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


1Division of Hematology and 2Department of Pathology and Laboratory Medicine, The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, USA. 3Division of Hematology, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School and Vascular Biology Program, Department of Surgery, Boston Children's Hospital, Boston, Massachusetts, USA. 4Lillehei Heart Institute and Department of Pediatrics, University of Minnesota, Minneapolis, Minnesota, USA. 5Department of Computational Biology, St. Jude Children's Research Hospital, Memphis, Tennessee, USA. 6Division of Hematology/Oncology, Boston Children's Hospital and Department of Pediatric Oncology, Dana-Farber Cancer Institute, Harvard Medical School, and Broad Institute of MIT and Harvard, Cambridge, Massachusetts, USA. 7Department of Hematology, St. Jude Children's Research Hospital, Memphis, Tennessee, USA.

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 cells–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 1013-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 GIME (for Gata1-deficient MEP) (10). Retroviral transduction of Gata1 cDNA into GIME 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 1014-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.

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

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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...
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 exhibited 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
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 2G and H, Supplemental Table 1, and ref. 20). Endogenous bone marrow 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).

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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 $10^{12}$-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 $10^3 \times 20\% \times 40 = 8 \times 10^{11}$ platelets. For comparison, there are $3\times10^{11}$ 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 iPSCs.

GATA1 facilitates hematopoietic development by activating erythro-megakaryocytic genes and repressing genes associated with stem/progenitor cells and alternate lineages (32, 33). Germ-line 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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Address correspondence to: Mitchell J. Weiss, St. Jude Children’s Research Hospital, 262 Danny Thomas Place, MS #355, Memphis, Tennessee 38105, USA. Phone: 901.595.2051; E-mail: mitch.weiss@stjude.org.

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