

# Expandable Megakaryocyte Cell Lines Enable Clinically Applicable Generation of Platelets from Human Induced Pluripotent Stem Cells

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## SUMMARY

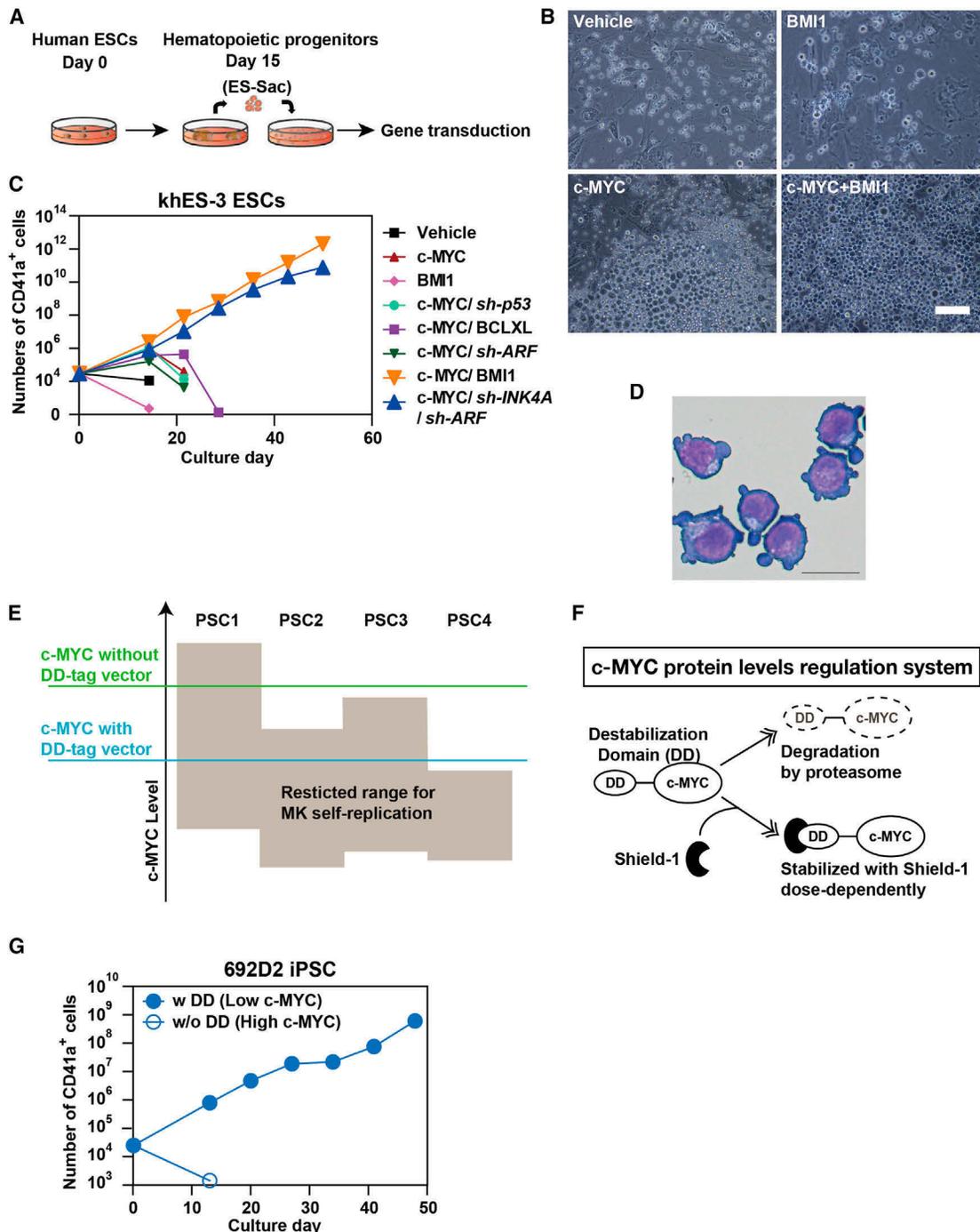
The donor-dependent supply of platelets is frequently insufficient to meet transfusion needs. To address this issue, we developed a clinically applicable strategy for the derivation of functional platelets from human pluripotent stem cells (PSCs). This approach involves the establishment of stable immortalized megakaryocyte progenitor cell lines (imMKCLs) from PSC-derived hematopoietic progenitors through the overexpression of BMI1 and BCL-XL to respectively suppress senescence and apoptosis and the constrained overexpression of c-MYC to promote proliferation. The resulting imMKCLs can be expanded in culture over extended periods (4–5 months), even after cryopreservation. Halting the overexpression of c-MYC, BMI1, and BCL-XL in growing imMKCLs led to the production of CD42b<sup>+</sup> platelets with functionality comparable to that of native platelets on the basis of a range of assays *in vitro* and *in vivo*. The combination of robust expansion capacity and efficient platelet production means that appropriately selected imMKCL clones represent a potentially inexhaustible source of hPSC-derived platelets for clinical application.

## INTRODUCTION

Platelets generated from megakaryocyte (MK) precursors are vital for the treatment of many hematological diseases and traumas. Currently, platelets can only be obtained through blood donation. Fresh single-donor platelets have a short shelf life and must be maintained with plasma at 20°C–24°C; they readily lose clotting activity when pooled from multiple donors and frozen or warmed to 37°C (Bergmeier et al., 2003; Nishikii et al., 2008). Moreover, repeated transfusion induces the production of anti-

bodies against allogenic human leukocyte antigen (HLA) or human platelet antigen (HPA) on the transfused platelets (Schiffer, 2001), which renders the patient unresponsive to platelet transfusion therapy. These supply logistics and practical limitations represent barriers to the widespread application of platelets as a resource for patients. In that context, human induced pluripotent stem cells (hiPSCs) (Takahashi et al., 2007) could represent a potent alternative source of platelet production. Because platelets do not contain nuclei, gamma irradiation before transfusion could be used to eliminate any residual contaminating hiPSCs and their derivatives, reducing the risk of tumorigenesis. Thus, the application of iPSC-based technology could potentially yield a consistent supply of HLA- and/or HPA-matched or even autologous platelets in a way that would address some of the major roadblocks in the current clinical approaches to platelet-based therapy.

Our group and another recently demonstrated that hiPSCs derived from human skin fibroblasts or blood cells or from human embryonic stem cells (hESCs) can be used to generate platelets *in vitro* and that these platelets appeared to function normally when transfused into mouse models (Takayama et al., 2010; Lu et al., 2011). However, the yield of platelets was still far below what would be required to generate even 1 u of platelet concentrate for patient transfusion. Recent studies have shown that self-replicating MK progenitors can be directly generated from murine hematopoietic stem cells (HSCs) within bone marrow (BM) *in vivo* (Yamamoto et al., 2013), but there was no evident way to sustain long-term self-replication of MK progenitors *in vitro*. We previously showed that the activation of c-MYC to a restricted level below that associated with senescence and apoptosis induction appears to lead to an increase in platelet generation (Takayama et al., 2010). In the present study, we show that co-overexpression of c-MYC and BMI1, a polycomb complex component that represses the *INK4A/ARF* gene locus (Oguro et al., 2006), enables megakaryocytic cell lines (MKCLs) derived from hiPSCs or hESCs to grow continuously for up to 2 months. A destabilization domain (DD) vector system (Banaszynski et al., 2006) enabled us to control exogenous c-MYC within the appropriate range, leading to successful induction of



**Figure 1. Induction of a Megakaryocyte Progenitor Cell Line from Human Embryonic Stem Cells and Induced Pluripotent Stem Cells Was Induced by Restricted Range of c-MYC Overexpression along with BMI1 Overexpression**

(A) Scheme for inducing megakaryocyte progenitor cell lines (MKCLs) from human PSCs. The targets for gene transduction are hematopoietic progenitors, including the CD34<sup>+</sup> population.

(B) Representative photomicrograph of cells transduced with vector alone, c-MYC alone, BMI1 alone, or a combination of c-MYC and BMI1 (200×). The scale bar represents 100 μm.

(C) Numbers of CD41a<sup>+</sup> cells after gene manipulation. Hematopoietic progenitor cells (HPCs) within human embryonic stem cell (hESC; KhES-3 clone) sacs were collected and transduced with noninducible retroviral vectors with vector alone (GFP alone), c-MYC alone, BMI1 alone, or combinations of c-MYC plus *p53* knockdown, c-MYC plus *ARF* knockdown, c-MYC plus *BCL-XL*, c-MYC plus *INK4A/ARF* knockdown (blue), or c-MYC plus BMI1 (orange). The combinations of c-MYC plus BMI1 and c-MYC plus *INK4A/ARF* knockdown induced exponential growth in CD41a<sup>+</sup> megakaryocytes (MKs). Results are expressed as means from two to three independent experiments.

(D) Representative image of May-Giemsa-stained MKCLs. The scale bar represents 20 μm.

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MKCLs. The addition of BCL-XL made it possible to obtain immortalized MKCLs (imMKCLs) that can be grown for more than 5 months and thus function as candidate cell banks. When the expression of c-MYC, BMI1, and BCL-XL is turned off, imMKCLs produce functional CD42b (glycoprotein Ib  $\alpha$  [GPIb $\alpha$ ] and receptor for von Willebrand factor [vWF])<sup>+</sup> platelet particles. The expression of CD42b on platelets is required for the initiation of clotting (Ware et al., 2000) and bacterial clearance in vivo (Wong et al., 2013). Although previous studies have reported that CMK, Meg01, and K562 cells, three well-known MK lineage leukemia cell lines, can become polyploid and release CD41a<sup>+</sup> particles in the presence of agonist stimulation, they do not provide a suitable source for a platelet supply because the particles are CD42b<sup>-</sup> (Isakari et al., 2009; Sato et al., 1989; Terui et al., 1998). In this report, we describe a strategy that involves MKCLs with long-term self-renewal capacity and the potential to provide an inexhaustible supply of CD42b<sup>+</sup> platelets; in that respect, they resemble endogenous self-replicating MK progenitors identified in vivo (Yamamoto et al., 2013). Clinical application of this technology could provide a plentiful supply of platelets from suitably screened and selected imMKCL clones to serve as cell bank stocks with minimal risk of adverse side effects.

