EUROPEAN HEMATOLOGY ASSOCIATION

Ferrata Storti Foundation

Haematologica 2017
Volume 102(5):826-834

Loss of Forkhead box M1 promotes erythropoiesis through increased proliferation of erythroid progenitors

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ABSTRACT

Forkhead box M1 (FOXM1) belongs to the forkhead/winged-helix family of transcription factors and regulates a network of proliferation-associated genes. Its abnormal upregulation has been shown to be a key driver of cancer progression and an initiating factor in oncogenesis. FOXM1 is also highly expressed in stem/progenitor cells and inhibits their differentiation, suggesting that FOXM1 plays a role in the maintenance of multipotency. However, the exact molecular mechanisms by which FOXM1 regulates human stem/progenitor cells are still uncharacterized. To understand the role of FOXM1 in normal hematopoiesis, human cord blood $CD34^+$ cells were transduced with FOXM1 short hairpin ribonucleic acid (shRNA) lentivirus. Knockdown of FOXM1 resulted in a 2-fold increase in erythroid cells compared to myeloid cells. Additionally, knockdown of FOXM1 increased bromodeoxyuridine (BrdU) incorporation in erythroid cells, suggesting greater proliferation of erythroid progenitors. We also observed that the defective phosphorylation of FOXM1 by checkpoint kinase 2 (CHK2) or cyclin-dependent kinases 1/2 (CDK1/2) increased the erythroid population in a manner similar to knockdown of FOXM1. Finally, we found that an inhibitor of FOXM1, forkhead domain inhibitor-6 (FDI-6), increased red blood cell numbers through increased proliferation of erythroid precursors. Overall, our data suggest a novel function of FOXM1 in normal human hematopoiesis.

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Received: September 7, 2016.

Accepted: January 24, 2017.

Pre-published: February 2, 2017.

doi:10.3324/haematol.2016.156257

Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures: www.haematologica.org/content/102/5/826

Introduction

Hematopoiesis is the critical process by which normal blood cells are derived from self-renewing and pluripotent hematopoietic stem cells (HSCs).[1,2] Erythropoiesis, which leads to the formation of mature red blood cells, requires that each cell division is simultaneously coupled with differentiation.[3,4] Erythropoiesis can be divided into 3 stages: early erythropoiesis, terminal erythroid differentiation, and reticulocyte maturation.[5,6] During early erythropoiesis, HSCs sequentially give rise to a common myeloid progenitor, megakaryocyte-erythrocyte progenitor, burst-forming unit-erythroid (BFU-E), and colony-forming unit-erythroid (CFU-E) cells that differentiate into proerythroblasts.[7-11] In terminal erythroid differentiation, morphologically recognizable proerythroblasts undergo sequential mitosis to become basophilic, polychromatic, and orthochromatic erythroblasts that expel their nuclei to become reticulocytes.[12] At the final step of erythropoiesis, multinucleated reticulocytes mature into red blood cells accompanied by the loss of intracellular organelles, a decrease in cell volume, and extensive membrane remodeling.[13-18]

Erythropoiesis is tightly regulated by various regulatory growth factors and transcription factors,[3,6] with erythropoietin (EPO) and GATA-1 playing essential roles.[7,10] The binding of EPO to its receptor (EPOR) activates the Jak2-Stat5 signaling path-

way to promote the proliferation of erythroid progenitor cells and to rescue erythroid progenitors from cell death.[19-21] GATA-1 leads to the expression of erythroid-specific genes including EPOR, adult globin genes, heme biosynthesis enzymes, and erythroid membrane proteins.[22] The expression or transcriptional activation of erythroid-specific growth and transcription factors is critical for normal erythropoiesis.

FOXM1 belongs to the forkhead/winged-helix family of transcription factors and binds to a specific DNA consensus sequence through a highly conserved DNA-binding domain (DBD).[23-26] It is a key transcription factor in the regulation of a network of proliferation-associated genes including the G1/S transition, S phase progression, G2/M transition, and M phase progression, and is also critical for DNA replication, mitosis, spindle assembly, and genomic stability.[24,27-30] Consistent with its role in cell-cycle progression, FOXM1 expression is highly upregulated in a number of human cancers, including liver, ovarian, breast, prostate, colon, and brain tumors.[31-32] Its abnormal upregulation has been shown to be a key driver of cancer progression and an initiating factor of oncogenesis.[33]

FOXM1 is also highly expressed in multipotent progenitor cells and inhibits differentiation of progenitors, suggesting that FOXM1 plays a role in the maintenance of multipotent progenitor cells.[34-36] It is reported that Foxm1 participates in the maintenance of pluripotency of mouse P19 embryonal carcinoma cells by directly regulating *Oct4* transcription.[37] In addition, the overexpression of Foxm1 alone in human newborn fibroblasts restarts the expression of pluripotent genes, including *Oct4, Nanog,* and *Sox2*. Recently, it was also reported that Foxm1 is essential for the quiescence and maintenance of hematopoietic stem cells in the mouse model.[38] Loss of Foxm1 induced the decrease of cyclin-dependent kinase inhibitors by suppressing the *Nurr1* gene (a critical regulator of HSC quiescence) expression. However, the exact molecular mechanisms by which FOXM1 regulates human hematopoietic stem and progenitor cells are still uncharacterized.

In this study, we have examined the role of FOXM1 in normal hematopoiesis using human cord blood CD34$^+$ cells and lentivirus to target FOXM1. We found that knockdown of FOXM1 resulted in an increase in the erythroid population compared to the myeloid population, with a higher expression of CD71$^+$ (erythroid) cells compared to CD11b$^+$ (myeloid) cells. We also found that FOXM1 knockdown increased BrdU incorporation in CD71$^+$ cells only, suggesting a greater proliferation of erythroid progenitors. Taken together, these studies suggest a novel function of FOXM1 in normal human hematopoiesis. Our data indicate that FOXM1 inhibitors, such as FDI-6, may be beneficial in treating patients with anemia due to decreased red blood production.

