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Research Article

Hematology

Congenital amegakaryocytic thrombocytopenia (CAMT) is caused by the loss of thrombopoietin receptor–mediated (MPL-mediated) signaling, which causes severe pancytopenia leading to bone marrow failure with onset of thrombocytopenia and anemia prior to leukopenia. Because *Mpl*<sup>-/-</sup> mice do not exhibit the human disease phenotype, we used an in vitro disease tracing system with induced pluripotent stem cells (iPSCs) derived from a CAMT patient (CAMT iPSCs) and normal iPSCs to investigate the role of MPL signaling in hematopoiesis. We found that MPL signaling is essential for maintenance of the CD34<sup>+</sup> multipotent hematopoietic progenitor (MPP) population and development of the CD41<sup>+</sup>GPA<sup>+</sup> megakaryocyte-erythrocyte progenitor (MEP) population, and its role in the fate decision leading differentiation toward megakaryopoiesis or erythropoiesis differs considerably between normal and CAMT cells. Surprisingly, complimentary transduction of *MPL* into normal or CAMT iPSCs using a retroviral vector showed that MPL overexpression promoted erythropoiesis in normal CD34<sup>+</sup> hematopoietic progenitor cells (HPCs), but impaired erythropoiesis and increased aberrant megakaryocyte production in CAMT iPSC–derived CD34<sup>+</sup> HPCs, reflecting a difference in the expression of the transcription factor *FLI1*. These results demonstrate that impaired transcriptional regulation of the MPL signaling that normally governs megakaryopoiesis and erythropoiesis underlies CAMT.

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# Congenital amegakaryocytic thrombocytopenia iPSC cells exhibit defective MPL-mediated signaling

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**Congenital amegakaryocytic thrombocytopenia (CAMT) is caused by the loss of thrombopoietin receptor-mediated (MPL-mediated) signaling, which causes severe pancytopenia leading to bone marrow failure with onset of thrombocytopenia and anemia prior to leukopenia. Because *Mpl*<sup>-/-</sup> mice do not exhibit the human disease phenotype, we used an in vitro disease tracing system with induced pluripotent stem cells (iPSCs) derived from a CAMT patient (CAMT iPSCs) and normal iPSCs to investigate the role of MPL signaling in hematopoiesis. We found that MPL signaling is essential for maintenance of the CD34<sup>+</sup> multipotent hematopoietic progenitor (MPP) population and development of the CD41<sup>+</sup>GPA<sup>+</sup> megakaryocyte-erythrocyte progenitor (MEP) population, and its role in the fate decision leading differentiation toward megakaryopoiesis or erythropoiesis differs considerably between normal and CAMT cells. Surprisingly, complimentary transduction of MPL into normal or CAMT iPSCs using a retroviral vector showed that MPL overexpression promoted erythropoiesis in normal CD34<sup>+</sup> hematopoietic progenitor cells (HPCs), but impaired erythropoiesis and increased aberrant megakaryocyte production in CAMT iPSC-derived CD34<sup>+</sup> HPCs, reflecting a difference in the expression of the transcription factor *FLI1*. These results demonstrate that impaired transcriptional regulation of the MPL signaling that normally governs megakaryopoiesis and erythropoiesis underlies CAMT.**

## Introduction

It has been well documented that thrombopoietin (TPO) plays an essential role in the self-renewal of HSCs (1, 2) and in the production of megakaryocytes (MKs) and platelets (3, 4). TPO acts via defined signaling pathways that include JAK-STAT, MAPK-ERK1/2, and PI3K-v-akt murine thymoma viral oncogene homolog 1 (PI3K-AKT) (5, 6). Congenital amegakaryocytic thrombocytopenia (CAMT) is a genetic disorder caused by the loss of function or deletion of myeloproliferative leukemia virus oncogene (*MPL*), the gene encoding the TPO receptor (7, 8). CAMT presents at birth with severe thrombocytopenia and absent bone marrow MKs and develops into bone marrow failure/aplastic anemia during the childhood years or even earlier. The disease is fatal unless successfully treated with HSC transplantation, which indicates that TPO/MPL signaling is indispensable for hematopoietic homeostasis in humans. Notably, most CAMT patients show a reduction in platelet and erythrocyte counts prior to the decrease in leukocyte counts (9), and repetitive transfusion of erythrocyte and/or platelets is usually necessary prior to curative bone marrow transplantation. These clinical features imply that each hematopoietic lineage has its own distinct dependency on MPL signaling. On the other hand, the precise roles of MPL at defined differentiation steps during normal hematopoiesis and the effects of its loss in CAMT patients remain unclear, in large part because of the difficulty of obtaining patients' HSCs for in vitro analysis. Although mouse *Tpo*<sup>-/-</sup> or *Mpl*<sup>-/-</sup> models show sustained thrombo-

cytopenia with smaller numbers of MKs and smaller myeloid and erythrocyte progenitor pools in the bone marrow (4, 10), they do not fully recapitulate the phenotype manifested in CAMT patients. For example, *Mpl*<sup>-/-</sup> mice have normal levels of erythrocytes and leukocytes in their peripheral blood throughout life, and live to an old age without developing bone marrow failure/aplastic anemia.

Disease-specific human induced pluripotent stem cells (iPSCs) are an attractive tool for elucidating the pathogenesis of hematological diseases (11–15), for validating gene therapy models (13, 15–17), and for drug screening. Of importance in the present study is that MKs and erythrocytes generated in vitro from disease-specific iPSCs are an effective tool for studying the mechanism of not only thrombopoiesis (11), but also erythropoiesis (18–20).

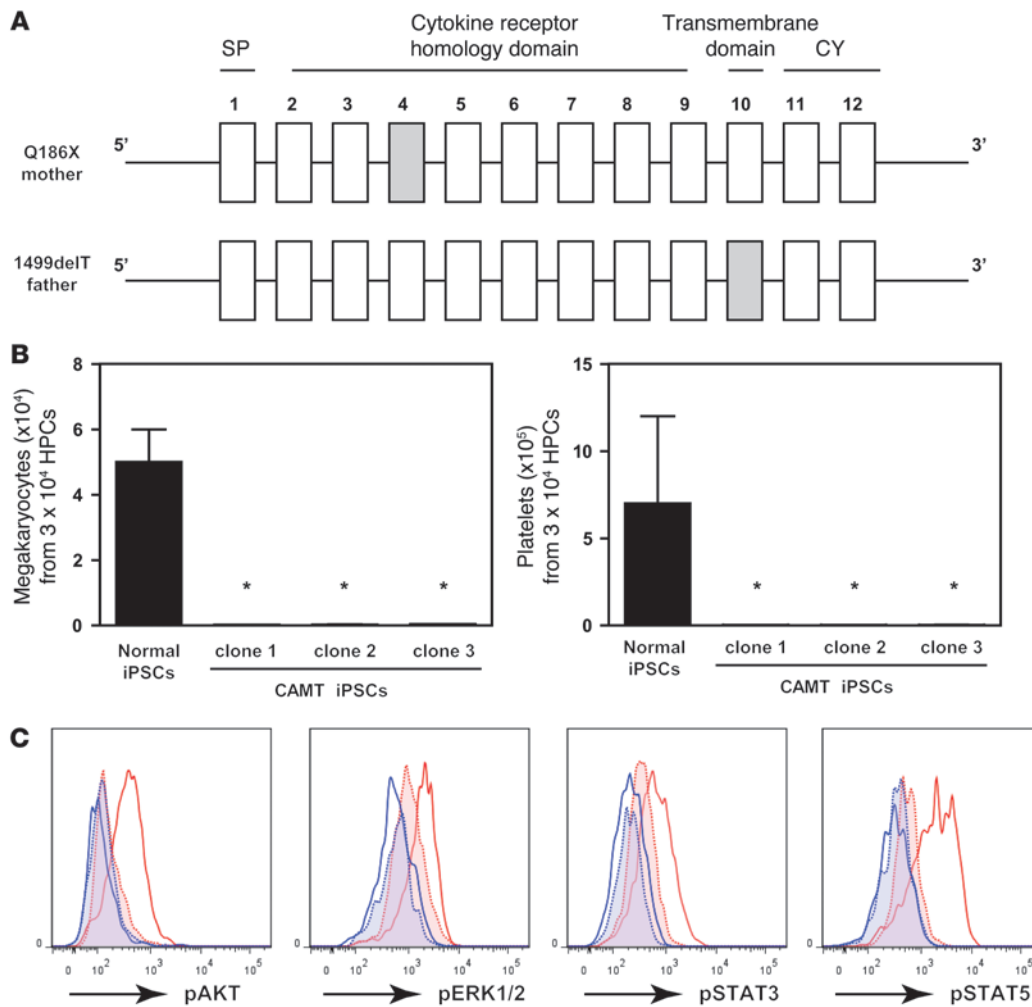
Here, we established iPSCs derived from a patient diagnosed with CAMT and treated with curative allogeneic stem cell transplantation (referred to herein as CAMT iPSCs) (7, 21). In several established CAMT iPSCs, the *MPL* mutations responsible for the complete loss of MPL expression were carried over. Using CAMT iPSCs and an in vitro disease tracking system we established previously (22–24), we determined the precise link between MPL signaling and development of a common MK/erythrocyte progenitor (MEP) and elucidated the pathogenesis of CAMT by recapitulating the clinical manifestations of the disease.