## RESULTS

### Induction of Expandable MK Progenitor Cells from Human PSCs with c-MYC and BMI1

We previously showed that, when expressed at an appropriate level, c-MYC acts as a growth mediator in normal megakaryopoiesis and thrombopoiesis from hESCs or hiPSCs, whereas excessive c-MYC expression in hematopoietic progenitor cells (HPCs) induces the activation of the INK4A and ARF pathways, leading to senescence and apoptosis (Takayama et al., 2010). Therefore, we hypothesized that c-MYC activation might contribute to self-replication at the MK progenitor stage. When we assessed the effects of c-MYC overexpression alone, BMI1 overexpression alone, c-MYC overexpression plus p53 knockdown, c-MYC plus BCL-XL overexpression, and c-MYC plus BMI1 overexpression in CD34<sup>+</sup>CD43<sup>+</sup>-containing HPCs derived from the KhES-3 hESC line, we found that c-MYC overexpression alone or the combination of c-MYC and BMI1 overexpression increased numbers of large cells expressing megakaryocytic CD41a<sup>+</sup>CD42a (GPIX)<sup>+</sup>CD42b<sup>+</sup>CD9<sup>+</sup> markers over a 2-week period (Figures 1A–1C; Figure S1A available online). With c-MYC and BMI1 overexpression (Figure 1C, orange triangles) or c-MYC overexpression and *INK4A/ARF* knockdown (Figure 1C, blue triangles), but none of the other aforementioned conditions, proliferation of this cell population was maintained at an exponential level for 2 months (MK progenitor cell line; Figure 1C, orange triangles) and was dependent upon the presence of thrombopoietin (TPO) with the help of

stem cell factor (SCF). This suggests that the effect of BMI1 may be to at least inhibit INK4A/ARF-dependent senescence and apoptosis during the initiation of self-replication, as confirmed by quantitative PCR (qPCR) analysis, which revealed BMI1 represses c-MYC-induced the upregulation of *INK4A/ARF* (Figure S1B). The CD41a<sup>+</sup> MKCLs derived from hESCs showed monoblastic morphology with basophilic cytoplasm (Figure 1D) and generated aberrant platelet-like particles expressing normal levels of CD41a but reduced levels of CD42b (Figure S1C). This is consistent with the requirement for the downregulation of c-MYC for maturation of MKs (Takayama et al., 2010).

### A Defined c-MYC Expression Level Is Important for MKCL Induction and Robust Expansion

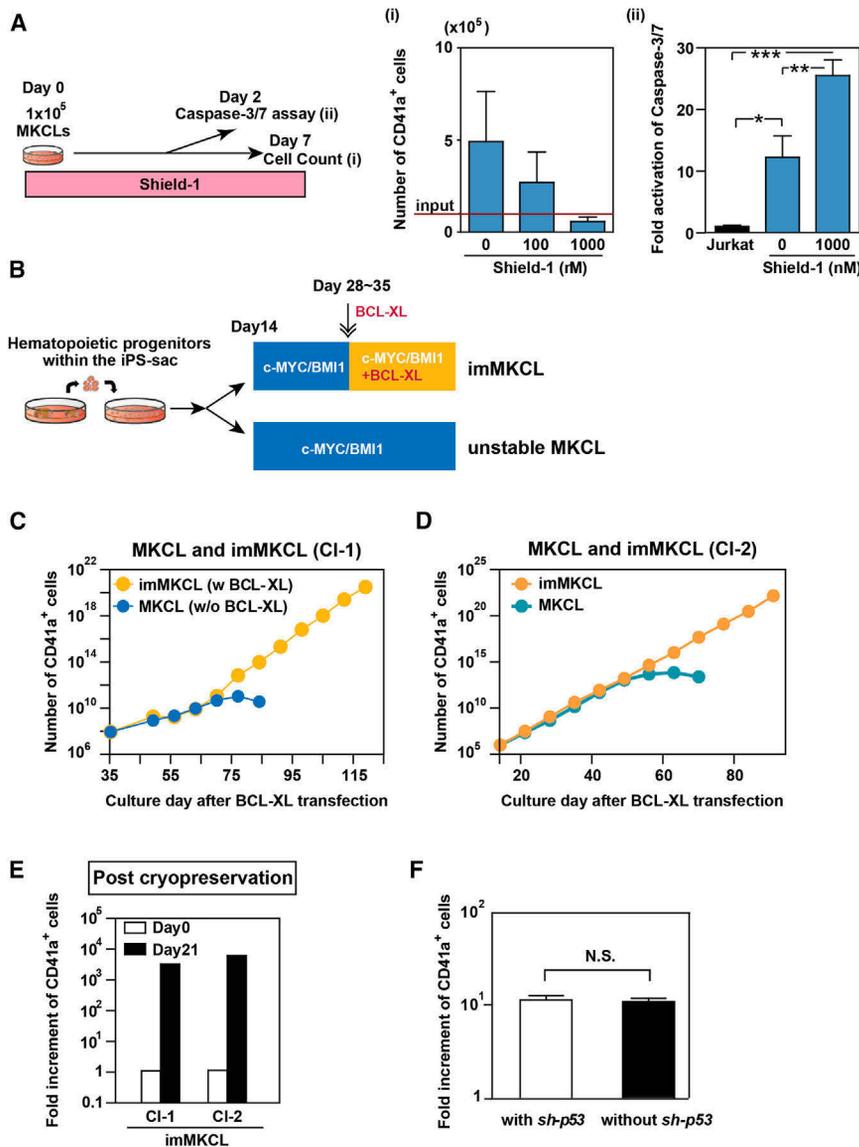
Our ability to induce MKCLs with c-MYC and BMI1 overexpression and then grow the cells for more than 2 weeks varied among individual PSC clones (data not shown). Therefore, we suspected that individual PSC clones require different levels of c-MYC. When we prepared an inducible all-in-one vector harboring c-MYC and BMI1 (Ohmine et al., 2001) (Figure S1D), we obtained clearer evidence that c-MYC levels are crucial for the sustained growth of MKCL, given that we saw different results with c-MYC-2A-BMI1 and BMI1-2A-c-MYC gene sequences in this vector (Figures S1E and S1F). Furthermore, to confirm the hypothesis that, in some individual PSC clones, the effective c-MYC expression level may be restricted to a specific range (Figure 1E), we used a vector tagged with a DD (Banaszynski et al., 2006) in order to reduce the level of c-MYC protein (Figure 1F). This system regulates protein stability, and thus the level of c-MYC expression, in a manner that depends on the Shield1 concentration (NIH 3T3 cells in Figure S1G). When we used this DD-tagged vector system to establish iPSC (692D2)-derived MKCLs, we found that 692D2 iPSCs showed no self-replication when transduced with c-MYC-2A-BMI1 overexpression without a DD tag, but transduction of the DD tag vector without the addition of Shield1 allowed clone 692D2 to grow for up to 50 days in culture (Figure 1G). This self-replication was inhibited by 100 or 1,000 nM Shield1 (Figure 2Ai), suggesting the total c-MYC level most likely blocked stable self-replication. This result was not due to nonspecific cell toxicity (Figure S2A) or activation of the *INK4A/ARF* gene locus by the high level of c-MYC, given that levels of *p14* and *p16* mRNA (derived *INK4A/ARF* locus gene) did not differ in the presence or absence of 1,000 nM Shield1 (Figure S2B).

Given that BMI1, which is also overexpressed in our system, represses the *INK4A/ARF* gene locus, the mechanism underlying the MYC-dependent failure of continuous self-replication was unclear. However, the well-known involvement of c-MYC in caspase-dependent apoptosis (Juin et al., 2002) prompted us to assess caspase activity in the MKCL clone. Caspase assays revealed that the activation of caspases 3 and 7 in DD

(E) Schematic diagram illustrating the hypothesis that there is an association between c-MYC levels and MK self-replication. Individual human PSC clones (hESCs or hiPSCs) define the c-MYC activation window (restricted range) needed to induce MK self-replication.

(F) Scheme for a c-MYC regulation system using a destabilization domain (DD). Proteins with a DD are rapidly destroyed via the ubiquitin-proteasome pathway. Administration of Shield1 putatively inhibits DD-mediated degradation in a concentration-dependent manner.

(G) Numbers of CD41a<sup>+</sup> MKs determined by flow cytometry. MKs were derived from iPSCs (692D2) transduced with inducible c-MYC-DD-2A-BMI1 (with [w] DD) or inducible c-MYC-2A-BMI1 retroviral vector (without [w/o] DD; see Figure S2A).



**Figure 2. Later Expression of BCL-XL Inhibited Caspase Activation Induced by Excessive c-MYC, Stabilizing Cell Growth and Contributing to the Establishment of imMKCLs**

(A) Relationship between the c-MYC expression level, cell viability, and caspase activity. (i) Numbers of live  $CD41a^+$  MKs derived from hiPSC (692D2)-based MKCLs generated with an inducible DD vector in the absence or presence of the indicated concentrations of Shield1. (ii) Caspase 3 and 7 activity in samples of Jurkat cells (control, fold = 1, black bar) or hiPSC (692D2)-derived MKCLs on day 2 of culture after transduction with a DD system in the absence or presence of 1,000 nM Shield1. \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001. Results are expressed as means  $\pm$  SE from five independent experiments.

(B) Scheme for generating unstable MKCLs with c-MYC and BMI1 (2F) or imMKCLs with c-MYC, BMI1, and BCL-XL (3F).

(C and D) Additional transduction of *BCL-XL* gene improved the growth curve for  $CD41a^+$  MKs derived from hiPSC (692D2)-derived MKCLs with a DD system (C; clone 1 [CI-1]) and from hESCs (KhES3)-derived MKCLs (D; CI-2). Yellow circles indicate the numbers of cells obtained with 3F, and blue circles indicate 2F. Cell numbers were calculated cumulative values.

(E) Increment in  $CD41a^+$  cells derived from CI-1 and CI-2 after cryopreservation. Results were an average of two independent experiments.

