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MEIS1 Regulates Hemogenic Endothelial Generation, Megakaryopoiesis, and Thrombopoiesis in Human Pluripotent Stem Cells by Targeting TAL1 and FLI1

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SUMMARY

Human pluripotent stem cells (hPSCs) provide an unlimited source for generating various kinds of functional blood cells. However, efficient strategies for generating large-scale functional blood cells from hPSCs are still lacking, and the mechanism underlying human hematopoiesis remains largely unknown. In this study, we identified myeloid ectopic viral integration site 1 homolog (MEIS1) as a crucial regulator of hPSC early hematopoietic differentiation. MEIS1 is vital for specification of APLNR$^+$ mesoderm progenitors to functional hemogenic endothelial progenitors (HEPs), thereby controlling formation of hematopoietic progenitor cells (HPCs). TAL1 mediates the function of MEIS1 in HEP specification. In addition, MEIS1 is vital for megakaryopoiesis and thrombopoiesis from hPSCs. Mechanistically, *FLI1* acts as a downstream gene necessary for the function of MEIS1 during megakaryopoiesis. Thus, MEIS1 controls human hematopoiesis in a stage-specific manner and can be potentially manipulated for large-scale generation of HPCs or platelets from hPSCs for therapeutic applications in regenerative medicine.

INTRODUCTION

Hematopoietic stem cell (HSC) transplantation has successfully been used to treat patients suffering from hematopoietic diseases and malignancies. However, the availability and immunological incompatibility of HSCs limit their clinical applications (Copelan, 2006). Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), are capable of long-term proliferation and can differentiate into all three embryonic germ layers *in vitro*, providing a potential renewable source for generating transplantable HSCs (Inoue et al., 2014). However, despite considerable progress in methods of optimizing hematopoietic differentiation, the efficient generation of HSCs and other genuine functional blood cells from hPSCs has been unsuccessful (Kaufman, 2009; Slukvin, 2016; Vo and Daley, 2015). Therefore, a better understanding of the hematopoietic differentiation progress of hPSCs and its regulatory mechanism is highly demanded before adequate numbers of robustly transplantable HSCs or other functional blood cells from hPSCs can be generated.

Hematopoietic differentiation from hPSCs goes through a sequential series of cell-fate decisions, including specification of mesoderm, lateralization of mesoderm, formation of the hemogenic endothelium progenitors (HEPs), and generation of hematopoietic progenitors through endothelial to hematopoietic transition (Slukvin, 2016). The differentiation process can be monitored by the sequential onset of markers including BRACHYURY, APLNR, CD31/CD34, and CD43/CD45 (Slukvin, 2016). Each stage in hematopoietic differentiation from hPSCs is precisely regulated by different transcriptional factors. Our study has demonstrated that MSX2 is essential for the induction of mesoderm from hPSCs (Wu et al., 2015). The reciprocal repression of NANOG and CDX2 directs mesoderm specification into anterior and posterior subtypes (Mendjan et al., 2014). It has been shown that ectopic expression of *RUNX1a* promotes hematopoietic commitment of hESCs by increasing the expression of mesoderm and hematopoietic development-associated genes (Ran et al., 2013). TAL1 has been reported to promote hematopoietic differentiation of hPSCs through accelerating the generation of HEPs (Real et al., 2012). In line with the critical roles of transcription factors in hematopoietic specification from hPSCs, a recent study demonstrates that the combination of *GATA2/ETV2* or *GATA2/TAL1* can directly program hPSCs to hemogenic endothelium-like cells with distinct hematopoietic potential (Elcheva et al., 2014). Therefore, exploring novel transcription factors implicated in hematopoietic commitment of hPSCs is highly beneficial for large-scale production of transplantable HSCs from hPSCs *in vitro*.

MEIS1 (myeloid ectopic viral integration site 1 homolog) belongs to the MEIS subfamily of TALE (three-amino-acid loop extension) homeodomain-containing transcription

factors. MEIS1 was first identified in BXH-2 leukemic mice, and its aberrant overexpression is required for the induction and maintenance of MLL-fusion-induced leukemia (Collins and Hess, 2016). *MEIS1* shows high expression in HSCs and is downregulated during differentiation except in the megakaryocytic lineage in which it is highly expressed (Pineault et al., 2002). Meis1-deficient zebrafish presents severely impaired primitive and definitive hematopoiesis (Cvejic et al., 2011). Mice lacking Meis1 show extensive hemorrhaging in the trunk and die at embryonic day 14.5. Furthermore, the number of HSCs in Meis1$^{-/-}$ fetal liver is dramatically reduced, and the cells fail to protect lethally irradiated mice (Azcoitia et al., 2005; Gonzalez-Lazaro et al., 2014; Hisa et al., 2004), suggesting the essential role of Meis1 in early mouse hematopoiesis. However, the stage at which Meis1 regulates early hematopoiesis and the underlying mechanism remain to be elucidated. In addition, the role of MEIS1 in early hematopoietic differentiation in humans is still undefined.

The embryonic lethality observed in Meis1$^{-/-}$ mice results from failure of lymphatic-venous separation during embryonic angiogenesis due to the absence of megakaryocytes (Carramolino et al., 2010). Elevated expression of *Meis1* in mouse embryonic stem cells promotes megakaryocytic progenitor differentiation while suppressing erythroid progenitor development at the megakaryocyte-erythroid progenitor (MEP) stage (Cai et al., 2012). *MEIS1* overexpression directs human hematopoietic progenitor cells (HPCs) toward the MEP fate and enhances megakaryocytic colony formation ability (Zeddies et al., 2014). Although these studies demonstrate the importance of MEIS1 in megakaryocytic differentiation, the precise roles of MEIS1 in megakaryocytic maturation, platelet formation, and the underlying mechanisms remain to be defined.

In this study, by taking advantage of a chemical-defined hematopoietic differentiation model, whole-genome gene profiling and the CRISPR/CAS9 technology, we identified MEIS1 as a crucial regulator for hPSC differentiation into functional hematopoietic cells. We also found that MEIS1 regulates hematopoietic differentiation in a stage-specific manner and by targeting the transcription factors TAL1 and FLI1. Together, we define a role of MEIS1 in human development, unveil new mechanisms for human hematopoiesis, and contribute potential new strategies to regenerative medicine.

RESULTS

MEIS1 as a Potential Regulator of Early Human Hematopoietic Differentiation

To identify key regulators of human early hematopoietic differentiation, we induced directed hematopoietic differentiation of H1 hESCs in a chemically defined system (CDS) by using a previously reported strategy with modifications (Pang et al., 2013; Wang et al., 2012) (Figure S1A). We performed time course RNA sequencing (RNA-seq) analysis in hESC samples collected from day 0 to day 4 after differentiation. Gene set enrichment analysis (GSEA) demonstrated that hematopoiesis-related genes were significantly enriched in the differentiated cells at day 4 compared with undifferentiated cells, thus validating our screening strategy for hematopoietic gene screening (Figure 1A). To identify key transcription factors governing differentiation, 68 transcriptional factors upregulated gradually and steadily during early hematopoietic differentiation of H1 hESCs were selected (see Table S5). After 4 days of differentiation, the mRNA levels of all these factors increased by more than 10-fold (Figure 1B and Table S5; false discovery rate [FDR] < 0.01). Interestingly, several previously reported genes crucial for mammalian hematopoiesis such as *GATA2, HOXA9, GFI1, HOXA7*, and *HOXA5* were identified (Dou et al., 2016; Huang et al., 2015; Ramos-Mejia et al., 2014; Sandler et al., 2014), thereby validating the screening strategy (Figure 1B). We were particularly interested in MEIS1 because of its previously documented roles in leukemogenesis and early hematopoiesis in animals (Azcoitia et al., 2005; Collins and Hess, 2016).