Methods

Cell culture

Primary human CD34$^+$ hematopoietic stem/progenitor cells were purified from umbilical cord blood units obtained from The National Cord Blood Program at the Howard P. Milstein Cord Blood Center of New York Blood Center. These are unprocessed, non-clinical grade cord blood units and are considered "Research Units". These materials are de-identified and are therefore considered not to involve human subjects as per the Stanford Human Research Protection Program and Institutional Review Board. CD34$^+$ cells were purified using MACS cell separation (Miltenyi Biotec) and cryopreserved. Primary human bone marrow CD34$^+$ cells were purchased from Lonza. Upon thawing, cells were cultured in x-Vivo15 medium (Lonza) containing 10% fetal bovine serum (FBS), 1x Penicillin-Streptomycin-Glutamine (PSG) (Invitrogen), FLT-3 (Miltenyi Biotec), thrombopoietin (TPO; Miltenyi Biotec), interleukin (IL)-3 (Miltenyi Biotec), IL-6 (Miltenyi Biotec), stem cell factor (SCF; Miltenyi Biotec), EPO, and transferrin (Sigma-Aldrich) by a 3 phase liquid culture system as described in the *Online Supplementary Table S1*. HEK 293 and K562 cell lines were cultured in Iscove's Modification of Dulbecco's Medium (Corning) containing 10% FBS and 1x PSG.

Colony assays

GFP$^+$ or mCherry$^+$ sorted hematopoietic cells were seeded in methylcellulose medium containing IL-3, SCF, granulocyte-macrophage colony-stimulating factor (GM-CSF), and EPO (H4434, StemCell Technologies), in triplicate, with a density of 1000 cells per plate. Erythroid (BFU-E and CFU-E) and myeloid colony-forming unit granulocyte/monocyte (CFU-GM) colonies were counted 2 weeks later by an investigator blinded to the conditions. Methylcellulose medium containing only EPO (H4330; StemCell Technologies) was used for erythroid colony (CFU-E) and colonies were counted 1 week later. Methylcellulose medium containing SCF, IL-3, granulocyte-colony stimulating factor (G-CSF), and GM-CSF (H4035; StemCell Technologies) was used for the myeloid colony, and colonies were counted 2 weeks later.

Flow cytometry

For cell surface flow cytometry, cells were washed with phosphate-buffered saline (PBS) and then incubated with indicated antibodies for 20 minutes at room temperature. After washing, cells were analyzed by fluorescence-activated cell sorting (FACS). For intracellular flow cytometry, cells were fixed in 3.2% paraformaldehyde for 10 minutes at 37°C, and permeabilized with 100% methanol for 30 minutes at -80°C. After washing, cells were incubated with indicated antibodies and then analyzed by FACS. Data were collected on a FACS Calibur (BD Biosciences) or a DxP10 (Cytek) flow cytometer and analyzed using FlowJo Software (v.10). All antibodies are listed in the *Online Supplementary Methods*.

BrdU incorporation

Cells were plated in a 48 well plate and incubated with 10 μl of 1 mM BrdU (BD Pharmingen) for 45 minutes. Cells were fixed in 3.2% paraformaldehyde for 10 minutes at 37°C, and permeabilized with 100% methanol for 30 minutes at -80°C. After washing, cells were treated with RQ1 RNase-free DNase (Promega) for 1 hour at 37°C. After washing, cells were incubated with BrdU and CD71-PE antibodies and then analyzed by FACS. Antibody against BrdU (Bu20A: APC-conjugated: 17-5071-42) was purchased from eBioscience.

Drug treatment

FDI-6 (Axon2384; Axon Medchem) was dissolved in dimethyl sulfoxide (DMSO) to create a 40 mM stock and added to cells at a final concentration of 10 μM. Roscovitine (S1153; Selleck Chemicals), U0126-EtOH (S1102; Selleck Chemicals), and ADZ7762 (S1532; Selleck Chemicals) were dissolved in DMSO and added to cells at a final concentration of 10 μM, 5 μM, and 50 nM, respectively. Cells were treated with the respective drugs from day 1 to day 5 after thawing; the drug was then washed out for the remainder of the culture time.