(F) The additional effect of *p53* knockdown on immortalization of MKCLs. Results are expressed as means  $\pm$  SE from three independent experiments.

self-replicable MKCLs derived from iPSC clone 692D2 was 2-fold higher in the presence of 1,000 nM Shield1 than in its absence. This increased caspase activation was also associated with reduced cell viability (Figures 2Ai and 2Aii), confirming that restricting caspase activation may be key to successfully establishing expandable MKCLs (Figure S2B). Notably, caspase activation in the absence of Shield1 was still 12.2 $\times$  higher than in Jurkat cells (Figure 2Aii). Some apoptotic events are transcriptionally determined (Chen et al., 2007; Kumar and Cakouros, 2004). Therefore, to further address the mechanism by which excessive c-MYC represses self-replication and induces apoptosis, we carried out a microarray analysis with iPSC-derived MKCLs treated with or without 1,000 nM Shield1. Gene ontology classification of genes differentially expressed in the presence and absence of Shield1 indicated a correlation between higher levels of c-MYC (1,000 nM Shield1) and the expression of the proapoptotic factors *BMF* and *BBC3* (*PUMA*), which can contribute to the release of cytochrome-c from mitochondria

via mitochondrial outer membrane permeabilization. High levels of c-MYC also influenced cell-cycle-related genes, increasing the expression of cyclin-dependent kinase inhibitors (*CDKN1B*, *p27*, and *Kip1*), which could arrest MKCL growth. These changes were also confirmed by qPCR analysis (Figures S2C and S2D). Thus, high c-MYC expression can lead to caspase-dependent MKCL apoptosis (Figure 2A), despite the suppression of the INK4A/ARF pathway by BMI1 (Figure S2B). These data again indicate that alternative apoptosis pathways, as well as the INK4A/ARF pathways, are independently induced by excessive c-MYC in individual PSC-derived MKCL clones.

### Suppression of Caspase Activation through BCL-XL Expression Promotes Immortalization

We noticed that MKCLs obtained from either ESC clone KhES3 or iPSC clone 692D2 with c-MYC plus BMI1 (Figure 2B, bottom line) exhibited limited growth potential and discontinuous cell growth that ceased at about 60 days, potentially reflecting an increase in caspase activation (692D2 cell growth in Figure 2C; KhES3 cell growth in Figure 2D; 0 nM Shield1 for 692D2 in Figure 2Ai and KhES-3 in Figure S2E). Thus, it appears that the expression of c-MYC and BMI1 alone are not suitable for

generation of an immortalized cell line. We therefore sought to examine the effect of BCL-XL on days 14–21 after transduction of c-MYC plus BMI1 (Figure 2B). We found that BCL-XL overexpression induced exponential growth of CD41a<sup>+</sup> cells derived from either 692D2 or KhES3 cells. This growth persisted for over 5 months, and we therefore deemed these cells to be self-renewing imMKCLs (Figures 2C and 2D for 692D2 [imMKCL clone 1, CI-1], and Figure 2D for KhES-3 ESCs [CI-2]; yellow circles). These two imMKCLs also showed comparable growth after cryopreservation (Figure 2E) as well as somewhat larger cell size and expression of CD41a, CD42a, CD42b, and CD9 (Figure S2F). Growth of these imMKCLs was sustained even with higher c-MYC expression in the presence of 100 or 1,000 nM Shield1 (Figures S2G–S2I), and the cells exhibited comparatively low levels of caspase 3 and 7 activation, similar to that of Jurkat cells (Figure S2I).

To further validate the function of BCL-XL in caspase regulation, we tested the effect of the caspase inhibitor Z-VAD FMK on proliferation. Caspase inhibition increased proliferation about 21-fold, whereas BCL-XL expression elicited a 64-fold increase. In contrast, the vehicle control (DMSO) enhanced apoptosis (Figures S3A–S3C). Altogether, these findings again confirm that BCL-XL inhibits apoptosis through caspase 3 and 7 inactivation and that c-MYC, BMI1, and BCL-XL are all required for the induction of imMKCLs from PSC clones. In addition, because it is well known that activation of p53 and p21 is also associated with c-MYC-dependent apoptosis (Hotti et al., 2000), we assessed the effect of inhibiting p53 on cell growth with imMKCL CI-1. Our results show no involvement of p53 in cells expressing c-MYC, BMI1, and BCL-XL (Figure 2F), and a similar result was obtained with KhES-3-imMKCL (CI-2; data not shown).

Next, we asked whether the simultaneous overexpression of c-MYC, BMI1, and BCL-XL was more suitable for establishing imMKCLs than overexpression of c-MYC plus BMI1 followed by later expression of BCL-XL. To address that question, we used four individual PSC clones to compare two protocols: simultaneous overexpression of all three genes or overexpression of c-MYC and BMI1 followed by BCL-XL 14–21 days later, counting from the HPC stage (days 28–35 from the hESC or iPSC stage; Figure 2B, top). Simultaneous addition of all three genes promoted maximal proliferation for only up to 40–50 days, whereas stepwise addition of c-MYC and BMI1 followed by BCL-XL consistently showed more sustained proliferation with all clones examined (Figure S3D–S3G).

One potential caveat to this approach is that long-term cultivation might lead to imMKCLs becoming oncogenic. Interestingly, after cultivation for 5 months, two of three imMKCLs, CI-1 and CI-2 but not CI-7, consistently showed a specific karyotypic abnormality: chromosomal translocation that included chromosome +1 or –5 (Figure S4A). When these two clones were separately infused into immunodeficient mice, one (n = 5) displayed leukemogenesis contributing to early death, but the other did not (Figure S4B). These results highlight the importance of transplantation studies with imMKCLs for clone selection. Interestingly, CI-7 exhibited no karyotypic abnormality (Figure S4A) and consistently showed no abnormalities in transplantation studies (up to 16 weeks; Figure S4B). Therefore, we suggest that combined analysis, including both karyotypic examination and transplantation of individual imMKCL clones,

would be useful for selecting imMKCLs as cell bank stock candidates.

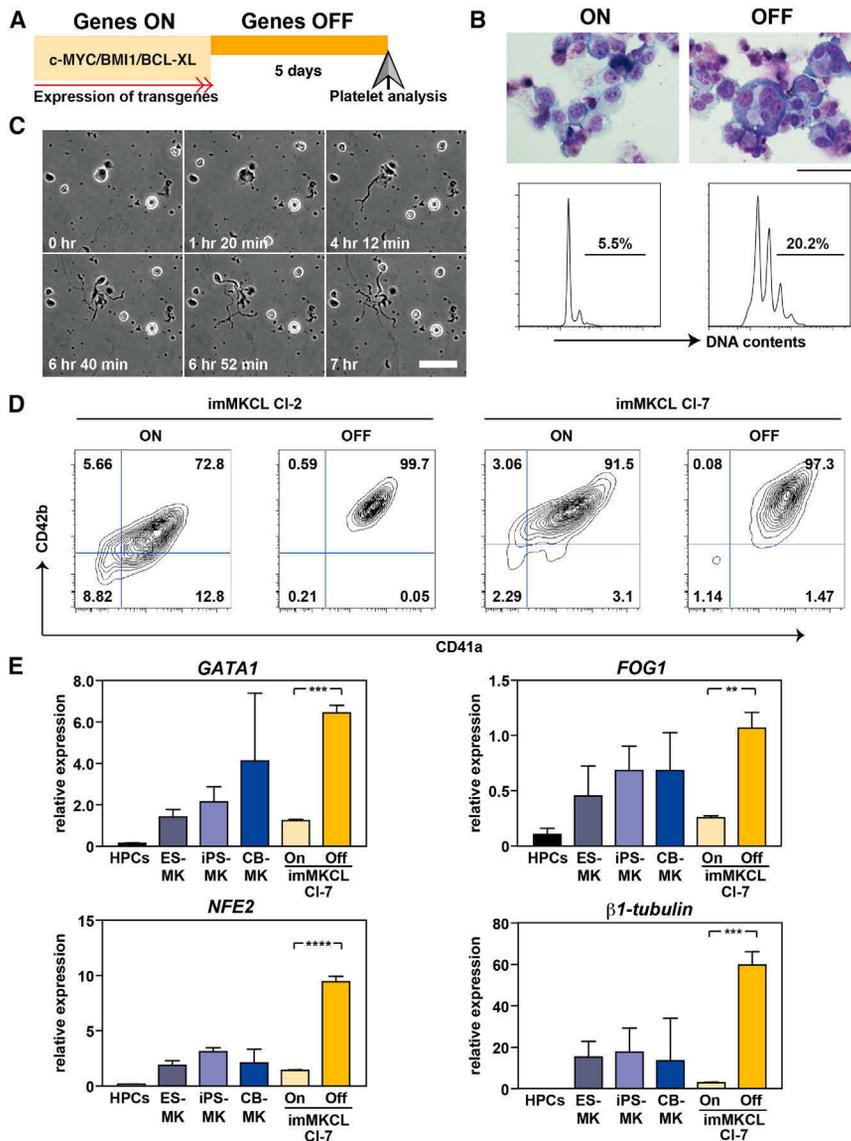
### Differentiation Phase of imMKCLs for Upregulation of CD42b during Maturation

The GPIb-V-IX complex, particularly CD42b (GPIb $\alpha$ ), on platelets is a key binding site for vWF and is required for the initial adhesion of platelets to an injured vessel wall (Ware et al., 2000) as well as normal circulation after transfusion (Leytin et al., 2004). Platelets lacking CD42b expression are quickly cleared from the circulation in vivo, leading to insufficient numbers of circulating platelets after transfusion (Nishikii et al., 2008). Assuming that the downregulation of c-MYC would be required for the generation of CD42b<sup>+</sup> platelets (Takayama et al., 2010), we turned off the expression of all three inducers (c-MYC, BMI1, and BCL-XL). Five days after these genes were turned off under serum-free conditions (Figure 3A), the iPSC-derived imMKCLs had changed to exhibit MK polyploidization (Figure 3B; on, 5.5% [left]; off, 20.2% [right]) as well as proplatelet formation (Figure 3C and Movie S1) with increased CD42b expression. This is exemplified by the two imMKCL clones in Figure 3D. Along with those changes, after turning off the expression of the exogenous inducing genes, both endogenous and exogenous c-MYC, BMI1, and BCL-XL expression declined (Figure S5A), and transcription factors associated with MK maturation, GATA1, FOG1, NFE2, and  $\beta$ 1-tubulin increased to levels comparable to or higher than those seen in cord blood (CB)-derived MKs (Figure 3E).

