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ARTICLE

Good Manufacturing Practice-Grade of Megakaryocytes Produced by a Novel *Ex Vivo* Culturing Platform

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Ex vivo **(EV)-derived megakaryocytes (MKs) have shown great promise as a substitute for platelets in transfusion medicine to alleviate a severe shortage of donor-platelets. Challenges remain that include poor efficiency, a limited scale of production, and undefined short-term storage conditions of EV-derived MKs. This study aims to develop a high-efficiency system for large-scale production of Good Manufacturing Practice (GMP)-grade MKs and determine the short-term storage condition for the MKs. A roller-bottle culture system was introduced to produce GMP-grade MKs from small-molecule/cytokine cocktail expanded hematopoietic stem cells. Various buffer systems and temperatures for the short-term storage of MKs were assessed by cell viability, biomarker expression, and DNA ploidy levels. MKs stored for 24 hours were transplanted into sublethally irradiated nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice to confirm their platelet-releasing and tissue-homing ability *in vivo*. A yield of ~ 2.5 × 10^4 $CD41a^+$/$CD42b^+$ MKs with purity of ~ 80% was achieved from one original cord blood $CD34^+$ cell. Compared with the static culture, the roller-bottle culture system significantly enhanced megakaryopoiesis, as shown by the cell size, DNA ploidy, and megakaryopoiesis-related gene expression. The optimal storage condition for the MKs was defined as normal saline with 10% human serum albumin at 22°C. Stored MKs were capable of rapidly producing functional platelets and largely distributing in the lungs of NOD/SCID mice. The novel development of efficient production and storage system for GMP-grade MKs represents a significant step toward application of these MKs in the clinic.**

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
☑ *Ex vivo* (EV)-derived megakaryocytes (MKs) potentially function as a substitute for donor-platelets in clinical transfusion, but the poor yield and purity, insufficient production scale, and undefined short-term storage conditions of the MKs limit their application.

WHAT QUESTION DID THIS STUDY ADDRESS?
☑ This study aimed to develop a system for production of high-yield and high-purity MKs, and determine the optimal short-term storage condition.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?
☑ This study developed a roller-bottle culture system that can produce high-yield and high-purity MKs from small-molecule/cytokine cocktail pre-expanded hematopoietic stem cells. In addition, the optimal storage condition for the MKs was defined as normal saline with 10% human serum albumin at 22°C.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?
☑ To reduce the dependence on donor-platelets, the development of efficient production and storage system for EV-derived MKs represents a significant step toward application of these MKs in the clinic.

Platelet transfusion is the most important treatment option to increase the number of circulating platelets, thus alleviating uncontrolled bleeding in patients with hematological diseases or after radiotherapy, chemotherapy, or hematopoietic stem cell transplantation.[1,2] As the population is aging coupled with an increased number of patients with cancer, the demand for platelets in the clinic has risen drastically, leading to a severe shortage of platelet resources around the world.[3,4] Besides, there are some distinct disadvantages in donor-derived platelets including the short-life span, potential refractoriness to repeated transfusions, and a risk of potential viral or bacterial contaminations. Thus, it is imperative that alternative strategies need to be developed for obtaining donor-independent platelets for clinical uses.[5,6]

Ex vivo (EV) production of megakaryocytes (MKs) and platelets has been successfully achieved from various stem origins, including hematopoietic stem cells (HSCs),

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embryonic stem cells, and induced pluripotent stem cells, which raises a possibility of applying donor-independent platelets in the clinic.[7–10] However, challenges remain regarding EV-produced platelets, which include a low yield of production and lack of full functional capacity. Only 5–35 platelets are released from one MK on average *in vitro*, which is far less than the platelet output of MKs from adult bone marrow (BM) where a mature MK is capable of producing > 2,000 platelets. Besides, > 50% of EV-produced platelets are $CD41^-$ with a weak agonist response and compromised thrombin-incorporated functions *in vivo*. In contrast, MKs can function not only as precursors of platelets but also as a direct substitute for platelet transfusion.[11,12] The research by Wang *et al*. demonstrated that platelets shed *in vivo* by EV-derived MKs are very similar to donor-derived platelets in terms of size distribution, circulating half-life, and functionality in the mouse model.[13] Moreover, EV-derived MKs have been successfully transfused into the human, thus demonstrating their safety.[11,14]

Despite the promise of EV-produced MKs as a substitute for donor-platelets in the clinic there are several challenges: first, HSCs usually need to expand before their differentiating into MKs. This expansion process is frequently done along with the nondirective differentiation, leading to a relatively low yield and purity of terminally differentiated MKs.[15–17] Second, to date, most production processes are carried out in a limited scale using culture dishes or flasks. Thus, it is necessary to develop a novel culture system that is suitable for a large-scale production of MKs. Finally, EV-produced MKs need to be preserved and stored, even for a short period of time, without a loss of functions before their usage in the clinic.

We previously reported an optimal small-molecule/cytokine cocktail (SMC) for HSC expansion via inhibition of differentiation.[18] After a 7-day culture, HSCs achieved an ~ 28-fold expansion with a purity of $CD34^+$ cells > 85%. In this study, we reported the production of MKs from SMC-expanded HSCs with a high yield and purity on a large scale based on Good Manufacturing Practice (GMP) standards. We also developed an optimal short-term storage condition for EV-produced MKs and verified their platelet-releasing ability and tissue distribution in a mouse model.

METHODS

Animal care and ethics statement

Nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice at 6–7 weeks of age were purchased from SLAC Laboratory Animal Company (Shanghai, China). The mouse experimental protocol was approved by the Institutional Animal Care and Use Committees of Soochow University (IACUC permit number: SYXK (Su) 2013-0064).

Human cord blood samples used in this study were obtained from healthy newborn donors in Suzhou Municipal Hospital (Suzhou, China) after obtaining the mothers' written informed consent. The study protocol was approved by the Hospital's Ethics Committee and Research Ethics Advisory Committee (Permit Number 2015SZSLK075).

Cord blood HSC collection

Cord blood mononuclear cells were isolated by a Ficoll-Hypaque (density 1077 g/cm^2; GE Healthcare, Oslo, Norway) density-gradient centrifugation. $CD34^+$ cells were enriched from mononuclear cells with immunomagnetic selection (Miltenyi Biotec, Bergisch Gladbach, Germany), as reported previously.[19] Enriched $CD34^+$ cells were confirmed by flow cytometry (BD Biosciences, San Jose, CA) with the purity > 90%.

HSC expansion

Freshly isolated cord blood HSCs were expanded with SMCs, as reported previously.[18] Specifically, $CD34^+$ cells were cultured in a 24-well plate in 1 mL of serum-free medium (Stemspan SFEM; Stem Cell Technologies, Vancouver, British Columbia, Canada) upon addition of SMC at 37°C in 5% CO_2 for 5 days. SMC was composed of stem cell factor (SCF 100 ng/mL; Biopharmagen, Suzhou, China), Fms-related tyrosine kinase 3 ligand (100 ng/mL; Biopharmagen), thrombopoietin (TPO 40 ng/mL; Biopharmagen), interleukin (IL) –6 (15 ng/mL; Biopharmagen), stemregenin1 (SR1 1 μM; Selleck Chemicals, Houston, TX), CAY10433 (C433, 0.1 μM; Cayman Chemical, Ann Arbor, MI), and valproic acid (VPA 0.2 mM; Sigma-Aldrich, St. Louis, MO).