We first confirmed *MEIS1* upregulation during early hematopoietic differentiation of both H1 hESCs and an hiPSC line, BC1 (Chou et al., 2011) (Figure 1C). *MEIS*1 upregulation was also observed in H1 and BC1 cells induced to undergo hematopoietic differentiation in the presence of the mouse AGM-S3 (mAGM-S3) feeder cells (Figure 1D), which have been shown to efficiently induce hematopoietic differentiation of hPSCs (Mao et al., 2016). With this method, CD43$^+$ and CD45$^+$ HPCs can be generated in a stepwise manner (Figure S1B). Furthermore, in both CDS and mAGM-S3 co-culture systems, CD43$^+$ cells could further differentiate into CD45$^+$ hematopoietic cells (Figure S1C) and generated all types of hematopoietic colonies including BFU-E, CFU-E, CFU-GM, and CFU-GEMM (Figure S1D). These data demonstrated that both methods led us to successfully generate functional hematopoietic cells from hPSCs. We therefore further measured the expression of *MEIS1* in different populations of hematopoietic cells. In CD43$^+$ and CD45$^+$ hematopoietic cells derived from H1 and BC1 cells, *MEIS1* showed expression levels comparable to that in human cord-blood-derived CD34$^+$ cells. In contrast, *MEIS1* expression was nearly undetectable in undifferentiated cells (Figures 1E and 1F). In addition, we also detected the expression of other hematopoietic differentiation-associated transcriptional factors listed in Figure 1B. As shown in Figure S1E, *GATA2* and *GFI1* showed comparable expression levels in the hematopoietic cells derived

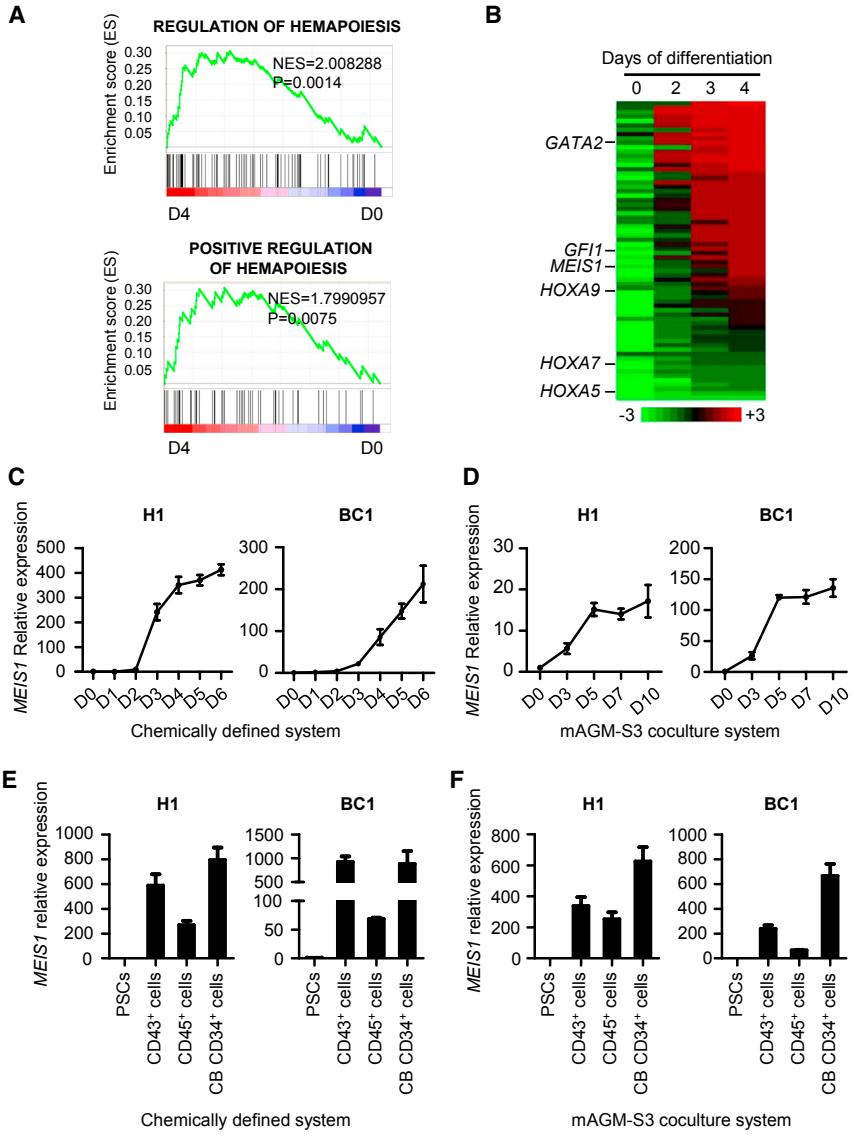

Figure 1. MEIS1 as a Potential Regulator of Early Hematopoietic Differentiation of hPSCs

(A) GSEA comparison of the differentiated cells at day 4 with undifferentiated cells: the enrichment of genes involved in regulation of hematopoiesis (top) and positive regulation of hematopoiesis (bottom) in the differentiated cells at day 4. NES, normalized enrichment score.

(B) Heatmaps of gradually increased transcriptional factors during early hematopoietic differentiation from H1 hESCs. RNA-seq analysis was performed on samples collected at day 0, day 2, day 3, and day 4 of differentiation.

(C and D) Time course analysis of *MEIS1* expression during hematopoietic differentiation of hESCs (H1) or hiPSCs (BC1) under chemically defined condition (C) or in mAGM-S3 co-culture (D).

(E and F) Real-time PCR analysis of *MEIS1* in $CD34^+$ cells from human cord blood and the hematopoietic cells derived from H1 or BC1 cells under chemically defined condition (E) or cultured with the mAGM-S3 co-culture system (F). Relative expression is normalized to the level (= 1) of mRNA in undifferentiated H1 or BC1 cells.

Error bars represent mean ± SEM of samples from three independent experiments.

from hPSCs and the $CD34^+$ cells isolated from human cord blood. However, the expression of *HOXA* genes (*HOXA5, HOXA7*, and *HOXA9*) in the hematopoietic cells derived from hPSCs was obviously lower than that of the $CD34^+$ cells isolated from human cord blood. Consistent with our results, low expression of *HOXA* genes can also be found in published transcriptome analysis of hPSC-derived hematopoietic cells (Dou et al., 2016; Ferrell et al., 2015; Ng et al., 2016). Thus, we identified MEIS1 as a potential regulator of early hematopoietic differentiation in hPSCs.

MEIS1 Deletion Impairs Early Hematopoietic Differentiation

We next addressed the potential role of MEIS1 in hematopoietic specification of hPSCs. We created targeted deletion for the *MEIS1* gene in H1 hESCs and BC1 cells using the CRISPR/CAS9 technology. Small guide RNAs (sgRNAs) targeting different exons of the human *MEIS1* gene were designed and tested for their genome editing efficacy (Figures 2A, S2A, and S2B). After several clone picking selections, we generated cell clones with homozygous MEIS1 deletion in both H1 hESCs and BC1 cells with E3G1 sgRNA. Expression of *MEIS1* was completely absent (Figure 2B), while sequencing analysis confirmed frameshifts due to deletion or insertion (Figure 2C). We also included a MEIS1 heterozygous clone derived from H1 hESCs in parallel with the MEIS1 homozygous clones for future analyses.

MEIS1 deletion had no effect on hPSC pluripotency. Both $MEIS1^{-/-}$ H1 cells and $MEIS1^{-/-}$ BC1 cells grew as compact and morphologically normal colonies. Real-time PCR,

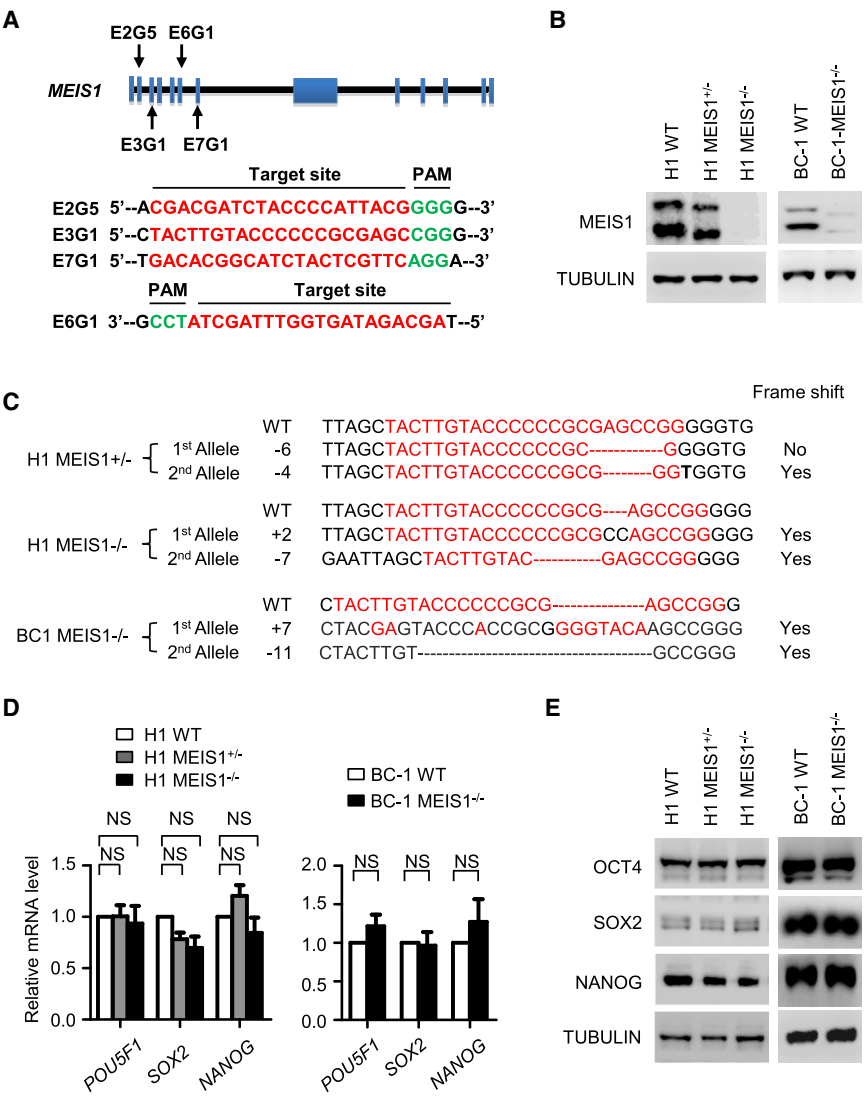

Figure 2. Establishment of MEIS1-Deleted hPSC Lines Using the CRISPR/CAS9 Technology

(A) Schematics of the Cas9/sgRNA-targeting sequences at the human MEIS1 locus.