## Results

*Disease-specific iPSCs from a CAMT patient failed to generate MKs and platelets.* A candidate patient was treated with bone marrow transplantation at 12 years of age (7, 21) after being diagnosed with

**Conflict of interest:** The authors have declared that no conflict of interest exists.

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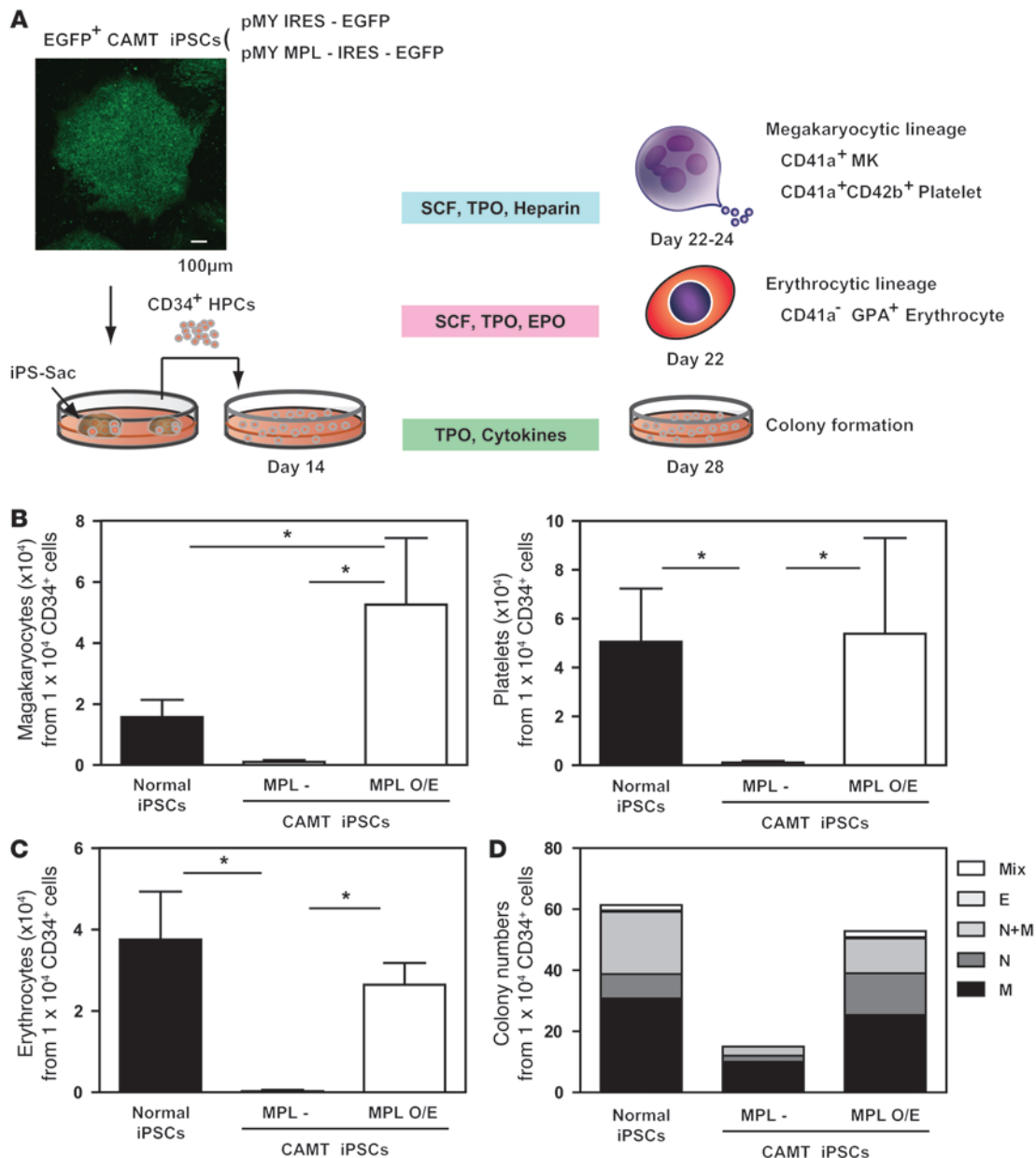
**Figure 1** Disease-specific iPSCs recapitulate the disease phenotype manifested in a patient with CAMT. **(A)** Maternal mutation Q186X (C-to-T transition at the cDNA nucleotide position 556) in exon 4 and paternal mutation 1,499delT (single nucleotide deletion of thymine at position 1,499) in exon 10 in a patient with CAMT. SP, signal peptide; CY, cytoplasmic domain. **(B)** Generation of MKs and platelets from normal or CAMT iPSC-derived HPCs cultured on C3H10T1/2 feeder cells for 10 days in the presence of SCF (50 ng/ml), TPO (100 ng/ml), and heparin (25 U/ml). All CAMT iPSC clones generated few MKs or platelets. **(C)** Flow cytometric analysis of MPL-mediated downstream signaling in normal iPSC- (red lines) or CAMT iPSC-derived (blue lines) CD34<sup>+</sup> HPCs stimulated with 100 ng/ml TPO for 10 minutes (solid lines) or with vehicle control (dotted lines). No response was observed with CAMT iPSCs. \**P* < 0.05.

CAMT. We used skin fibroblasts from the patient to create iPSCs with normal karyotypes using the previously established method with G glycoprotein of the vesicular stomatitis virus (VSV-G) pseudotyped retroviruses (23, 25) harboring 4 (*OCT3/4*, *SOX2*, *KLF4*, and *c-MYC*) or 3 (*OCT3/4*, *SOX2*, and *KLF4*) reprogramming factors (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI64721DS1). The resultant CAMT iPSCs exhibited mutations corresponding to the original donor skin, including compound heterozygous point mutations in the *MPL* locus: a C-to-T transition at the cDNA nucleotide position 556 in exon 4, and a single nucleotide deletion of thymine at position 1,499 in exon 10 (Figure 1A and ref. 7). The following parameters were taken as evidence of the pluripotency of CAMT iPSCs: alkaline phosphatase staining; immunostaining for SSEA-4, TRA1-60, and TRA1-81 (Supplemental Figure 1B); gene expression (data not shown); and the capacity for teratoma formation in

NOD/SCID mice (Supplemental Figure 1C). We also confirmed that the exogenous reprogramming factors were all silenced in the established iPSCs (data not shown).

To explore the hematopoietic differentiation potential of CAMT iPSCs, we evaluated 3 CAMT iPSC clones and compared them with normal iPSCs (clone TkDA3-4; see Methods) previously established from age-matched dermal fibroblasts using 4 reprogramming factors (23). Using our recently established in vitro differentiation system (22–24), we confirmed that all of the CAMT iPSC clones generated few MKs or platelets, even in the presence of 100 ng/ml TPO, 50 ng/ml stem cell factor (SCF), and 25 U/ml heparin (Figure 1B).

CAMT patients are indeed thrombocytopenic at diagnosis: their platelet counts range 20,000–50,000 platelets/mm<sup>3</sup>, equivalent to 5%–10% of that in healthy individuals. Conversely, platelet numbers from CAMT iPSCs in this study were less than 1% of that obtained with normal iPSCs (0.51% ± 0.29%, 0.62% ± 0.42%, and