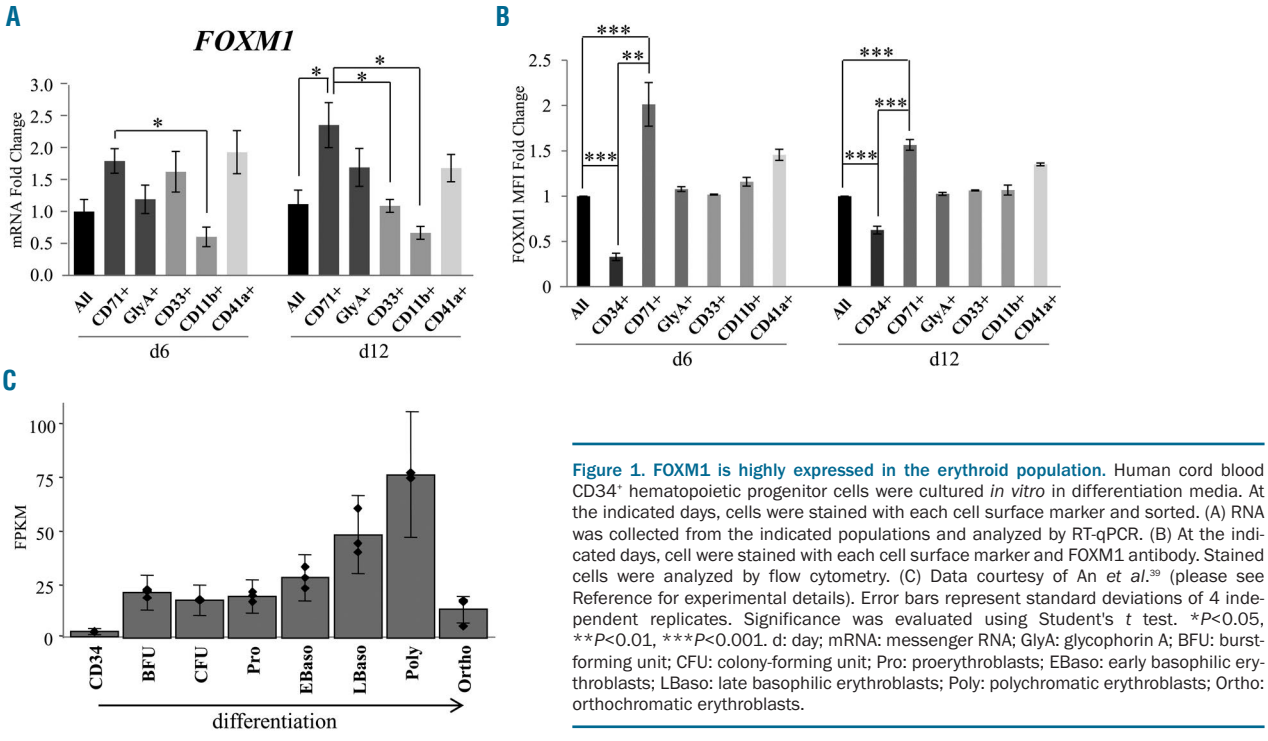

Figure 1. FOXM1 is highly expressed in the erythroid population. Human cord blood CD34⁺ hematopoietic progenitor cells were cultured *in vitro* in differentiation media. At the indicated days, cells were stained with each cell surface marker and sorted. (A) RNA was collected from the indicated populations and analyzed by RT-qPCR. (B) At the indicated days, cell were stained with each cell surface marker and FOXM1 antibody. Stained cells were analyzed by flow cytometry. (C) Data courtesy of An *et al.*[39] (please see Reference for experimental details). Error bars represent standard deviations of 4 independent replicates. Significance was evaluated using Student's *t* test. **P*<0.05, ***P*<0.01, ****P*<0.001. d: day; mRNA: messenger RNA; GlyA: glycophorin A; BFU: burst-forming unit; CFU: colony-forming unit; Pro: proerythroblasts; EBaso: early basophilic erythroblasts; LBaso: late basophilic erythroblasts; Poly: polychromatic erythroblasts; Ortho: orthochromatic erythroblasts.

Results

FOXM1 is highly expressed in the erythroid population

To understand the role of FOXM1 in hematopoietic cells, we first investigated the expression profiles of FOXM1 in individual cell populations. Trilineage differentiation of human cord blood CD34⁺ hematopoietic progenitor cells was induced *in vitro* using a 3-phase liquid culture system, as described in the *Online Supplementary Table S1*. We used the following cell surface markers: CD71 for early erythroid, glycophorin A (GlyA) for late erythroid, CD33 for early myeloid, CD11b for late myeloid, and CD41a for megakaryocytes. Populations were analyzed on day 6 or day 12 of culture. Sorting efficiencies for each population were confirmed by running reverse transcription-quantitative polymerase chain reaction (RT-qPCR) with each cell marker (*Online Supplementary Figure S1*). We found that *FOXM1* had a 3-fold increased RNA level in CD71⁺ cells compared to CD11b⁺ cells (Figure 1A). To observe the effects on the protein level, we performed intracellular flow cytometry for FOXM1 with each cell surface marker. Consistent with the RNA levels, FOXM1 protein was high in the CD71⁺ population, suggesting that FOXM1 plays a specific role in erythropoiesis (Figure 1B and *Online Supplementary Figure S2*).

Data from a previously published paper confirms our findings that FOXM1 has a role in erythropoiesis. The authors performed a detailed transcriptome analysis of the various stages of normal human erythropoiesis including BFU-E, CFU-E, proerythroblasts (Pro), Early and Late basophilic erythroblasts (E Baso and L Baso), polychromatic erythroblasts (Poly), and orthochromatic erythroblasts (Ortho).[39] Interestingly, *FOXM1* expression was elevated in erythroid cells, suggesting a functional role for FOXM1 in erythroid population (Figure 1C; data courtesy of An *et al.*[39]). We chose to focus on the earlier stages of erythropoiesis which also demonstrated a higher expression of FOXM1, since it was challenging to study the later stages of erythropoiesis in our liquid culture system using shRNA knockdown.

FOXM1 downregulation increases the erythroid population

To understand the function of FOXM1 in human erythropoiesis, we examined whether FOXM1 knockdown affects normal hematopoiesis. We confirmed the knockdown efficiency of two different shRNAs against FOXM1 and observed significantly decreased RNA levels in human cord blood CD34⁺ cells (Figure 2A). We also confirmed decreased protein levels with FOXM1 knockdown in the K562 cell line by immunoblot analysis and in the human cord blood CD34⁺ cells by intracellular flow cytometry (*Online Supplementary Figure S3A,B*). After transduction of human CD34⁺ cells with lentivirus expressing FOXM1 shRNA, we studied hematopoietic differentiation using FACS analysis with a range of cell surface markers. We found that knockdown of FOXM1 resulted in an increase of the erythroid population as measured by CD71 and GlyA and a decrease in the myeloid population as measured by CD11b (Figure 2B, *Online Supplementary Figures S4* and *S5*). Additionally, we detected the effects on specific stages of erythroid differentiation by using GlyA, CD49d, and Band3 as markers.[40] Consistent with our findings in the CD71⁺/GlyA⁺ population, the populations of Pro, E Baso, and L Baso stages were increased by FOXM1 knockdown (*Online Supplementary Figure S6A,B*). Similarly, methylcellulose colony assays demonstrated increased numbers of CFU-E colonies and decreased numbers of

