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Brief Report **Hematology** **Stem cells**

Transfusion of donor-derived platelets is commonly used for thrombocytopenia, which results from a variety of clinical conditions and relies on a constant donor supply due to the limited shelf life of these cells. Embryonic stem (ES) and induced pluripotent stem (iPS) cells represent a potential source of megakaryocytes and platelets for transfusion therapies; however, the majority of current ES/iPS cell differentiation protocols are limited by low yields of hematopoietic progeny. In both mice and humans, mutations in the gene-encoding transcription factor GATA1 cause an accumulation of proliferating, developmentally arrested megakaryocytes, suggesting that GATA1 suppression in ES and iPS cell–derived hematopoietic progenitors may enhance megakaryocyte production. Here, we engineered ES cells from WT mice to express a doxycycline-regulated (dox-regulated) shRNA that targets *Gata1* transcripts for degradation. Differentiation of these cells in the presence of dox and thrombopoietin (TPO) resulted in an exponential (at least 10^{13}-fold) expansion of immature hematopoietic progenitors. Dox withdrawal in combination with multilineage cytokines restored GATA1 expression, resulting in differentiation into erythroblasts and megakaryocytes. Following transfusion into recipient animals, these dox-deprived mature megakaryocytes generated functional platelets. Our findings provide a readily reproducible strategy to exponentially expand ES cell–derived megakaryocyte-erythroid progenitors that have the capacity to differentiate into functional platelet-producing megakaryocytes.

Inducible *Gata1* suppression expands megakaryocyte-erythroid progenitors from embryonic stem cells

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Transfusion of donor-derived platelets is commonly used for thrombocytopenia, which results from a variety of clinical conditions and relies on a constant donor supply due to the limited shelf life of these cells. Embryonic stem (ES) and induced pluripotent stem (iPS) cells represent a potential source of megakaryocytes and platelets for transfusion therapies; however, the majority of current ES/iPS cell differentiation protocols are limited by low yields of hematopoietic progeny. In both mice and humans, mutations in the gene-encoding transcription factor GATA1 cause an accumulation of proliferating, developmentally arrested megakaryocytes, suggesting that GATA1 suppression in ES and iPS cell–derived hematopoietic progenitors may enhance megakaryocyte production. Here, we engineered ES cells from WT mice to express a doxycycline-regulated (dox-regulated) shRNA that targets *Gata1* transcripts for degradation. Differentiation of these cells in the presence of dox and thrombopoietin (TPO) resulted in an exponential (at least 10^{13}-fold) expansion of immature hematopoietic progenitors. Dox withdrawal in combination with multilineage cytokines restored GATA1 expression, resulting in differentiation into erythroblasts and megakaryocytes. Following transfusion into recipient animals, these dox-deprived mature megakaryocytes generated functional platelets. Our findings provide a readily reproducible strategy to exponentially expand ES cell–derived megakaryocyte-erythroid progenitors that have the capacity to differentiate into functional platelet-producing megakaryocytes.

Introduction

Platelets and megakaryocytes generated in vitro from embryonic stem (ES) or induced pluripotent stem (iPS) cells are potentially useful for treating thrombocytopenia and for delivering pro- or antithrombotic proteins to sites of vascular injury (1–4). However, such therapies are impeded by relatively low yields of megakaryocytes from standard ES/iPS cell differentiation protocols. One potential solution is to overexpress transcription factors that expand hematopoietic progenitors, although these factors must be carefully titrated to optimize megakaryocyte yields (5, 6). Based on prior observations of *GATA1*-mutated patients, mice, and cell lines, we assessed whether reversible suppression of this essential hematopoietic transcription factor can enhance the production of functional megakaryocytes by ES cells. Mice and humans with *GATA1* mutations accumulate hematopoietic cells resembling immature megakaryocytes and/or megakaryocyte-erythroid progenitors (MEPs) (7–9). Previously, we demonstrated that in vitro hematopoietic differentiation of murine *Gata1*$^-$ ES cells generates self-renewing MEP-like cells, termed G1ME (for Gata1-deficient MEP) (10). Retroviral transduction of *Gata1* cDNA into G1ME cells triggered the formation of erythroblasts and megakaryocytes. However, these lineage-committed cells failed to mature fully in vitro, consistent with findings that their optimal development requires precise timing and levels of GATA1 that cannot be achieved by standard retroviral transfer (11–15). Thus, we generated ES cells harboring a doxycycline-inducible (dox-inducible) *Gata1* shRNA transgene. In vitro hematopoietic differentiation with dox resulted in greater than 10^{13}-fold expansion of MEP-like cells. Dox removal restored endogenous GATA1 expression, thereby triggering differentiation into erythroblasts and mature megakaryocytes capable of generating functional platelets in vivo. Our findings illustrate how clinical studies of rare genetic blood disorders inspired an approach to enhancing the production of megakaryocytes from pluripotent stem cells.