### Induction of Efficient Yield of CD41a<sup>+</sup>CD42b<sup>+</sup> Platelets

Turning c-MYC, BMI1, and BCL-XL off with a doxycycline-regulated system increased the CD42b<sup>+</sup> platelet yield from imMKCLs and upregulated CD42b expression in CD41a<sup>+</sup> platelets (Figures 4A and 4B), in comparison to maintaining the overexpression of the three factors or BCL-XL alone (Figure S5B). In addition, whereas imMKCLs generated significant numbers of CD41a<sup>+</sup> CD42b<sup>+</sup> particles that closely resembled endogenous platelets, the well-known MKCLs Meg01, CMK, and K562, produced mostly CD41a<sup>+</sup>, but CD42b<sup>–</sup>, platelet-like particles (Figure S5C). We estimated production to be three CD42b<sup>+</sup> platelets per CI-2 imMKCL-MK and 10 platelets per CI-7 imMKCL-MK after the induction of differentiation (5 days after exogenous expression was turned off; Figure S5D) under serum-free conditions, which is a suitable level for clinical application. In a 10-cm dish scale (10 ml),  $4 \times 10^6$  and  $2 \times 10^6$  platelets per ml were obtained from imMKCL CI-7 and CI-2, respectively (Figure 4C). Therefore, our proposed system could theoretically yield  $10^{11}$  platelets (equivalent to one transfusion) within 5 days with 25–50 l medium.

Characterization of platelet yields using flow cytometry revealed that the expression levels of platelet-functional molecules, including CD42b, CD61 ( $\beta$ 3 integrin), protease-activated receptor 1 (PAR1; thrombin receptor), CD49b ( $\alpha$ 2 integrin), and CD29 ( $\beta$ 1 integrin), were mostly comparable to those seen in fresh human peripheral blood (PB)-derived platelets and higher than in human endogenous pooled platelets, although the expression of GPVI (GP6) was a little weaker than it was in fresh PB (Figure 4D), possibly because of receptor shedding at 37°C (Gardiner et al., 2012). At the ultrastructural level, transmission



**Figure 3. Turning Off 3F Transgenes Promoted the Maturation of MKs from imMKCLs**

(A) MKs and platelets were analyzed 5 days after turning off 3F (cMYC, BMI1, and BCL-XL) expression.

(B) Giemsa staining (top pictures) and flow cytometric analysis of ploidy among imMKCLs with and without 3F expression (genes on or genes off). The scale bar represents 50  $\mu$ m.

(C) imMKCL CI-7 examined with time-lapse microscopy 4 days after genes were turned off. Sequential images showing proplatelet formation. The scale bar represents 50  $\mu$ m.

(D) Representative contour plots for imMKCL CI-2 and CI-7 (MK populations are shown in side and forward scatter contour plots in a flow cytometer) derived from ESCs and iPSCs (KhES3 and DN-SeV2, respectively).

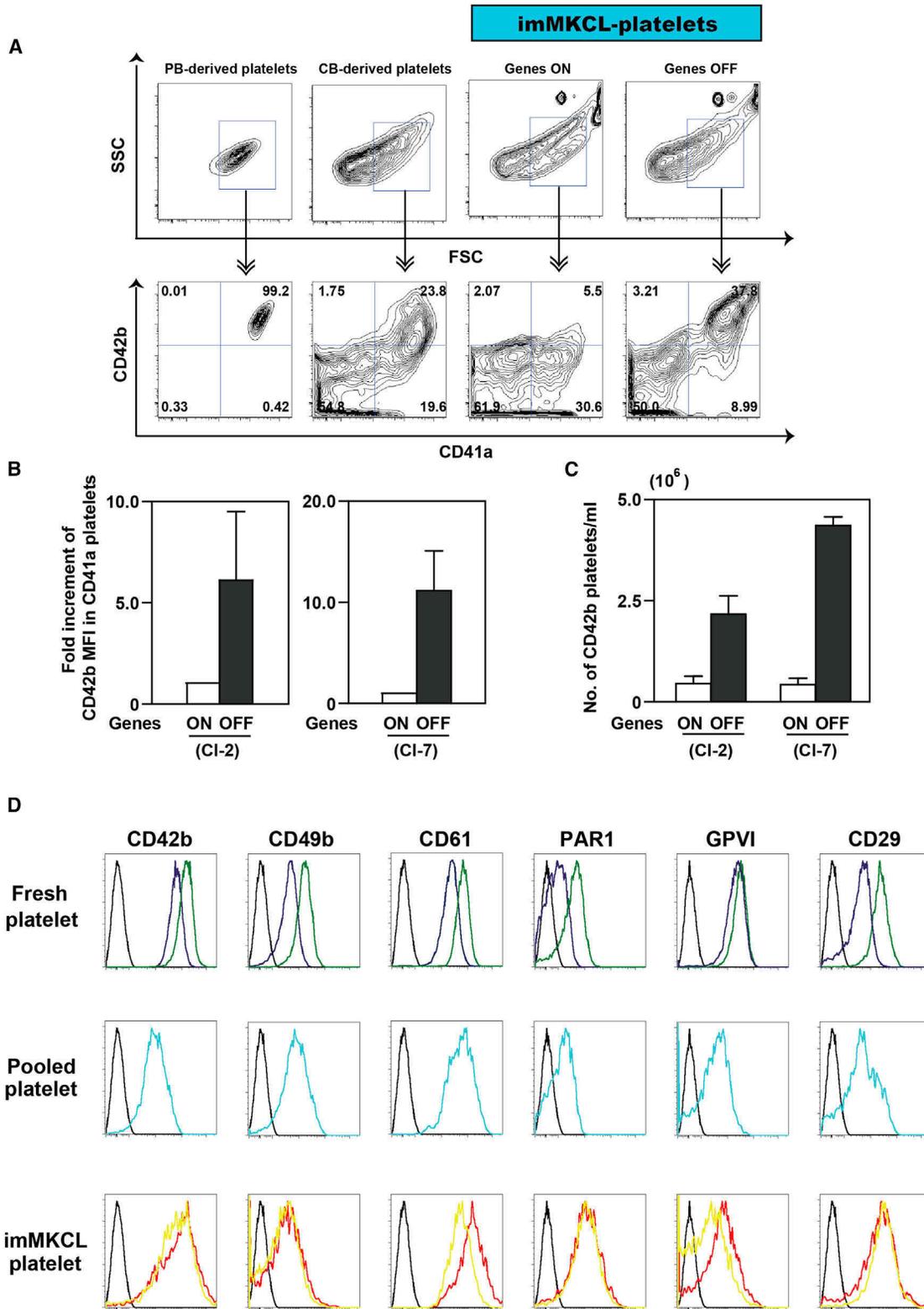
(E) qPCR analysis of *GATA1*, *p45 NF-E2*, *FOG1*, and  $\beta$ 1-tubulin gene expression. Samples were obtained from hiPSC-derived CD34<sup>+</sup>/CD43<sup>+</sup>/CD41a<sup>-</sup>/GPA<sup>-</sup> HPCs (Sac-HPCs), Sac-dependent MKs from ESCs and iPSCs, MKs derived from cord blood (CB)-CD34<sup>+</sup> cells, and imMKCL CI-7 (DN-SeV2 iPSC clone) in the presence (on) or absence (off) of 3F. Gene expression was normalized to GAPDH expression. 3F was transduced into Sac-HPCs as described in the [Experimental Procedures](#). Results are expressed as means  $\pm$  SE from three independent experiments.

electron microscopy revealed frequent generation of platelets through cytoplasmic fragmentation (Figure S6A) and proplatelets in the culture dish (Figure 3C and Movie S1). Individual imMKCL-derived platelets showed normal microtubules but fewer granules when compared to fresh human donor platelets (Figure 5A and S6A). To explore the functionality of imMKCL-derived platelets, we used flow cytometry to examine integrin  $\alpha$ IIb $\beta$ 3 activation (inside-out signaling) or platelet aggregation after stimulation with platelet agonists (De Cuyper et al., 2013). Agonist stimulation increased PAC-1 binding mean fluorescent intensity, given that this antibody binds to the activated form of  $\alpha$ IIb $\beta$ 3 integrin (Figures 5B and 5C) as well as platelet aggregation (Figure 5D), clot retraction (Figure 5E), actin cytoskeletal changes (Figure S6B), and vWF or ADP secretion (Figure S6C). Collectively, most in vitro functional parameters indicated that imMKCL platelets gave less robust responses than fresh human platelets, but, comparison to pooled human endogenous platelets (Figures 5C and 5D) or iPSC-derived platelets generated

reversed this adhesion (Figure 5F). With our current protocol, final platelet collection takes place during the final 5 days in the absence of serum at 37°C. Altogether, these two conditions may account for the relatively low granule content (Figure S6C) and diminished aggregation (Figure 5D).

### imMKCL-Derived Platelets Show Thrombogenic Activity in Mouse Models of Thrombocytopenia

Next, we evaluated the in vivo circulation of imMKCL platelets with previously optimized transfusion models (Takayama et al., 2010). Using NOD/SCID/IL-2Rg-null (NOG) mice with irradiation-induced thrombocytopenia, flow cytometric analyses were carried out 30 min, 2 hr, and 24 hr after transfusion (1 or 6  $\times$  10<sup>8</sup> platelets per mouse). The posttransfusion kinetics of imMKCL-derived platelets were nearly the same as those obtained with fresh human platelets (n = 4 individual groups in two independent experiments; Figures 6A and 6B). To further assess the functionality of imMKCL platelets in vivo, we used



**Figure 4. imMKCL-Derived Mature MKs Generated CD41a<sup>+</sup>CD42b<sup>+</sup> Platelets upon Turning Off 3F Transgenes**

(A) Representative contour plots for human peripheral blood (PB)-, CB-CD34<sup>+</sup>HS/PC-, and imMKCL (3F on and off)-derived platelets (platelet population are shown in side and forward scatter contour plots).

(B) Fold increment in CD42b<sup>+</sup> mean fluorescent intensity (MFI) among CD41a<sup>+</sup> imMKCL CI-2 (KhES3)- and CI-7 (DN-SeV2)-derived platelets. White bars, genes on; black bars, genes off. Results are expressed as means ± SE from three independent experiments. The mean value of the samples with genes on is assigned as 1.0.