MK differentiation

For the initial evaluation of MK differentiation from SMC pre-expanded HSCs, 1×10^5 uncultured $CD34^+$ cells or SMC pre-expanded $CD34^+$ cells were cultured in a 24-well plate in 1 mL of serum-free medium supplemented with SCF (25 ng/mL) and TPO (50 ng/mL) for 12 days. To determine the effect of SR1, VPA, and C433 on MK differentiation, SMC pre-expanded $CD34^+$ cells were cultured with SCF (25 ng/mL) and TPO (50 ng/mL) upon addition of 1 μM SR1, 0.1 μM C433, 0.2 mM VPA, or dimethyl sulfoxide (DMSO; Sigma-Aldrich) for 12 days.

Scalable production of MKs in the roller-bottle culture system

One unit of fresh isolated cord blood HSCs was first cultured with SMC in a T-25 flask, as described above. After 5 days of culture, cells were transferred into a 2-liter roller bottle with 400–600 mL medium at the density of 1.5×10^5/mL and cultured in a customized incubator for an additional 9 days. The culture medium was modified IMDM supplemented with SCF (100 ng/mL), TPO (100 ng/mL), IL-3 (15 ng/mL), IL-6 (25 ng/mL), IL-11 (25 ng/mL; PeproTech, Rocky Hill, NJ), low-density lipoprotein (40 μg/mL; Stem Cell Technologies), and granulocyte-macrophage colony-stimulating factor (15 ng/mL; Biopharmagen).[20] Cells were subcultured according to the cell density and fresh medium with cytokines was added every 3 days.

MK characterization

Cells were collected and labeled with fluorescein isothiocyanate (FITC)-CD41a and allophycocyanin (CD42b antibodies (BD Biosciences) for 30 minutes in dark and then analyzed using a FACSVerse flow cytometer (BD Biosciences). When analyzing, the "cell population" was first gated by using the FSC-SSC dot plot and all the

positive events were based on the "cell population." For pro-platelet-like structure analysis, MKs were seeded on fibronectin (50 μg/mL; Sigma-Aldrich) coated dishes and incubated for 8–12 hours in culture medium for adhesion. The next day, phase contrast microscopy was used to observe the attached pro-platelet-like structures. The number of extending shafts from the main cell body per MK was counted.[21] For DNA content analysis, 1×10^6 cells were first incubated with the CD42b antibody for 30 minutes in the dark. After incubation, cells were fixed with 1% paraformaldehyde for 1 hour and then treated with 100 μg/mL RNase A for 10 minutes. Subsequently, cells were stained with 40 μg/mL propidium iodide for 10 minutes in the dark. For analysis of megakaryopoiesis-related gene expression, cDNA was reverse-transcribed using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher, Pittsburgh, PA) and real-time quantitative polymerase chain reaction (PCR) was performed with the Power SYBR Green PCR Master Mix (Thermo Fisher) on the Step-One Plus real-time PCR system (Applied Biosystems, Thermo Fisher). All experiments were conducted in triplicate and the primer sequences are listed in **Supplementary Table S1**.

Analysis of storage conditions

Harvested mature MKs were washed 3 times with phosphate-buffered saline (PBS) and resuspended in normal saline (NS) at 1×10^7 cells/mL in a 5 mL syringe with void air removed, and kept at 22°C or 4°C for 7 days. Human serum albumin (HSA; 4% v/v or 10% v/v; Tonrol Biopharmaceutical) and acid-citrate-dextrose (ACD) solution (14% v/v, including 2.5% trisodium citrate, 2.0% d-glucose, and 1.5% citric acid) were added alone or in combination for testing their effect on short-term storage of MKs. Cell viability analysis was performed by flow after incubating with the 7-Amino-Actinomycin (7-AAD; BD Biosciences) and PE-Annexin V (BD Biosciences) as per manufacturer's instructions.

In vivo analysis in mice

After sublethal irradiation (2.5 Gy), NOD/SCID mice were intravenously administered with 1×10^6 $CD41a^+/CD42b^+$ MKs with (experimental group, $n = 12$) or without (control group, $n = 3$) green fluorescent protein (GFP) labeling, or 100 μL PBS (blank group, $n = 3$). For GFP labeling, GFP lentivirus was prepared as previously described.[20] MKs were transduced with GFP lentivirus for 8 hours and then cultured for 24–48 hours. To detect the presence of human platelets in the circulation system, mouse peripheral blood samples were collected from the retro-orbital plexus after transplantation using heparinized microcapillaries (Thermo Fisher) and stained with anti-human FITC-CD41a and APC-CD42b antibodies before analysis by flow cytometry. For assessing human platelet activation, 10 μL peripheral blood was incubated with or without 50 μM adenosine diphosphate (ADP; Sigma-Aldrich) at 37°C for 10 minutes, after which flow analysis was done for detecting CD62P surface exposure with anti-human FITC-CD41a and phycoerythrin-CD62P antibodies. For observing the tissue distribution of transplanted MKs in mice, recipient mice were euthanized at various times after transplantation. Mouse tissues, including heart, liver, spleen, lungs, and kidneys, were isolated and analyzed by the *in vivo* fluorescence imaging system.

Statistical analysis

Statistical analysis was performed by using the one-way analysis of variance test and Student's *t*-test, performed with GraphPad Prism 5 software. Results were considered statistically significant when the $P < 0.05$.