▶ Related Commentary: p. 2261

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Results and Discussion

We introduced 3 tandem *Gata1* shRNAs or a scrambled control shRNA downstream of a dox-regulated promoter embedded within the *Hprt* gene of murine ES cells (ref. 16 and Supplemental Figure 1, A and B; supplemental material available online with this

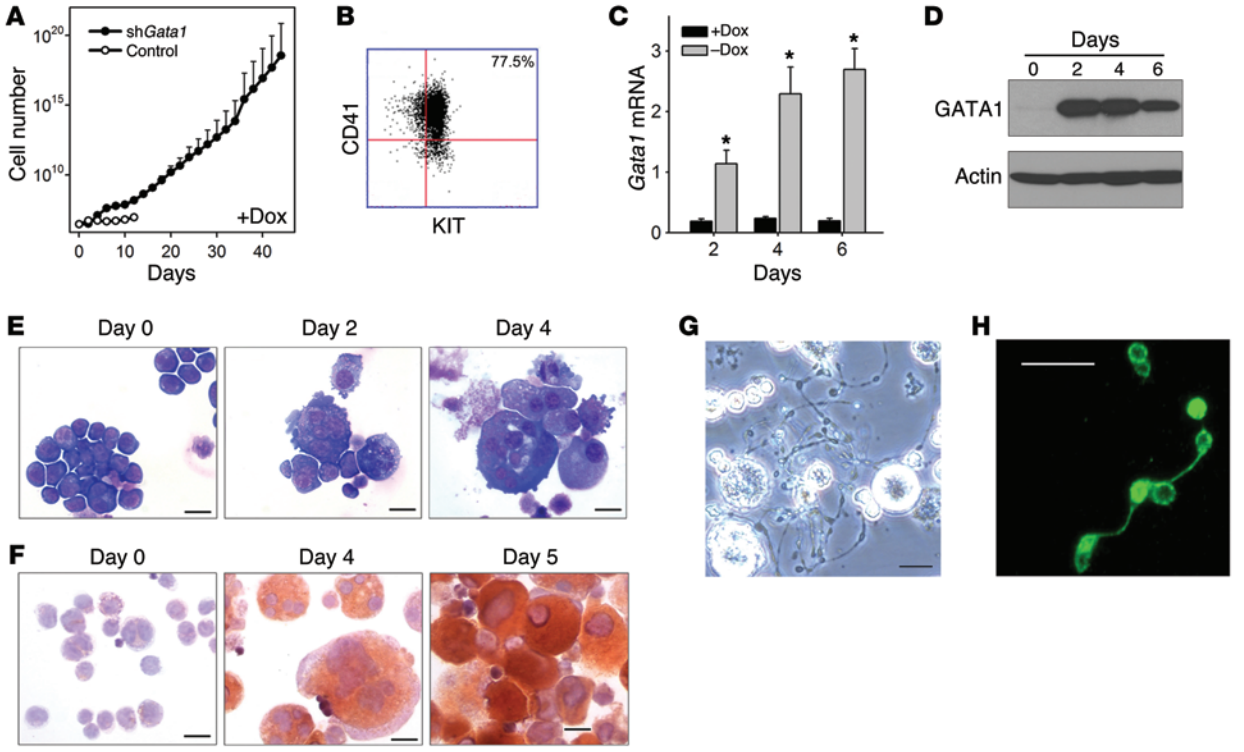

Figure 1. Dox-regulated suppression of *Gata1* generates self-renewing MEPs from ES cells. (**A**) Murine ES cells containing dox-inducible *Gata1* shRNA or control transgenes were differentiated into embryoid bodies for 6 days, disaggregated, and cultured with dox, TPO, and SCF. Cumulative numbers of nonadherent cells are plotted versus time (n = 4 experiments). (**B**) Flow cytometry showing expression of KIT and CD41 in G1ME2 cells. (**C**) Semiquantitative RT-PCR showing induction of *Gata1* mRNA after dox withdrawal (n = 3 experiments). *$P < 0.05$ versus +dox group by Student's t test. Mean ± SEM. (**D**) Western blotting for GATA1 protein after dox removal. (**E**) May-Grünwald Giemsa–stained G1ME2 cells after culture in TPO following dox removal. Scale bars: 20 μm. (**F**) Acetylcholinesterase expression. Scale bars: 20 μm. (**G**) Phase contrast microscopy showing proplatelet formation by G1ME2-derived megakaryocytes 6 days after dox removal. Original magnification, ×400. Scale bar: 20 μm. (**H**) Proplatelet formation by G1ME2-derived megakaryocytes illustrated by anti–β1-tubulin immunofluorescence microscopy. Original magnification, ×900. Scale bar: 10 μm. All representative data are shown from 3 to 4 experiments.

article; doi:10.1172/JCI77670DS1). Recombinant ES cells were differentiated into embryoid bodies, disaggregated with trypsin, and seeded into liquid culture with stem cell factor (SCF), thrombopoietin (TPO), and dox (Supplemental Figure 1C). Semiquantitative real-time PCR (RT-PCR) showed reduced *Gata1* mRNA in cells expressing the corresponding shRNAs (Supplemental Figure 1D). Hematopoiesis from control ES cells ceased after about 2 weeks, while *Gata1*-suppressed ES cells produced a homogeneous nonadherent cell population that expanded logarithmically for more than 40 days (Figure 1A). The expanded cell population that emerged from *Gata1*-deficient ES cells, termed G1ME2, exhibited immature morphology (Figure 1E) and expressed CD41 and KIT (Figure 1B), similarly to embryonic/fetal hematopoietic progenitors (17, 18). Dox withdrawal from G1ME2 cells cultured in erythropoietin (EPO) and TPO derepressed *Gata1* mRNA (Figure 1C) and protein (Figure 1D), with accompanying proliferation arrest (not shown) and erythro-megakaryocytic differentiation (Supplemental Figure 2, A–F). In methylcellulose with multilineage cytokines, individual G1ME2 cells gave rise to colonies containing erythroblasts and/or megakaryocytes, but not granulocytes or macrophages, indicating bilineage potential resembling MEPs (Supplemental Figure 2, D–F). After dox withdrawal with EPO or TPO, G1ME2 cells formed mainly erythroblasts or megakaryocytes, respectively (Supplemental Figure 2, A–C). Thus, shRNA suppression of *Gata1* enhances the production of MEP-like cells from ES cells. These findings are similar to what we observed for *Gata1*$^-$ ES cells (10) and, therefore, not likely caused by shRNA off-target effects.

This study focuses on the production of functional megakaryocytes and platelets from amplified G1ME2 cells. Approximately 5 to 6 days after dox withdrawal with TPO and SCF present, most G1ME2 cells differentiated into megakaryocytes, as evidenced by morphology (Figure 1E), acetylcholinesterase expression (ref. 19 and Figure 1F), and proplatelet formation (Figure 1, G and H). We compared megakaryocytes generated from murine fetal liver (FL) hematopoietic progenitors (20), G1ME2 cells, and G1ME cells (ref. 10 and Figure 2). The kinetics and levels of *Gata1* mRNA and protein expression differed greatly between G1ME cells after retroviral transfer of *Gata1* cDNA versus G1ME2 cells after dox withdrawal (Figure 2, A and B), with the latter approximating more closely FL-derived megakaryocytes. *Gata2* mRNA declined in all samples, consistent with prior studies on megakaryocytic maturation (21). This decline was more precipitous in G1ME cells, perhaps due to relative overexpression of viral vector–encoded GATA1, which represses *Gata2* transcription (22). The megakaryocytic genes *Vwf*, *Pf4*, *Gp1ba*, *Selp*, and *Ppbp* were expressed at similar levels in FL- and G1ME2-derived megakaryocytes, but to a lesser extent in megakaryocytes generated from G1ME cells (Figure 2, A and B). Compared with G1ME cells, G1ME2-derived megakaryocytes