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thrombocytopenic NOG mice and high-spatiotemporal-resolution confocal laser microscopy to visualize the initial adhesion of individual platelets to laser-exposed vessel walls and the subsequent thrombus formation under flow conditions without apparent endothelial disruption (Takizawa et al., 2010; Nishimura et al., 2012). Using fresh human platelets or imMKCL-derived platelets labeled with carboxyfluorescein diacetate and succinimidyl ester, along with injection of Texas Red dextran to visualize blood cell kinetics, we calculated the numbers of platelets adhering to the endothelium after laser injury in the NOG system (Movie S2). We confirmed that single imMKCL-derived platelets adhere to the vessel without forming aggregates with host platelets. AK4 antibody (antihuman P-selectin) partly reversed the platelet adhesion (i.e., >60% for Cl-2 platelets; Figure 6C), indicating that P-selectin contributed to the initial adhesion of the imMKCL platelets and that endothelial-derived vWF and P-selectin are relevant in our mouse models (Nishimura et al., 2012). We also confirmed the contribution of these platelets to thrombi in vivo. We found that platelets from four different imMKCL clones differentially contributed to the developing thrombi to a degree that was at a minimum better than human endogenous pooled platelets ( $n = 40$  vessels from three to five animals individually,  $p < 0.0001$ ; Figures 6D and 6E and Movie S3), suggesting that transfer to in vivo conditions may further improve the functionality of imMKCL platelets, perhaps through rejuvenation via endocytosis of granules. Accordingly, our results suggest that, although imMKCL platelets display a smaller capacity for adhesion and aggregation than fresh donor platelets in vitro and in vivo, the functional capacity observed is at a useful level and could potentially be improved by further optimization of the culture conditions and/or the collection method.

## DISCUSSION

For successful clinical application of hiPSC technology to platelet transfusion, it will be necessary to produce very large quantities of platelets. To achieve this goal, we will need to boost production efficiency at two stages: the transition from HSCs or myeloid lineage HPCs to MKs and the transition from MKs to platelets (Fuentes et al., 2010; Lu et al., 2011; Takayama et al., 2010; Lambert et al., 2013; Yamamoto et al., 2013). In the present report, we focused on a strategy of generating self-renewing immortalized MKs (Yamamoto et al., 2013), and we have succeeded in establishing imMKCLs with in vitro long-term expansion capacity from human ESCs or iPSCs with three defined factors (c-MYC, BMI1, and BCL-XL) with a temporally hierarchical overexpression protocol (Figures 2 and S3D–S3G). Sustained expansion of these imMKCLs relied on carefully regulated expression of the inducing genes to balance proliferative and apoptotic signals and optimize consistent proliferation. These self-replicating MKs may be applicable in the clinic as a

source for a continuous and virtually inexhaustible supply of platelets.

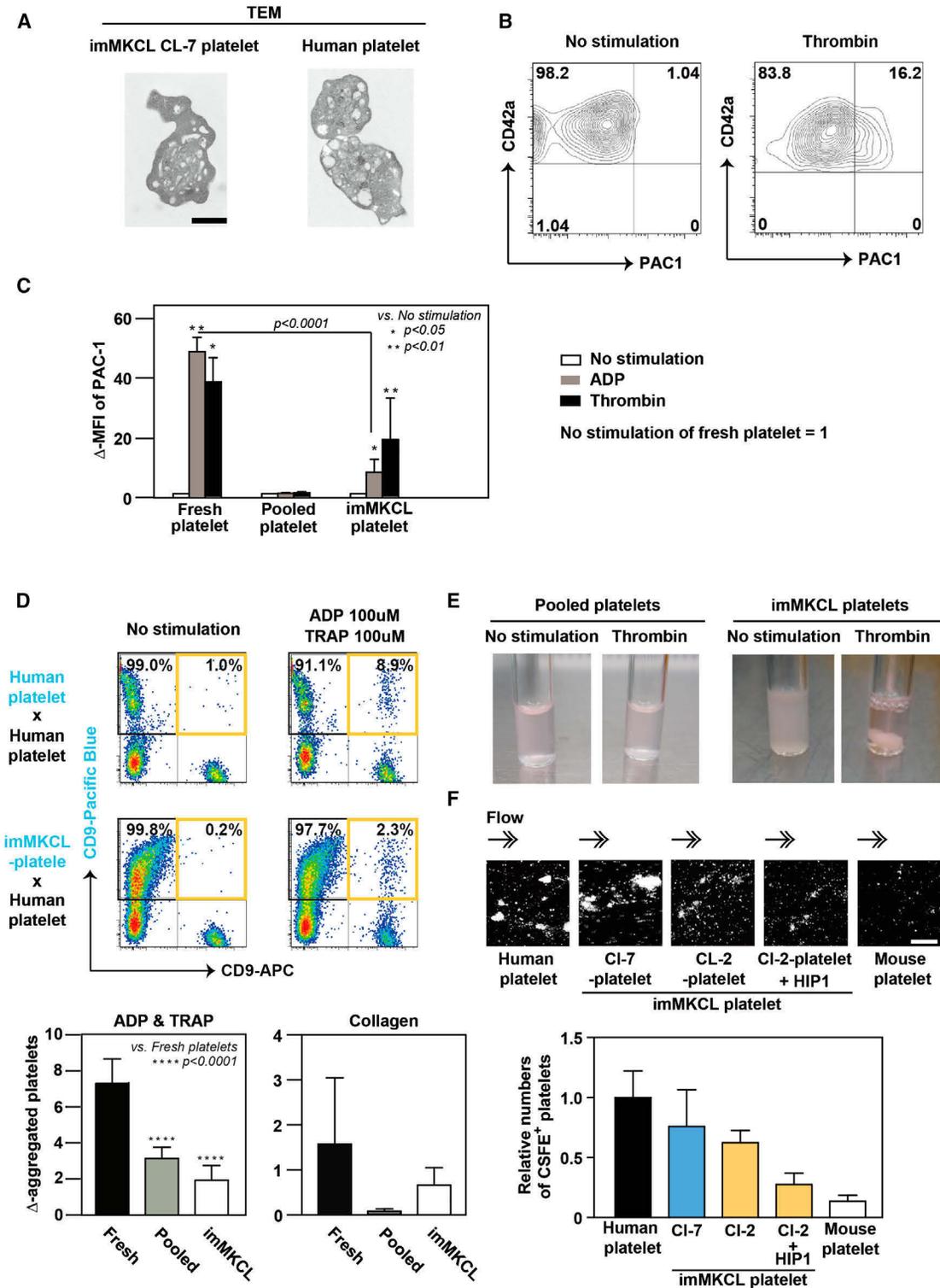
We also showed that the INK4A/ARF and caspase 3 and 7 pathways are activated at the MK progenitor stage when c-MYC levels are too high. The DD derived from mutant human FKBP12 contributes to instability of the tagged protein, but the effect is attenuated by addition of Shield1 (Banaszynski et al., 2006). By using a DD tag vector system as a tool for investigating the impact of restricted c-MYC levels (Figures 1E–1G and S1G), we confirmed the importance of reducing caspase activity during immortalized self-replication of the MK lineage in the presence of TPO and SCF, which suggests that caspase activity plays a role in the previously observed unstable proliferation of MKs in vitro (Figures 7A–7C). This “constrained protein expression” system could also be useful for studying genes that must be strictly regulated. These findings also suggest the existence of a regulatory pathway distinct from the INK4A/ARF and p53 pathways that exerts a protective effect against oncogenic stress in the MK lineage (Figure 2F).

Exogenous overexpression of BCL-XL contributed to long-term self-replication by attenuating caspase 3 and 7 activation, even in the presence of higher c-MYC levels (Figure 7C), and endogenous BCL-XL may be required for the survival of imMKCLs yielding platelets (Figures S5A and S5B). Consistent with that idea, results obtained with BCL-XL-deficient mice indicate that this protein is required for MK survival and platelet release (Josefsson et al., 2011). By contrast, sustained BCL-XL overexpression reportedly has a negative effect on the development of demarcation membranes in MKs and on platelet production (Kaluzhny et al., 2002). However, c-MYC-dependent MK proliferation was also TPO-dependent and required BMI1 to be present prior to BCL-XL (Figures 1C, 2C, 2D, and S3D–S3G). Interestingly, we recently showed that the combination of c-MYC and BCL-XL overexpression in CD34<sup>+</sup>CD43<sup>+</sup>-containing HPCs leads to stable erythroblast self-replication induced by erythropoietin (Hirose et al., 2013) but not MK lineage growth (Figure 1C). Thus, distinct combinations of c-MYC and BCL-XL and c-MYC and BMI1 appear to provide context-dependent expansion capacity for erythroblast or MK lineages, respectively (Hirata et al., 2013; Yamamoto et al., 2013). It has also been reported that BMI1 directly binds to RUNX1 and core binding transcriptional factor  $\beta$  and acts as a regulator during megakaryopoiesis (Yu et al., 2012), suggesting that BMI1 may have an alternative function in imMKCL development. The MK proliferation program may be disrupted when the effects of BCL-XL dominate before the MK proliferation program is fully established.

For clinical application, the quality of expandable imMKCLs must be strictly validated, and they must be cryopreserved as master cell banks (MCBs) matched to the required HLA and HPA type. After thawing, MCB-derived working cells would be expected to grow within an appropriate and sufficient term and

(C) Numbers of CD41a<sup>+</sup>CD42b<sup>+</sup> platelets generated from imMKCL Cl-2 (KhES3) and Cl-7 (DN-SeV2) in a 1 ml culture volume. White bars, genes on; black bars, genes off. Results are expressed as means  $\pm$  SE from three independent experiments.

(D) Histograms show CD42b, CD49b, CD61, PAR1, GPVI, and CD29 expression on platelets (fresh and pooled) derived from normal donors (two different donors) and imMKCL Cl-2 and Cl-7. Donor-derived pooled platelets were pooled at 37°C for 5 days. Fresh human platelets were from donors 1 (purple) or 2 (green). Pooled platelets were from donor 1. imMKCL platelets were from Cl-2 (yellow) and Cl-7 (red). Black lines in all panels indicate IgG control. x axes, MFI (log scale); y axes, counts.



**Figure 5. Characterization of imMKCL-Derived Platelets In Vitro**

(A) Transmission electron micrographs of an imMKCL CL-7-derived platelet 5 days after genes off (left) and two fresh donor platelets (right). The imMKCL platelet shows fewer a-granules and dense granules than fresh platelets. The scale bar represents 1  $\mu$ m.

(B) Representative contour plots for imMKCL platelets showing CD42a (GPIX) and PAC1 bound in the absence or presence of thrombin (1 u/ml).