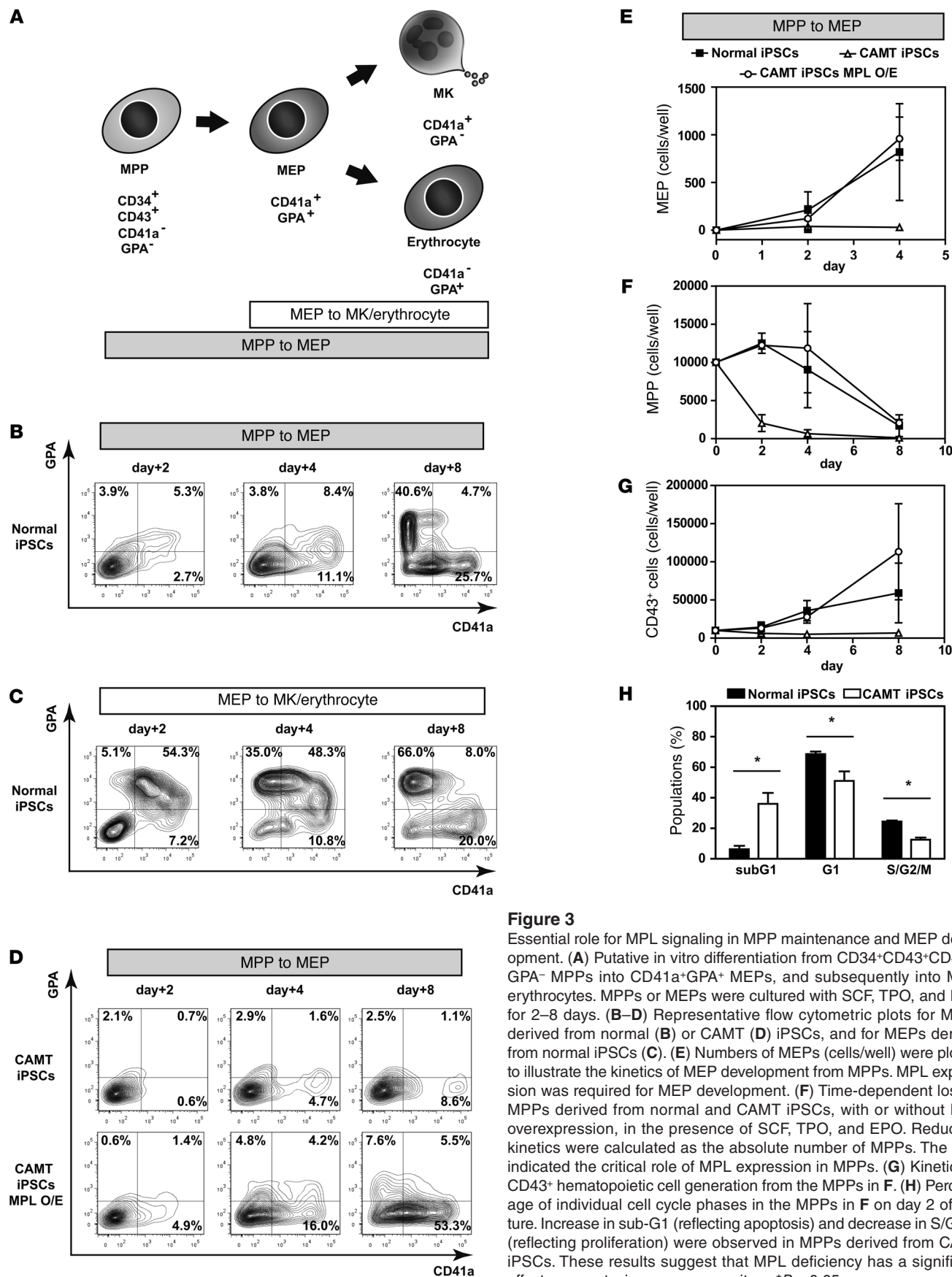


**Figure 2**

Disease phenotype of CAMT iPSCs was seemingly restored by MPL supplementation. (A) In vitro differentiation protocol using CAMT iPSCs transduced with *MPL*. CAMT iPSCs were transduced using a pMY retroviral vector harboring *EGFP* and *MPL* or *EGFP* alone and then selected as *EGFP*<sup>+</sup> populations. Using the indicated cytokines, CAMT iPSC-derived *EGFP*<sup>+</sup> CD34<sup>+</sup> HPCs overexpressing *MPL* were subjected to in vitro differentiation toward the MK or erythrocyte lineage or colony formation. Scale bar: 100 μm. (B and C) Numbers of MKs and platelets (B) cultured in SCF, TPO, and heparin for 8–10 days and erythrocytes (C) cultured with SCF (50 ng/ml), TPO (10 ng/ml), and EPO (6 U/ml) for 8 days. Cells were cultured without or with supplemental *MPL* to induce overexpression (O/E). (D) Colony-forming potential for myeloid lineage in normal iPSC-derived CD34<sup>+</sup> HPCs, and CAMT iPSC-derived *EGFP*<sup>+</sup> CD34<sup>+</sup> HPCs with or without *MPL* supplementation, in MethoCult H4434 semisolid medium containing TPO (50 ng/ml), SCF, EPO, IL-3, and GM-CSF. M, macrophage; N, neutrophil; N+M, neutrophil and macrophage; E, erythrocyte. \**P* < 0.05.

0.56% ± 0.21%; Figure 1B). However, when we reduced the TPO concentration to a more physiological level (0.1–1 ng/ml) (8), platelet numbers from CAMT iPSCs reached 5%–10% of those obtained with normal iPSCs (Supplemental Figure 2); i.e., they approximated the relative numbers obtained in vivo. This finding suggested that CAMT iPSCs recapitulate the thrombocytopenia seen in the CAMT patient, yielding a useful CAMT disease model.

These results were further confirmed by the failure of TPO stimulation to induce phosphorylation of mediators downstream of *MPL* (i.e., pAKT, pERK1/2, pSTAT3, and pSTAT5) in CD34<sup>+</sup> HPCs (Figure 1C) and by the absence of *MPL* mRNA and *MPL* protein expression (Supplemental Figure 3, A–C), which corresponded to the complete absence of *MPL* expression in bone marrow cells from the patient (26).



**Figure 3**

Essential role for MPL signaling in MPP maintenance and MEP development. (A) Putative in vitro differentiation from CD34<sup>+</sup>CD43<sup>+</sup>CD41a<sup>-</sup>GPA<sup>-</sup> MPPs into CD41a<sup>+</sup>GPA<sup>+</sup> MEPs, and subsequently into MKs/erythrocytes. MPPs or MEPs were cultured with SCF, TPO, and EPO for 2–8 days. (B–D) Representative flow cytometric plots for MPPs derived from normal (B) or CAMT (D) iPSCs, and for MEPs derived from normal iPSCs (C). (E) Numbers of MEPs (cells/well) were plotted to illustrate the kinetics of MEP development from MPPs. MPL expression was required for MEP development. (F) Time-dependent loss of MPPs derived from normal and CAMT iPSCs, with or without MPL overexpression, in the presence of SCF, TPO, and EPO. Reduction kinetics were calculated as the absolute number of MPPs. The data indicated the critical role of MPL expression in MPPs. (G) Kinetics of CD43<sup>+</sup> hematopoietic cell generation from the MPPs in F. (H) Percentage of individual cell cycle phases in the MPPs on day 2 of culture. Increase in sub-G1 (reflecting apoptosis) and decrease in S/G2/M (reflecting proliferation) were observed in MPPs derived from CAMT iPSCs. These results suggest that MPL deficiency has a significant effect on apoptosis among progenitors. \*P < 0.05.





*Retroviral complementation of WT MPL restored hematopoiesis to CAMT iPSCs.* To determine whether complementation of WT MPL in CAMT iPSCs could restore blood generation, CAMT iPSCs were transduced with *MPL* and *EGFP* using retroviral vectors and then purified by sorting out EGFP<sup>+</sup> cells using the differentiation protocol depicted in Figure 2A. CAMT iPSCs transduced with vehicle vector showed severely impaired generation of MKs, platelets, and erythrocytes, although they retained reduced granulocyte and macrophage differentiation potential in colony formation assays (Figure 2, B–D). Conversely, overexpression of MPL in CAMT iPSCs restored the differentiation potential of all myeloid cell lineages to levels comparable to those seen in normal iPSCs (Figure 2, B–D).

*TPO/MPL signaling was indispensable for MPP maintenance and transition to common MEPs.* Earlier studies demonstrated that, in an *in vitro* hematopoietic differentiation system using human ES cells (ESCs), CD34<sup>+</sup>CD43<sup>+</sup>CD41a<sup>-</sup>GPA<sup>-</sup> and CD41a<sup>+</sup>GPA<sup>+</sup> populations represent multipotent hematopoietic progenitors (MPPs) (27) and MEPs (28), respectively (Figure 3A). We evaluated whether human iPSCs exhibit traceable differentiation steps, similar to those seen with normal dermal fibroblast-derived iPSCs, in our differentiation system. CD34<sup>+</sup>CD43<sup>+</sup>CD41a<sup>-</sup>GPA<sup>-</sup> MPPs sorted on day 14 of culture differentiated into CD41a<sup>+</sup>GPA<sup>+</sup> MEPs on additional day +4, which in turn differentiated into CD41a<sup>+</sup>GPA<sup>-</sup> MKs (25.7%) and CD41a<sup>-</sup>GPA<sup>+</sup> erythrocytes (40.6%) on additional day +8 (Figure 3B). We then further confirmed that the selected CD41a<sup>+</sup>GPA<sup>+</sup> MEPs could be differentiated into both CD41a<sup>+</sup>GPA<sup>-</sup> MKs (20.0%) and CD41a<sup>-</sup>GPA<sup>+</sup> erythrocytes (66%) on additional day +8 (Figure 3C). In our culture system, therefore, CD34<sup>+</sup>CD43<sup>+</sup>CD41a<sup>-</sup>GPA<sup>-</sup> MPPs derived from normal iPSCs had the potential to generate CD41a<sup>+</sup>GPA<sup>+</sup> MEPs that preferentially differentiated into erythrocytes rather than MKs (Figure 3, B and C).