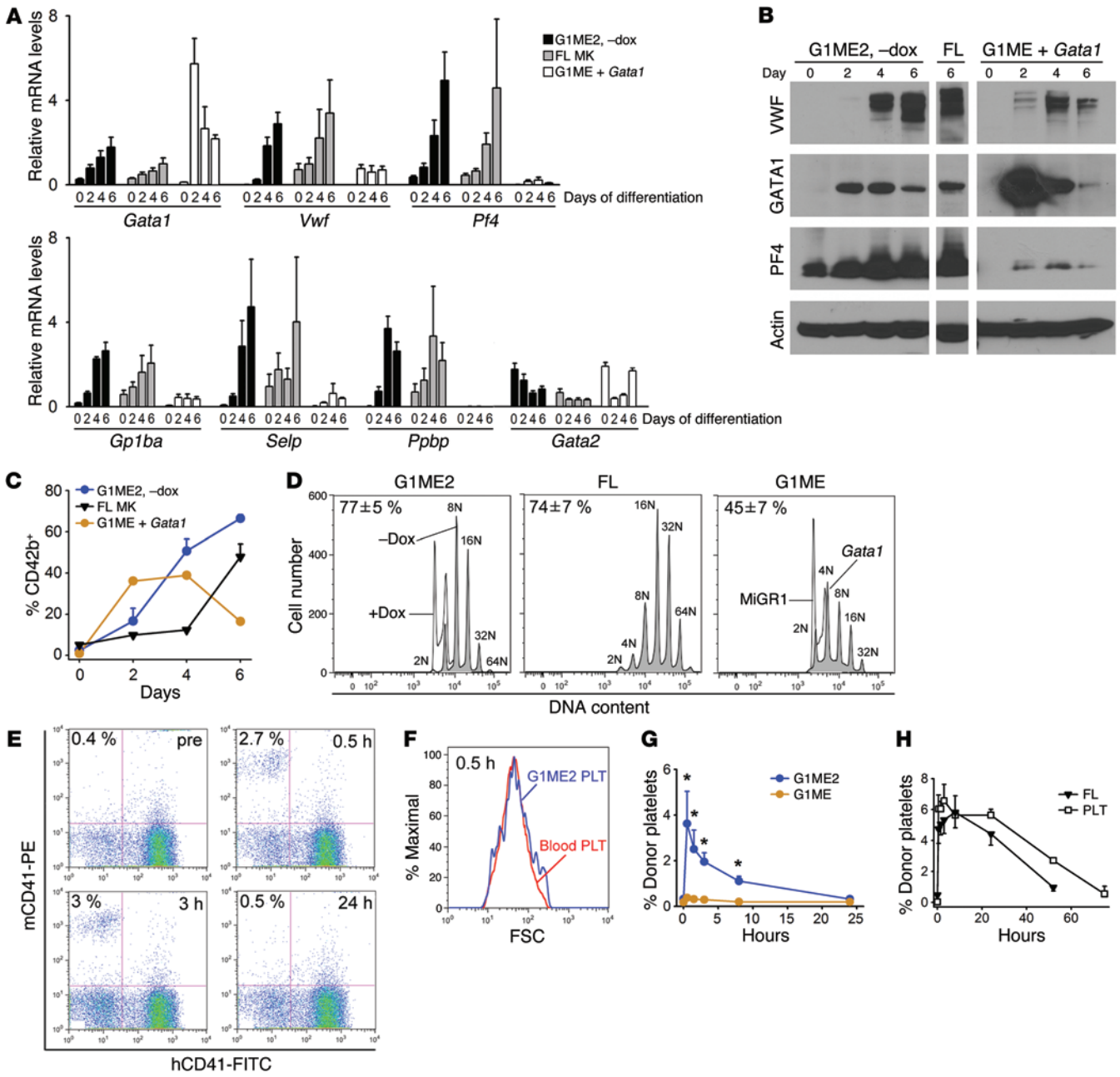

Figure 2. Physiological restoration of *Gata1* optimizes megakaryocytic maturation. Megakaryocytes were generated from G1ME2 cells after dox withdrawal, from FL progenitors after culture in TPO, and from G1ME cells after retroviral transfer of *Gata1* cDNA (10). (**A**) Quantification of megakaryocyte-expressed mRNAs. Increased *Gata2* expression in G1ME cells at day 6 likely represents outgrowth of uninfected cells (n = 3–6 experiments). (**B**) Western blotting for VWF, GATA1, and PF4. Representative of 3 experiments. (**C**) CD42b$^+$ cell surface expression measured by flow cytometry (n = 4–6 experiments). (**D**) Megakaryocyte DNA content at the optimal times after initiation of megakaryocyte differentiation: G1ME2 cells, 5 days; FL, 6 days; G1ME cells, 4 days. Megakaryocyte percentages with more than 4 N DNA are indicated in the upper left corners of the panels. Mean ± SEM, 3 experiments. (**E**) Megakaryocytes generated from G1ME2 cells 5 days after dox withdrawal were injected intravenously into mice expressing human αIIb (hCD41) in place of both endogenous mouse (mCD41) genes. G1ME2-derived platelets (mCD41$^+$) were distinguished from endogenous platelets (hCD41$^+$) by species-specific antibodies. (**F**) Platelet forward scatter (FSC) distribution of G1ME2 cell–derived and endogenous platelets. (**G**) Approximately equal numbers of G1ME2 or G1ME cell–derived megakaryocytes were injected intravenously, and their circulating platelet progeny were quantified (n = 4 experiments). *P < 0.05 vs. preinfusion group by Student's t test. Mean ± SEM. (**H**) FL-derived megakaryocytes or normal mouse platelets (PLTs) were injected intravenously, and circulating donor platelets were quantified (n = 3 experiments). The numbers of infused megakaryocytes for each experiment are shown in Supplemental Table 1.

exhibited stronger, more sustained expression of the maturation marker CD42b (GP1bα) (Figure 2C) and increased DNA content, reflecting endoreduplication of normal megakaryopoiesis (Figure 2D). Transcriptome studies demonstrated that G1ME2 cells exhibit a robust megakaryocyte maturation program that is more similar to FL megakaryocytes than those generated from G1ME cells (Supplemental Figure 3). Thus, restoration of *Gata1* expression, either by retroviral transduction of G1ME cells or reversal of RNA