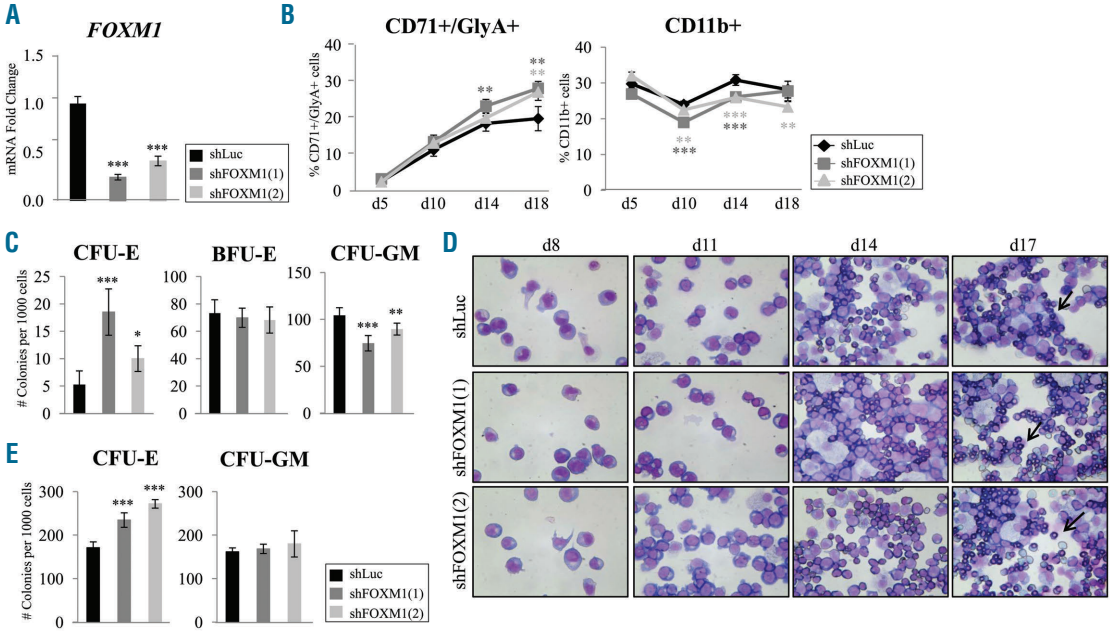

Figure 2. FOXM1 downregulation increases the erythroid population. Human cord blood CD34⁺ hematopoietic progenitor cells were transduced with lentivirus carrying shRNA against FOXM1 or luciferase (Luc) control. (A) Cells were sorted for GFP⁺ at 5 days after transduction. RNA was collected and analyzed by RT-qPCR. (B) Transduced cells were analyzed for CD71, GlyA, and CD11b expression by flow cytometry at the indicated days after transduction. (C) 1000 cells of GFP⁺ cells were plated in methylcellulose media and cultured for 2 weeks. Colonies were counted by an investigator blinded to the conditions. (D) GFP⁺ cells following cytospin and Wright-Giemsa staining at the indicated days post-transduction, and imaged at 63 x magnification. Arrows indicate a representative erythrocyte. (E) Cells in erythroid media or myeloid media were transduced with lentivirus and then were sorted for GFP⁺ at 5 days after transduction. 1000 cells of each GFP⁺ cells were plated in methylcellulose media for detection of CFU-E or CFU-GM, and cultured. Colonies were counted by an investigator blinded to the conditions. **P*<0.05, ***P*<0.01, ****P*<0.001. sh: short hairpin; d: day; GlyA: glycophorin A; CFU-E: colony-forming unit-erythroid; BFU-E: burst-forming unit-erythroid; CFU-GM: colony-forming unit granulocyte/monocyte.

CFU-GM colonies, but we did not note any morphological changes in FOXM1 knockdown cells (Figure 2C,D and *Online Supplementary Figure S7*). To determine whether the increase in the erythroid population resulting from FOXM1 knockdown was not due to a decrease in myelopoiesis, we cultured human cord blood CD34⁺ cells in medium supporting either erythroid or myeloid differentiation as described in the *Online Supplementary Tables S2* and *S3*. After transduction with lentivirus expressing FOXM1 shRNA, we measured the colony forming activity of cells cultured in methylcellulose media specific for either erythroid progenitors or myeloid progenitors. We observed an increase in CFU-E colonies with erythroid specific medium in CD34⁺ FOXM1 knockdown cells, but no change in CFU-GM colonies with myeloid media (Figure 2E). We observed similar results using FACS analysis (*Online Supplementary Figure S8*). These results suggest that FOXM1 knockdown directly affects erythropoiesis.

To study the effects of FOXM1 overexpression during erythropoiesis, we cloned full-length cDNA of FOXM1 into a lentiviral vector (*Online Supplementary Figure S9A,B*). Human CD34⁺ cells were transduced with lentivirus expressing FOXM1 cDNA, and FACS analysis and colony assays were performed. FOXM1 overexpression did not affect erythroid or myeloid differentiation (*Online Supplementary Figure S9C,D*).

Taken together, these results suggest that FOXM1 knockdown directly increases the erythroid population and overexpression does not increase erythroid or myeloid colony formation.

FOXM1 downregulation increases the proliferation of erythroid progenitors

FOXM1 is known to play a critical role in cell proliferation.[37] In order to understand its potential mechanism of action in erythropoiesis, we investigated its role on the proliferation of hematopoietic progenitor cells. We again used an *in vitro* liquid culture system to differentiate and separate transduced CD34⁺ cells into erythroid and non-erythroid or myeloid populations using CD71 and CD11b. Viable cells were counted every 3 days to observe effects on proliferation. We found that FOXM1 knockdown increased the number of CD71⁺ cells, but not CD71-, CD11b⁺, or total cell populations after 11 days in culture (Figure 3A,B). This is similar to the increase in the CD71⁺/GlyA⁺ population, which we noted in earlier experiments (Figure 2B). To verify that the increased cell number is caused by increased cell proliferation, we performed BrdU incorporation assays at 13 days after transduction and found that knockdown of FOXM1 resulted in increased BrdU positive cells in the CD71⁺ population only, indicating more rapid proliferation of erythroid progenitors (Figure 3C left panel and *Online Supplementary Figure S10*). Similarly, H3P-ser10, which is a specific marker for M phase, was increased in FOXM1 knockdown CD71⁺ cells (Figure 3C right panel), indicating increased mitotic activity in the erythroid population with knockdown of FOXM1. Additionally, we transduced sorted CD71⁺ erythroid cells with FOXM1 shRNA lentivirus and observed increased BrdU positive cells (*Online Supplementary Figure S11*), confirming the direct action of