RESULTS

Differentiation of MKs from SMC-expanded HSCs

Cord blood-derived $CD34^+$ cells with or without SMC pre-expansion were used to generate MKs in serum-free medium supplemented with SCF and TPO. For SMC pre-expansion, $CD34^+$ cells were cultured with SMC for 5 days, which yielded 18.2 ± 1.5-fold expansion with 91.4 ± 2.1% of $CD34^+$ purity (**Figure 1a,b**). Subsequently, the differentiation efficiency of MKs from SMC-expanded $CD34^+$ cells were confirmed with a majority of cells positive for $CD41a^+/CD42b^+$ (73.2 ± 2.3%) after 9 days of culture, whereas unexpanded $CD34^+$ cells yielded a similar population (76.5 ± 3.6%) of cells positive for $CD41a^+/CD42b^+$ after 12 days of culture (**Figure 1c**). There were no significant differences in the purity of generated MKs between SMC-expanded $CD34^+$ cells and unexpanded $CD34^+$ cells. In terms of the MK yield, the maximal number of total cells and $CD41a^+/CD42b^+$ MKs induced from SMC-expanded $CD34^+$ cells were achieved on day 9 and then declined (**Figure 1d,e**, SMC-expanded $CD34^+$ cells). In contrast, unexpanded $CD34^+$ cells continued to proliferate from day 0 to day 12 (**Figure 1d,e**, unexpanded $CD34^+$ cells). The total cell number from unexpanded $CD34^+$ cells was about 5.5 times higher than that of SMC-expanded $CD34^+$ cells. Considering that SMC $CD34^+$ cells had already expanded about 18-fold before cell seeding, the MK yield is that 953.4 ± 58.8 $CD41a^+/CD42b^+$ MKs were generated from per original $CD34^+$ cell with SMC pre-expansion; On the other hand, only 276.1 ± 23.8 $CD41a^+/CD42b^+$ MKs were obtained from one original $CD34^+$ cells without SMC pre-expansion (**Figure 1f**). Therefore, the total number of culture days was 14 (5 days of SMC expansion plus 9 days of differentiation) for original $CD34^+$ cells to produce the maximal yield and purity of MKs. These results indicate that SMC-expanded HSCs can generate MKs with a purity similar to the unexpanded HSCs but with 3.5 times higher yield.

Effect of three small molecules on MK differentiation and maturation

Previous studies demonstrated that SR1 promoted MK differentiation and maturation in addition to its effect on HSC expansion.[22,23] On the other hand, little is known about the effect of VPA and C433 on megakaryopoiesis. We then focused on the assessment of VPA and C433 on differentiating SMC-expanded $CD34^+$ cells toward the megakaryocytic lineage. After 9 days of culture, SR1 treatment enhanced the yield of MKs with a purity of $CD41a^+$ and $CD42b^+$ cells similar to that of control (**Figure 2a,b**, SR1). However, the population of $CD41a^+$ and $CD42b^+$ cells decreased after VPA or C433 treatment, leading to a reduction

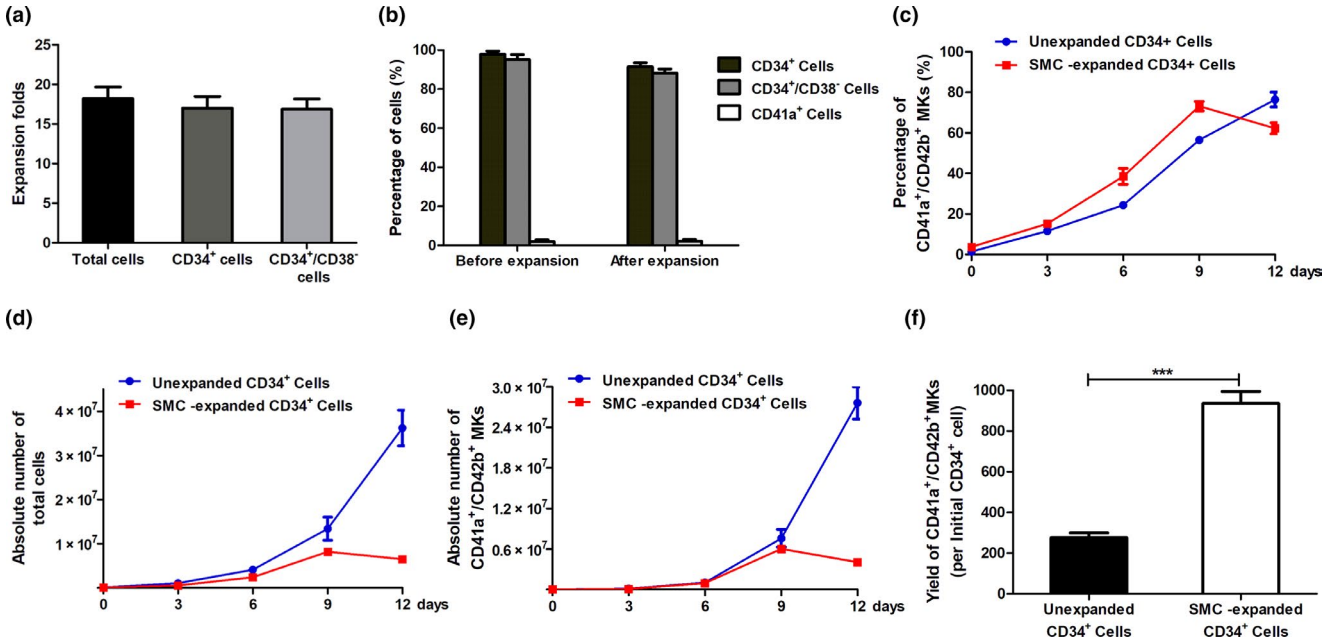

Figure 1 Confirmation of MK differentiation effect from SMC-expanded hematopoietic stem cells. (**a**) Expansion folds of total cells, $CD34^+$ cells, and $CD34^+/CD38^-$ cells after 5 days of expansion with SMC combination. (**b**) Percentage of $CD34^+$ cells, $CD34^+/CD38^-$ cells, and $CD41a^+$ cells after 5 days of expansion with SMC combination. (**c**) Purity of $CD41a^+/CD42b^+$ MKs differentiated from unexpanded or SMC-expanded $CD34^+$ cells over time. (**d**) Absolute number of total cells cultured from unexpanded or SMC-expanded $CD34^+$ cells over time. (**e**) Absolute number of $CD41a^+/CD42b^+$ MKs cultured from unexpanded or SMC-expanded $CD34^+$ cells over time. (**f**) Yield of $CD41a^+/CD42b^+$ MKs per input cord blood hematopoietic stem cells. Data are shown as mean ± SD, $n = 3$. $^{***}P < 0.001$. MKs, megakaryocytes; SMC, small-molecule/cytokine cocktail.

of total MK numbers (**Figure 2a,b**, VPA and C433). These results strongly suggest that VPA and C433 do not have an effect on MK production. Thus, these three small molecules were removed in the subsequent production of MKs.

Optimization of culture protocols for increase of MK yield

Given a limited effect of SCF and TPO in stimulating production of MKs, we applied an optimal cytokine cocktail (CC)[20] to support the production of MKs from SMC-expanded HSCs. As shown in **Figure 2c**, CC induced a very large population of cells expressing megakaryocytic markers with $CD41a^+$ cells at 82.7 ± 1.03% and $CD42b^+$ cells at 78.5 ± 1.90% on day 9 of culture. Significantly, the absolute number of $CD41a^+/CD42b^+$ MKs with CC treatment was ~ 15-fold higher than that with the addition of SCF and TPO alone (**Figure 2d**). Therefore, a 9-day culture protocol with CC is optimal to produce from SMC-expanded HSCs a maximal number of MKs with high purity.

