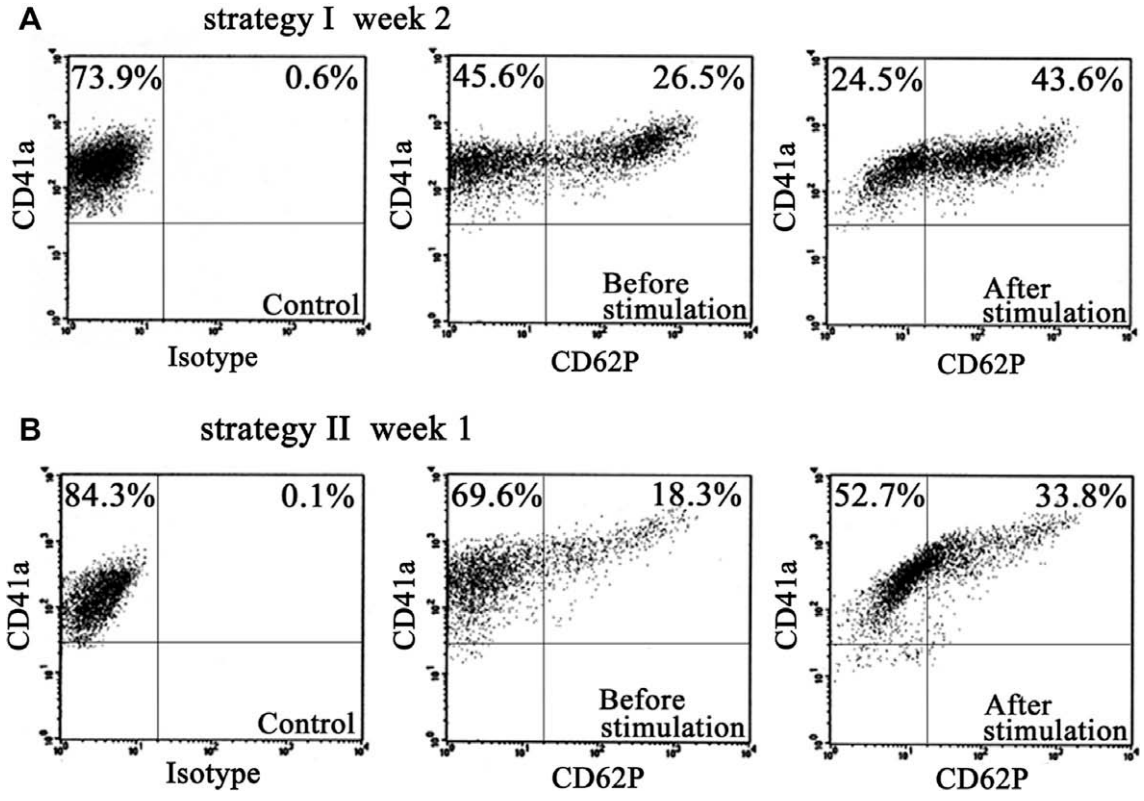

Fig. 4. *In vitro* activation of platelets ($n = 4$). Isolated $CD41a^+$ cells were generated from fresh $CD34^+$ cells *via* (A) strategy I at week 2 and (B) strategy II at week 1, and their isotype control. After stimulation with the platelet activation reagent, CD62P was significantly up-regulated in the $CD41a^+$ cells. The numbers within dot plots indicate the percentages of marker expressions.

expansion of HSCs and further differentiation HSCs into Mks become important clinical issues.

We previously demonstrated that $CD34^+$ cells can be successfully expanded in an optimized serum-free medium [17]. The results showed that expanded $CD34^+$ cells could restore the hematopoiesis of irradiated NOD/SCID mice [18] and efficiently differentiate into lymphoid lineages [19]. In this study, we further investigated whether serum-free expanded $CD34^+$ cells could differentiate *in vitro* into Mks, one of myeloid lineages. Many studies have reported that fresh $CD34^+$ cells can differentiate into Mks and evaluated the effect of single cytokines on Mk growth [12,16,22]. However, few papers discussed how to generate large amounts of Mks from serum-free expanded $CD34^+$ cells or the interaction of cytokine combinations on Mk growth. In this study, we developed an optimized Mk induction medium with cytokine cocktail and compared the growth kinetics and characteristics of generated Mks that were induced from fresh and serum-free expanded $CD34^+$ cells.

Our results showed that SCF, TPO, IL-6, IL-3, FL, IL-9, and GM-CSF were necessary for Mks generation, and IL-11 had a significant negative individual effect on Mk generation. This result is consistent with those presented by Taguchi et al., who proved that IL-11 mainly stimulates Mks maturation and not proliferation [10]. We assayed ploidy level, transcription factor (GATA-1 and NF-E2) expressions, and platelet activation ability to confirm that the generated Mks were functional Mks [1]. Our results showed that both GATA-1 and NF-E2 were highly expressed in generated Mks and CD62P was up-regulated after stimulation. Some reporters demonstrated that Mks induced from CB HSCs exhibit lower hyperploidy levels than those induced from MPB and BM HSCs [3,23]. We observed that the highest percentage of induced $CD41a^+$ cells from serum-free expanded $CD34^+$ cells that exhibited hyperploidy level ($>4n$) was almost 9%, which is superior/comparable to previously reported data [24]. Besides, Mks derived from serum-free expanded $CD34^+$ cells showed higher percentages of hyperploidy and platelet activation ability than those derived from fresh $CD34^+$ cells.

Finally, the Mk medium was developed by the systematic designs and has a low concentration of cytokines, low-induction period and high-induction efficiency in contrast to other reports [4,25]. Importantly, this study showed serum-free expanded $CD34^+$ cells retain their hematopoietic multipotency to differentiate into functional myeloid lineages. After HSC expansion and Mk induction, the increase of Mk numbers was almost 5000-fold. The combination of an HSC expansion system and Mk induction system can provide a promising source of Mks for clinical application and cell therapy in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.11.019.

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