(B) Western blotting analysis of MEIS1 in H1, H1 MEIS1$^{+/-}$, and H1 MEIS1$^{-/-}$ cells or BC1 and BC1 MEIS1$^{-/-}$ cells. WT and MEIS1-deleted hPSCs were treated with 10 μM retinoic acid for 4 days. Tubulin was used as a loading control.

(C) DNA-sequencing results of H1 MEIS1$^{+/-}$, H1 MEIS1$^{-/-}$, and BC1 MEIS1$^{-/-}$ cells.

(D) Real-time PCR analysis of *POU5F1*, *SOX2*, and *NANOG* in undifferentiated H1, H1 MEIS1$^{+/-}$, and H1 MEIS1$^{-/-}$ cells or BC1 and BC1 MEIS1$^{-/-}$ cells. Relative expression is normalized to the level (= 1) of mRNA in H1 or BC1 cells.

(E) Western blotting analysis of OCT4, SOX2, and NANOG in undifferentiated H1, H1 MEIS1$^{+/-}$, and H1 MEIS1$^{-/-}$ cells or BC1 and BC1 MEIS1$^{-/-}$ cells. Tubulin was used as a loading control.

Error bars represent mean ± SEM of samples from three independent experiments. NS, not significant.

western blot, and immunofluorescence analyses further showed that MEIS1 deletion did not alter the expression of pluripotency markers such as NANOG, OCT4, and SOX2 (Figures 2D, 2E, and S2C).

In contrast, MEIS1 deletion profoundly impaired hematopoietic differentiation of hPSCs. In experiments with the mAGM-S3 co-culture system, MEIS1 deletion reduced the number of CD43^{+} HPCs by 2-fold, as assessed with flow cytometry and immunofluorescence analysis in both H1 cells (wild-type [WT] 9.17% ± 0.65% versus MEIS1$^{-/-}$ 4.20% ± 0.52%, $p < 0.01$) and BC1 (WT 5.57% ± 0.42% versus MEIS1$^{-/-}$3.03% ± 0.07%, $p < 0.05$) cells (Figures 3A and S2D). Similar decrease in CD43^{+} HPCs was observed in cells produced under the CDS condition (H1, WT 5.20% ± 0.15% versus MEIS1$^{-/-}$ 2.24% ± 0.27%, $p < 0.001$; BC1, WT 7.84% ± 0.62% versus MEIS1$^{-/-}$ 3.44% ± 0.48%, $p < 0.01$) (Figure S2E). To further demonstrate that MEIS1 deletion impairs hematopoietic differentiation of hPSCs, we measured the induction of CD45^{+} hematopoietic cells, which arise from CD43^{+} HPCs (Slukvin, 2016). Indeed, the number of CD45^{+} cells was much lower with MEIS1 deletion in both H1 hESCs (H1 WT 7.70% ± 0.76% versus H1 MEIS1$^{-/-}$ 2.55% ± 0.92%, $p < 0.05$) and BC1 cells (BC1 WT 5.06% ± 0.36% versus BC1 MEIS1$^{-/-}$ 2.48% ± 0.20%, $p < 0.01$) (Figure 3B). The decrease in CD43^{+} and CD45^{+} HPCs was also observed in MEIS1 heterozygous cells (Figures 3A and 3B). Thus, MEIS1 deletion impairs hematopoietic differentiation of hPSCs.

MEIS1 Deletion Suppresses HEP Specification

The decrease in production of hematopoietic cells from hPSCs caused by MEIS1 deletion may result from (1)

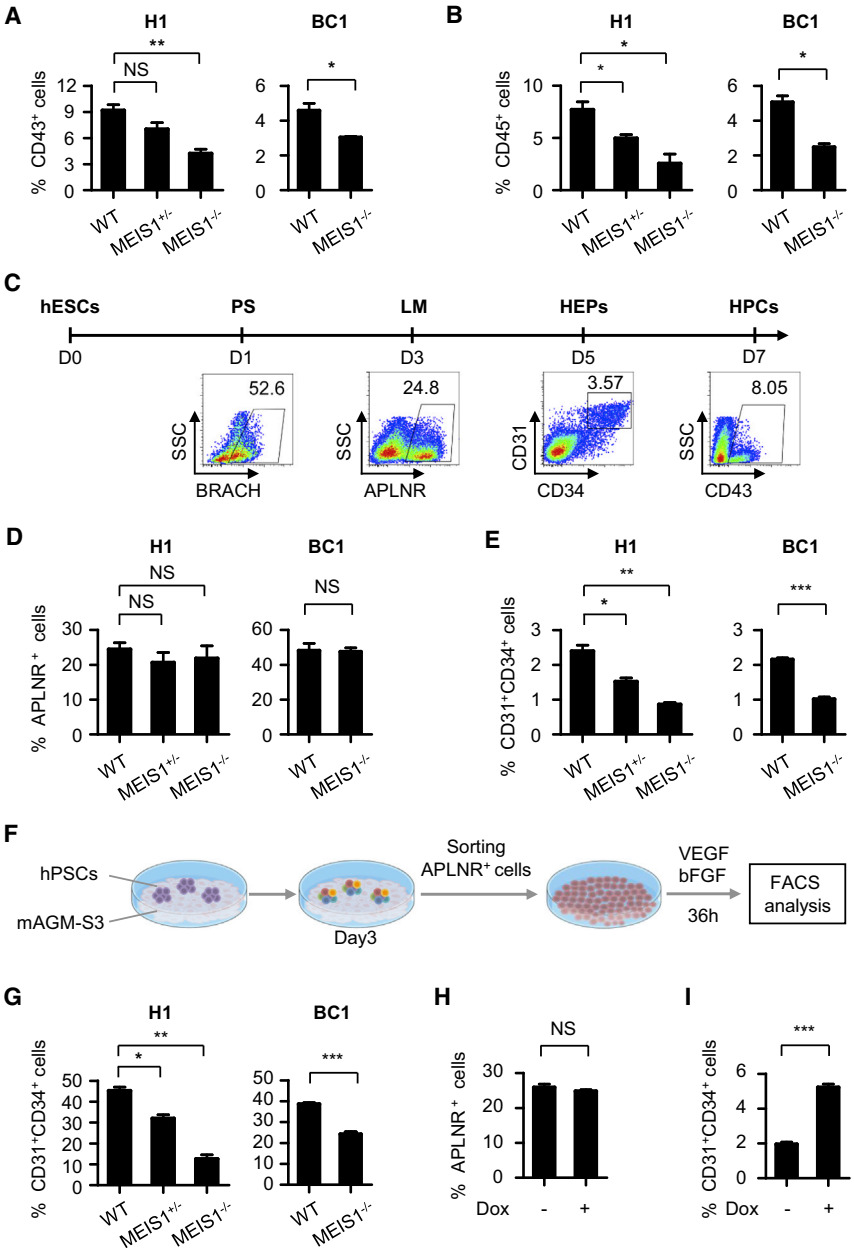

Figure 3. MEIS1 Deletion Impairs Early Hematopoietic Differentiation of hPSCs by Suppressing HEP Specification

(A) Flow cytometry analysis of the percentage of $CD43^+$ hematopoietic precursors at day 7 of differentiation from WT and MEIS1-deleted hPSCs in mAGM-S3 co-culture.

(B) Flow cytometry analysis of the percentage of $CD45^+$ blood cells at day 12 of differentiation from WT and MEIS1-deleted hPSCs in mAGM-S3 co-culture.

(C) Representative flow cytometry dot plots showing the sequential emergence of $brachyury^+$ mesoderm cells, $APLNR^+$ lateral mesoderm cells, $CD31^+CD34^+$ HEPs, and $CD43^+$ hematopoietic cells during hPSC hematopoietic differentiation in the mAGM-S3 co-culture system.

(D) Flow cytometry analysis of the percentage of $APLNR^+$ lateral mesoderm cells at day 3 of differentiation from WT and MEIS1-deleted hPSCs in mAGM-S3 co-culture system.

(E) Flow cytometry analysis of the percentage of $CD31^+CD34^+$ HEPs at day 5 of differentiation from WT and MEIS1-deleted hPSCs in mAGM-S3 co-culture.

(F) Schematic diagram showing the HEP potential analysis of $APLNR^+$ cells at day 3 of differentiation in mAGM-S3 co-culture system. FACS, fluorescence-activated cell sorting.

(G) Flow cytometry analysis of the percentage of $CD31^+CD34^+$ HEPs transited from $APLNR^+$ cells.