Scalable production of mature MKs in a roller-bottle culture system

Clinical applications of EV-produced MKs call for the development of a standardized and large-scale production platform. A roller-bottle culture system that comprised a customized CO_2/O_2 adjustable incubator and roller bottles (2-liter capacity) were applied, which offered a 3D environment with a large culture volume. The stepwise culture protocol was described in **Figure 3a** and CC was used for the production of MKs from day 5 to day 5 + 9. Cells cultured in a T-75 flask that was placed in the same incubator were used as a control (static culture). Compared with the control, the roller-bottle culture system significantly increased the number of $CD41a^+/CD42b^+$ MKs by about 1.8-fold (**Figure 3b**); MKs were larger cells (**Figure 3c**) with more extending shafts in the pro-platelet-like structures (red arrows in **Figure 3d** and **Figure** 3e); Moreover, as shown in **Figure 3f**, DNA ploidy levels of MKs using the roller-bottle culture of MKs were much higher than those of the static culture (2N, 52.0 ± 2.6% vs. 62.0 ± 4.0%; 4N, 24.7 ± 1.2% vs. 21.0 ± 2.4%; and 8N and $8N^+$, 22.3 ± 1.4 vs. 16.0 ± 1.5%). Furthermore, expression of key genes related to MK differentiation and maturation (*GATA1*, *FOG1*, *NF-E2*, and *β-tubulin*) were higher in the roller-bottle culture condition (**Figure 3g**). Thus, these results indicate that the roller-bottle culture system cannot only enlarge the scale of MK production but also enhance the efficiency of mature MK differentiation. Therefore, after 5 days of pre-expansion with SMC followed by an additional 9-day culture with the roller-bottle culture system, the normalized yield of $CD41a^+/CD42b^+$ MKs was around 2.5×10^4 from one initial cord blood HSC (**Figure 3h**).

Optimal condition for short-term storage of MKs

The short-term storage and/or transportation are indispensable for most cell products, including MKs. Therefore, it is important to determine buffer conditions and temperatures for optimal preservation and storage of mature MKs.

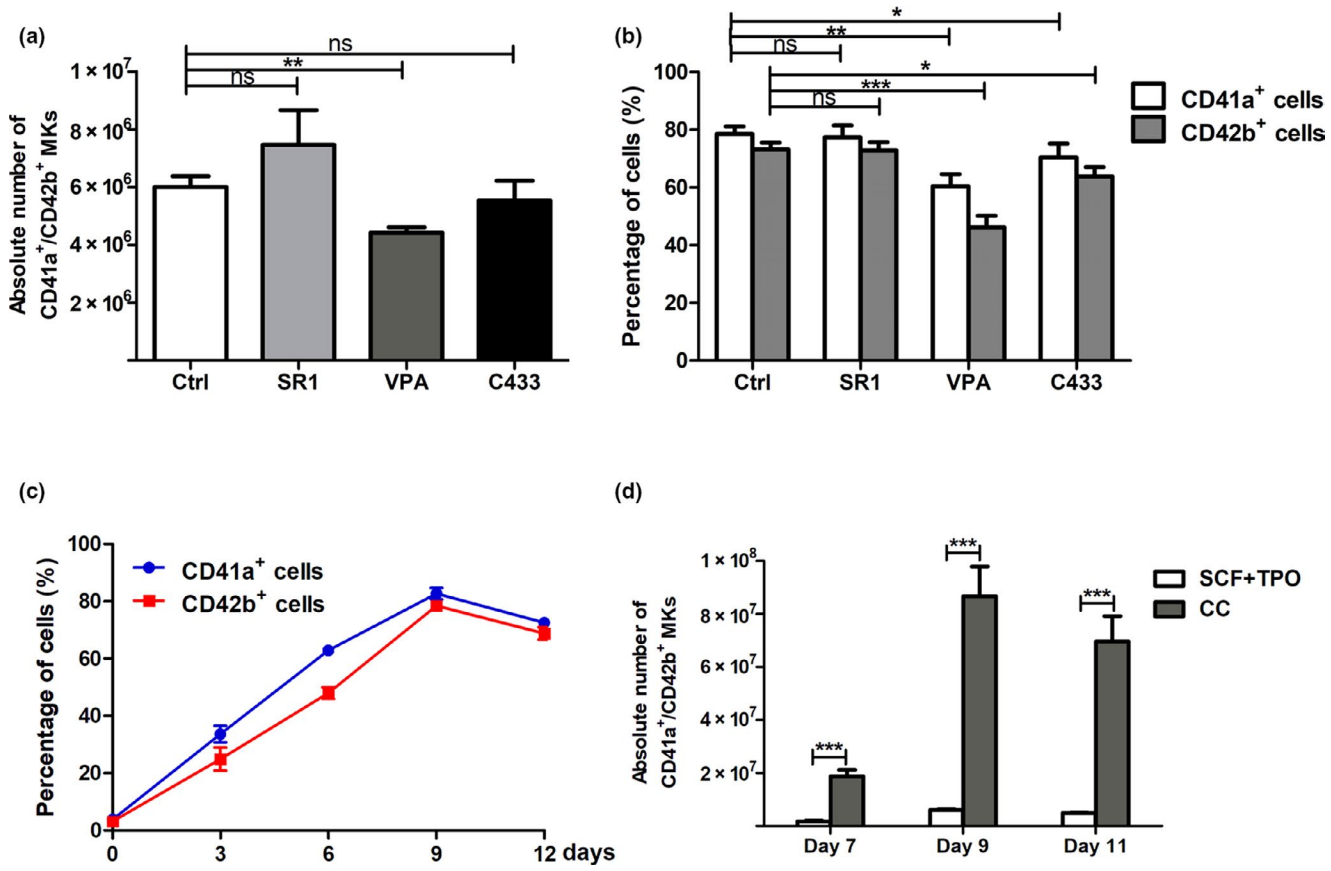

Figure 2 Optimization of culture conditions for producing MKs from small-molecule/cytokine cocktail-expanded hematopoietic stem cells. (**a**) Absolute number of $CD41a^+/CD42b^+$ MKs after 9 days of culture with the indicated treatments. (**b**) Percentage of $CD41a^+$ and $CD42b^+$ cells after 9 days of culture with the indicated treatments. (**c**) Percentage of $CD41a^+$ and $CD42b^+$ cells over time induced by CC. (**d**) Absolute number of $CD41a^+/CD42b^+$ MKs from day 7 to day 9 induced by SCF + TPO or CC. Data are shown as mean ± SD, $n = 3$. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. CC, cytokine cocktail; MKs, megakaryocytes; SCF, stem cell factor; SR1, stemregenin1; TPO, thrombopoietin; VPA, valproic acid.