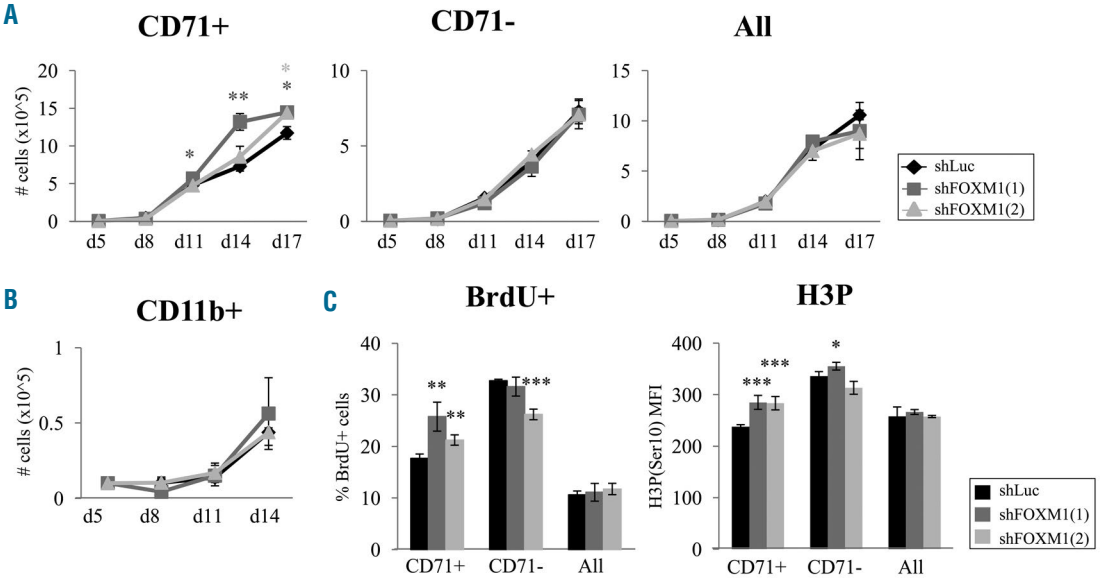

Figure 3. FOXM1 downregulation increases the proliferation of erythroid progenitors. Human cord blood CD34$^+$ hematopoietic progenitor cells were transduced with lentivirus carrying shRNA against FOXM1 or Luc control. Cells were sorted for CD71$^+$/GFP$^+$ (for the CD71$^+$ group), CD71$^-$/GFP$^+$ (for the CD71$^-$ group), CD11b$^+$/GFP$^+$ (for the CD11b$^+$ group), or GFP$^+$ (for the All group) cells at 5 days after transduction. (A and B) 4000 sorted cells were cultured for an additional 12 days or 9 days. Cells were counted every 3 days. (C) Sorted cells were cultured for an additional 8 days. At 13 days post-transduction, cells were incorporated with BrdU, and then stained with BrdU and CD71 antibodies. Or, cells were stained with H3P-ser10 and CD71 antibodies. Stained cells were analyzed by flow cytometry. *P<0.05, **P<0.01, ***P<0.001. sh: short hairpin; d: day; BrdU: bromodeoxyuridine; Luc: luciferase.

FOXM1 knockdown in erythroid proliferation.

In summary, these data suggest that FOXM1 downregulation increases the proliferation of the erythroid progenitors.

FOXM1 effects on erythropoiesis are independent of the p53 pathway

Previous reports established that p53 and FOXM1 negatively regulate each other's activity.[27,41] In our system, we confirmed that FOXM1 expression was decreased in CD34$^+$ progenitor cells treated with Nutlin-3, a drug that leads to p53 stabilization by blocking MDM2 (*Online Supplementary Figure S12A,B*). It has also been reported that Foxm1-deficient MEFs have increased transcriptional activity of p53 with corresponding stimulation of their target gene expression.[27] This suggests the possibility that p53 activity is involved in regulating FOXM1 activity during erythropoiesis. To test this hypothesis, we studied the expression of p53 target genes in FOXM1 knockdown cells. FOXM1 downregulation did not affect the expression of *p21, WIG-1, BAX*, and *GADD45A* in either the early or late stages of erythropoiesis (*Online Supplementary Figure S13A*). The expression of *GATA1* (a canonical erythroid transcription factor) and *TNF-α* (whose role in erythropoiesis we and others have studied) were also not affected (*Online Supplementary Figure S13B*).[42] Finally, we could not detect any increase in the p53 protein level and cPARP$^+$ apoptotic cells in FOXM1 knockdown cells, although their levels were increased in RPS19 knockdown cells, which were used as a positive control (*Online Supplementary Figure S13C,D*).[42,43]

Taken together, these results suggest that the effects of FOXM1 on erythropoiesis are p53-independent.

FOXM1 function on erythroid proliferation requires its phosphorylation by CHK2 or CDK1/2 kinases

It is known that FOXM1 activity is regulated by phosphorylation involving multiple regulatory kinases. For example, FOXM1 transcriptional activity requires binding of the CDK-Cyclin complexes and subsequent phosphorylation to regulate G2/M cell cycle regulatory genes.[44-46] Additionally, FOXM1 is phosphorylated by DNA damage-induced CHK2, resulting in the stabilization of the FOXM1 protein.[27]

To understand the effects of the upstream signaling pathways on FOXM1 during erythroid proliferation and differentiation, we studied three representative kinases, checkpoint kinase 2 (CHK2), cyclin-dependent kinase 1/2 (CDK1/2), and polo-like kinase 1 (PLK1). We examined their expression levels at various time points during hematopoiesis using our *in vitro* CD34$^+$ system. All observed kinases were expressed at the protein level throughout hematopoiesis, with a relatively high expression in the earlier stage (Figure 4A). We then made alanine-substituted FOXM1 mutants on serine or threonine residues that would be predicted to affect normal phosphorylation (Figure 4B). We confirmed that mRNA and protein of the FOXM1 mutants were normally expressed in CD34$^+$ cells by RT-qPCR and in HEK 293 cells by immunoblot analysis (*Online Supplementary Figure S14A,B*). We then transduced CD34$^+$ progenitor cells with these constructs and performed methylcellulose colony assays as described previously. We found increased BFU-E and CFU-E colonies in mt1, mt2/3, and mt1-5 but not mt4/5 (Figure 4C), suggesting that FOXM1 phosphorylation by CHK2 and CDK1/2 kinases is important in its role in regulating the proliferation of erythroid progenitors.

