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Epigenetic control of *Apolipoprotein E* expression mediates gender-specific hematopoietic regulation

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Abstract

Epigenetic alterations play a central role in the control of normal and malignant blood cell development. We demonstrate here that expression of a truncated DNA methyltransferase 3B isoform DNMT3B7, which has been shown to alter cellular epigenetic patterns, decreases the overall number of hematopoietic stem and progenitor cells (HSPCs), and markedly diminishes blood cell reconstitution within the female hormonal microenvironment. Gene expression profiling of HSPCs isolated from *DNMT3B7* transgenic embryos identified *Apolipoprotein E* (*Apoe*) as overexpressed. The CpG island controlling *Apoe* expression had lower levels of modified cytosines in *DNMT3B7* transgenic HSPCs, corresponding with the observed increase in gene expression. Furthermore, we observed that spleens and bone marrows of female mice transplanted with *DNMT3B7* transgenic HSPCs express very high levels of *Apoe*. Finally, the introduction of *Apoe*-overexpressing HSPCs into male recipients decreased bone marrow engraftment, recapitulating our original observations in female recipients. Our work reveals a dynamic interplay between the intrinsic epigenetic changes in HSPCs and extrinsic endocrine factors acting on these cells to regulate the efficiency of hematopoietic stem and progenitor cell engraftment and reconstitution. We have identified a novel mechanism by which gender-specific hormones modulate HSPC function, which could serve as a target for augmenting hematopoiesis in cases with limited HSC functionality.

Keywords

Hematopoiesis; Epigenetics; DNA methyltransferase 3B; Gender-specific hormones; Apolipoprotein E

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Author contributions

AV, CAR, and LAG designed the research; AV, HZ, JBL, KT, and NY performed research; AV, HZ, JA, and LAG analyzed data; AV and LAG wrote the paper. All authors edited and approved the final manuscript.

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Introduction

Hematopoietic stem cell (HSC) self-renewal and differentiation are exquisitely controlled developmental processes that are regulated by several factors modifying signaling pathways and transcription factor binding, leading to gene expression changes. Signaling via gender-specific nuclear hormone receptors is known to regulate hematopoiesis. Androgens and estrogens regulate lineage-specific differentiation, although the results regarding the specific direction of this regulation are conflicting [1–5]. Despite extensive studies on the relationship of gender-specific hormones with HSC function, the molecular mechanism behind these interactions has not been defined.

Gene expression changes that regulate hematopoietic self-renewal and differentiation pathways are driven in part by epigenetic modifications. Deficiency of DNA methyltransferase 3a (*Dnmt3a*), one of the enzymes that converts cytosine to 5-methylcytosine (5-mC), has been associated with the expansion of the HSC pool and a progressive loss of their differentiation capacity [6]. Sequencing of genomes of patients with acute myeloid leukemia (AML) identified the presence of *DNMT3A* mutations in ~20% of cytogenetically normal patients [7–9]. Subsequent studies indicated that at least some of these mutations conferred a dominant-negative phenotype to the mutated *DNMT3A* allele [10]. In addition to the *de novo* DNA methyltransferases, the maintenance methyltransferase Dnmt1 has been demonstrated to be required for HSC self-renewal, niche retention, and lineage-specific differentiation [11, 12]. We showed recently that erythroid differentiation is associated with dynamic changes in the levels of a second modified cytosine base, 5-hydroxymethylcytosine (5-hmC), with altered erythroid and myeloid differentiation observed in the context of mutations in Ten-Eleven Translocase protein 2 (*TET2*), the enzyme that converts 5-mC to 5-hmC [13]. These observations illustrate the central role played by epigenetic modifiers in the self-renewal and differentiation of hematopoietic stem and progenitor cells (HSPCs).

We showed previously that the expression of a truncated DNA methyltransferase 3B (DNMT3B) isoform, DNMT3B7, which is expressed in cancer cell lines and tissues isolated from cancer patients, promotes developmental defects and accelerates tumorigenesis in *Eμ-Myc* mice, which are predisposed to developing B-cell lymphomas [14]. Subsequently, we demonstrated that DNMT3B7 likely acts as a dominant negative inhibitor of full-length Dnmt3b, since *Dnmt3b* heterozygosity within *Eμ-Myc* mice accelerated tumorigenesis similar to Dnmt3b heterozygosity [15]. *Dnmt3b* knockout mice demonstrate developmental defects and have characteristics similar to patients who have Immunodeficiency, Centromeric instability, Facial anomalies (ICF) syndrome [16]. Given that *DNMT3B7* transgenic mice demonstrate defects in hematopoiesis and lineage-specific differentiation that are very similar to *Dnmt3b* deficiency [14], we tested the effect of *DNMT3B7* expression on hematopoiesis. Our work reveals a mouse model in which recipient gender modulates the rate of engraftment and reconstitution of HSPCs. Using this novel experimental model, we sought to identify the molecular mechanisms that operate in the hormone-mediated control of hematopoiesis.