Two storage buffers, HSA and ACD of various concentrations and combinations, were first evaluated at 22℃ (**Table 1**). A direct comparison of cell viability over time in 5 groups revealed that viable cells decreased significantly in NS alone or supplemented with ACD with < 15% of viable cells remaining on day 1 of storage (**Figure 4a**, groups NS, ACD, and HSA + ACD). In the buffer of NS with the addition of HSA (**Figure 4a**, groups 4% HSA and 10% HSA), MK viability was decreased much slower with over 80% of cell viability after 1-day storage (82.9 ± 1.82% in 4% HSA and 85.6 ± 3.19% in 10% HSA) and over 60% of cell viability after 2-day storage (65.2 ± 5.25% in 4% HSA and 73.6 ± 4.82% in 10% HSA). The detection of 7-AAD and Annexin V by flow suggests cell death via apoptosis. In the FSC-SSC flow dot plots, compared with the MKs before storage, the NS and ACD groups showed obviously more impurities and cell debris (the population outside the "Cell gate" in the left panel of **Figure 4c**), resulting in very few cell populations for subsequent analysis. Meanwhile, the storage buffer containing ACD resulted in significantly more $7\text{-AAD}^+/\text{Annexin V}^+$ cells than the groups with no ACD (the right panel of **Figure 4c**), further demonstrating the injury effect caused by ACD. When increasing the concentration of HSA from 4% to 10%, the proportion of viable cells increased, especially on the third or fifth days of storage (**Figure 4a**, groups 4% HSA and 10% HSA). Consistent with this observation, 7-AAD^+ and Annexin V^+ cells in 10% HSA were less than those in 4% HSA (**Figure 4c**). Therefore, the optimal buffer for the short-term storage of MKs is 10% HSA.

Subsequently, we tested the storage temperature(s) with the use of NS with 10% HSA as the storage buffer. As shown in **Figure 4b**, the viability of MKs decreased to 50.2 ± 5.72% after 1 day of storage at 4℃, which was significantly lower than that of MKs at 22℃. MK viability at 22℃ remained higher than that at 4℃. On the other hand, CD41a and CD42b surface markers and the DNA ploidy of MKs did not exhibit significant change when mature MKs were stored at 22℃ (**Figure 4d,e**). Therefore, the optimal conditions for the short-term storage of mature MKs are defined as NS with 10% HSA at 22℃.

Platelet release and tissue distribution studies of MKs in xenotransfused mice

Platelet-releasing capacity and tissue distributions of EV-produced MKs were evaluated in NOD/SCID mice. MKs

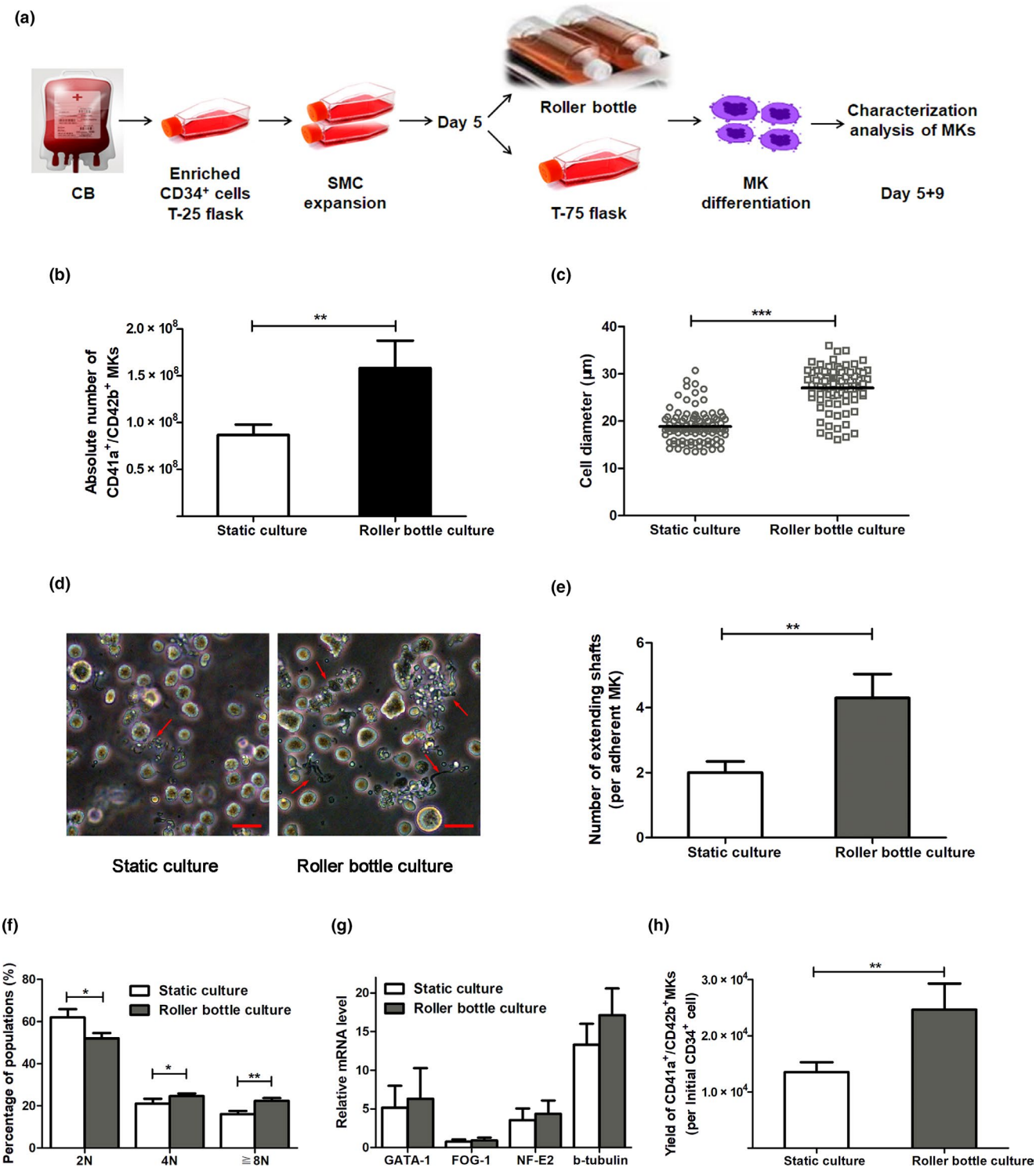

Figure 3 Scalable production and characterization analysis of mature MKs. (**a**) Schematic diagram of the stepwise culture protocol from cord blood $CD34^+$ cells to MKs in the roller-bottle culture system. (**b**) Absolute number of $CD41a^+/CD42b^+$ MKs at the end of culture. (**c**) Cell size distribution of MKs measured by microscopy. Digital images of cells were captured and cell diameters were measured by Image J software. (**d**) Representative cell morphology of MKs and pro-platelet-like structures (red arrows). Scale bar = 20 μm. (**e**) Number of extending shafts from one adherent MK with ≧ 30 cells counted per condition. (**f**) DNA ploidy distribution of MKs. (**g**) The mRNA levels of megakaryopoiesis-related gene assessed by real-time quantitative polymerase chain reaction. *β-actin* was used as an internal control. (**h**) Yield of $CD41a^+/CD42b^+$ MKs per input cord blood hematopoietic stem cell. All data are shown as means ± SD, $n = 3$. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. CB, cord blood; MKs, megakaryocytes; SMC, small-molecule/cytokine cocktail.