(H) Flow cytometry analysis of the percentage of $APLNR^+$ lateral mesoderm cells at day 3 of differentiation from H1 hESCs without doxycycline (Dox −) or with 1 μg/mL doxycycline (Dox +), which induces MEIS1 expression in mAGM-S3 co-culture.

(I) Flow cytometry analysis of the percentage of $CD31^+CD34^+$ HEPs at day 5 of differentiation from H1 hESCs without or with MEIS1 overexpression in mAGM-S3 co-culture.

Error bars represent mean ± SEM of samples from three independent experiments. NS, not significant, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

suppressed proliferation or increased apoptosis of hematopoietic cells or (2) decreased generation of hematopoietic cell precursors. To distinguish between these possibilities, we assessed the rates of proliferation and apoptosis of $CD43^+$ HPCs. No significant changes in the fraction of cycling or apoptotic cells were detected in $CD43^+$ hematopoietic cells with MEIS1 deletion in both H1 hESCs and BC1 cells (Figures S2F and S2G). These results led us to hypothesize that the impaired generation of early hematopoietic cell precursors with MEIS1 deletion might result from decreased numbers of $CD43^+$ and $CD45^+$ HPC generation.

The entire hematopoietic differentiation process from H1 hESCs could be monitored in both the CDS and the mAGM-S3 culture system (Figures 3C and S1A). Furthermore, $CD31^+CD34^+$ cells derived from H1 hESCs could further differentiate into both endothelial cells and hematopoietic cells in the OP9 co-culture model (Uenishi et al., 2014) (Figure S3A). These results confirmed

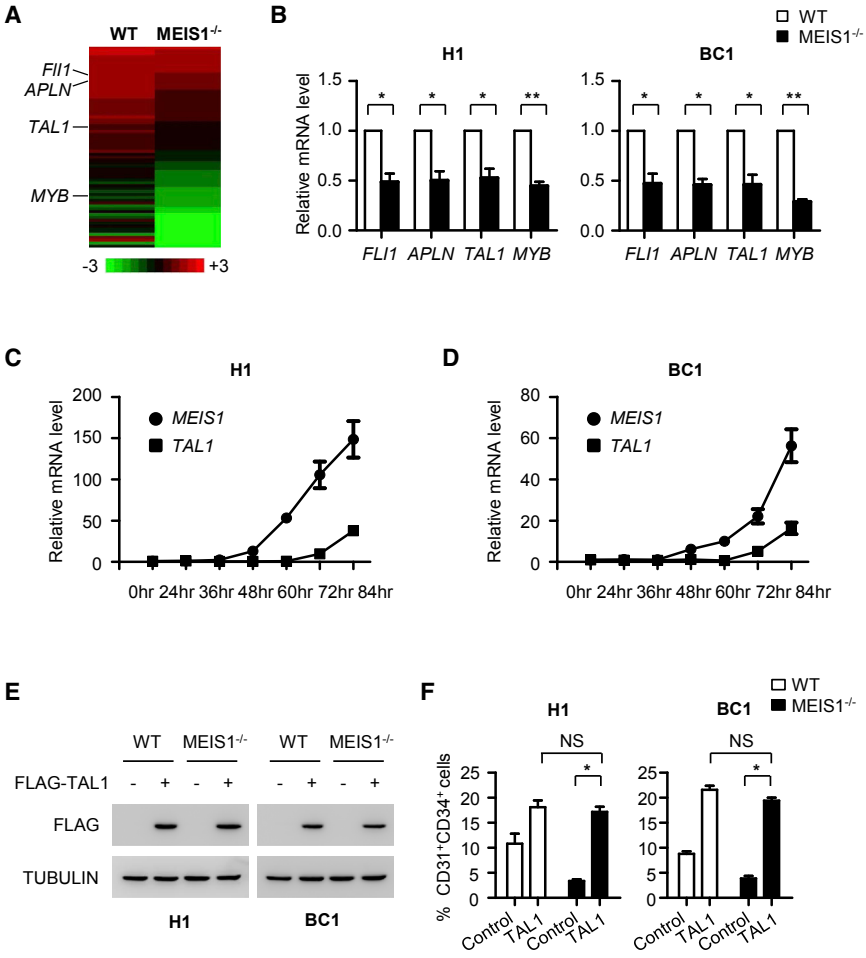

Figure 4. MEIS1 Regulates the Specification of HEP Cells by Targeting TAL1

(A) Heatmaps of 70 downregulated genes in cells differentiated from H1-MEIS1$^{-/-}$ compared with those from differentiated H1 cells.

(B) Real-time PCR analysis of *TAL1* in cells differentiated from WT and MEIS1-deleted hPSCs at day 3 of differentiation in chemically defined medium.

(C and D) Time course analysis of gene expression of *MEIS1* and *TAL1* in H1 (C) or BC1 (D) during hematopoietic differentiation in chemically defined medium with real-time PCR. All values are normalized to the level (= 1) of mRNA in cells cultured in mTeSR1 before differentiation (0 hr).

(E) Expression of FLAG-MEIS1 protein in hPSCs infected with a lentivirus carrying the MEIS1-2A-GFP cassette. Vector carrying only GFP was used as a control.

(F) Flow cytometry analysis of the percentage of CD31^{+}CD34^{+} HEPs differentiated from WT and MEIS1-deleted hPSCs without or with *TAL1* overexpression. GFP^{+} gated events are shown.

Error bars represent mean ± SEM of samples from three independent experiments. NS, not significant, *p < 0.05 and **p < 0.01.

CD31^{+}CD34^{+} cells generated in both cultures are functional intact HEPs. No significant changes in the fraction of brachyury^{+} mesoderm cells or APLNR^{+} lateral mesoderm cells were observed with MEIS1 deletion (Figures 3D and S3B). In contrast, MEIS1 deletion in H1 hESCs caused the population of CD31^{+}CD34^{+} HEPs to reduce by approximately 3-fold (2.39% ± 0.17% versus 0.86% ± 0.05%, $p < 0.01$) (Figure 3E, left). A similar decrease was also observed in BC1 cells (Figure 3E, right). Similar decreases in CD31^{+}CD34^{+} HEPs were also observed in H1 hESCs and BC1 cells under CDS conditions (Figure S3C). Furthermore, the defects of HEP generation caused by MEIS1 deletion could be rescued by forced expression of *MEIS1* (Figure S3D).

To directly test whether MEIS1 deletion impairs HEP specification, we sorted APLNR^{+} cells and induced them to HEPs by adding vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) to the culture (Figure 3F). As expected, MEIS1 deletion profoundly inhibited the transition of CD31^{+}CD34^{+} HEPs from APLNR^{+} cells (Figure 3G). Interestingly, MEIS1 level was much higher in CD31^{+}CD34^{+} cells than in APLNR^{+} cells derived from H1 hESCs and BC1 cells (Figure S3E).

Finally, we asked whether MEIS1 plays a causal role in HEP specification, and we overexpressed *MEIS1* in H1 hESCs. *MEIS1* overexpression significantly enhanced the production of CD31^{+}CD34^{+} HEPs while exerting little effect on APLNR^{+} cell induction (Figures 3H and 3I). Together, our findings demonstrated that MEIS1 acts as a pivotal regulator of hPSC early hematopoietic differentiation and specifically controls HEP specification from APLNR^{+} lateral mesoderm cells.

MEIS1 Controls HEP Specification by Targeting TAL1

To investigate the molecular mechanism by which MEIS1 controls HEP specification, we performed RNA-seq analysis of cells undergoing HEP transition, with or without MEIS1 deletion. After differentiation, a large number of genes were downregulated in MEIS1-deleted H1 hESCs compared with the WT cells (Figure 4A, Table S5, FDR < 0.01). Among

those, a number of mammalian hematopoiesis-associated genes such as *FLI1*, *APLN*, *TAL1*, and *MYB* were significantly downregulated, again showing that hematopoiesis was impaired with MEIS1 deletion. Decreased gene expression of *FLI1, APLN, TAL1*, and *MYB* was further validated in both H1 hESCs and BC1 cells with real-time PCR analysis (Figure 4B). Due to their previously documented roles in human hematopoiesis (Real et al., 2012; Yu et al., 2012), we hypothesized that APLN or TAL1 might mediate the function of MEIS1 during HEP specifications. To test this hypothesis, we first applied APLN directly to MEIS1$^{-/-}$ H1 hESCs induced to undergo hematopoietic differentiation. Surprisingly, APLN addition failed to rescue the decrease in CD31^{+}CD34^{+} HEPs caused by MEIS1 deletion (Figure S4A). We next focused on the role of TAL1 in mediating the function of MEIS1. Time course analysis revealed that *MEIS1* was upregulated within 36–48 hr and by approximately 10-fold at 48 hr after differentiation, when APLNR^{+} lateral mesoderm cells emerged (Figure 4C). In contrast, *TAL1* began to increase after 60–72 hr of differentiation induction. Similar results were observed in BC1 cells (Figure 4D). In addition, *MEIS1* was significantly upregulated in APLNR^{+} cells compared with undifferentiated hPSCs. In contrast, expression of *TAL1* was extremely low with little difference between APLNR^{+} cells and undifferentiated hPSCs (Figures S4B and S4C). These results suggested that the expression of *MEIS1* preceded the induction of *TAL1* expression during hPSC early hematopoietic differentiation. Meanwhile, forced expression of *MEIS1* significantly increased the level of *TAL1* at day 3 after differentiation (Figure S4D), further implying that MEIS1 might act as a potential upstream regulator of TAL1. We next tested the role of TAL1 by expressing *TAL1-2A-GFP* in hPSCs via lentiviral infection. Overexpression of *TAL1* at both the protein and the mRNA level in H1 hESCs and BC1 cells was verified (Figures 4E and S4E). Strikingly, flow cytometry analysis demonstrated that *TAL1* overexpression completely reversed the decrease in CD31^{+}CD34^{+} HEPs caused by MEIS1 deletion in both H1 hESCs and BC1 cells (Figure 4F). Thus, TAL1 mediates the function of MEIS1 in converting APLNR^{+} cells to HEPs during hPSC hematopoietic differentiation.