Materials and Methods

Mice and transplantations

DNMT3B7 transgenic mice were maintained as described previously [14, 15, 17]. *Dnmt3b* knockout mice were a kind gift from En Li [16, 18]. *Apoe*$^{-/-}$ mice on the C57Bl/6 background were obtained and maintained as described [19, 20]. Transplantations were performed using ten million E14.5 fetal liver cells derived from timed mating of *DNMT3B7*-transgenic mice. Competitive transplantations were performed using 2 million cells from E14.5 CD45.2-bearing WT or *DNMT3B7*-transgenic fetal livers or from E12.5 fetal liver cells derived from timed mating of *Dnmt3b*-heterozygous mice, mixed with 0.5 million E14.5 fetal liver CD45.1-bearing cells as the competitor cells. Bone marrow transplantations using WT or *Apoe*$^{-/-}$ mice were performed using 1.25 million experimental cells mixed with 1.25 million competitor cells derived from bone marrows of CD45.1 mice. Recipient C57Bl/6 mice carrying either CD45.1 or CD45.1/CD45.2 markers were conditioned for transplantation with lethal irradiation (960 rads) using a cesium source. Four hours after irradiation, cells for transplantation were injected intravenously into the retro-orbital sinus of recipient mice under Ketamine/Xylazine anesthesia. Irradiated mice were provided with antibiotics (Bactrim) for 2 weeks after transplantation. Blood counts were measured at 2 weeks, 4 weeks, and monthly thereafter on the Hemavet CBC analyzer (CDC technologies). All mice were maintained in pathogen-free conditions according to an IACUC-approved protocol at The University of Chicago.

Flow cytometry

For analysis of stem cell numbers, E14.5 fetal livers from WT or *DNMT3B7*-transgenic embryos were made into single cell suspensions, and stained for lineage markers using FITC-tagged antibodies. The lineage negative population was further analyzed for Sca-1 and c-Kit positive populations or for the SLAM markers CD48 and CD150 [21]. For competitive transplantation experiments, engraftment potential was analyzed in the blood of mice at 2, 4 and 8 weeks after transplantation using antibodies against CD45.1 and CD45.2.

RNA-Sequencing

LSK cells were isolated from wild-type, *DNMT3B7* hemizygous and *DNMT3B7* homozygous embryos at E14.5. We obtained between 10,000 and 100,000 cells per embryo and pooled the cells from four to five embryos of each genotype to perform RNA-Sequencing. Total RNA was isolated using the RNeasy Micro Kit (Qiagen), and 1–3 μg of total RNA used to isolate mRNA by polyA selection. Libraries were generated, and single end sequencing was performed on the Illumina HiSeq 2000 machine.

The RNA-Sequencing reads for pooled LSK cells derived from WT, *DNMT3B7*-hemizygous, or *DNMT3B7*-homozygous E14.5 fetal livers were mapped to the reference genome NCBI37/mm9 using Tophat [22, 23]. Gene expression analysis was performed using Cufflinks [24]. Detailed description of RNA-Sequencing analysis and quantification is included in Supplemental Methods.

Analysis and visualization of RNA-Sequencing data

Using the FPKM values generated from the above analyses, an 'adjusted' gene list was generated for WT, *DNMT3B7* hemizygous and *DNMT3B7* homozygous LSK cells, consisting of genes that are uniquely expressed, or are 2-fold overexpressed in that genotype. To visualize these lists and compare the genes that are expressed in each genotype, a 3-way Venn diagram was generated using BioVenn [25]. More detailed descriptions are included in the Supplemental Methods.

From the Venn diagram, lists of genes that are uniquely expressed or overexpressed in a particular genotype were analyzed using gene ontology analysis pathways, specifically in the WebGestalt website [26]. Genes were subsequently ordered by the *DNMT3B7* dose-dependent decrease or increase in FPKM values, and the ontology of these genes was analyzed similar to above.

Analysis of modified cytosine levels

Bisulfite sequencing was performed on fetal liver cell DNA isolated from WT, DNMT3B7 hemizygous, and DNMT3B7 homozygous embryos at E14.5 as described [13, 14] using primers listed in Supplemental Table 1.

Reverse transcription and PCR amplification

Total RNA was made using Trizol (Invitrogen). Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Invitrogen). Real time PCR was performed using the Applied Biosystems 7500 Fast PCR system using the Power SYBR Green PCR Master Mix (Applied Biosystems). Primer sequences are listed in Supplemental Table S1. Genotyping of *DNMT3B7* mouse embryos was performed by real-time PCR using the TaqMan mastermix, using primers and probes as listed in Supplemental Table S1. Genotyping of Dnmt3b mouse embryos was performed by end-point PCR using the primers listed in Supplemental Table S1.

GEO Accession number

The data corresponding to the RNA-Sequencing run are provided in GEO accession number GSE56170.