Table 1 Buffer optimization for short-term storage of mature MKs

Groups	Storage buffer content			Storage temperature
	NS	HSA (v/v)	ACD (v/v)	
NS	+	–	–	22°C
4% HSA	+	4%	–	22°C
10% HSA	+	10%	–	22°C
ACD	+	–	14%	22°C
HSA + ACD	+	10%	14%	22°C

Freshly harvested MKs were aliquoted and stored in the indicated buffer at 22°C.
ACD, acid-citrate-dextrose; HSA, human serum albumin; MK, megakaryocyte; NS, normal saline.

stored for 24 hours in the optimal condition were used to perform this evaluation. Human platelets released by infused MKs could be detected in mouse peripheral blood during the next 48 hours. As show in **Figure 5a**, in the first 0.5 hours, human $CD41a^+/CD42b^+$ platelets occurred with a low level of 0.5 ± 0.1%, after which the human platelet level increased rapidly, peaking at 4 hours (3.2 ± 0.6%). The percentage of human platelets declined gradually to < 1% after 24 hours. The half-life of released human platelets in mice was ~ 6 hours (**Figure 5b**). It has been reported that although NOD/SCID mice are immunodeficient, their macrophages still have a strong clearance effect on human platelets, which probably is a main reason for the limited detection window of human platelets in the NOD/SCID mouse model.[13,24]

The responsiveness of released human platelets to agonists was also investigated to assess their activity. After simulation with ADP (50 μM), platelet activation could be detected by CD62P (P-selectin) surface antigen expression (**Figure 5c**). Before activation, human platelets isolated from murine blood were relatively quiescent with low mean fluorescence intensity of CD62P expression. After ADP incubation, mean fluorescence intensity increased approximately threefold. These results indicate that human platelets released by produced MKs in mice can be activated.

Tissue distributions of the infused MKs in mice were examined by a live fluorescent microscopy. Prior to transplantation, cells were labeled with GFP via lentiviral transduction (> 90% efficiency). As shown in **Figure 5d**, most infused MKs were entrapped in the murine lung with strong GFP signals from 0.5–3 hours post-transplantation and GFP signals gradually decreased after 6 hours. These results were in agreement with the kinetics of human platelets in murine peripheral blood (**Figure 5a**), which also suggests that, after infusion, MKs were first entrapped by the pulmonary microvasculature, where they temporarily settled down, releasing platelets under the blood flow shear force. MKs subsequently underwent apoptosis. Other than the lungs, small amounts of MKs (GFP signals) were present in the kidneys, which might be related to the clearance process. No fluorescence signals could be detected in the control group (administered with 100 μL PBS) whereas only weak autofluorescent signals were detected in the lungs of the control group (administered with MKs without GFP labeling).

DISCUSSION

In the current study, we have successfully developed a novel roller-bottle culture system for the large-scale production of high-yield and high-purity MKs based on GMP standards. Approximate 6.25×10^{10} $CD41a^+/CD42b^+$ MKs can be obtained from one cord blood unit (2.5×10^6 $CD34^+$ cells), which can theoretically meet the demand of transfusion for 45 patients (with an average weight of 70 kg and transfusion of 2×10^7 MKs per kg) in the clinic. Furthermore, we have optimized conditions of the buffer system and temperature for short-term storage of EV-produced MKs, which are important approaches for clinical applications.

In terms of the EV production of MKs, various approaches have been reported, including different cell sources, culture protocols, and production scales. In **Table 2**, we summarize typical strategies of MK production in literature and make a comparison to our present work.[7,8,12,25–27] It has been reported that the MK yield obtained by one-step differentiation of HSCs was inadequate for characterizing MKs *in vitro* and applications in the clinic.[28,29] To circumvent the deficiency, culture protocols in most studies include the step of HSC expansion prior to megakaryocytic differentiation. Moreover, stromal cells, animal components, or any gene manipulations need to be avoided in entire cell culture processes to minimize potential risks in clinical applications. Because of these precautions, a panel of recombinant cytokines, including SCF, Fms-related tyrosine kinase 3 ligand, IL-3, granulocyte-macrophage colony-stimulating factor, granulocyte colony-stimulating factor are commonly used for HSC expansion.[30,31] However, it is well known that the pleiotropic effects of most cytokines allow HSCs to be expanded in large quantities along with nondirective differentiation into various blood lineages, resulting in the low production efficiency of MKs in the subsequent procedure.[32,33]

In previously reported culture strategies, the EV production of MKs (without gene manipulations) is ~ 330 MKs from 1 seeded $CD34^+$ cell with ~ 45% of purity.[7,12,17,26–28] In order to increase the yield, HSCs usually need to expand before their specific differentiation into MKs. First, we have developed SMC that can efficiently expand HSCs and meanwhile inhibit their nonspecific differentiations. The differentiation capacity of SMC-expanded $CD34^+$ cells toward the MK lineage is shown to be similar to that of unexpanded $CD34^+$ cells. Second, we have used the combination of an optimal CC and a roller-bottle culture system to achieve high-efficient production of MKs in the differentiation phase. Finally, we have made great efforts to explore the optimal nutrition, cytokine concentrations, and culture conditions (including the rotate speed, culture volume, PH, oxygen, carbon dioxide percentages, etc.) to provide the best micro-environment conditions for a custom-built roller-bottle culture system. By using this novel protocol and culture system, 2.5×10^4 $CD41a^+/CD42b^+$ MKs can be obtained from one seeded cord blood $CD34^+$ cell with a purity of ~ 80%. As far as we know, this is the first report for EV producing MKs in such a large scale. Most importantly, this novel technology and culturing system provide a significant approach toward the clinical application of EV-derived MKs.