MEIS1$^{-/-}$ Megakaryocytes Fail to Undergo Polyploidization

The important roles of MEIS1 in hPSC early hematopoietic differentiation led us to ask whether it might play additional roles in HPC differentiation into multilineage functional blood cells. We enriched CD43^{+} HPCs produced after 12-day differentiation from hPSCs and assessed their behavior in the colony-forming unit (CFU) assay, and no significant differences in colony numbers and types were detected in cells with or without MEIS1 deletion (Figure S5A).

It was previously reported that Meis1 deletion disrupts the generation of megakaryocytes *in vivo* (Azcoitia et al., 2005; Carramolino et al., 2010; Gonzalez-Lazaro et al., 2014; Hisa et al., 2004), leading us to determine the megakaryocytic potential of MEIS1$^{-/-}$ HPCs in a more specialized culture system containing thrombopoietin (TPO) and other cytokines. This was also because the CFU assay was not well suited for assessing the differentiation potential of HPCs to the megakaryocytic lineages. In this culture system, large differentiated cells emerged at day 3 and peaked in number at day 6, while proplatelets were also observed at day 6 (Figure S5B). Flow cytometry, May-Grünwald Giemsa (MGG) staining, and the spreading assay further confirmed the generation of functional megakaryocytes (Figures S5B and S5C). Similar to platelets from peripheral blood, hPSC-derived platelet-sized particles (PLPs) responded normally to agonist stimulation as demonstrated by adhesion, spreading, aggregation, and α-granule release (Figures S5D–S5G), indicating that hPSC-derived megakaryocytes in our systems can produce viable and functional intact PLPs. Interestingly, *MEIS1* was upregulated during the process (Figure S5H), suggesting that it might also play a role in megakaryocytic differentiation. Indeed, by using flow cytometry and immunostaining, we found that MEIS1 deletion robustly inhibited the generation of CD41a^{+}CD42b^{+} megakaryocytes in both differentiating H1 hESCs and BC1 cells (Figures 5A and S6A). In addition to the overall decrease in CD41a^{+}CD42b^{+} megakaryocytes, quantification of cell diameters demonstrated that MEIS1-deleted cells differentiated from HPCs were much smaller compared with the WT cells (H1 hESCs 18.86 ± 0.45 μm versus 29.07 ± 0.66 μm; BC1 cells 20.06 ± 0.48 μm versus 29.33 ± 0.78 μm, $p < 0.001$, $n = 100$) (Figures 5B and S6B). The decrease in cell volume with MEIS1 deletion was also shown in experiments with flow cytometry (Figure 5C). Thus, MEIS1 is pivotal for hPSC megakaryocytic differentiation.

The decrease in cell size caused by MEIS1 deletion suggested that the CD41^{+} megakaryocytes produced from MEIS1$^{-/-}$ HPCs might fail to mature properly. In general, the process of megakaryocyte maturation consists of three major steps: the emergence and expansion of the demarcation membrane system (DMS), polyploidization mediated by endomitosis, and production of proplatelets followed by the release of platelets (Machlus and Italiano, 2013). To reveal the ultrastructure of the megakaryocytes derived from hPSCs, we used thin-section electron micrography to observe granules, mitochondria, DMS, and lobulated nuclei, allowing us to clearly visualize these structures in WT hPSC-derived megakaryocytes (Figure S6C, left). In contrast, the granules and DMS in MEIS1$^{-/-}$ megakaryocytes were largely absent (Figure S6C, right), suggesting defects in the differentiation program. We therefore

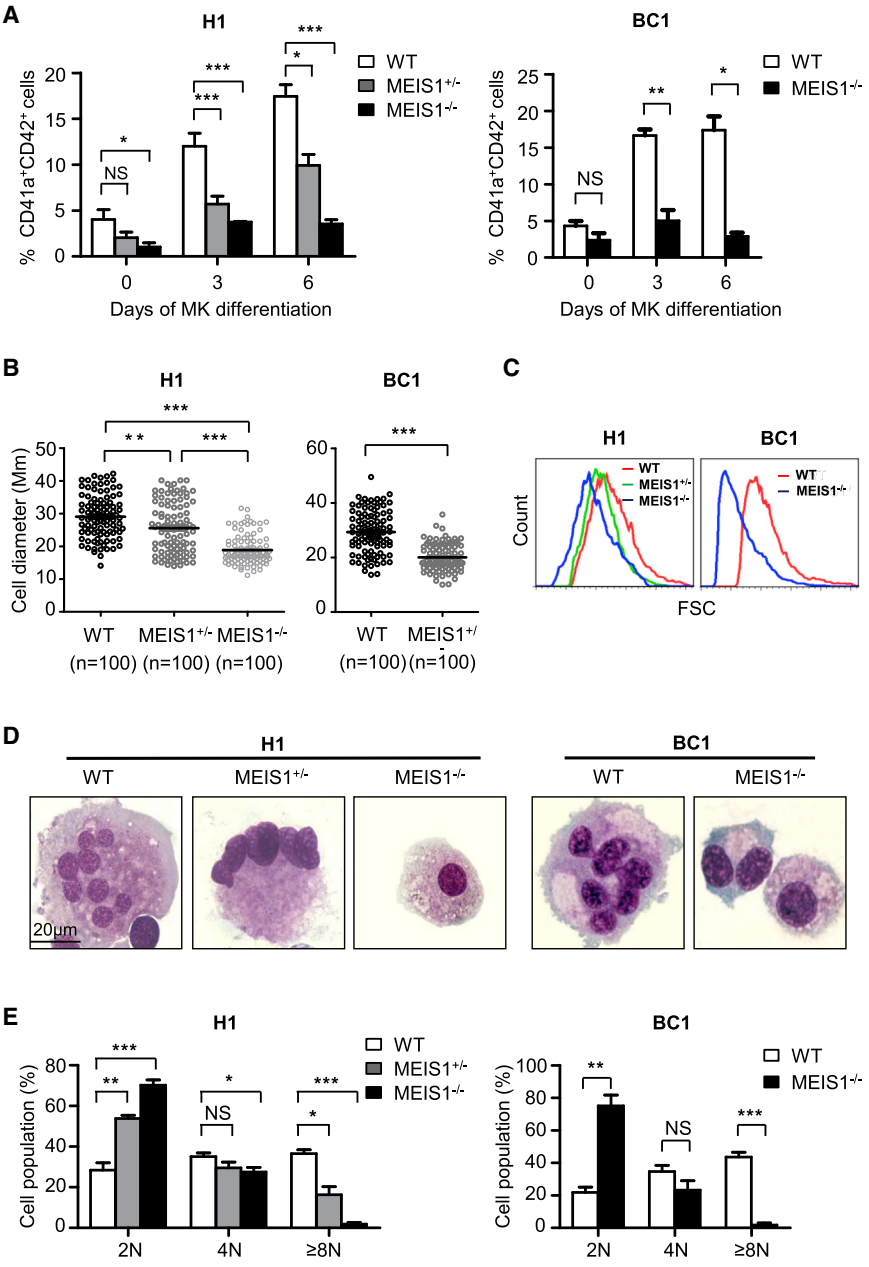

Figure 5. Megakaryocytes Generated from MEIS1$^{-/-}$ HPCs Fail to Undergo Polyploidization

(A) Flow cytometry analysis of the percentage of CD41a^{+}CD42b^{+} megakaryocytes at various stages of megakaryocytic differentiation.

(B) Distribution of cell sizes (100 cells) in each group as measured by a hemocytometer.

(C) The cell size at day 3 of megakaryocytic differentiation as measured by flow cytometry.

(D) Morphology analysis of megakaryocytes at day 3 of megakaryocytic differentiation by MGG staining (scale bar, 20 μm).

(E) Ploidy distribution of megakaryocytes at day 3 of megakaryocytic differentiation was analyzed by staining cellular DNA with propidium iodide.