(C) PAC1 binding to human fresh platelets, human pooled platelets, or imMKCL platelets was quantified by flow cytometry. Data depict means ( $\pm$  SEM) from three independent experiments. y axis indicates  $\Delta$ MFI calculated as agonist (+) minus no agonist (-). The MFI of agonist (-) was 1.0 in individual samples. ADP (200  $\mu$ M) or thrombin (1 u/ml) was the agonist.

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generate large numbers of platelets. In this context, continuous culture after cryopreservation (Figure 2E) may enable a continuous supply of platelets through gene manipulation (i.e., a drug-induced genes-off condition). Furthermore, after we had established stably expandable imMKCLs in the presence of serum, we identified two clones that were capable of growing well in liquid culture after adaptation to serum- and feeder-free conditions (Figure S6D). This development suggests that it may well be feasible to use this type of system to generate platelets on the type of industrial scale that can be achieved with tanks.

We compared our current imMKCL/MCB system with our earlier strategy (Takayama et al., 2010). Figure S7 depicts the advantages of the current strategy and its feasibility in terms of three parameters: manipulation, duration, and culture scale. For each parameter in the figure, the top panels show our current protocol, and the bottom panels show the earlier protocol (Takayama et al., 2010). It is important to consider the need to prepare and provide more than  $10^{11}$  platelets, given that  $3 \times 10^{11}$  platelets are required for 1 u of platelet concentrate for transfusion in the United States. We suggest using  $10^8$  imMKCL cells per vial as a MCB-derived working cell stock, which will produce  $2.5 \times 10^{10}$  MKs in around 14 days (Figures 2C, 2D, and S3D–S3G). In addition, much less medium is required in the current system (Figure S7), and there might be no requirement for mouse feeder cells or serum (Figure S6D). However, when looking at the similarity between human endogenous platelets and imMKCL platelets, there were several discrepancies between the functional parameters in vitro and in vivo (Figures 6C and 6D). In general, platelet concentrate is supplied in highly concentrated serum for transfusion. This suggests that supplementing with serum or a serum replacement could help to increase the functional capacity of imMKCL-derived platelets. Our data already suggest that this approach could be helpful; for example, serum supplementation improved clot retraction with imMKCL-derived platelets (Figure 5E). This is in contrast to endogenous pooled platelets, which did not form clots at all, even with serum supplementation. Therefore, although further optimization of this final step is needed (e.g., through the induction of differentiation at room temperature (20°C –24°C) before imMKCL platelets are clinically useful, we conclude that an imMKCL system could potentially provide useful platelets in large quantities.

As mentioned, another area for further optimization is the platelet yield per MK, the improvement of which would contribute to further reducing the volume. Several earlier reports

on in vitro platelet production systems documented similarly limited efficiency of platelet production from single MKs (Ono et al., 2012; Takayama et al., 2008, 2010). In contrast, it is thought that each MK generates several thousand platelets in vivo (Patel et al., 2005). One approach could be to use a bioreactor that mimics the conditions within BM (Patel et al., 2005), where shear stress from blood flow might accelerate platelet biogenesis from MKs (Junt et al., 2007). On the basis of that idea, we recently demonstrated the feasibility of an artificially produced bioreactor with 2D microfluid circulation and biodegradable scaffolds, which increased platelet yield from hESC- and hiPSC-derived MKs (Nakagawa et al., 2013).

In conclusion, the technology outlined in this study sheds light on an exciting avenue for potential iPSC-based platelet supply. This protocol is also being developed for an Investigational New Drug Application to the United States Food and Drug Administration. To achieve that, further optimization of MK maturation and the efficiency of platelet release in a liquid culture system will help to improve the technology as it develops toward clinical application. Ultimately, we believe that, through the integration of a broad range of technologies, it will be feasible through to achieve clinically effective platelet transfusion without a requirement for donor blood.

## EXPERIMENTAL PROCEDURES

### Ethical Review

KhES-3 hESC clone (Institute for Frontier Medical Science, Kyoto University) was used with approval from the Ministry of Education, Culture, Sports, Science and Technology of Japan. Collection of PB from healthy volunteers was approved by the ethics committee of the Institute of Medical Science at the University of Tokyo and the Kyoto University Committee for Human Sample-Based Experiments. All studies involving the use of human samples were conducted in accordance with the Declaration of Helsinki.

### Cells, Reagents, and Mice

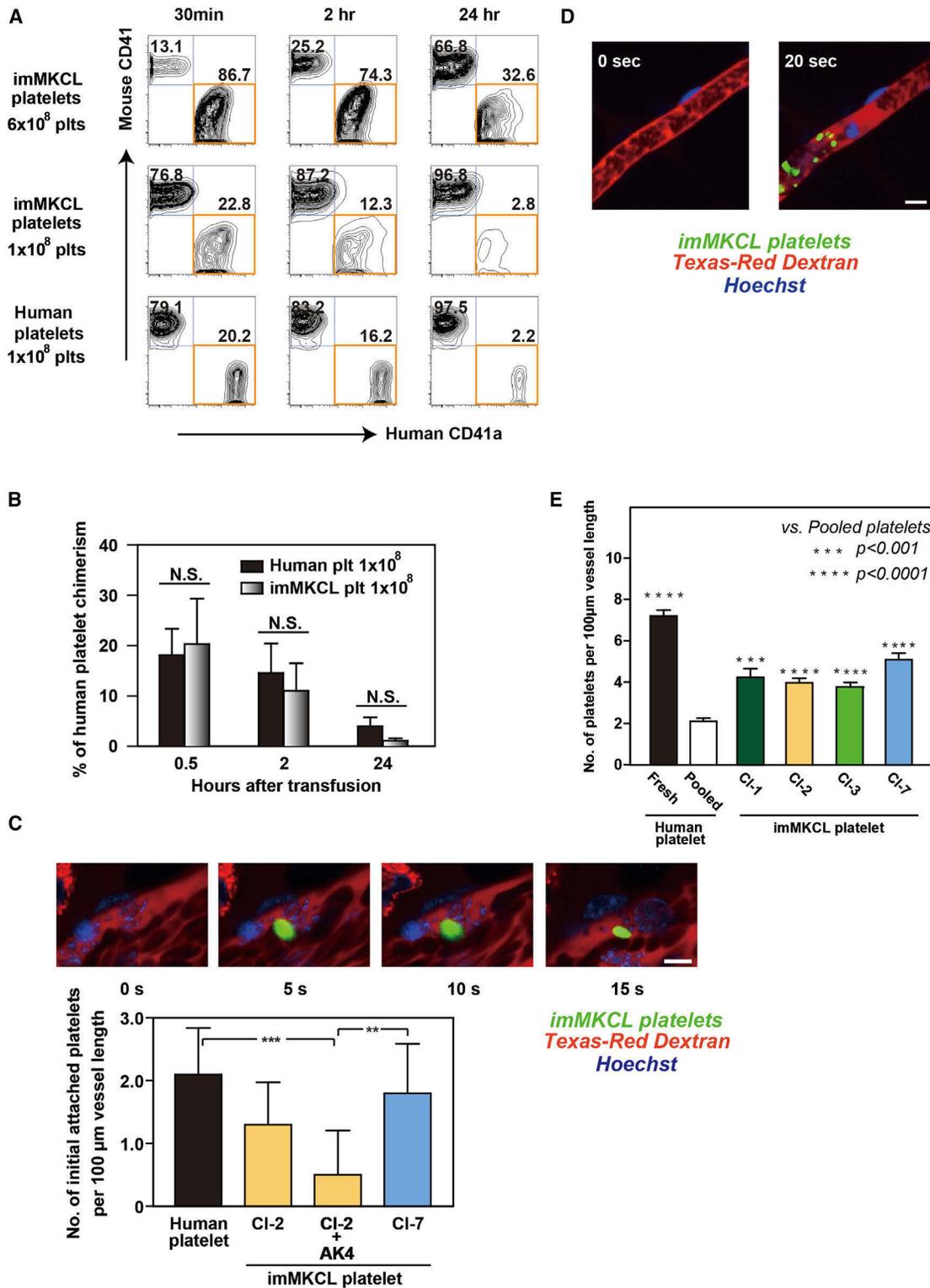
KhES-3 hESCs from H. Suemori (Suemori et al., 2006) and human iPSC clones (585A1, 585B1, 606A1, 648B1, 692D2, and DNSeV-2) were used (Okita et al., 2013). Six-week-old NOG mice were purchased from the Central Institute for Experimental Animals. The mice were irradiated at 2.4 Gy in order to induce thrombocytopenia 9 days before transfusion. Then, selected mice (platelets =  $5\text{--}20 \times 10^7/\mu\text{l}$ ) were used for studies of posttransfusion platelet kinetics and in vivo imaging of thrombogenicity.

The following vectors were used: pMXs retroviral vector, pGCDNsam retroviral vector, modified pMXs Tet off-inducible retroviral vector (Ohmine et al., 2001), and CSLI-based all-in-one inducible lentiviral vector (Ai-LV) (Takayama et al., 2010). Retrovirus production with a 293 Gag, Pol, VSV-G (vesicular stomatitis virus G) system and lentiviral production were as described previously

(D) Flow cytometric detection of aggregated platelets as a double-positive population among human fresh donor platelets stained with CD9-APC or among human fresh donor platelets (top panel in the dot plot) or with imMKCLs platelets (bottom panel in the dot plot) stained with CD9 Pacific Blue after agonist stimulation. In the absence of agonist stimulation, the double-positive population was small. The lower graph contains summarized results showing the percent double-positive platelets (human fresh platelets, pooled platelets, or imMKCL platelets). y axis indicates D-aggregated percentage calculated as agonist (+) minus agonist (–). Results are expressed as means  $\pm$  SE from seven independent experiments for ADP&TRAP (left) or four independent experiments for collagen (right).

(E) Pictures of clot retraction. Human endogenous pooled or imMKCL (CI-7) platelets were suspended in 20% platelet-depleted human plasma containing Iscove's modified Dulbecco's medium ( $1.6 \times 10^8$  platelets/ml). Even when supplemented with human plasma, pooled platelets stored without serum showed no clot retraction, whereas imMKCL platelets did.