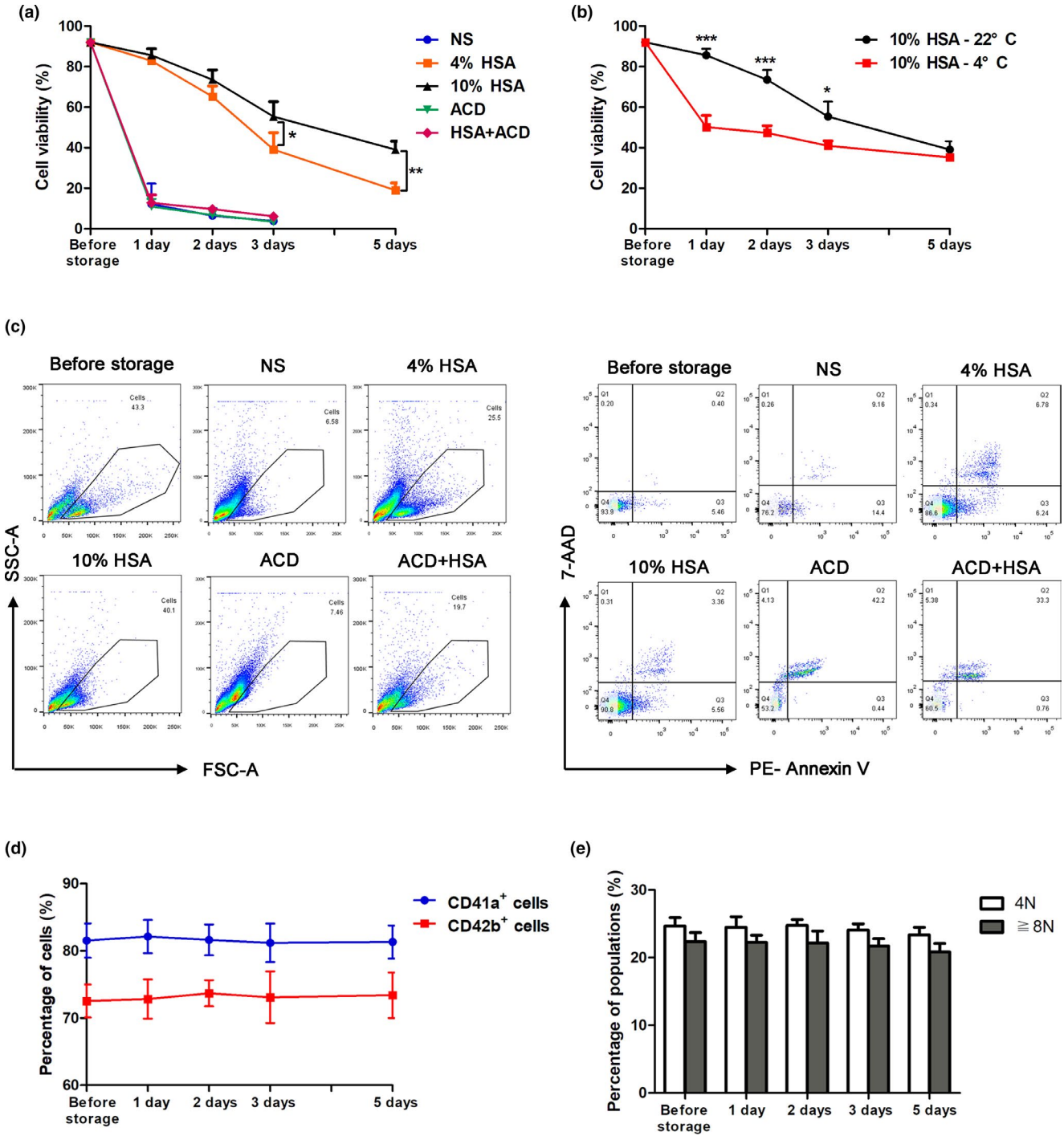

Figure 4 Optimization of conditions for short-term storage of MKs. (**a**) MK cell viability during storage in the indicated buffer at 22°C. (**b**) MK cell viability during storage in the buffer of NS with 10% HSA at 22°C or 4°C. (**c**) Representative flow cytometry profiles for the expression of 7-Amino-Actinomycin and Annexin V of MKs before storage and on day 1 of storage. (**d**) Percentage of $CD41a^+$ and $CD42^+$ cells during storage in the buffer of NS with 10% HSA at 22°C. (**e**) DNA ploidy distribution during storage in the buffer of NS with 10% HSA at 22°C. Data are shown as mean ± SD, $n = 3$. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. ACD, acid-citrate-dextrose; HSA, human serum albumin; MK, megakaryocyte; NS, normal saline.

The novel roller-bottle culture system achieves much higher yields of MKs and greatly promotes megakaryopoiesis than the conventional culture using flasks.[27] Possible reasons are as follows: (i) rotary cell culture can prevent cell sedimentation, facilitate cell-cell interaction, and better supply nutrients and oxygen, thus promoting cell viability and proliferation more efficiently than the traditional static culture; (ii) the microgravity generated during the rotary process

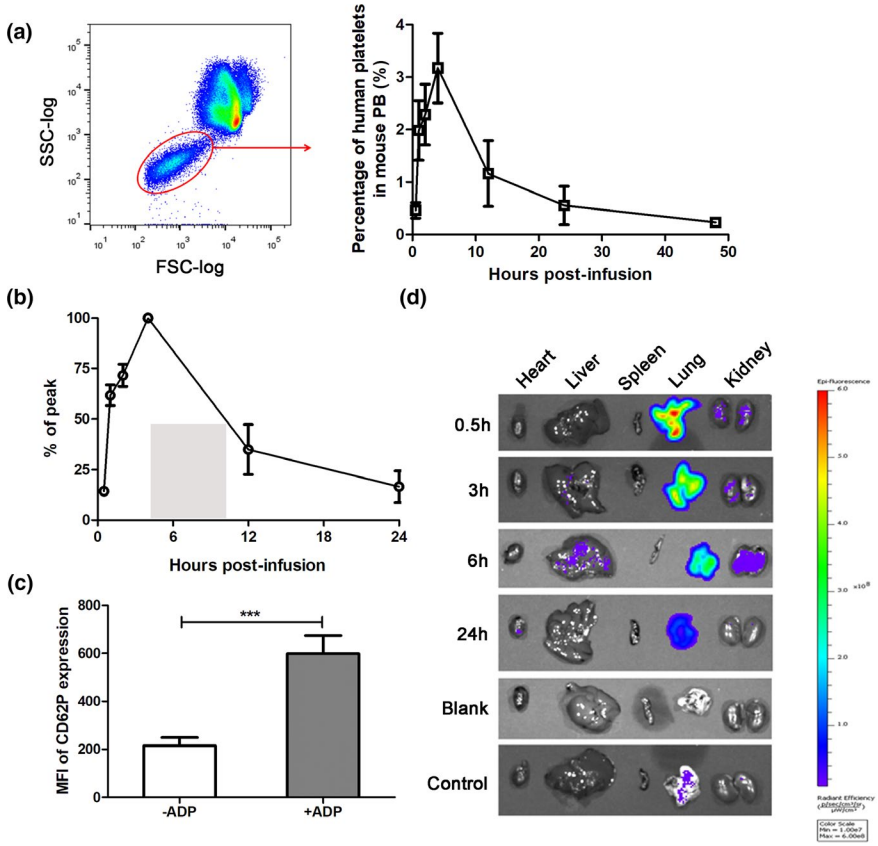

Figure 5 *In vivo* platelet release and tissue distribution of MKs in NOD/SCID mice. (**a**) Percentage of human CD41a$^+$/CD42b$^+$ platelets detected in mouse peripheral blood over the first 48 hours after infusion. (**b**) Percentage of peak human platelet counts calculated according to **a**. The half-life is indicated by a gray rectangle beginning at peak platelet count. Data are shown as mean ± SD, $n = 3$ at each time point assayed. (**c**) CD62P expression MFI of human platelets in the presence or absence of adenosine diphosphate (50 μM) at 4 hours after MK infusion. Data are shown as mean ± SD, $n = 3$. ***$P < 0.001$. (**d**) Fluorescence imaging of tissues in NOD/SCID mice infused with MKs. NOD/SCID mice were infused with green fluorescent protein-labeled human MKs and tissues, including heart, liver, spleen, lungs, and kidneys, were observed by the *in vivo* fluorescence imaging system at indicated time points. ADP, adenosine diphosphate; MFI, mean fluorescence intensity; MK, megakaryocyte; NOD/SCID, nonobese diabetic/severe combined immunodeficient.