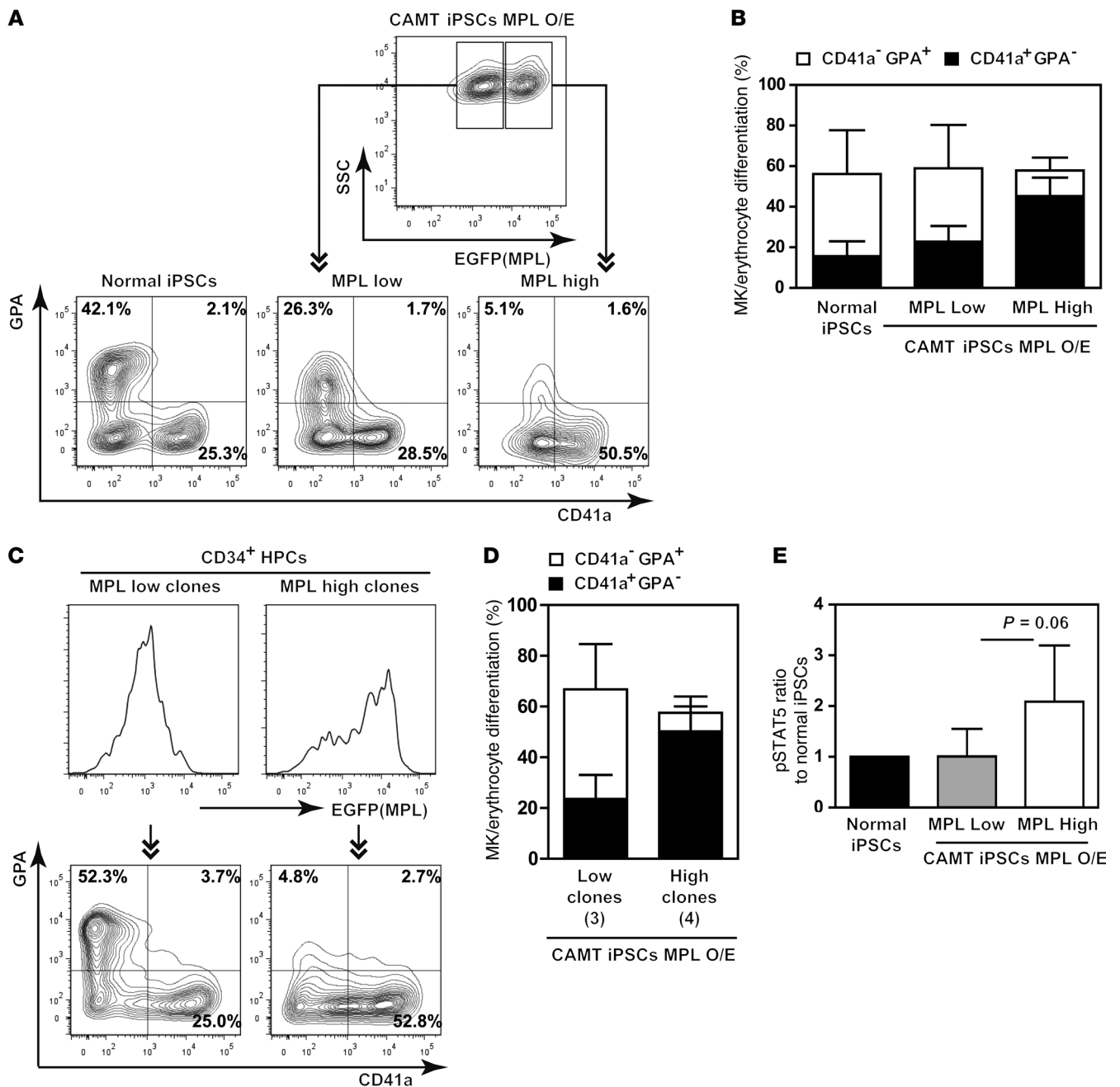
CAMT iPSCs transduced with vehicle vector showed severely defective transition from MPPs to MEPs, which was corrected in CAMT iPSCs overexpressing MPL (Figure 3, D and E). In addition, whereas the number of MPPs derived from normal or CAMT iPSCs overexpressing MPL was maintained at least until day 4, the number of cells derived from untreated CAMT iPSCs declined rapidly, even in the presence of SCF and TPO (Figure 3F). Consequently, fewer CD43<sup>+</sup> pure hematopoietic cells were derived from CAMT iPSCs (Figure 3G). In addition, serial replating assays revealed that CD34<sup>+</sup> HPCs derived from CAMT iPSCs generated a few colonies exclusively in the first replating trial, whereas CD34<sup>+</sup> HPCs from normal iPSCs or from ESCs produced much larger numbers of colonies, even after the second replating trial (Supplemental Figure 4), which implies impairment of self-replication by CAMT iPSC-derived MPPs. The smaller numbers of MPPs could potentially be accounted for by increased apoptosis (sub-G1 population) and/or decreased proliferation (S/G2/M populations) (Figure 3H and Supplemental Figure 5); however, this was more likely due to the loss of MPL. Collectively, these results indicate that MPL signaling is essential for the transition of MPPs to MEPs as well as for MPP maintenance, and that MPL also contributes to their differentiation into granulocytes and macrophages (Figure 2D). Notably, this differentiation profile was consistent with the clinical course in CAMT patients, who typically show early onset of severe thrombocytopenia and anemia prior to leukopenia (9) and HSC exhaustion.

*Supplementing CD34<sup>+</sup> HPCs derived from CAMT iPSCs with normal levels of MPL contributed to lineage commitments to both MKs and erythrocytes, but higher levels blocked erythropoiesis.* Retroviral gene transduction is a powerful tool for studying the actions by a gene of interest. We noted that

the expression levels obtained after MPL supplementation in CAMT iPSCs paralleled fluorescence intensity after retroviral transduction-mediated EGFP expression (Supplemental Figure 6, A and B). On day 22 of culture, normal iPSC-derived MPPs could be differentiated into both erythrocytes and MKs in the presence of SCF, TPO, and EPO. Under these conditions, they preferentially differentiated into erythrocytes (Figure 4, A and B). Flow cytometric analysis showed that a CAMT iPSC clone overexpressing MPL produced 2 distinct populations exhibiting different levels of EGFP expression (EGFP<sup>hi</sup> and EGFP<sup>lo</sup>; indicative of high and low MPL expression, respectively) on day 22 of culture (Figure 4A). The EGFP<sup>lo</sup> population showed nearly equal levels of erythrocyte and MK differentiation, similar to normal iPSCs. However, the EGFP<sup>hi</sup> population showed apparently increased MK differentiation, with few erythrocytes (Figure 4, A and B). These results were identical to results obtained in a prospective analysis in which CD34<sup>+</sup> HPCs derived from 7 individual CAMT iPSC clones were EGFP<sup>hi</sup> or EGFP<sup>lo</sup> on day 14, faithfully reflecting MPL expression level — i.e., EGFP<sup>hi</sup> and EGFP<sup>lo</sup> populations equalized at 10 times normal and at normal levels of MPL, respectively (Supplemental Figure 6), and were respectively subjected to the differentiation protocol (Figure 4, C and D). We also confirmed that the intensity of EGFP expression, as detected by flow cytometry, corresponded well to the level of pSTAT5 (Figure 4E). Based on our results thus far, we concluded that the intensity of MPL signaling determines the fate of erythrocyte and MK differentiation at the MPP stage and that excessive MPL signaling may block erythrocyte differentiation in CAMT iPSCs.

*Excessive TPO/MPL signaling in MKs induces aberrant megakaryopoiesis, leading to generation of CD42b<sup>-</sup> platelets.* As shown in Figure 2B, 3 times as many MKs were generated from CAMT iPSCs overexpressing MPL than from normal iPSCs. The level of MPL expression in CD34<sup>+</sup> HPCs derived from CAMT iPSCs overexpressing MPL was greater than in those from normal iPSCs (Supplemental Figure 6B); however, the numbers of CD42b<sup>+</sup> platelets were similar, which suggests that excessive MPL signaling might adversely influence megakaryopoiesis. Flow cytometric analysis on day 24 of culture revealed that CAMT iPSCs overexpressing MPL generated greater numbers of MKs showing incomplete maturation — i.e., with both CD41a<sup>+</sup>CD42b<sup>-</sup>CD42a<sup>-</sup> and CD41a<sup>+</sup>CD42b<sup>-</sup>CD42a<sup>+</sup> populations and low ploidy (Figure 5, A–C). Moreover, the dysregulated MKs appeared to release CD41a<sup>+</sup>CD42b<sup>-</sup>CD42a<sup>-</sup> platelets (Figure 5, D and E), even in the presence of GM-6001 (Supplemental Figure 7), which we previously showed to induce retention of CD42b on platelets by preventing its metalloproteinase-catalyzed shedding (29).

We also previously showed that excessive c-MYC activation in MKs blocks MK maturation and leads to CD42b<sup>lo</sup> platelet generation (23). We therefore tested whether overexpression of MPL in human ESCs also blocks MK maturation in association with reduced platelet generation. Indeed, ESCs overexpressing MPL did generate the immature type of CD41a<sup>+</sup>CD42b<sup>-</sup>CD42a<sup>-</sup> MKs with lower ploidy and CD41a<sup>+</sup>CD42b<sup>-</sup>CD42a<sup>-</sup> platelets (Figure 5, F and G). We therefore concluded that excessive MPL signaling induces dysregulation of thrombopoiesis, which might be related to the MPL signaling level, as evidenced by the increased TPO sensitivity of CAMT iPSCs overexpressing MPL (Supplemental Figure 8). In addition, flow cytometric analysis suggested that the augmented MPL signaling might be associated with increased pAKT, pSTAT3, or pSTAT5A, but not pERK1/2, in CD41a<sup>+</sup> MKs (Supplemental Figure 9), which indicates that excessive signaling impairs normal MK development.