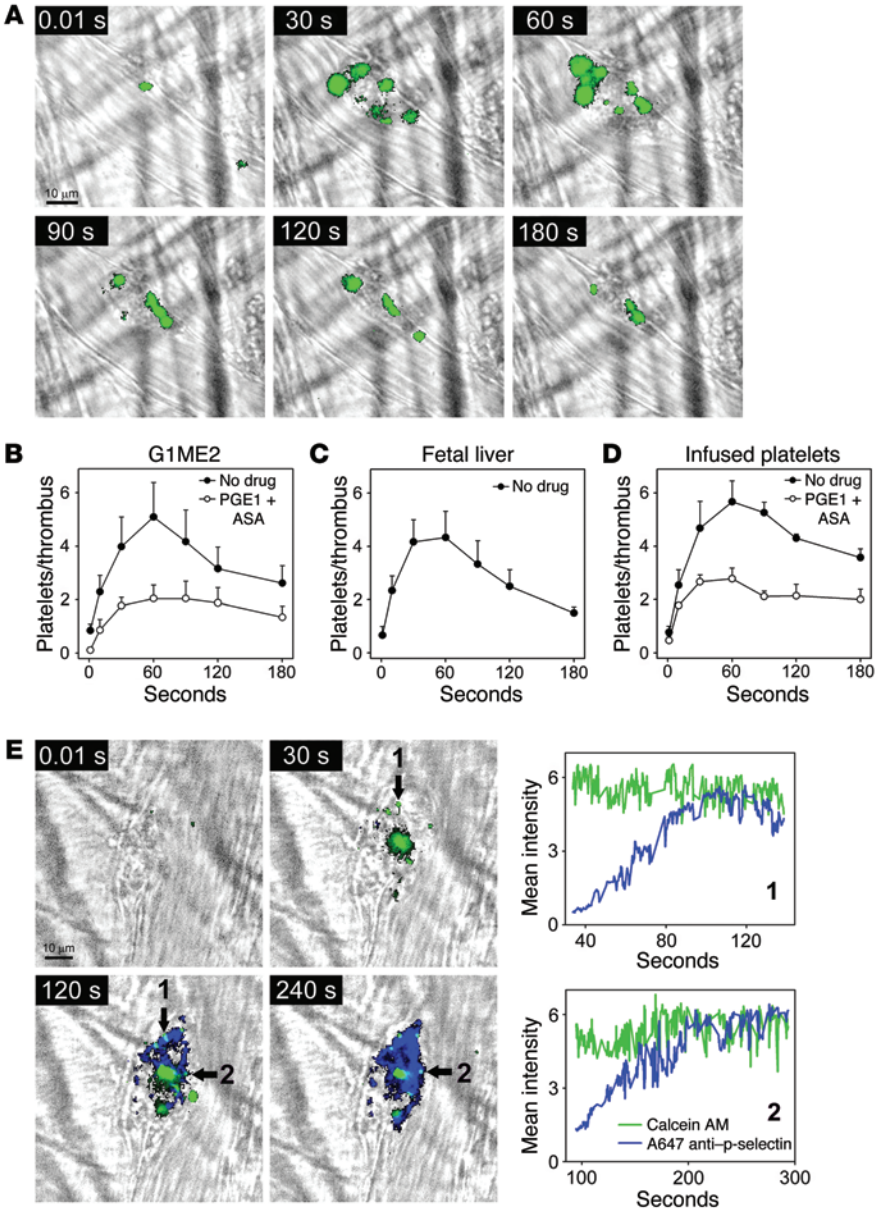

Figure 3. G1ME2-derived megakaryocytes produce functional platelets in vivo. The cremaster muscle artery injury model (20). (**A**) Calcein AM loaded, BSA-purified G1ME2 cell–derived megakaryocytes (0.5 to 1 × 10^6 cells) were infused into C57BL/6 mice. Thereafter, a cremaster arteriole was laser pulsed and monitored by video microscopy. Incorporation of G1ME2-derived platelets (green) into a thrombus after injury is shown. Representative of 10 experiments. 4 to 7 separate laser injuries were performed for each experiment. (**B**–**D**) 0.5 to 1 × 10^6 megakaryocytes (**B** and **C**) or 1 × 10^7 washed mouse platelets (**D**) were incubated with or without platelet inhibitor drugs and tested for adherence to fresh laser-induced thrombi (n = 3–6 experiments). PGE1, 1 μM; ASA, 1 mM. (**E**) Alexa Fluor 647–conjugated anti–P-selectin antibody was infused after G1ME2-derived megakaryocytes and before laser injury. Left panels: confocal fluorescent video microscopy of thrombi. Endogenous calcein$^-$, P-selectin$^+$ platelets are blue. The arrows show 2 G1ME2-derived platelets visualized over time by single particle tracking. These platelets were green initially (calcein$^+$ P-selectin$^-$), then became cyan (green + blue) following P-selectin induction. Representative images are shown from 3 independent experiments. Scale bar: 10 μm. Right: calcein (green lines) and P-selectin expression (blue lines) versus time after laser injury in platelets 1 and 2 from the left panels.

interference in G1ME2 cells, induced megakaryopoiesis. However, derepressing endogenous *Gata1* by silencing of dox-regulated shRNAs in G1ME2 cells produced superior megakaryocyte maturation that should translate to more functional platelets.