(F) Ex vivo flow chamber system within which human vWF (10  $\mu\text{g}/\text{ml}$ ) was immobilized, and the shear rate was  $1,600 \text{ s}^{-1}$ . Human and mouse PB platelets and imMKCL CI-2 and CI-7 platelets were stained with carboxyfluorescein diacetate, succinimidyl ester (CFSE) and analyzed. Top: representative immunofluorescence micrograph of the chamber. Bottom: relative number of CSFE<sup>+</sup> platelets adhering to human vWF. HIP-1, human CD42b antibody. The scale bar represents 100  $\mu\text{m}$ . Results are expressed as means  $\pm$  SE of a total of 20 trials from three independent experiments. The mean value of human platelets is assigned as 1.0.

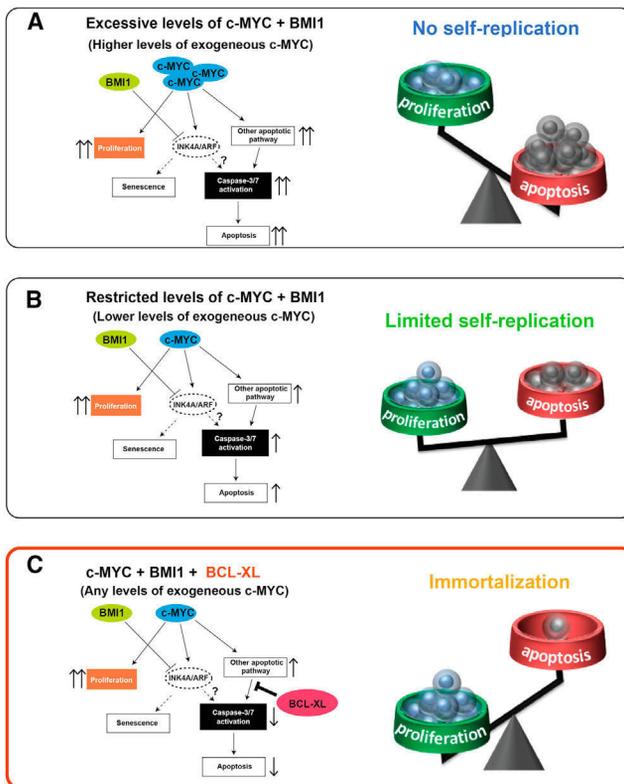


**Figure 6. imMKCL-Derived Platelets Show Circulation Potential and Intact In Vivo Functionality in a Thrombocytopenic Mouse Model**

(A) Platelet transfusion model in which NOG mice were irradiated (2.4 Gy) in order to induce thrombocytopenia. Nine days later, imMKCL-derived platelets ( $6 \times 10^8$  or  $1 \times 10^8$ ) and human PB-derived platelets ( $1 \times 10^8$ ) were injected via the tail vein. Shown are representative contour plots of samples from a transfused mouse. Detected are mouse CD41<sup>+</sup> and human CD41a<sup>+</sup> cells 30 min, 2 hr, and 24 hr after transfusion.

(B) Platelet chimerism was quantified by flow cytometry. Circulation of injected platelets was evaluated after 30 min, 2 hr, and 24 hr. N = 4 individual groups from two independent experiments.

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**Figure 7. Models of MKCL Self-Replication Systems**

(A) Excessive c-MYC induces strong activation of caspase 3 and 7 and apoptotic pathways, despite the suppression of INK4A/ARF by ectopic BMI1, leading to no self-replication.

(B) Appropriate activation of c-MYC induces a relatively low level of caspase 3 and 7 activation, leading to limited self-replication.

(C) Added BCL-XL acts in concert with BMI1 to suppress c-MYC-related apoptotic pathways, leading to MKCL immortalization.

(Eto et al., 2007; Takayama et al., 2010). We amplified the DD-encoding sequence from pPTunerC vector and Shield1 (Clontech and Takara Biotechnology) to generate the cMYC-DD construct. Shield1 was used to block DD-domain-mediated protein degradation. The use of viral vectors was approved by committees at the University of Tokyo or Kyoto University.

#### Cell Culture

On day 14 of culture, during hematopoietic differentiation from hESCs and hiPSCs (Takayama et al., 2008), HPCs were collected and transferred onto irradiated C3H10T1/2 cells in the presence of 50 ng/ml human SCF (R&D Systems) and 50 ng/ml human TPO (R&D) (Takayama et al., 2010). Gene expression was controlled by the presence or absence of 2  $\mu$ M

$\beta$ -estradiol or 1  $\mu$ g/ml doxycycline (Clontech) for modified pMXs inducible vector or the presence of 1  $\mu$ g/ml doxycycline for Ai-LV, respectively. MK differentiation from CB CD34<sup>+</sup> cell was accomplished as described previously (Proulx et al., 2004).

#### In Vitro Analysis of imMKCLs, imMKCL Platelets, and Human Platelets

qRT-PCR, cell-surface markers of imMKCLs and platelets, electron microscopy, and PAC-1 binding were examined as described previously (Takayama et al., 2010). Flow-cytometry-based platelet aggregation assays were performed as described previously (De Cuyper et al., 2013). Clot retraction assays were carried out as described previously (Takizawa et al., 2010). An ex vivo flow chamber precoated with human vWF (10  $\mu$ g/ml, provided by K. Soejima, Kaketsuken) was utilized to observe shear (1,600 s<sup>-1</sup>)-dependent thrombus formation under an inverted microscope equipped with fluorescence (Nikon A1R) and microfluid systems (Ibidi Products). Platelets were stained with CFSE dye (5  $\mu$ M, Invitrogen) and used in the presence of CD42b (HIP1; Abcam; 10  $\mu$ g/ml) or isotype-matched antibodies (BioLegend). Human pooled platelets were prepared by 5-day culture at 37°C under serum-free conditions.

#### In Vivo Analysis in Mice

Nine days later, irradiation in NOG mice, fresh or pooled human platelets from a donor, or imMKCL-derived platelets were intravenously administered (100  $\mu$ l). Then, blood samples (50–100  $\mu$ l) were collected from the retro-orbital plexus in the mice 30 min, 2 hr, and 24 hr after transfusion. The samples were labeled with human CD41a-APC and mouse CD41-PE antibodies (EMFRET Analytics), after which chimerism was analyzed. Similar NOG mice with thrombocytopenia were also used for in vivo imaging studies. To visually analyze thrombus formation in the microcirculation of the mesentery in living animals, we used in vivo laser- and reactive-oxygen-species-induced injury with a visualization technique developed through modification of conventional methods (Nishimura et al., 2012; Takizawa et al., 2010). Some experiments were performed with AK4 (human P-selectin antibody; Abcam; 20  $\mu$ g/ml). Additional details of the methods and other information for imaging are presented in the Supplemental Information.

#### Statistical Analysis

All data are presented as means  $\pm$  SEM. The statistical significance of the observed differences was determined with one-way ANOVA followed by Tukey's multiple comparison test and two-tailed Student's t tests for pairwise comparisons. Values of  $p < 0.05$  were considered significant.

#### ACCESSION NUMBERS

Raw and normalized microarray data have been deposited in the NCBI Gene Expression Omnibus database under accession number GSE54168.

#### SUPPLEMENTAL INFORMATION

Supplemental Information contains Supplemental Experimental Procedures, seven figures, and three movies and can be found with this article online at <http://dx.doi.org/10.1016/j.stem.2014.01.011>.

(C) Time-lapse confocal microscopy showing potential in vivo platelet function. Mice were transfused with  $1 \times 10^8$  platelets derived from different groups. Top: representative sequential images showing initial adhesion by imMKCL-derived platelets onto injured vessel walls. CFSE-labeled imMKCL platelets (Cl-2 and Cl-7; green) along with dextran (Texas Red) are shown. Bottom: the number of initially attached platelets per 100  $\mu$ m vessel length were counted. Actual results are shown in Movie S2. The results are averaged data from 40 vessels from three to five animals in each group. N.S., not significant; \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ . AK4; human P-selectin antibody. The scale bar represents 10  $\mu$ m.

(D) Representative sequential images showing thrombus formation by imMKCL-derived platelets within a small capillary and artery. CFSE-labeled imMKCL platelets (green) along with dextran (Texas Red) are shown. Hematoporphyrin was administered in order to induce thrombus formation prior to laser-induced injury. Platelets adhered to the site of laser injury, finally contributing to the complete occlusion of vessels. Original videos are available as Movie S3. The scale bars represent 10  $\mu$ m.

(E) Fresh donor platelets, pooled donor platelets, and imMKCL-derived platelets incorporated into thrombi were quantified. Pooled platelets were stored for 5 days at 37°C. Platelets from imMKCL Cl-1, Cl-2, Cl-3, and Cl-7 were used. The results summarize four independent experiments ( $n = 40$  vessels from three to five animals for each group). N.S., not significant; \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