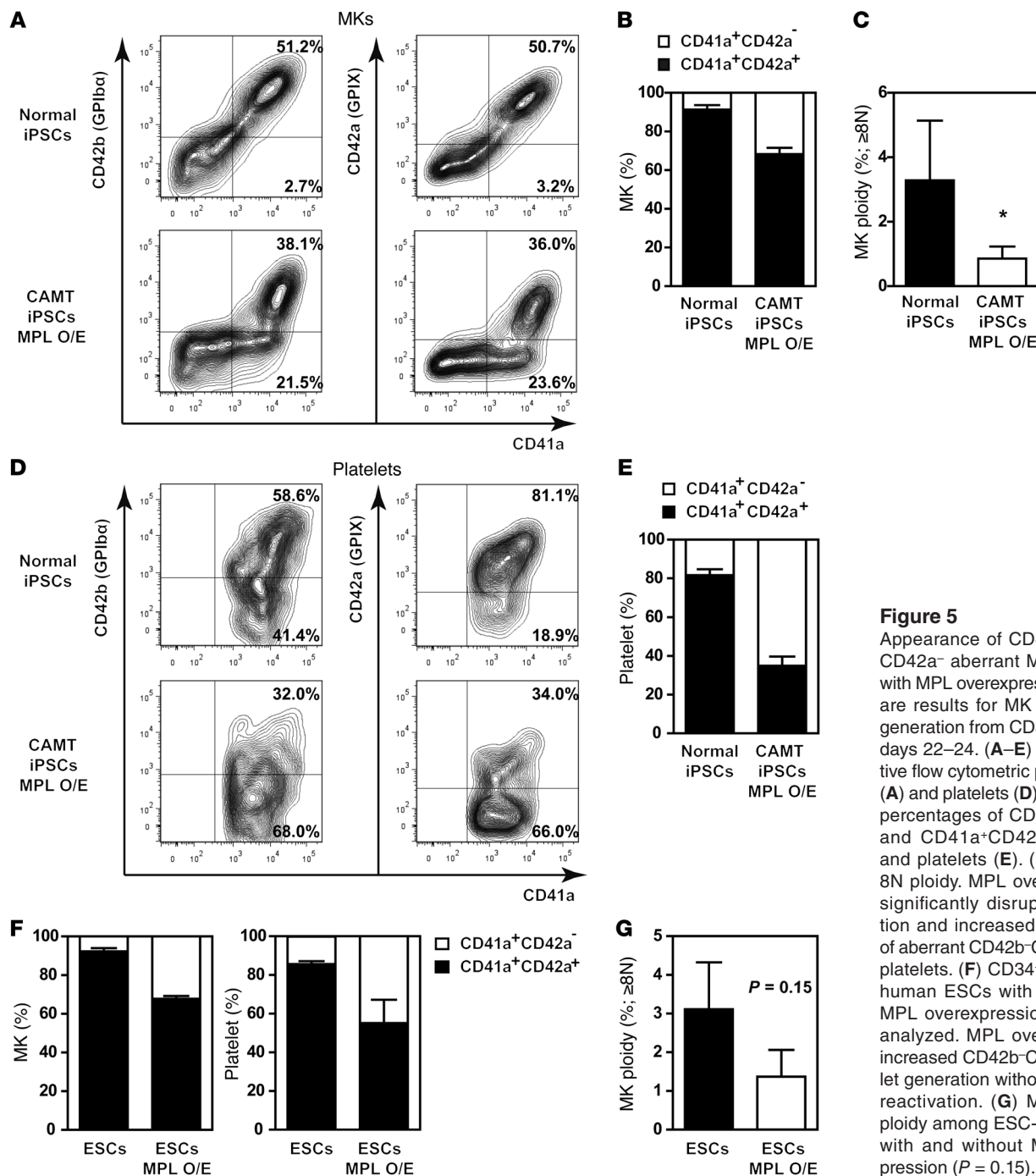


**Figure 4**

MPL expression levels may determine MK and erythrocyte specification. **(A)** Representative flow cytometric plots of MK and erythrocyte differentiation on day 22. EGFP<sup>hi</sup> and EGFP<sup>lo</sup> populations of CAMT iPSCs overexpressing MPL were retrospectively reanalyzed for CD41a and GPA expression. **(B)** Percent CD41a<sup>-</sup>GPA<sup>+</sup> erythrocytes and CD41a<sup>+</sup>GPA<sup>-</sup> MKs from the indicated iPSCs. High MPL expression led to preferential differentiation into MKs. **(C)** EGFP<sup>hi</sup> and EGFP<sup>lo</sup> CD34<sup>+</sup> HPC populations derived from several CAMT iPSC clones with MPL overexpression are shown as histograms and flow cytometric plots. Representative plots after induction of differentiation are shown for each clone (high and low MPL intensity). **(D)** Percent MK/erythrocyte differentiation. The number of clones is indicated in parentheses. MPL expression level affected the MK/erythrocyte lineage commitment of CD34<sup>+</sup> HPCs. **(E)** CD34<sup>+</sup> HPCs were incubated with TPO and analyzed for pSTAT5. Results are presented as fluorescence intensity relative to that of normal iPSCs (assigned as 1). The population with higher MPL overexpression showed a markedly augmented TPO-mediated response ( $P = 0.06$ ).

*Loss of MPL signaling in CAMT iPSCs impairs FLI1-mediated lineage determination toward erythrocytes or MKs.* As shown in Figure 4, the strong intensity of TPO/MPL signaling influenced dominant differentiation into MKs from CAMT iPSC-derived CD34<sup>+</sup> HPCs at

the expense of erythropoiesis. However, in normal hematopoiesis, some reports showed that TPO/MPL signaling contributed to erythropoiesis (30, 31). To assess the relationship between MPL signal intensity and erythropoiesis in normal cells, the differentiation

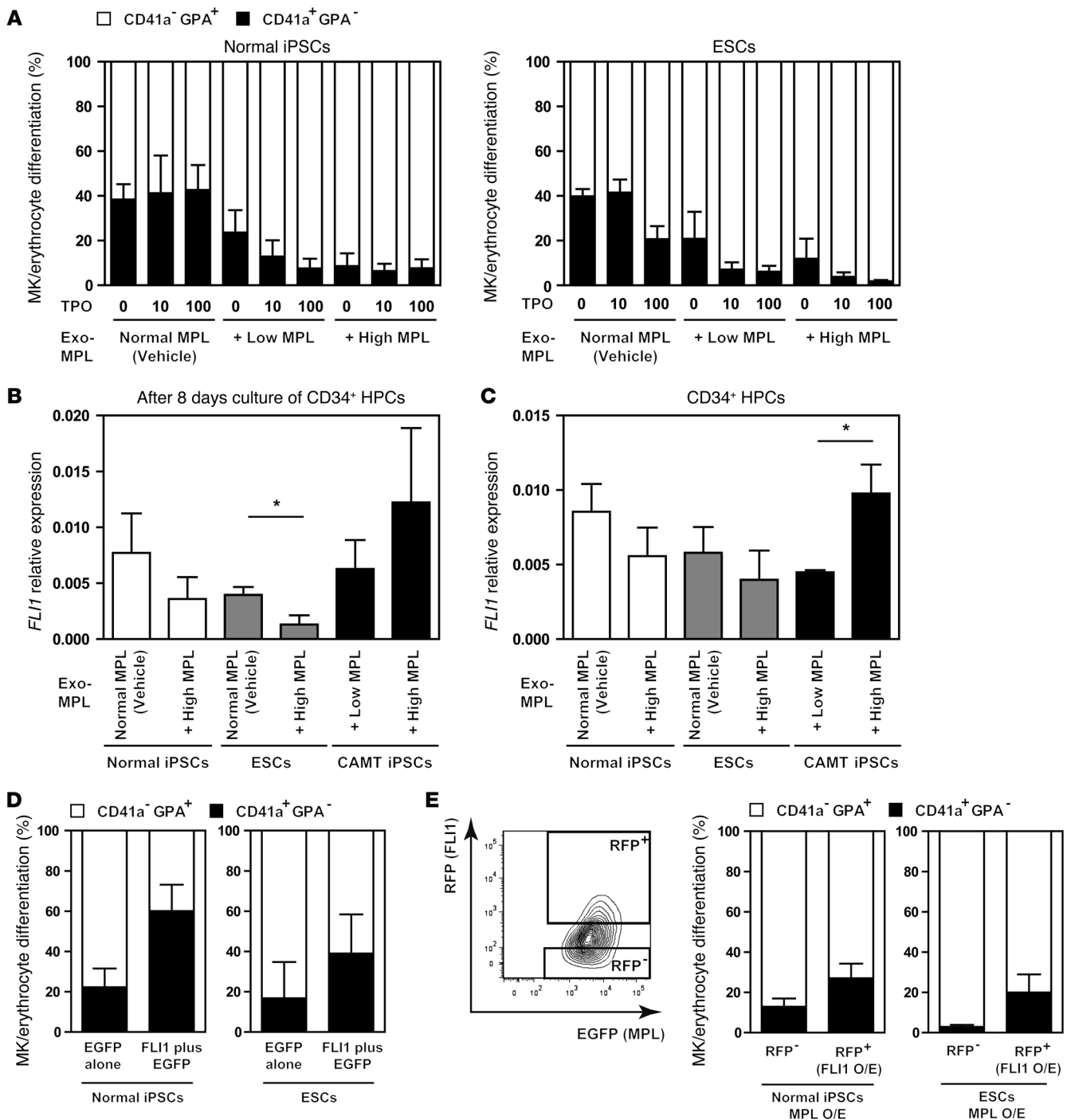


**Figure 5** Appearance of CD41a<sup>+</sup>CD42b<sup>-</sup>CD42a<sup>-</sup> aberrant MKs/platelets with MPL overexpression. Shown are results for MK and platelet generation from CD34<sup>+</sup> HPCs on days 22–24. (A–E) Representative flow cytometric plots for MKs (A) and platelets (D) and the final percentages of CD41a<sup>+</sup>CD42a<sup>-</sup> and CD41a<sup>+</sup>CD42a<sup>+</sup> MKs (B) and platelets (E). (C) MKs with 8N ploidy. MPL overexpression significantly disrupted maturation and increased populations of aberrant CD42b<sup>-</sup>CD42a<sup>-</sup> MKs/platelets. (F) CD34<sup>+</sup> HPCs from human ESCs with and without MPL overexpression were also analyzed. MPL overexpression increased CD42b<sup>-</sup>CD42a<sup>-</sup> platelet generation without transgene reactivation. (G) MKs with 8N ploidy among ESC-derived MKs with and without MPL overexpression ( $P = 0.15$ ). \* $P < 0.05$ .