Error bars represent mean ± SEM of samples from three independent experiments. NS, not significant, *p < 0.05, **p < 0.01, ***p < 0.001. MK, megakaryocytic.

determined polyploidization in hPSC-derived megakaryocytes with or without MEIS1 deletion. MGG staining showed that large-size megakaryocytes with high degree of polyploidy were generated from WT H1 hESCs and BC1 cells (Figure 5D). In contrast, the megakaryocytes derived from MEIS1$^{-/-}$cells were much smaller in size and mostly contained two or even fewer nuclei (Figure 5D). Analysis of DNA content further revealed that most megakaryocytes differentiated from MEIS1$^{-/-}$ H1 hESCs were arrested at 2N or 4N stage, with very few cells beyond >8N stage (≥8N, WT 36.56% ± 2.31% versus MEIS1$^{-/-}$1.86% ± 0.72%, p < 0.001) (Figures 5E and S6D, left). The same deficiency was also observed in megakaryocytes derived from BC1 cells (≥8N, WT 43.52% ± 5.90% versus MEIS1$^{-/-}$ 1.76% ± 1.27%, p < 0.001) (Figures 5E and S6D, right). Together, our results demonstrated that MEIS1 is vital for DMS development and polyploidization during megakaryocyte maturation.

MEIS1 Deletion Abolishes Thrombopoiesis

Because DMS development and polyploidization are indispensable for thrombopoiesis, we next explored the effects

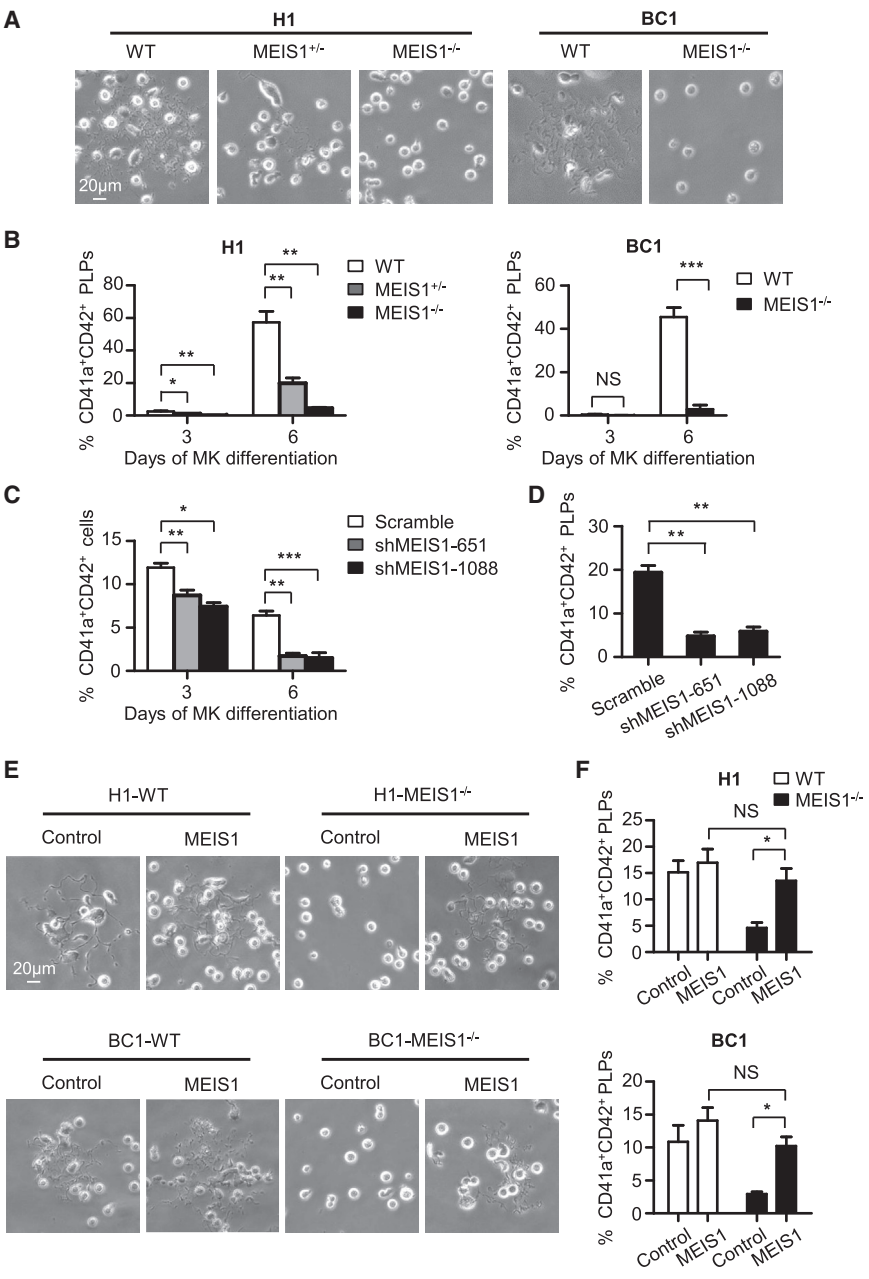

Figure 6. MEIS1 Deletion Completely Abrogates Thrombopoiesis

(A) The morphology of representative proplatelets at day 6 of megakaryocytic differentiation (scale bar, 20 μm).

(B) Flow cytometry analysis of the percentage of $CD41a^+CD42b^+$ PLPs in the culture supernatant at day 6 of megakaryocytic differentiation.

(C) Generation of $CD41a^+CD42b^+$ megakaryocytes from HPCs with MEIS1 depletion induced by shMEIS1-651 or shMEIS1-1088. A scramble shRNA was used as a control.

(D) Generation of $CD41a^+CD42b^+$ PLPs derived from HPCs without or with MEIS1 depletion.

(E) Phase-contrast images of proplatelet formation from WT and MEIS1-deleted hPSCs without or with *MEIS1* overexpression. Cells were cultured on plates precoated with 100 mg/mL fibrinogen for convenient observation. Vector carrying GFP was used as a control (scale bar, 20 μm).

(F) Flow cytometry analysis of the percentage of $CD41a^+CD42b^+$ PLPs derived from WT and MEIS1-deleted hPSCs without or with *MEIS1* overexpression.

Error bars represent mean ± SEM of samples from three independent experiments. NS, not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. MK, megakaryocytic.

of MEIS1 deletion on thrombopoiesis. While a large number of proplatelet-forming megakaryocytes were found from WT H1 hESCs and BC1 cells (Figure 6A, Movie S1), no proplatelets were detected in megakaryocytes derived from MEIS1-deleted cells (Figure 6A and Movie S2). Thus, MEIS1 deletion completely abolishes thrombopoiesis. In keeping with the results from MEIS1-deleted cells, many fewer proplatelets were produced from $MEIS1^{+/-}$ megakaryocytes (Figure 6A). We next assessed platelet production directly by measuring the percentages of PLPs collected from culture supernatant at day 6 after differentiation. Consistent with the earlier findings, $CD41a^+CD42b^+$ PLPs were nearly undetectable from culture supernatant of megakaryocytes with MEIS1 deletion (H1 hESCs 57.26% ± 6.13% versus 4.53% ± 0.57%; BC1 cells 45.38% ± 4.33% versus 2.90% ± 1.81%; $p < 0.01$) (Figure 6B).

To exclude the possibility that the decreased megakaryocytic differentiation might result from earlier HEP defects caused by MEIS1 deletion, we depleted MEIS1 directly in $CD43^+$ HPCs using small hairpin RNAs (shRNAs) and determined the impact on megakaryocytic differentiation and platelet generation (Figure S6E). Indeed, *MEIS1* knockdown

from CD43$^+$ HPCs caused the same phenotype as observed in MEIS1-knocked-out H1 hESCs (Figures 6C, 6D, and S6F). These results indicated that MEIS1 deletion indeed impaired megakaryopoiesis and thrombopoiesis, independent of the earlier HEP defects.

To further confirm that the deficiency in thrombopoiesis was due to the loss of MEIS1, we determined whether *MEIS1* ectopic expression could rescue the defects caused by MEIS1 deletion (Figure S6G). As shown in Figures 6E and 6F, overexpression of *MEIS1* in WT HPCs failed to further improve the efficiency of platelet generation. In contrast, overexpression of *MEIS1* markedly increased generation of PLPs from MEIS1$^{-/-}$ HPCs. Strikingly, there was no statistical difference between WT and MEIS1$^{-/-}$cells when *MEIS1* was overexpressed. Together, we provide evidence for an essential role of MEIS1 in human thrombopoiesis.