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## REFERENCES

- Banaszynski, L.A., Chen, L.C., Maynard-Smith, L.A., Ooi, A.G., and Wandless, T.J. (2006). A rapid, reversible, and tunable method to regulate protein function in living cells using synthetic small molecules. *Cell* 126, 995–1004.
- Bergmeier, W., Burger, P.C., Piffath, C.L., Hoffmeister, K.M., Hartwig, J.H., Nieswandt, B., and Wagner, D.D. (2003). Metalloproteinase inhibitors improve the recovery and hemostatic function of in vitro-aged or -injured mouse platelets. *Blood* 102, 4229–4235.
- Chen, C., Fuhrken, P.G., Huang, L.T., Apostolidis, P., Wang, M., Paredes, C.J., Miller, W.M., and Papoutsakis, E.T. (2007). A systems-biology analysis of isogenic megakaryocytic and granulocytic cultures identifies new molecular components of megakaryocytic apoptosis. *BMC Genomics* 8, 384.
- De Cuyper, I.M., Meinders, M., van de Vijver, E., de Korte, D., Porcelijn, L., de Haas, M., Eble, J.A., Seeger, K., Rutella, S., Pagliara, D., et al. (2013). A novel flow cytometry-based platelet aggregation assay. *Blood* 121, e70–e80.
- Eto, K., Nishikii, H., Ogaeri, T., Suetsugu, S., Kamiya, A., Kobayashi, T., Yamazaki, D., Oda, A., Takenawa, T., and Nakauchi, H. (2007). The WAVE2/Abi1 complex differentially regulates megakaryocyte development and spreading: implications for platelet biogenesis and spreading machinery. *Blood* 110, 3637–3647.
- Fuentes, R., Wang, Y., Hirsch, J., Wang, C., Rauova, L., Worthen, G.S., Kowalska, M.A., and Poncz, M. (2010). Infusion of mature megakaryocytes into mice yields functional platelets. *J. Clin. Invest.* 120, 3917–3922.
- Gardiner, E.E., Al-Tamimi, M., Andrews, R.K., and Berndt, M.C. (2012). Platelet receptor shedding. *Methods Mol. Biol.* 788, 321–339.
- Hirata, S., Takayama, N., Jono-Ohnishi, R., Endo, H., Nakamura, S., Dohda, T., Nishi, M., Hamazaki, Y., Ishii, E.I., Kaneko, S., et al. (2013). Congenital amegakaryocytic thrombocytopenia iPS cells exhibit defective MPL-mediated signaling. *J. Clin. Invest.* 123, 3802–3814.
- Hirose, S., Takayama, N., Nakamura, S., Nagasawa, K., Ochi, K., Hirata, S., Yamazaki, S., Yamaguchi, T., Otsu, M., Sano, S., et al. (2013). Immortalization of erythroblasts by c-MYC and BCL-XL enables large-scale erythrocyte production from human pluripotent stem cells. *Stem Cell Reports* 1, 499–508.
- Hotti, A., Järvinen, K., Siivola, P., and Hölttä, E. (2000). Caspases and mitochondria in c-Myc-induced apoptosis: identification of ATM as a new target of caspases. *Oncogene* 19, 2354–2362.
- Isakari, Y., Sogo, S., Ishida, T., Kawakami, T., Ono, T., Taki, T., and Kiwada, H. (2009). Gene expression analysis during platelet-like particle production in phorbol myristate acetate-treated MEG-01 cells. *Biol. Pharm. Bull.* 32, 354–358.
- Josefsson, E.C., James, C., Henley, K.J., Debrincat, M.A., Rogers, K.L., Dowling, M.R., White, M.J., Kruse, E.A., Lane, R.M., Ellis, S., et al. (2011). Megakaryocytes possess a functional intrinsic apoptosis pathway that must be restrained to survive and produce platelets. *J. Exp. Med.* 208, 2017–2031.
- Juin, P., Hunt, A., Littlewood, T., Griffiths, B., Swigart, L.B., Korsmeyer, S., and Evan, G. (2002). c-Myc functionally cooperates with Bax to induce apoptosis. *Mol. Cell. Biol.* 22, 6158–6169.
- Junt, T., Schulze, H., Chen, Z., Massberg, S., Goerge, T., Krueger, A., Wagner, D.D., Graf, T., Italiano, J.E., Jr., Shivdasani, R.A., and von Andrian, U.H. (2007). Dynamic visualization of thrombopoiesis within bone marrow. *Science* 317, 1767–1770.
- Kaluzhny, Y., Yu, G., Sun, S., Toselli, P.A., Nieswandt, B., Jackson, C.W., and Ravid, K. (2002). BclxL overexpression in megakaryocytes leads to impaired platelet fragmentation. *Blood* 100, 1670–1678.
- Kumar, S., and Cakouros, D. (2004). Transcriptional control of the core cell-death machinery. *Trends Biochem. Sci.* 29, 193–199.
- Lambert, M.P., Sullivan, S.K., Fuentes, R., French, D.L., and Poncz, M. (2013). Challenges and promises for the development of donor-independent platelet transfusions. *Blood* 121, 3319–3324.
- Leytin, V., Allen, D.J., Gwozdz, A., Garvey, B., and Freedman, J. (2004). Role of platelet surface glycoprotein Ibalpha and P-selectin in the clearance of transfused platelet concentrates. *Transfusion* 44, 1487–1495.
- Lu, S.J., Li, F., Yin, H., Feng, Q., Kimbrel, E.A., Hahm, E., Thon, J.N., Wang, W., Italiano, J.E., Cho, J., and Lanza, R. (2011). Platelets generated from human embryonic stem cells are functional in vitro and in the microcirculation of living mice. *Cell Res.* 21, 530–545.
- Nakagawa, Y., Nakamura, S., Nakajima, M., Endo, H., Dohda, T., Takayama, N., Nakauchi, H., Arai, F., Fukuda, T., and Eto, K. (2013). Two differential flows in a bioreactor promoted platelet generation from human pluripotent stem cell-derived megakaryocytes. *Exp. Hematol.* 41, 742–748.
- Nishikii, H., Eto, K., Tamura, N., Hattori, K., Heissig, B., Kanaji, T., Sawaguchi, A., Goto, S., Ware, J., and Nakauchi, H. (2008). Metalloproteinase regulation improves in vitro generation of efficacious platelets from mouse embryonic stem cells. *J. Exp. Med.* 205, 1917–1927.
- Nishimura, S., Manabe, I., Nagasaki, M., Kakuta, S., Iwakura, Y., Takayama, N., Ooehara, J., Otsu, M., Kamiya, A., Petrich, B.G., et al. (2012). In vivo imaging visualizes discoid platelet aggregations without endothelium disruption and implicates contribution of inflammatory cytokine and integrin signaling. *Blood* 119, e45–e56.
- Oguro, H., Iwama, A., Morita, Y., Kamijo, T., van Lohuizen, M., and Nakauchi, H. (2006). Differential impact of Ink4a and Arf on hematopoietic stem cells and their bone marrow microenvironment in Bmi1-deficient mice. *J. Exp. Med.* 203, 2247–2253.
- Ohmine, K., Ota, J., Ueda, M., Ueno, S., Yoshida, K., Yamashita, Y., Kiritto, K., Imagawa, S., Nakamura, Y., Saito, K., et al. (2001). Characterization of stage progression in chronic myeloid leukemia by DNA microarray with purified hematopoietic stem cells. *Oncogene* 20, 8249–8257.
- Okita, K., Yamakawa, T., Matsumura, Y., Sato, Y., Amano, N., Watanabe, A., Goshima, N., and Yamanaka, S. (2013). An efficient nonviral method to generate integration-free human-induced pluripotent stem cells from cord blood and peripheral blood cells. *Stem Cells* 31, 458–466.
- Ono, Y., Wang, Y., Suzuki, H., Okamoto, S., Ikeda, Y., Murata, M., Poncz, M., and Matsubara, Y. (2012). Induction of functional platelets from mouse and human fibroblasts by p45NF-E2/Maf. *Blood* 120, 3812–3821.
- Patel, S.R., Hartwig, J.H., and Italiano, J.E., Jr. (2005). The biogenesis of platelets from megakaryocyte proplatelets. *J. Clin. Invest.* 115, 3348–3354.
- Proulx, C., Dupuis, N., St-Amour, I., Boyer, L., and Lemieux, R. (2004). Increased megakaryopoiesis in cultures of CD34-enriched cord blood cells maintained at 39 degrees C. *Biotechnol. Bioeng.* 88, 675–680.
- Sato, T., Fuse, A., Eguchi, M., Hayashi, Y., Ryo, R., Adachi, M., Kishimoto, Y., Teramura, M., Mizoguchi, H., Shima, Y., et al. (1989). Establishment of a human leukaemic cell line (CMK) with megakaryocytic characteristics from a Down's syndrome patient with acute megakaryoblastic leukaemia. *Br. J. Haematol.* 72, 184–190.

- Schiffer, C.A. (2001). Diagnosis and management of refractoriness to platelet transfusion. *Blood Rev.* 15, 175–180.
- Suemori, H., Yasuchika, K., Hasegawa, K., Fujioka, T., Tsuneyoshi, N., and Nakatsuji, N. (2006). Efficient establishment of human embryonic stem cell lines and long-term maintenance with stable karyotype by enzymatic bulk passage. *Biochem. Biophys. Res. Commun.* 345, 926–932.
- Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131, 861–872.
- Takayama, N., Nishikii, H., Usui, J., Tsukui, H., Sawaguchi, A., Hiroyama, T., Eto, K., and Nakauchi, H. (2008). Generation of functional platelets from human embryonic stem cells in vitro via ES-sacs, VEGF-promoted structures that concentrate hematopoietic progenitors. *Blood* 111, 5298–5306.
- Takayama, N., Nishimura, S., Nakamura, S., Shimizu, T., Ohnishi, R., Endo, H., Yamaguchi, T., Otsu, M., Nishimura, K., Nakanishi, M., et al. (2010). Transient activation of c-MYC expression is critical for efficient platelet generation from human induced pluripotent stem cells. *J. Exp. Med.* 207, 2817–2830.
- Takizawa, H., Nishimura, S., Takayama, N., Oda, A., Nishikii, H., Morita, Y., Kakinuma, S., Yamazaki, S., Okamura, S., Tamura, N., et al. (2010). Lnk regulates integrin  $\alpha$ IIb $\beta$ 3 outside-in signaling in mouse platelets, leading to stabilization of thrombus development in vivo. *J. Clin. Invest.* 120, 179–190.
- Terui, Y., Furukawa, Y., Kikuchi, J., Iwase, S., Hatake, K., and Miura, Y. (1998). Bcl-x is a regulatory factor of apoptosis and differentiation in megakaryocytic lineage cells. *Exp. Hematol.* 26, 236–244.
- Ware, J., Russell, S., and Ruggeri, Z.M. (2000). Generation and rescue of a murine model of platelet dysfunction: the Bernard-Soulier syndrome. *Proc. Natl. Acad. Sci. USA* 97, 2803–2808.
- Wong, C.H., Jenne, C.N., Petri, B., Chrobok, N.L., and Kubes, P. (2013). Nucleation of platelets with blood-borne pathogens on Kupffer cells precedes other innate immunity and contributes to bacterial clearance. *Nat. Immunol.* 14, 785–792.
- Yamamoto, R., Morita, Y., Ooehara, J., Hamanaka, S., Onodera, M., Rudolph, K.L., Ema, H., and Nakauchi, H. (2013). Clonal analysis unveils self-renewing lineage-restricted progenitors generated directly from hematopoietic stem cells. *Cell* 154, 1112–1126.
- Yu, M., Mazor, T., Huang, H., Huang, H.T., Kathrein, K.L., Woo, A.J., Chouinard, C.R., Labadorf, A., Akie, T.E., Moran, T.B., et al. (2012). Direct recruitment of polycomb repressive complex 1 to chromatin by core binding transcription factors. *Mol. Cell* 45, 330–343.