of normal iPSC- or ESC-derived CD34<sup>+</sup> HPCs into MKs or erythrocytes in the presence of SCF, TPO, and EPO was manipulated by varying the level of exogenous MPL expression. Consistent with an earlier report (31), normal CD34<sup>+</sup> HPCs strongly expressing MPL showed erythrocyte-biased differentiation that was dependent on TPO/MPL signaling (Figure 6A and Supplemental Figure 10). This suggests that the program of differentiation toward MKs or erythrocytes distinctly differs between normal and CAMT iPSC-derived CD34<sup>+</sup> HPCs and that the lineage balance in normal iPSC-derived HPCs may correspond to normal erythropoiesis in vivo and in vitro (28, 32, 33). Although the underlying mechanism governing lineage

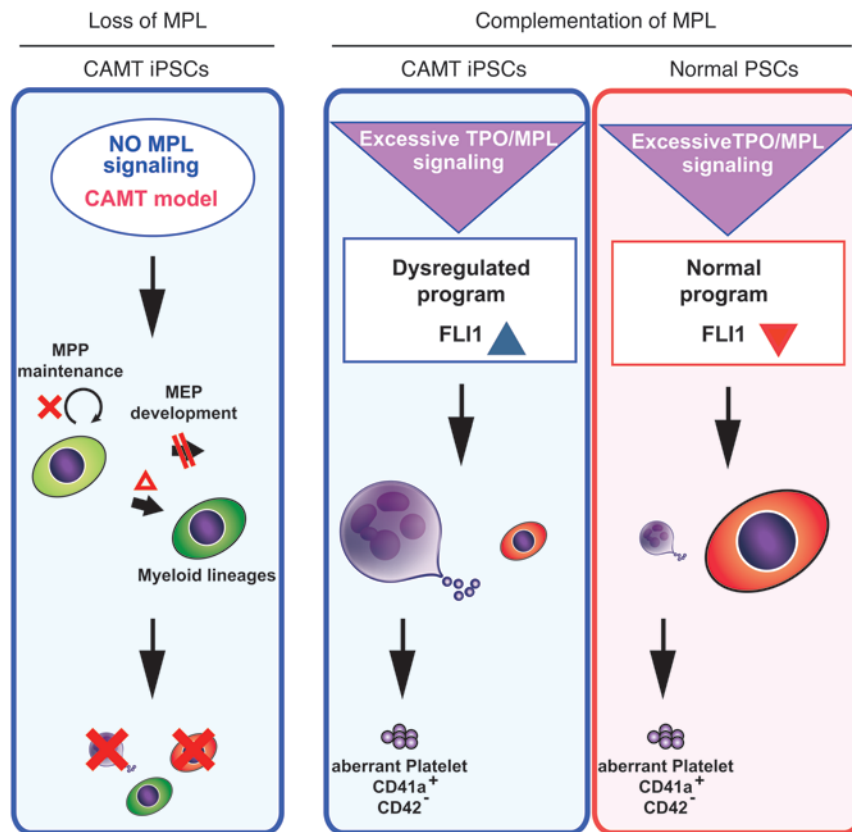
commitment toward MKs or erythrocytes is not fully understood, it appears that Friend leukemia virus integration 1 (FLI1) and Kruppel-like factor 1 (KLF1) are potential fate-determining transcriptional factors at the MEP stage and that they mutually antagonize one another (34, 35). Therefore, to evaluate the fate determination program in normal and CAMT iPSC-derived CD34<sup>+</sup> HPCs, we used quantitative PCR to assess *FLI1* and *KLF1* expression. Consistent with the mutually antagonistic relationship between *FLI1* and *KLF1* as well as with earlier reports (28, 34), higher *KLF1* and lower *FLI1* expression were observed with erythrocyte-biased differentiation in normal iPSC-derived cells, whereas lower *KLF1* and higher





**Figure 6**

FLI1-mediated MK/erythrocyte differentiation differed between normal and CAMT iPSCs. Shown are results for MK and erythrocyte generation from CD34<sup>+</sup> HPCs on day 22. **(A)** Percent CD41<sup>+</sup>GPA<sup>-</sup> MKs and CD41<sup>-</sup>GPA<sup>+</sup> erythrocytes derived from normal iPSCs (left) or ESCs (right) and transduced with vehicle or MPL expression vector in the presence of 0, 10, or 100 ng/ml TPO with SCF and EPO. Exogenous MPL expression was assessed based on EGFP fluorescence intensity. Erythropoiesis was enhanced in a TPO/MPL signaling–dependent manner. **(B and C)** *FLI1* expression in CD34<sup>+</sup> HPCs before **(C)** and after **(B)** differentiation. EGFP<sup>hi</sup> CD34<sup>+</sup> HPCs derived from normal iPSCs and ESCs with or without MPL overexpression, or EGFP<sup>lo</sup> or EGFP<sup>hi</sup> CD34<sup>+</sup> HPCs from CAMT iPSCs overexpressing MPL, were sorted and cultivated for an additional 8 days. **(D)** Percent MK and erythrocyte differentiation from CD34<sup>+</sup> HPCs derived from normal iPSCs or ESCs overexpressing EGFP alone (vehicle) or FLI1 plus EGFP. **(E)** Percent MK and erythrocyte differentiation from normal iPSCs or ESCs overexpressing MPL-EGFP and FLI1-RFP. CD34<sup>+</sup> HPCs derived from normal iPSCs or ESCs overexpressing MPL were transfected with FLI1-RFP or vector and cultivated for an additional 8 days. Exogenous FLI1 expression was assessed based on RFP expression (shown in the contour plot). FLI1 overexpression attenuated erythrocyte-biased differentiation in normal iPSCs and ESCs. \**P* < 0.05.



**Figure 7**

Model of human megakaryopoiesis and erythropoiesis regulated by MPL signaling. CAMT iPSC behavior showed that MPL signaling is essential for MPP maintenance and the development of MEPs from MPPs. In addition, MPL contributes to further myeloid development, but it is not indispensable, which indicates that the dependency on MPL signaling varies among lineages. MPL complementation experiments yielded 2 important findings. First, CAMT iPSCs exhibit dysregulated differentiation toward megakaryopoiesis and erythropoiesis, which is characterized by MK-biased differentiation due to intrinsically dominant *FLI1* expression accelerated by ectopic TPO/MPL signaling. Conversely, *FLI1* expression is diminished and erythropoiesis is dominant in normal PSCs. Second, excessive TPO/MPL signaling impairs MK maturation and facilitates generation of aberrant CD41a<sup>+</sup> and CD42-null platelets from both normal and CAMT iPSCs.

*FLI1* were observed with MK-biased differentiation in CAMT iPSC-derived cells (Figure 6B and Supplemental Figure 11A). It is noteworthy that the *FLI1* expression profile at the CD34<sup>+</sup> progenitor stage was completely reversed between normal and CAMT iPSC-derived HPCs: *FLI1* expression was reduced in normal iPSC-derived CD34<sup>+</sup> HPCs but markedly elevated in CAMT iPSC-derived CD34<sup>+</sup> HPCs in a manner dependent on exogenous MPL expression, and this profile persisted even after differentiation (Figure 6, B and C). On the other hand, *KLF1* expression behavior appeared to show a similar pattern, but only after differentiation (Supplemental Figure 11, A and B). This suggests that *FLI1* is primarily involved in the fate decision from pluripotent stem cell-derived (PSC-derived) MEPs, which may subsequently affect *KLF1* function.

We next confirmed that overexpression of *FLI1* plus EGFP in CD34<sup>+</sup> HPCs derived from normal iPSCs facilitated MK-biased differentiation of the CD34<sup>+</sup> HPCs compared with HPCs overexpressing EGFP alone (Figure 6D). More importantly, lentiviral transduction of the same normal CD34<sup>+</sup> HPC population overexpressing MPL plus EGFP with red fluorescent protein-labeled (RFP-labeled) *FLI1* diminished erythrocyte-biased differentiation (Figure 6E). Furthermore, *FLI1* mRNA levels were well correlated with RFP levels in PSCs, even in the presence of elevated MPL, whereas *KLF1* levels were inversely related to RFP levels (Supplemental Figure 12). These results indicate that CD34<sup>+</sup> HPCs derived from CAMT iPSCs exhibit MK-biased differentiation due to impairment of *FLI1* transcriptional factor. Hypermethylation of the *FLI1* promoter reportedly suppresses *FLI1* expression, leading to reduced collagen synthesis in fibroblasts derived from patients with scleroderma fibrosis (36). However, bisulfite sequence analy-

sis of the *FLI1* promoter region revealed that most CpGs in CD34<sup>+</sup> HPCs derived from normal and CAMT iPSCs overexpressing MPL were unmethylated and did not significantly differ (data not shown). We therefore conclude that the opposite behavior of CAMT versus normal iPSC-derived HPCs is associated with hyperactivation of *FLI1*, independent of its DNA methylation status.