Circulating megakaryocytes lodge in pulmonary vascular beds where they produce functional platelets (20, 23, 24). We harvested mature megakaryocytes from G1ME2 cells, G1ME cells, and FL progenitor cultures at optimal maturation time points, as assessed by CD42b expression and ploidy (Figure 2, C and D, and not shown), and injected approximately equal numbers into mice engineered to express the human platelet integrin subunit αIIb (CD41) in place of the endogenous protein (25). Circulating donor megakaryocyte–derived platelets were distinguished from endogenous ones using species-specific anti-CD41 antibodies. G1ME2-derived megakaryocytes gave rise to circulating (mouse) CD41$^+$ platelets similar in size to native mouse platelets (Figure 2, E and F). No circulating platelets were detected from G1ME-derived megakaryocytes (Figure 2G), consistent with their suboptimal in vitro maturation. FL-derived megakaryocytes produced platelets with a circulating half-life about 4- to 5-fold greater than those generated from G1ME2 cells (Figure 2H).

We used the cremaster arteriole laser injury model (20) to assess the functionality of platelets generated from G1ME2 megakaryocytes. Fluorescent video microscopy detected G1ME2-derived platelets in nascent thrombi within 30 seconds after laser injury (Figure 3A and Supplemental Video 1). Platelets from G1ME2 megakaryocytes, FL megakaryocytes, and normal mouse blood incorporated into thrombi with similar kinetics (Figure 3, B–D). Deposition of G1ME2 platelets or control platelets into thrombi was reduced after preincubation with the activation inhibitors prostaglandin E1 (PGE1) and aspirin (ASA) (Figure 3, B and D). Endogenous platelets within thrombi expressed the activation marker P-selectin (Figure 3E and Supplemental Video 2). G1ME2-derived platelets expressed surface P-selectin about 1 minute after lodging in clots, similar to normal platelets (26). Thus, G1ME2-derived platelets home to sites of vascular injury, where they subsequently become activated.