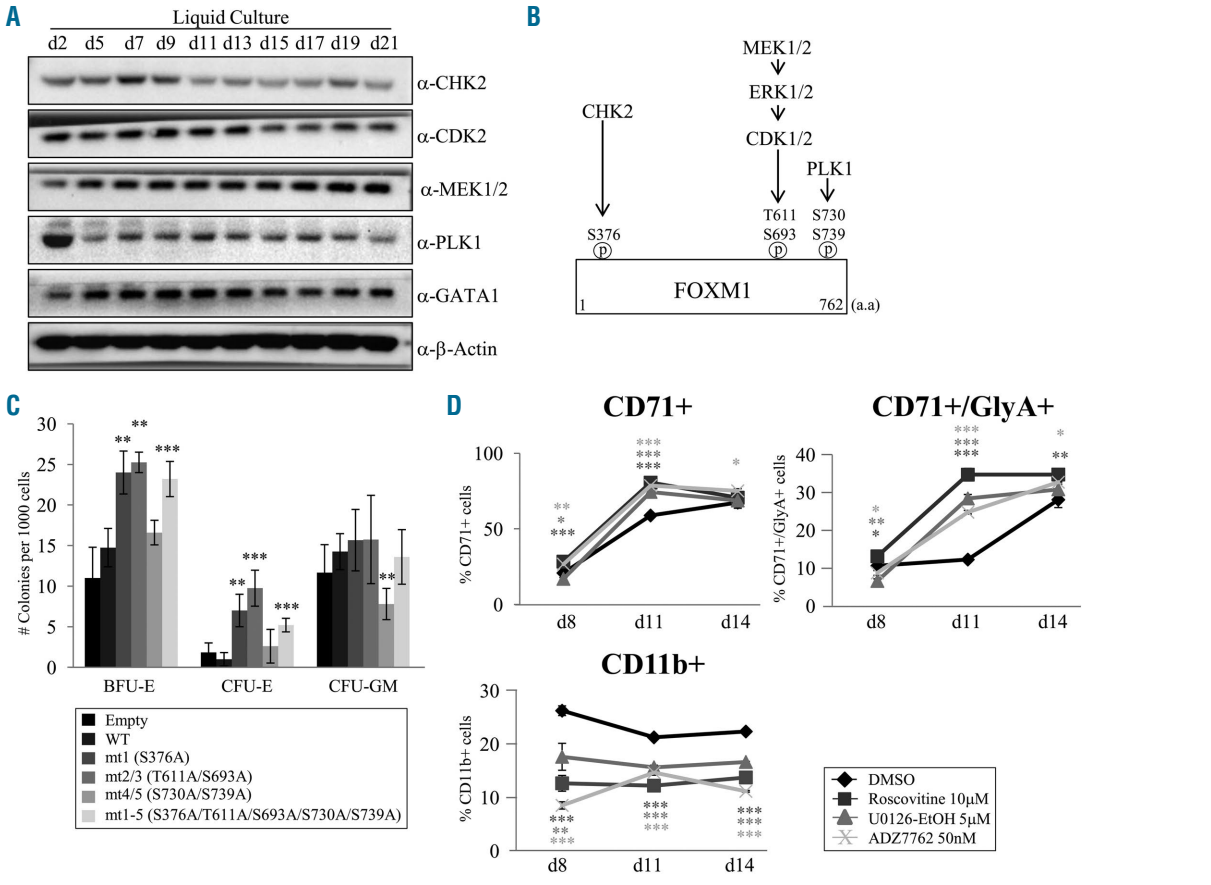

Figure 4. FOXM1 function on erythroid proliferation requires its phosphorylation by CHK2 or CDK1/2 kinases. (A) Human cord blood CD34⁺ hematopoietic progenitor cells were cultured for 21 days. At the indicated days, cells were harvested. Protein was collected and analyzed by immunoblot analysis. GATA1 was used as an erythroid-specific marker protein. β-Actin was used as a loading control. (B) The schema of human FOXM1 phosphorylation sites by CHK2, CDK1/2, and PLK1. (C) Human cord blood CD34⁺ hematopoietic progenitor cells were transduced with lentivirus carrying FOXM1 mutant cDNA. Cells were sorted for mCherry⁺ at 5 days after transduction. 1000 cells of mCherry⁺ cells were plated in methylcellulose media and cultured for 2 weeks. Colonies were counted by an investigator blinded to the conditions. (D) Human cord blood CD34⁺ hematopoietic progenitor cells were treated with the each kinase inhibitor for 5 days. Cells were analyzed for CD71, GlyA, and CD11b expression by flow cytometry at the indicated days after washing out the drug. *P<0.05, **P<0.01, ***P<0.001. WT: wild-type; d: day; CHK2: checkpoint kinase 2; CDK1/2: cyclin-dependent kinase 1/2; PLK1: polo-like kinase 1; GlyA: glycophorin A; DMSO: dimethyl sulfoxide; mt: mutant.

Similarly, we observed increased BFU-E at 5 days, increased CFU-E with decreased BFU-E at 8 days, and increased Pro at 11 days after culture with all mutant forms, indicating an effect on promoting erythropoiesis (*Online Supplementary Figure S15*). Finally, we studied whether these kinase inhibitors show similar effects to FOXM1 mutants. We treated CD34⁺ cells with roscovitine (CDK1/2 inhibitor) or ADZ7762 (CHK1/2 inhibitor) and then performed FACS analysis on the indicated days. We observed an increase in the CD71⁺/GlyA⁺ population and a decrease in the CD11b⁺ population upon treatment with the kinase inhibitors (Figure 4D and *Online Supplementary Figure S16*). Additionally, we found similar results with U0126-EtOH, an inhibitor of MEK1/2, which is the upstream kinase of CDK1/2.

These results indicate that CHK2 or CDK1/2 signaling pathways are required for FOXM1 function on erythroid differentiation.

FDI-6 increases the erythroid population through increased proliferation of its progenitors

Recently, Gormally *et al*. identified a small molecule, FDI-6, as a specific inhibitor of FOXM1.[47] FDI-6 inhibits the transcriptional activity of FOXM1 by its displacement from target gene promoters. To observe whether FDI-6 shows the same effects to FOXM1 downregulation on erythroid cells, we treated cord blood CD34⁺ cells with 10 μM of FDI-6 for 5 days. We confirmed that 10 μM of FDI-6 was enough to inhibit FOXM1 transcriptional activity in cord blood CD34⁺ cells by observing the expression of several known FOXM1 target genes, *FOXM1, CDC25B, CyclinD1, CENPF, CyclinB2*, and *p27* (*Online Supplementary Figure S17*). We performed FACS analysis every 3 days and found dramatic increases of CD71⁺and CD71⁺/GlyA⁺ populations with FDI-6 treatment (Figure 5A and *Online Supplementary Figure S18*). Similar results were observed in methylcellulose colony assays containing 10 μM of FDI-6, with increased numbers of CFU-E colonies and decreased numbers of CFU-GM colonies with treatment (Figure 5B). Finally, we studied the effects of FDI-6 on cellular proliferation as described in Figure 3, and noted that FDI-6 increased the number of CD71⁺ cells and BrdU positive CD71⁺ cells (Figure 5C,D and *Online Supplementary Figure S19*).