## Discussion

Disease-specific iPSCs are a powerful tool for replacing invaluable cellular resources that are difficult or impossible to obtain from patients, enabling one to recapitulate a disease phenotype in stepwise fashion during differentiation in vitro (11). For example, the difficulty in obtaining living neurons from patients limits understanding of the pathogenesis of degenerative nerve diseases. On the other hand, in vitro neuronal differentiation systems using patient-derived iPSCs have contributed to clarifying the developmental pathogenesis of some diseases (37–40). Similarly, the rarity of MKs within bone marrow is closely associated with our inability to study normal and pathological megakaryopoiesis and platelet generation directly, especially in patients with severely reduced megakaryopoiesis. In that context, we used an in vitro disease tracing system with CAMT iPSCs to make 2 novel findings about the effect of MPL signaling on human hematopoiesis (Figure 7). First, a loss-of-function study revealed the diversity of MPL signaling required among hematopoietic lineages and recapitulated the clinical course of CAMT. Second, a gene complementation (gain-of-function) study using 2 normal iPSC clones and 4 CAMT iPSC clones overexpressing MPL showed that the program governing differentiation toward MKs or erythrocytes was impaired in CAMT iPSC-derived



HPCs, and that the effect might be determined primarily by FLI1 expression downstream of TPO/MPL signaling. Another important finding of this study was that complimentary transduction of *MPL* into CAMT iPSCs using a retroviral vector enabled expression of different levels of MPL and revealed the critical importance of MPL-mediated signaling. Taken together, these findings indicate that the strength of the intracellular signaling of TPO/MPL to downstream mediators may determine lineage-dependent fate and behavior and that this important process is completely dysregulated by loss of MPL in CAMT patients.

Patients with CAMT exhibit severe thrombocytopenia, which most often begins at birth and worsens into bone marrow failure with aplastic anemia during childhood or sooner. These features ultimately lead to death unless the patient is successfully treated with HSC transplantation. King et al. provided detailed descriptions of the clinical symptoms and disease courses in 20 CAMT patients (9). These patients were divided into 2 groups, CAMT-I and CAMT-II, based on the time course of the platelet counts during their first year of life, which correlated well with the behavior of the pancytopenia and the type of MPL mutations (9). CAMT-I patients exhibited biallelic nonsense or frameshift mutation of *MPL*, leading to complete null expression of the mRNA. These patients had very low platelet counts and developed severe bone marrow failure during childhood. CAMT-II patients exhibited single amino acid substitutions in the intracellular domain of MPL but retained some residual MPL function, which appeared to delay the onset of bone marrow failure compared with CAMT-I patients. The most critical manifestation in both types of this disease was severe thrombocytopenia and anemia, which were evident prior to leukopenia (9). These findings are consistent with the notion that individual hematopoietic lineages have different dependencies on MPL signaling; however, the precise link between MPL and lineage commitment and blood cell generation at defined differentiation steps remains unclear, in large part because of the difficulty of examining HSCs obtained directly from CAMT patients. The *Mpl*<sup>-/-</sup> mouse model, which has normal erythrocyte and leukocyte counts throughout life despite sustained thrombocytopenia, does not recapitulate the human phenotype, illustrating the difference in the MPL signal dependencies of mice and humans. We therefore created human iPSCs using fibroblasts from a CAMT-I patient (7, 21) with compound heterozygous nonsense and frameshift mutations in the *MPL* locus (Supplemental Figure 3). These mutations generated a premature stop codon, leading to rapid degradation of the mutated mRNA through nonsense-mediated mRNA decay (41). In fact, none of the truncated MPL proteins or mRNAs could be detected in CD34<sup>+</sup> HPCs derived from CAMT iPSCs (Supplemental Figure 3, A–C).

In vitro hematopoietic differentiation assays to track the progression of blood generation revealed that the CD34<sup>+</sup>CD43<sup>+</sup>CD41a<sup>-</sup>GPA<sup>-</sup> MPPs derived from iPSC clones created from healthy human specimens can be differentiated into CD41a<sup>+</sup>GPA<sup>+</sup> cells, which contain a MEP population (28) able to give rise to a proper balance of CD41a<sup>+</sup>GPA<sup>-</sup> MKs and CD41a<sup>-</sup>GPA<sup>+</sup> erythrocytes (Figure 3, B and C). In contrast, maintenance of the MPP population and the transition from MPPs to MEPs were completely blocked in CAMT iPSCs (Figure 3, D–F), although some potential for differentiation into the granulocyte and macrophage lineages was retained (Figure 2D). Unlike MPPs derived from normal iPSCs, those from CAMT iPSCs produced smaller numbers of CD43<sup>+</sup> cells (Figure 3G), consistent with the pancytopenia seen in CAMT patients and

indicative of the role of human MPL signaling in MPPs. Moreover, administration of a nonpeptide TPO mimetic, eltrombopag, improved pancytopenia in patients with aplastic anemia (42), suggesting the potential importance of MPL signaling in human HSCs/HPCs. This is consistent with our novel finding that MPL was indispensable for normal erythropoiesis. Thus, by using CAMT iPSCs, we were able to recapitulate the clinical course of CAMT patients, including the distinct features of their blood cell generation and maintenance during childhood (9).

It was previously suggested that in order to keep platelet production in the normal range, MPL signaling must fall within a window that is presumably regulated by either definite serum level of TPO (43, 44), level of MPL expression and its subsequent dimerization and internalization (45), or various inhibitory signaling molecules (5). One possible mechanism for the dysregulation of CAMT iPSC-derived HPCs is a deficiency in MPL signaling, which could also account for the childhood thrombocytopenia observed in patients. Interestingly, experiments involving MPL expression in normal and CAMT iPSCs strongly implied that regulation of the transcriptional factor FLI1, downstream of TPO/MPL at the CD34<sup>+</sup> HPC level, is impaired in CAMT. For dominant production of erythrocytes (rather than MKs) through normal hematopoiesis in humans, erythrocytes must outnumber platelets 20:1. In that case, erythrocytes would likely outnumber MKs 40,000:1, as platelet/MK generation is estimated to be about 2,000:1 (46). Under the conditions of normal human cells, stronger TPO/MPL signaling may suppress FLI1 activity (Figure 6, B and C); determining the mechanism underlying the aberrant FLI1 expression in CAMT iPSCs will require further study.

Our present study also showed that excessive MPL signaling in both normal and CAMT iPSCs led to deleterious megakaryopoiesis and production of CD41a<sup>+</sup>CD42b<sup>-</sup>CD42a<sup>-</sup> MKs and platelets (Figure 5). Furthermore, we confirmed that these aberrant CD42-null platelets were dysfunctional in terms of inside-out signaling (data not shown). Similar aberrant hematopoiesis with greater numbers of immature MKs and reduced erythropoiesis seen with CAMT iPSCs overexpressing MPL is observed clinically in myeloproliferative disease (MPD) patients carrying the MPLW515 mutant, a constitutively active, TPO-hypersensitive mutant that transduces a signal stronger than that of WT MPL upon TPO stimulation (47–49). The degree of similarity between HPCs from MPLW515 patients and the CAMT iPSCs in this study remains uncertain, but both cells are expected to exhibit hematopoiesis that differs from normal.

MKs derived from both normal and CAMT iPSC-derived HPCs overexpressing MPL in the presence of TPO showed augmented pSTAT3, pSTAT5A, and pAKT, but not pERK. However, we were unable to identify the specific downstream signaling molecule(s) responsible for the aberrant megakaryopoiesis through single transduction of a constitutively active form of AKT (50) or STAT5A (Stat5 1\*6) (51, 52) into CD34<sup>+</sup> HPCs derived from human ESCs (data not shown). This implies that enhanced signaling by STAT3 and/or other mediators is involved in the blockade of MK maturation and that MPL expression level governs MK maturation.

CAMT patients require HSC transplantation to survive, but a suitable donor is not always available. iPSC technology may enable development of new gene correction/administration therapies using the patients' own HSCs. In that regard, induction of adult-type HSCs from human PSCs was recently accomplished in vivo through teratoma formation in NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/SzJ (NSG) and NOD/SCID mice (53, 54).



In summary, we used CAMT iPSCs to demonstrate that MPL signaling is indispensable for maintenance of MPPs and for transition from MPPs to MEPs during early hematopoiesis. Its absence led to deficiencies in both erythropoiesis and megakaryopoiesis, although some development of myeloid cells was retained (Figure 7). This constellation of effects recapitulates the clinical course seen in CAMT patients. We were also able to show the true pathogenesis of CAMT, whereby lineage commitment by MEPs toward erythropoiesis is the result of a dysregulated program of activated modification of *FLI1* gene expression. These findings also provide a rationale for the use of MPL-stimulating drugs (i.e., TPO mimetics) in the treatment of anemia as well as other ailments, such as thrombocytopenia and stem cell suppression.

## Methods

Further information can be found in Supplemental Methods.

**Cells, reagents, and viral vectors.** pMX and pMY retroviral vectors were a gift from T. Kitamura (The University of Tokyo, Tokyo, Japan). pGCDNsam IRES EGFP retroviral vector was from M. Onodera (National Children's Research Center, Tokyo, Japan). The CSII lentiviral vector was from T. Yamaguchi (The University of Tokyo, Tokyo, Japan). pMX mRFP1 retroviral vector was from A. Hotta (CiRA, Kyoto University, Kyoto, Japan). The human ESC line was from N. Nakatsuji and H. Suemori (Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan). A normal human iPSC clone, TkDA3-4, was used as a reference (23). The UT-7/TPO cell line was from N. Komatsu (Juntendo University, Tokyo, Japan).