G1ME2-derived megakaryocytes gave rise to approximately 40 circulating platelets/cell with a half-life of 5 to 6 hours, while FL-derived megakaryocytes generated approximately 50 to 100 platelets/cell with a half-life of 20 to 30 hours (Figure 2, G and H, Supplemental Table 1, and ref. 20). Endogenous bone marrow

megakaryocytes produce an estimated 10^3 to 10^4 platelets/cell (24) with a half-life of about 72 hours (27). The reduced quantity and/or longevity of platelets generated in vitro from ES (or G1ME2) cell– and FL-derived megakaryocytes could be due to suboptimal culture conditions that fail to support full maturation. Alternatively, megakaryocytes from ES cells and fetal tissues are biologically distinct from adult megakaryocytes and their platelet progeny may have inherently reduced life spans (28–30). Human ES cell–derived megakaryocytes are believed to approximate those derived from early embryonic yolk sacs (31). Whether this is true for G1ME2 cell–derived megakaryocytes requires further investigation.

Although megakaryocytes generated from ES cells produce fewer platelets with a shorter in vivo life span, G1ME2 cells can be expanded at least 10^{13}-fold over 40 days (Figure 1A). Assuming yield of approximately 20% mature megakaryocytes that each produces 40 platelets, 1 G1ME2 cell could theoretically give rise to $10^{13} \times 20\% \times 40 = 8 \times 10^{13}$ platelets. For comparison, there are 3×10^{11} platelets in a single donor apheresis unit. Undifferentiated G1ME2 cells can also be cryopreserved for expansion and manipulation at a later date (not shown). Future technical advances in pluripotent stem cell differentiation and megakaryocyte culture should improve the yield and longevity of platelets generated from ES and iPS cells.

GATA1 facilitates hematopoietic development by activating erythro-megakaryocytic genes and repressing genes associated with stem/progenitor cells and alternate lineages (32, 33). Germline mutations in *GATA1* cause anemia and/or thrombocytopenia, usually associated with arrested precursor development (34). While it should be possible to treat these diseases through gene therapy approaches, our findings indicate that faithful erythro-megakaryopoiesis depends on precise physiological levels of GATA1, which cannot be achieved through heterologous regulatory elements. Thus, viral vector–based gene replacement strategies may require the use of endogenous GATA1 regulatory elements that more faithfully recapitulate natural expression of GATA1 (35, 36).

While reversible GATA1 suppression could be useful for expanding megakaryocytes/platelets from ES cells for transfusion therapies, the relatively short circulating life span of such products may necessitate more frequent administration than standard donor-derived platelets. On the other hand, ES cells are amenable to genetic manipulation, which can be used to reduce the immunogenicity of platelet progeny and/or engineer them for targeted drug delivery to thrombi. Nakamura et al. showed that ectopic expression of dox-regulated *MYC*, *BMI1*, and *BCL2L1* transgenes expands ES cell–derived megakaryocyte precursors that give rise to functional platelets after dox withdrawal (6). These observations likely overlap mechanistically with our approach, as GATA1 represses *Myc* (37) and *Myc* levels decrease approximately 3-fold after dox withdrawal in G1ME2 cells (not shown). However, shRNA suppression of *Gata1* expands MEPs consistently in bulk polyclonal cell populations, obviating potential problems associated with over- or underexpression of lentiviral-transduced immortalizing genes (6). Moreover, induced GATA1 deficiency expands bipotential MEPs that could potentially be used to manufacture either red blood cells or megakaryocytes/platelets for transfusion therapies. Here we provide proof of principle for the latter. Now it is important to investigate whether GATA1 suppression expands MEPs from human ES cells.

Methods

Detailed protocols are described in Supplemental Methods. Transcriptome data were deposited in the NCBI's Gene Expression Omnibus (GEO GSE66479).

Statistics. The mean and SEM were calculated for all experimental groups and analyzed using 2-tailed Student's *t* test.

Study approval. The IACUC of The Children's Hospital of Philadelphia approved all animal protocols.

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