These results suggest that inhibition of FOXM1 by FDI-6 increases erythroid cells by enhancing erythroid progenitor proliferation.

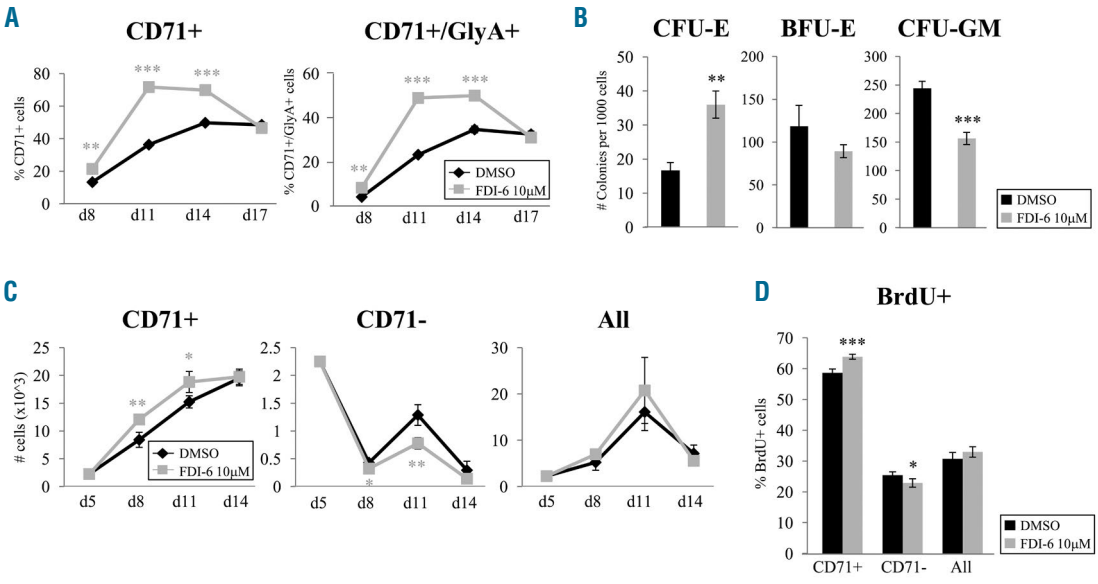

Figure 5. FDI-6 increases the erythroid population through increased proliferation of its progenitors. Human cord blood CD34⁺ hematopoietic progenitor cells were treated with FDI-6 for 5 days. (A) The drug was washed out after 5 days. Cells were analyzed for CD71 and GlyA expression by flow cytometry at the indicated days after culture. (B) 1000 drug-treated cells were plated in methylcellulose media containing 10 μM of FDI-6 and cultured for 10 days. Colonies were counted by an investigator blinded to the conditions. (C) Cells were sorted for CD71⁺ or CD71- cells at 5 days after drug treatment. 2250 sorted cells were cultured for an additional 9 days in differentiation media. Cells were counted every 3 days. (D) Sorted cells were cultured for an additional 4 days in differentiation media. At 9 days post-culture, cells were incorporated with BrdU, and then stained with BrdU and CD71 antibodies. Stained cells were analyzed by flow cytometry. *P<0.05, **P<0.01, ***P<0.001. d: day; CFU-E: colony-forming unit-erythroid; BFU-E: burst-forming unit-erythroid; CFU-GM: colony-forming unit granulocyte/monocyte; BrdU: bromodeoxyuridine; DMSO: dimethyl sulfoxide.

In summary, inhibition of FOXM1 activity either through decreased phosphorylation, expression (knockdown) or transcriptional activity (chemical inhibitor), increased proliferation of erythroid progenitors, thereby promoting erythropoiesis (Figure 6).

Discussion

In the study herein, we found that decreased levels of the FOXM1 protein increased red blood cell production through increased proliferation of human erythroid progenitors. We found that knockdown of FOXM1 by shRNA resulted in an increased number of erythroid cells with decreased myeloid cells. FOXM1 expression was relatively low in the myeloid population, and we suspect that the reduction in the myeloid population is an indirect effect from the promotion of erythropoiesis in our *in vitro* system. Indeed, we observed that the myeloid population was not affected by FOXM1 downregulation in isolation (Figures 2E and 3B and *Online Supplementary Figure S8*). In contrast, there is a significant expression of FOXM1 throughout normal human erythropoiesis, suggesting that FOXM1 plays a critical role and may be a unique target for certain forms of anemia.

Since FOXM1 is normally associated with proliferation, we would predict that knockdown would inhibit clonal expansion of stem/progenitor cells inhibiting differentiation. Interestingly, our results demonstrated that knockdown of FOXM1 increased proliferation of erythroid progenitors. Our findings suggest that FOXM1 could be a repressor of erythropoiesis. FOXM1 has been shown to function as a transcriptional repressor to inhibit mammary luminal cell differentiation.[48] FOXM1 directly represses the messenger RNA (mRNA) expression of GATA3, a master regulator of luminal cell differentiation, through a RB-dependent and DNMT-dependent mechanism. We tested whether FOXM1 also has a role as repressor in our *in vitro* model of hematopoiesis. We observed mRNA levels of known FOXM1 target genes, such as proliferation-associated genes including the cell cycle regulatory genes (*CyclinD1, p27, CyclinB1/B2, CyclinA1/A2, PCNA, CDC25b, PLK1, NEK2, CENPF*) and the proliferation genes (*TGF-α, JNK1, IGF-1, NEDD4*) in FOXM1 knockdown cells. However, we did not observe any significant changes in their gene expression by FOXM1 knockdown in the pure CD71⁺ erythroid population as well as in the all mixed CD71⁺ and CD71⁻ population (*data not shown*). We therefore suspect that the effects of FOXM1 on the proliferation of erythroid progenitors are due to alternative mechanisms.

We studied the effects of FOXM1 on erythroid population using shRNA knockdown, mutant proteins, and drug inhibitors. There was some discordance among the phenotypes, specifically with regard to the colony forming assays. While FOXM1 knockdown increases CFU-E numbers only, FOXM1 mutants exhibit increased BFU-E and CFU-E. Although we examined three representative kinases, there are still multiple regulatory pathways for FOXM1 activity such as protein stability by ubiquitin-proteasome pathway and protein expression by microRNAs (miRNAs). Therefore, it is conceivable that regulation of FOXM1 function in erythroid progenitors may not solely rely on its phosphorylation.