MEIS1 Controls Megakaryocytic Specification by Enhancing *FLI1* Expression

We next assessed the mechanism by which MEIS1 controls megakaryocytic maturation and thrombopoiesis. We conducted RNA-seq analysis, aiming to identify the downstream targets that mediate the function of MEIS1. We detected several hundreds of downregulated genes in cells with MEIS1 deletion ($p < 0.001$, FDR< 0.001, Table S5). GO analysis showed that 8 of the top 10 most significant biological processes were associated with platelet functions (Figure 7A). The GSEA based on KEGG gene sets also showed significant enrichment in platelet-specific genes in WT cells, whereas such enrichment was not seen in MEIS1-deleted cells (Figure S7A). The results from large-scale genomic analysis again demonstrated the reliability of our platelet generation system and verified that MEIS1 deletion indeed severely impairs thrombopoiesis.

Among the genes with significantly altered expression, a number of hematopoiesis-associated genes such as *PBX1* and *FLI1* were identified (Figure 7B and Table S5). We focused on FLI1 because of its previously reported roles in megakaryopoiesis (Li et al., 2015). Real-time PCR analysis confirmed downregulation of *FLI1* in MEIS1-deleted H1 hESCs and BC1 cells (Figure 7C). We then tested the function of FLI1 by overexpressing *FLI1-P2A-GFP* by using the lentivirus system (Figure S7B). Ectopic expression of *FLI1* was confirmed by real-time PCR and western blot analysis (Figures S7C and S7D). Remarkably, *FLI1* overexpression nearly completely rescued the decrease in CD41a$^+$CD42b$^+$ megakaryocytes and the reduction in cell sizes caused by MEIS1 deletion in H1 hESCs and BC1 cells (Figures 7D and S7E). Thus, FLI1 is vital to mediate the function of MEIS1 during megakaryopoiesis. Interestingly, in contrast to the strong effect in rescuing megakaryocytic differentiation, *FLI1* overexpression only moderately reversed the defects in proplatelet generation caused by MEIS1 deletion (Figures 7E and S7F), suggesting that FLI1 only partially mediates the function of MEIS1 during thrombopoiesis.

DISCUSSION

In summary, we identified MEIS1 as a potential regulator of hPSC early hematopoietic differentiation. MEIS1 controls specification of APLNR$^+$ mesoderm progenitors to HEPs, and TAL1 mediates this function of MEIS1. In addition, MEIS1 is vital for megakaryocytic differentiation, polyploidization, and subsequent platelet production from hPSCs. FLI1 acts as a downstream gene necessary for the function of MEIS1 during megakaryopoiesis and thrombopoiesis (Figure 7F). Together, we define a role of MEIS1 in human development, unveil new mechanisms for human hematopoiesis, and contribute potential new strategies to regenerative medicine.

In addition to its function as an accelerator in the pathogenesis of leukemia, MEIS1 has been shown to play crucial roles in normal hematopoietic development. Embryonic lethality at day 14.5 and hematopoietic, vascular, and retinal defects were observed in MEIS1-deleted homozygous animals (Azcoitia et al., 2005; Gonzalez-Lazaro et al., 2014; Hisa et al., 2004). Meis1 conditional knockout in adult mice also caused defects in adult erythropoiesis, megakaryopoiesis, and HSC maintenance (Ariki et al., 2014; Kocabas et al., 2012; Miller et al., 2016; Unnisa et al., 2012). Despite these animal studies, the role of MEIS1 in human early development has not been reported to date. Furthermore, how MEIS1 controls early hematopoiesis is even less understood. Here by using the CRISPR/CAS9 technology, we successfully created MEIS1 knockout cell lines in hPSCs, leading us to define an essential role for MEIS1 in human early hematopoiesis. In addition, we performed a detailed analysis of the potential function of MEIS1 in early hematopoietic development and presented evidence for a critical and specific role of MEIS1 in transition of APLNR$^+$ cells to CD31$^+$CD34$^+$ HEPs. Thus, the HSC decrease observed *in vivo* in Meis1 knockout animals might also result from the decrease in HEP generation.

By performing large-scale gene profiling analysis and subsequent functional studies, we identified TAL1 as the downstream transcription factor that mediates the function of MEIS1 in controlling HEP generation. Although the role of TAL1 as a master regulator in HEP generation in hESCs has been reported (Real et al., 2012), we established a functional link between MEIS1 and TAL1 and unveiled a new signaling mechanism that controls early hematopoiesis. Our results also suggest that MEIS1 acts as another master regulator of HEP generation and does so by acting upstream of TAL1.

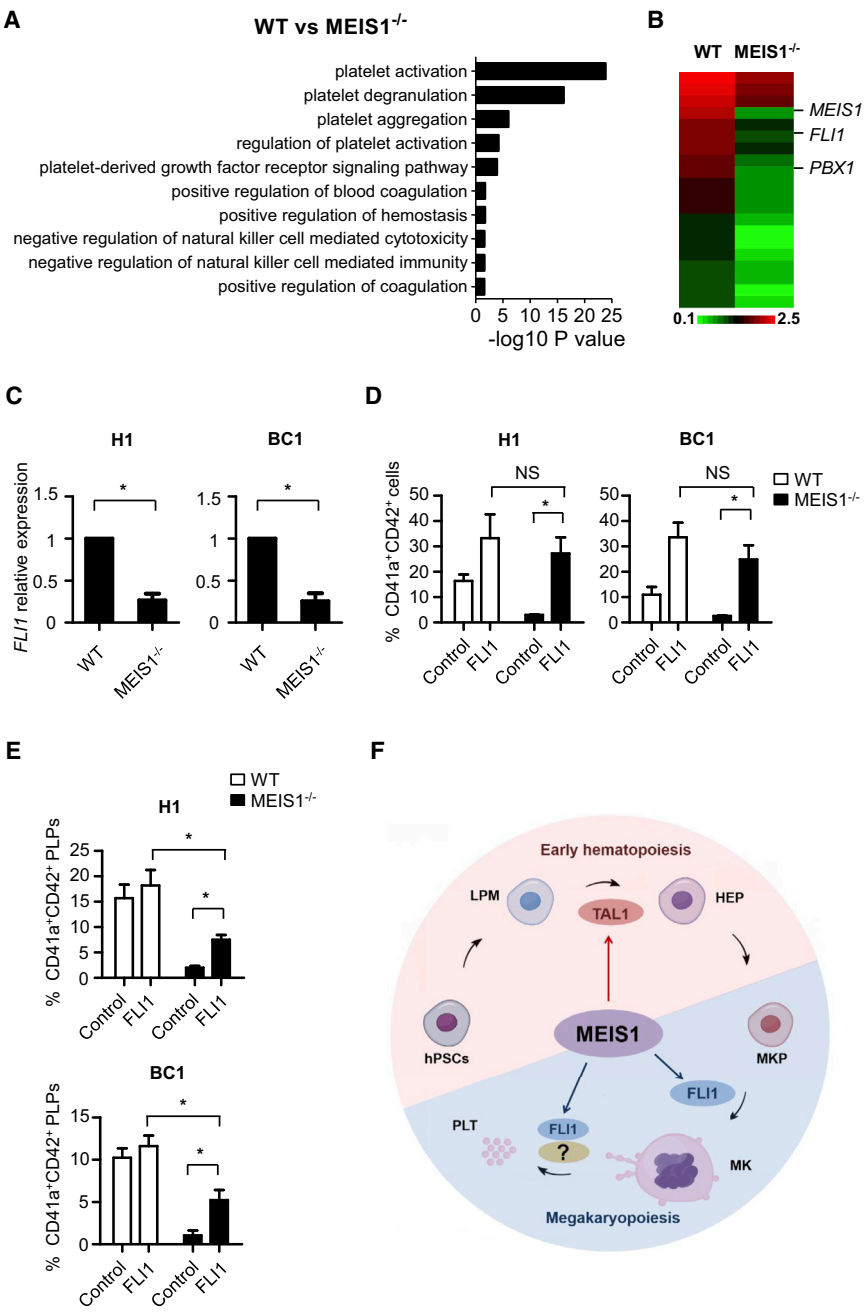

Figure 7. MEIS1 Controls Megakaryocytic Specification by Enhancing *FLI1* Expression

(A) Top 10 biological functions of downregulated genes in cells with MEIS1 deletion. RNA-seq analysis was performed on samples collected from at day 0, day 3, and day 6 of megakaryocytic differentiation.

(B) Heatmaps of top 20 downregulated transcriptional factors in cells with MEIS1 deletion during megakaryocytic differentiation from H1 hESCs.

(C) Real-time PCR analysis of *FLI1* in cells differentiated from WT and MEIS1-deleted hPSCs during megakaryocytic differentiation. GAPDH was an internal control. All values were normalized to the level (= 1) of mRNA in the cells derived from WT hPSCs.

(D) Flow cytometry analysis of the percentage of $CD41a^+CD42b^+$ megakaryocytes differentiated from WT and MEIS1-deleted hPSCs without or with *FLI1* overexpression. GFP^+ gated events are shown.

(E) Flow cytometry analysis of the percentage of $CD41a^+CD42b^+$ PLPs differentiated from WT and MEIS1-deleted hPSCs cultured on plates precoated with fibrinogen without or with *FLI1* overexpression. GFP^+ gated events are shown.