can potentially play an active role in enhancing DNA ploidy levels of MKs[34]; and (iii) the shear force produced during rotation may promote the formation of pro-platelet-like structures.[35,36]

Several studies regarding the storage and transportation for HSCs or mesenchymal stromal/stem cells (MSCs) have been investigated and proved that the viability of short-term liquid-stored HSCs/MSCs is enhanced by the presence of HSA.[37,38] ACD, an anticoagulation solution, is frequently included for preserving blood. In the current study, we tested the effect of HSA and ACD on mature MKs during storage with NS as the basal buffer. We have observed that HSA significantly preserves the viability of MKs whereas ACD has no positive effect on MK storage. Cold temperature has been shown to be beneficial for most cell-based products, including fresh blood, HSCs, and MSCs. However, 22°C is optimal for the storage of mature MKs in our study, which is similar to the preserving temperature of donor-platelets. Low temperature seems to accelerate the degradation of cell surface makers of MKs and platelets including CD42b.[39] In addition, EV-produced MKs are pure cell products and no plasma component is involved. After comparison studies with different storage buffers, the optimal storage buffer of MKs is normal saline with 10% HSA, so antibodies and pathogens that widely exist in human plasma are excluded from the MK products, which is translated to be safer for the clinical application.

Platelet release *in vivo* is very important for assessing the function of EV MKs. In this study, the platelet release potential of stored MKs has been evaluated in the mouse model. Although there are pro-platelet-like structures and even released platelets existing in the culture medium, the cells are washed three times with PBS and centrifuged at 300g to get rid of the released platelets and other medium components before storage. Therefore, the human platelets detected in the mouse blood are platelets that were released by transfused MKs. We have shown that human platelets are detected in mouse peripheral blood as short as 0.5 hours after MK injection, peaking at 4 hours. This observation suggests that a rapid platelet supply can be achieved in patients transfused with MKs. In terms of the number of platelets released from transfused MKs, it

Table 2 Summary of *ex vivo* production of human MKs

Cell source	Culture scale	Culture steps			Total culture time (d)	MK ploidy	MK purity	MK yield per input cell	References
		1	2	3					
CB $CD34^+$ cells	24-well plate	MGDF 14 days	N/A	N/A	14	$8N^+$: < 5%	$CD41a^+$: 73.5%	27	[25]
CB $CD34^+$ cells	24-well plate	TPO, IL-3, Flt-3L, IL-6 5 days	TPO, SCF, IL-6, IL-9 9 days	N/A	14	$8N^+$: 5–15%	$CD41a^+$: 97% $CD42b^+$: 86%	500	[26]
CB $CD34^+$ cells	75-cm^2 flask	hTERT stroma cells + SCF, Flt-3L, TPO 14 days	hTERT stroma cells + SCF, Flt-3L, TPO, IL-11 14 days	SCF, Flt-3L, TPO, IL-11 5 days	33	$4N^+$: < 10%	$CD41a^+$: 0.5%	500	[7]
CB $CD34^+$ cells	10-mL rotary culture vessel	TPO, SCF, IL-3 6 days	TPO, IL-11 9 days	N/A	15	$4N^+$: 45% $8N^+$: 18%	$CD41a^+$: 90% $CD42b^+$: 86%	50	[27]
iPSCs	50-mL microcarrier beads-assisted stirred bioreactors	BMP4, VEGF 4 days	SCF, TPO, IL-3, 8 days	SCF, TPO 10	22	$8N^+$: 51.8%	$CD41^+CD42a^+CD61^+$: 51.3 ± 11.5%	30	[12]
iPSCs	6-well plates	OP9 stroma cells + VEGF	Overexpression of c-MYC, BMI1 14 days	Overexpression of c-MYC, BMI1, BCL-XL > 4 months	4–5 months	$8N^+$: 20.2%	$CD41^+CD42a^+$: > 90%	Immortalized MK progenitor cell lines	[8]
CB $CD34^+$ cells	2-liter roller bottles	SCF, TPO, SR1, C433, VPA 5 days	SCF, TPO, IL-3, IL-6, IL-11, LDL, GM-CSF 9 days	N/A	14	$8N^+$: 22.3%	$CD41a^+$: 82.7% $CD42b^+$: 78.5%	2.5×10^4	Present work

BMP4, bone morphogenetic protein-4; C433, CAY10433; CB, cord blood; Flt-3L, Fms-related tyrosine kinase 3 ligand; IL, interleukin; iPSC, induced pluripotent stem cell; MGDF, megakaryocyte growth and development factor; MK, megakaryocyte; N/A, not applicable; SCF, stem cell factor; SR1, stemregenin1; TPO, thrombopoietin; VEGF, vascular endothelial growth factor; VPA, valproic acid.

peaks around 30 platelets per MK (calculated based on the peak percentage of platelets that were released with assumption that a mouse has a blood volume of 2 mL; **Figure 5a**). Although clearance by mouse macrophages may account for a low platelet yield, it is likely that EV MKs have a weaker capacity to release platelets than BM MKs, which is also reported by others.[24,40,41] The DNA ploidy of BM MKs can be as high as 128N, with an average of 16N, but the ploidy level of cultured MKs is mostly 2N and 4N, indicating a limited megakaryocytic maturation that may account for poor platelet release *in vivo*.[41,42] In this study, although the DNA ploidy levels of MKs are enhanced due to the positive role of microgravity generated during the rotary process, the percentage of $8N^+$ MKs is still low. We are currently trying to increase the ploidy level of EV MKs without decreasing their yield. It is our hope that the promising approach would further increase the DNA ploidy level of MKs without decreasing the production yield.

We also detected the *in vivo* distribution of transplanted MKs by using a live fluorescence imaging system. Most of the MKs were accumulated in the lungs. The pulmonary circulatory system has a strong shear force, probably allowing MKs to release platelets here. Recently, Lefrançais *et al*. observed that a large number of MKs were present in the lungs of mice for the first time by using the "two-photon intravital imaging" technique, and these MKs generated platelets at a rate of 10 million every hour.[43] Although whether human lungs support megakaryopoiesis remains to be further studied, our findings do provide an impetus for further investigating megakaryocytic differentiation *in vitro* and megakaryopoiesis *in vivo*.

In conclusion, high-yield and high-purity production of EV-derived MKs can be achieved in a novel roller-bottle culture system based on GMP standard. In addition, optimal short-term storage conditions have been obtained for preservation of EV MKs, which possess *in vivo* platelet-releasing ability in the mouse model. These advances lay a solid foundation to further explore these MKs as substitutes for donor-platelets in the clinic.

Supporting Information. Supplementary information accompanies this paper on the *Clinical and Translational Science* website (www.cts-journal.com).

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