We also studied the effects of CHK2 and CDK1/2 kinase inhibitors on erythropoiesis. These kinases are well-known to regulate various pathways, including cell cycle pathways and we, therefore, expected that it would be difficult to observe the effects of kinase inhibition on ery-

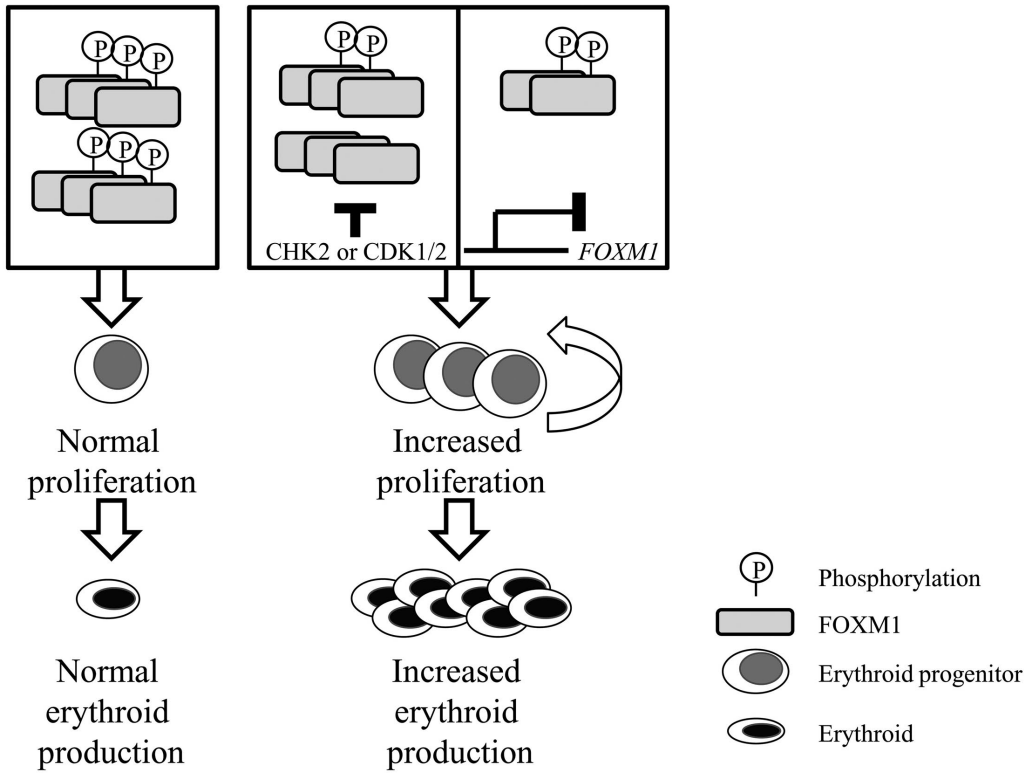

Figure 6. Model of increased erythroid production by FOXM1 deficiency. FOXM1 is functionally activated through the CHK2 or CDK1/2 kinase cascade. When FOXM1 cannot be activated because of certain mutations or defective expression, the proliferation of erythroid progenitors is amplified, resulting in increased erythroid cell production. We also observed that the FOXM1 inhibitor, FDI-6, shows a similar effect to FOXM1 downregulation. CHK2: checkpoint kinase 2; CDK1/2: cyclin-dependent kinase 1/2.

thropoiesis. For this reason, we initially used a range of drug concentrations and found that most of the cells were dead or arrested at the higher concentrations. However, we did observe an increase in the erythroid population and a decrease in the myeloid population without any cell growth defects at the lower concentrations, which is similar to our results with the FOXM1 mutants which cannot be phosphorylated by these kinases.

Previous work in zebrafish models using morpholino antisense against *foxm1* showed a blood defect in 70% of embryos, which is not consistent with our results.[49] We hypothesize that FOXM1 may have different functions during embryogenesis in zebrafish or due to effects in the bone marrow microenvironment. There are heterogeneous cells in the bone marrow microenvironment, and they regulate each other through paracrine as well as autocrine mechanisms. Currently, optimal human cell model systems to study the extrinsic effects of FOXM1 *in vitro* are not available.

In addition, Hou *et al*. previously reported that *in vivo* deletion of Foxm1 in mice leads to more proliferation and fewer quiescent cells of CD34$^+$ cells, but did not affect the differentiation of mature blood cells or the frequency of erythroid blasts.[38] One consideration is the source of CD34$^+$ cells (i.e., cord blood for our studies and bone marrow for previous studies). We did examine the effects of FOXM1 knockdown and FOXM1 inhibitor FDI-6 in human bone marrow CD34$^+$ cells and we could still observe the same effects (*Online Supplementary Figure S20*). Therefore, the difference in our findings is likely due to the variability of FOXM1 expression. The aforementioned paper showed higher expression of Foxm1 in primitive hematopoietic cells than in differentiated cells including myeloid cells, B cells, erythroblasts, and T cells. However, we observed that FOXM1 is highly expressed in more differentiated hematopoietic progenitors, especially the erythroid progenitor. Our data suggest that FOXM1 plays a unique role in erythroid cells rather than in CD34$^+$ cells. In addition, there may be differences between the mouse and human hematopoietic systems.[39]

In summary, our results demonstrate a novel function of FOXM1 in normal human hematopoiesis, in which FOXM1 deficiency leads to an increased proliferation of erythroid progenitors, resulting in an increased erythroid differentiation. These results suggest that FOXM1 inhibitors, such as FDI-6, may be beneficial in treating certain patients with anemia.

Acknowledgments

The authors would like to thank Narla Mohandas for critical discussion, reading of our manuscript, and sharing the RNA-seq data. This research was funded by NIH R01HL097561, R01DK107286, Department of Defense BM110060 (KMS), K08 DK090145-06 (AN), the Child Health Research Institute at Stanford Postdoctoral Fellowship 1111239-280-JHACT (MY), Stanford Bio-X Undergraduate Summer Research Program (SK), and USHHS Ruth L. Kirschstein Institutional National Research Service Award # T32 CA009056 (EB).

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