CAMT fibroblasts were cultivated in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (all from Invitrogen). Disease-specific iPSCs derived from dermal fibroblasts from a CAMT patient (CAMT iPSCs) were established using retroviral vectors harboring 4 reprogramming factors (*OCT3/4*, *SOX2*, *KLF4*, and *c-MYC*; clones 1 and 2) or 3 reprogramming factors (*OCT3/4*, *SOX2*, and *KLF4*; clone 3), as previously described (23). All PSCs were maintained as described previously (23). The mouse C3H10T1/2 cell line was purchased from the RIKEN Bio-Resource Center and was cultured as described previously (23). Retroviral supernatants for establishing iPSCs were obtained from a 293 GPG system (provided by R.C. Mulligan, Children's Hospital Boston, Boston, Massachusetts, USA) (25). Human VEGF, TPO, and SCF were from R&D Systems. Human EPO was from Kyowa Hakko Kirin Co. Heparin was from Ajinomoto Pharmaceuticals Co. The metalloproteinase inhibitor GM-6001 was from Cosmobio Co. The following antibodies were used (from BD, unless indicated otherwise): allophycocyanin- (APC)- or PE/Cy7-conjugated anti-CD34, APC-conjugated anti-CD41a (integrin  $\alpha$ IIb; HIP8 clone), eFluor 450-conjugated anti-CD42a (GPIX) (eBioscience), PE-conjugated anti-CD42b (GPIIb), PE-conjugated anti-CD43 (eBioscience), Alexa Fluor 405-conjugated anti-glycophorin A (GPA) (Biolegend), PE-conjugated anti-pAKT, anti-pERK1/2, anti-pSTAT3, and anti-pSTAT5.

**Hematopoietic differentiation of human ESCs and iPSCs.** Differentiation of human ESCs and iPSCs into hematopoietic cells was performed as described previously (22–24). In brief, small clumps of human PSCs (<100 cells treated with PBS containing 0.25% trypsin (Invitrogen), 1 mM CaCl<sub>2</sub> (Sigma-Aldrich), and 20% KSR (Invitrogen) were transferred onto mitomycin-treated or irradiated C3H10T1/2 cells and cocultured in hematopoietic cell differentiation medium, which was replaced every 3 days. On days 14–15 of culture, the HPCs within ESC- and iPSC-Sacs were collected and then sorted into CD34<sup>+</sup> HPCs, CD34<sup>+</sup>CD43<sup>+</sup>CD41a<sup>+</sup>GPA<sup>+</sup> MPPs, and CD41a<sup>+</sup>GPA<sup>+</sup> MEPs using an Aria flow cytometer (BD). They were then transferred onto freshly mitomycin-treated or irradiated feeder cells and further cultivated in differentiation medium supplemented with TPO and combinations of other cytokines/mediators/inhibitors (50 ng/ml SCF, 25 U/ml heparin sodium, 6 U/ml EPO, and 50  $\mu$ M GM-6001),

as described previously (22–24, 29). The medium was refreshed every 3 days, and nonadherent cells were collected and analyzed daily from day 16 to day 24.

**Retroviral and lentiviral vectors and infection.** Full-length *MPL* was subcloned into the retroviral vector pMY IRES EGFP (55). Full-length *FLI1* was subcloned into the retroviral vector pGCDNsam IRES EGFP or lentiviral vector pCSII harboring 2A mRFP1. Viral supernatant was generated as previously described (56). CAMT iPSCs, normal iPSCs, or ESCs were transduced with either vehicle vector or pMY-MPL-EGFP, after which EGFP<sup>+</sup> iPSCs were sorted using a flow cytometer. CD34<sup>+</sup> HPCs derived from normal iPSCs, with or without MPL overexpression, were also transduced with vehicle, pGCDNsam *FLI1*-EGFP, or pCS2 *FLI1*-RFP. This was followed by 8 days of culture in the presence of SCF, TPO, and EPO.

**Hematopoietic colony-forming cell assay.** Hematopoietic colony-forming cell assays were performed as described previously (23). In brief, CD34<sup>+</sup> HPCs from within iPSC-Sacs were cultivated for 14 days in MethoCult H4434 semisolid medium (Stem Cell Technologies Inc.) supplemented with 50 ng/ml human TPO. The colonies were then collected, stained with HemaColor (Merck), and observed under a microscope.

**Flow cytometric analysis of hematopoietic cells.** Nonadherent cells on days 16–24 of culture were prepared in PBS containing 3% FBS (staining medium) and stained with combinations of antibodies for 30 minutes on ice. Samples were then washed with staining medium and analyzed by flow cytometry. Dead cells were identified using PI and excluded. Precise numbers of the cells were estimated using True Count Beads (BD Biosciences).

**Flow cytometric analysis of ploidy in MKs.** Nonadherent cells on days 22–24 were prepared in staining medium and stained with 25  $\mu$ g/ml Hoechst 33342 (Sigma-Aldrich) for 45 minutes at 37°C, followed by anti-human CD41a-APC and CD42b-PE for 30 minutes. Samples were then washed with cold staining medium and analyzed by flow cytometry in the presence of PI.

**Flow cytometric analysis of platelets.** Washed platelets were prepared as described previously (22). The resultant platelet pellets were resuspended with staining medium and stained with anti-human CD41a-APC, CD42a-Pacific blue, and CD42b-PE for 30 minutes at room temperature. The platelets were then diluted in 200  $\mu$ l staining medium and analyzed by flow cytometry. Platelet numbers were estimated using True Count Beads.

**Flow cytometric analysis of the cell cycle.** Nonadherent cells from among MPPs on days 14 + 2 of culture were prepared in staining medium and stained with Vybrant DyeCycle Violet Stain (Invitrogen) for 30 minutes at 37°C. Samples were then stained with PI and analyzed by flow cytometry.

**Phosphorylation of downstream molecules of MPL.** CD34<sup>+</sup> HPCs derived from human iPSCs were starved overnight in differentiation medium containing 0.5% FBS, stained with CD34-APC for 30 minutes at 37°C, and then stimulated for 10 minutes with 100 ng/ml TPO. Immediately thereafter, they were fixed with Lyse/Fix buffer (BD) for 10 minutes at 37°C, permeabilized with Perm buffer III (BD) for 30 minutes on ice, and immunostained for pAKT, pERK1/2, pSTAT3, and pSTAT5 using PE-conjugated antibodies at room temperature for 1 hour. Samples were then washed with staining medium and analyzed by flow cytometry. Similarly, MKs in differentiation medium supplemented with SCF, TPO, and heparin on days 22–24 were stained with anti-CD41a-APC for 30 minutes at 37°C. They were then fixed, permeabilized, stained for downstream mediators, and analyzed as the HPCs were.

**Quantitative RT-PCR.** After sorting CD34<sup>+</sup> HPCs using flow cytometry, RNA was extracted and cDNA was synthesized as described previously (22). Real-time PCR was performed using an EagleTaq Master Mix with ROX and Universal Probe Library kit (Roche Applied Science) according to the manufacturer's instructions. Signals were detected using an ABI7900HT Real-Time PCR System (Applied Biosystems). Primer sets for *GAPDH*, *MPL*, and *KLF1* were determined using the Universal Probe Library Set for humans (<http://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=UP030000>). Primer sets for *FLI1* (Hs00956711\_m1) were purchased from Applied





Biosystems. The following primer sequences were used: *GAPDH* forward, 5'-AACAGCCTCAAGATCATCAGC-3'; *GAPDH* reverse, 5'-TTGGCAG-GTTTTCTAGACGG-3'; *MPL* primer 1 forward, 5'-CAGCGAGTCTCTTT-GTGG-3'; *MPL* primer 1 reverse, 5'-CCCAGCTGATCTGAAGTCC-3'; *MPL* primer 2 forward, 5'-AGGCCATCAGGACTGGAA-3'; *MPL* primer 2 reverse, 5'-CAGCTGTAACGGTAGCGAGA-3'; *MPL* primer 3 forward, 5'-GGTGACCGCTCTGCATCTA-3'; *MPL* primer 3 reverse, 5'-CAGGGCAT-GCCTCAGTCT-3'; *KLF1* forward, 5'-ACACCAAGAGCTCCACCT-3'; *KLF1* reverse, 5'-GTAGTGCGGGTCTCAGCTC-3'.

**Statistics.** All data are presented as mean  $\pm$  SD. The statistical significance of the observed differences was determined using 1-way ANOVA for multiple comparisons and 2-tailed Student's *t* tests for pairwise comparisons. A *P* value less than 0.05 was considered significant.

**Study approval.** The human ESC clone Kyoto hESC-3 (KhES-3) was obtained from the Institute for Frontier Medical Sciences of Kyoto University after approval for human ESC use was granted by the Minister of Education, Culture, Sports, Science, and Technology of Japan (MEXT). Dermal fibroblasts derived from a CAMT patient were obtained at University of Saga School of Medicine (Saga, Japan) under informed consent. The Review Boards for ethics at the Institute of Medical Science, The University of Tokyo, and University of Saga School of Medicine approved this research protocol, including the informed consent. The entire study was conducted in accordance with the Declaration of Helsinki.

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