(F) A working model for MEIS1 function and mechanism in hPSC early hematopoietic and megakaryocytic differentiation. LPM, lateral plate mesoderm; HEP, hemogenic endothelium progenitor; MKP, megakaryocyte progenitor; MK, megakaryocyte; PLT, platelet. Error bars represent mean ± SEM of samples from three independent experiments. NS, not significant, *$p < 0.05$.

In addition to the defects in HEP generation, we also identified defects in megakaryocytic differentiation and platelet generation in cells with MEIS1 deletion, consistent with the previously documented defects in megakaryocyte lineage development in *Meis1* mutant animals (Azcoitia et al., 2005; Carramolino et al., 2010; Gonzalez-Lazaro et al., 2014; Hisa et al., 2004). However, in contrast to the *in vivo* observations, generation of $CD41^+$ megakaryocytes from human hPSCs was impaired but not completely abrogated. Instead, the primary defect caused by MEIS1 deletion in our system was the failure of the cells to undergo polyploidization. This function of MEIS1 has not been reported earlier. Strikingly, nearly none of $CD41^+$ megakaryocyte generated from $MEIS1^{-/-}$ HPC can develop beyond the 4N stage, while live-imaging analysis clearly demonstrated that despite a small portion of $CD41^+$ megakaryocytes generated, no platelets can be produced from any $MEIS1^{-/-}$ megakaryocytes. These phenotypic defects point

to an equally important role for MEIS1 in thrombopoiesis aside from its function in megakaryopoiesis. Together, our studies markedly extend previous findings that overexpression of MEIS1 enhances MEP generation (Zeddies et al., 2014), providing the direct evidence for a critical and specific function of MEIS1 in megakaryocyte polyploidization and platelet derivation. At the mechanistic level, we found that FLI1 acts downstream of MEIS1 to mediate the function of MEIS1 in megakaryopoiesis and thrombopoiesis. Interestingly, *FLI1* overexpression nearly completely rescued differentiation of $CD41^+$ megakaryocytes but only partially increased platelet generation. Thus, a more complete delineation of the detailed mechanisms underlying MEIS1's actions awaits future studies.

hPSCs have long been recognized as a potential source for large-scale platelet generation *in vitro* for transfusion, and large numbers of megakaryocytes can indeed be derived from hiPSCs via reprogramming with various gene combinations (Sim et al., 2016). Our findings highlight the stage-specific functional significance of MEIS1, TAL1, and FLI1 transcription factors during early hematopoiesis, megakaryopoiesis, and thrombopoiesis. This knowledge can be proved invaluable for manipulating cell fate during hematopoietic differentiation and for large-scale megakaryocyte and platelet generation for future clinical applications.

EXPERIMENTAL PROCEDURES

$CD34^+$ Cell Preparation

Umbilical cordblood (CB) units were obtained from healthy full-term neonates with informed consent from the parents and approved by the ethics committee of the Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences. $CD34^+$ cells were isolated from CB using Ficoll-Hypaque density centrifugation medium (Sigma-Aldrich, St. Louis, MO) and MiniMACS $CD34^+$ isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions.

hPSC Hematopoietic Differentiation in Chemically Defined Conditions

hPSC hematopoietic differentiation was carried out under chemically defined conditions as previously described with some modification (Pang et al., 2013; Wang et al., 2012). hPSCs were dissociated into a single-cell suspension using 1 mg/mL Accutase (Gibco) and plated on Matrigel-coated dishes at a density of 3.5×10^4 cells/well (12-well plate) in mTeSR1 medium with 10 μM Y27632 (Calbiochem). After 24 hr, hPSCs were induced for stepwise differentiation. First, cells were cultured in Custom mTeSR1 medium supplemented with 40 ng/mL activinA (Peprotech) and 50 ng/mL BMP4 (Peprotech) for 2 days. Second, cells were incubated with Custom mTeSR1 medium supplemented with 40 ng/mL VEGF (Peprotech) and 50 ng/mL bFGF (Peprotech) for 2 days. Third, cells were incubated with Custom mTeSR1 medium supplemented with 40 ng/mL VEGF, 50 ng/mL bFGF, and 20 μM SB 431542 (STEMGENT) for 3 days. Finally, differentiated cells were transferred to low-attachment plates and cultured for 6 days in mTeSR1 medium containing 50 ng/mL stem cell factor (SCF) (Peprotech), 50 ng/mL TPO (Peprotech) and 50 ng/mL interleukin-3 (IL-3) (Peprotech), 1 mM GlutaMAX (Gibco), 2% B27 (Gibco), 0.1 mM monothioglycerol (Sigma-Aldrich), 1% insulin-transferrin-selenium (Gibco), 1% N-acetylaspartate (Gibco), 1% penicillin/streptomycin.

hPSC Hematopoietic Differentiation in the mAGM-S3 Co-culture

Hematopoietic differentiation of hPSCs co-cultured with the AGM-S3 cells was performed as previously described (Mao et al., 2016). Before co-culture with hPSCs, mAGM-S3 cells were grown to form an overgrown monolayer and treated with 5 ng/mL mitomycin C (Sigma-Aldrich) at 37°C for 2 hr. The hPSCs were prepared as a suspension of small aggregates using 2 U/mL dispase and plated on inactivated mAGM-S3 cells at a density of 20–30 aggregates/well (six-well plate) in mTeSR1 medium. On the next day, the medium was replaced with Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum (Gibco/Life Technologies), 15 ng/mL VEGF (Peprotech), 1% nonessential amino acid solution (Gibco), 100 μM 2-mercaptoethanol (Sigma-Aldrich) and 1 mM L-glutamine (Gibco), 50 μg/mL L-ascorbic acid (Sigma-Aldrich), 7 μg/mL human transferrin (Sigma-Aldrich). The medium was changed every day.

Megakaryocytic Differentiation from hPSCs

After 10 days of hematopoietic differentiation of hPSCs under the mAGM-S3 co-culture, cobblestone-like cells were mechanically detached and replated onto mitotically inactivated mAGM-S3 cells in the hematopoietic differentiation medium plus Y-27632 (10 μM), TPO (50 ng/mL), SCF (20 ng/mL), IL-3 (20 ng/mL), IL-6 (10 ng/mL), IL-9 (20 ng/mL), and IL-11 (20 ng/mL, Peprotech). Fresh medium was used every 3 days.

Establishing MEIS1 Knockout hPSC Lines Using CRISPR/CAS9 Technology

Lentivirus containing MEIS1-E3G1 was infected into H1 hESCs or BC1 hiPSCs, which were subsequently selected with puromycin (1 μg/mL, Sigma). After the genome editing efficacy was accessed using Surveyor assay, the cells were dissociated into single cells with Accutase (Gibco). Small colonies emerging from single cells were picked and expanded. MEIS1 knockout hESC lines were identified using western blotting assays and gene-sequencing analysis.

RNA-Seq

RNA-seq analysis was performed by BGI Company (BGI, Shenzhen, China) as previously described. The mRNA expression levels were visualized with a heatmap built based on the value of $\log_{10}$ (fragments per kilobase of transcript per million mapped reads + 1) using MultiExperiment Viewer v.4.2. GO enrichment was performed using Gene Ontology (http://geneontology.org/). The results are available at Gene Expression Omnibus (GEO: GSE92245).

Lentivirus Production and Infection

Lentiviruses for gene knockdown or overexpression were packaged using the Viralpower Lentivirus Packaging System (Invitrogen) according to the manufacturer's instruction. For early hematopoiesis, lentivirus for *MEIS1* or *TAL1* was added into mTeSR1 medium with small colonies of hPSCs and Polybrene (3 μg/mL) for 96 hr before the hematopoietic differentiation of hPSCs. Then GFP^+ cells (4×10^4/mL) were sorted for further hematopoietic differentiation. For megakaryopoiesis, lentivirus for *MEIS1* or *FLI1* was mixed with hematopoietic differentiation medium with HPCs and Polybrene (6 μg/mL) for 10 hr. Then the megakaryocytic medium was changed to fresh and cells were replated onto mitotically inactivated mAGM-S3 stromal cells for further differentiation. GFP^+ events were gated to analyze the megakaryocytic potential every 3 days.

Statistical Analysis

At least three independent experiments were performed for each analysis, and two-tailed Student's t test was used to compare the differences between two groups using the GraphPad Prism software. All data are expressed as the mean ± SD. Differences were considered statistically significant when $p < 0.05$.

ACCESSION NUMBERS

The accession number for the RNA-seq reported in this paper is GEO: GSE92245.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, five tables, and two movies and can be found with this article online at https://doi.org/10.1016/j.stemcr.2017.12.017.

AUTHOR CONTRIBUTIONS

H.T.W., C.C.L., X.L., F.M., and J.X.Z. coordinated and designed the project; H.T.W., C.C.L., X.L, M.G.W., J.G., and P.S. performed the experiments; H.T.W., C.C.L., X.L., D.W., and J.X.Z. analyzed the data; T.N., W.Z., Y.F.X., and L.H.S. contributed new reagents/analytic tools; H.T.W., C.C.L., and J.X.Z. wrote the manuscript.

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