Results

Embryonic fetal livers with altered epigenetic patterns contain fewer HSPCs

Given that alterations in DNA methylation patterns disrupt normal proliferation and differentiation of hematopoietic stem and progenitor cells [6, 11, 12], and that *DNMT3B7* transgenic mice display defective B-lymphopoiesis [14, 15], we sought to assay the total number of stem and progenitor cells produced in *DNMT3B7* transgenic mice. Flow cytometry was used to analyze the number of HSPCs in E14.5 fetal livers using Lineage negative (Lin-) Sca1+ c-Kit+ (hereafter referred to as LSK) cells as well as the SLAM markers, CD48 and CD150 [21]. Using the LSK population, we found 3-fold lower numbers of phenotypic stem and progenitor cells in embryos expressing *DNMT3B7* (Fig. 1A). Representative flow plots are shown in Fig. 1B. The number of later, committed progenitor

cells, marked by the SLAM marker CD48 within the Lin-population, is reduced by more than 3-fold in the *DNMT3B7*-expressing embryos (Fig. 1C). Representative flow plots are shown in Fig. 1D. We did not observe any significant difference in numbers of LT-HSC, marked by CD150+ CD48-SLAM markers (Supplemental Fig. S1A). This observation illustrates that the introduction of *DNMT3B7* results in a fewer number of HSPC and committed progenitor cells within the fetal livers, and argues that the predominant effect of *DNMT3B7* expression is within the committed progenitor cell compartment.

DNMT3B7 transgenic HSPCs are outcompeted in competitive transplantation experiments

To measure the functionality of HSPCs isolated from CD45.2-expressing *DNMT3B7* transgenic embryonic fetal livers, we performed competitive transplantation experiments in female CD45.1/CD45.2 recipient mice, using cells from wild-type CD45.1-expressing E14.5 embryonic fetal livers as competitor cells (Fig. 2A). The presence of both CD45.1 and CD45.2 markers on recipient mice helps distinguish endogenous cells within the recipients from the transplanted competitor (CD45.1) and experimental (CD45.2) cells. To give a competitive advantage to the experimental cells, only a quarter of the total number of cells injected comprised of competitor cells. As expected, we observed that donor-repopulation of CD45.2-expressing wild-type (WT) cells obtained from the *DNMT3B7* transgenic breeding was 4-fold better than the competitor CD45.1-expressing WT cells (Fig. 2B). The donor repopulation of CD45.2-expressing cells from *DNMT3B7* hemizygous (Hemiz) embryos demonstrated an early defect, which was overcome by two months post-transplantation (Fig. 2C). The most striking observation came from the transplantation of CD45.2-expressing *DNMT3B7* homozygous (Homoz) cells, which were outcompeted by the CD45.1-expressing WT cells that were provided at a quarter of the total number of cells (Fig. 2D).

The diminished repopulation of donor cells in *DNMT3B7* homozygous cells raised the possibility that the phenotype is due to the loss of expression of one or more genes due to the transgene insertion site, rather than the expression of *DNMT3B7* itself. Therefore, we performed competitive transplantation experiments using fetal liver cells retrovirally transduced with *DNMT3B7*. Consistent with our observation that the expression of DNMT3B7 is responsible for the observed phenotype, the introduction of *MigR1-DNMT3B7* into CD45.2-bearing wild-type fetal liver cells diminished the engraftment of CD45.2+ cells in irradiated female mice relative to those that received only *MigR1* (vector only). Furthermore, cells expressing *MigR1-DNMT3B7* were outcompeted by the CD45.1-expressing competitor cells at two weeks post-transplantation (Supplemental Fig. S1B–C). Thus, transgenic or retroviral expression of *DNMT3B7* compromises the ability of HSPCs to engraft and reconstitute in recipients.

To determine whether the altered engraftment and reconstitution of *DNMT3B7* transgenic cells was a result of a dominant negative effect on Dnmt3b activity [14–16], we performed competitive transplantation experiments using cells isolated from E12.5 fetal livers of WT, *Dnmt3b*$^{+/-}$, or *Dnmt3b*$^{-/-}$ embryos (Supplemental Fig. S2A–C). We observed that regardless of *Dnmt3b* expression, the CD45.2-expressing experimental cells engrafted better than CD45.1-expressing WT competitor cells within female recipient mice (Supplemental Fig. S2A–C), consistent with previous work [6]. In the control of engraftment and

reconstitution, *DNMT3B7* transgenic cells act differently than *Dnmt3b* knockout cells, suggesting that the effects of DNMT3B7 on hematopoiesis is not due to a dominant negative phenotype.

DNMT3B7-expressing HSPCs exhibit delayed reconstitution of blood counts in female mice

To assess the effects of *DNMT3B7* expression on peripheral blood cell counts, we performed transplantation experiments using cells collected from fetal livers of WT, DNMT3B7 hemizygous or DNMT3B7 homozygous embryos at embryonic day E14.5. Since *DNMT3B7* homozygous mice do not survive past the day of birth, transplantation is the most effective method to monitor hematopoietic effects of DNMT3B7. We observed that multilineage reconstitution was compromised in female recipients of *DNMT3B7* homozygous cells, especially in the first two months after transplantation (Fig. 3, Supplemental Fig. S3). Specifically, female mice receiving *DNMT3B7* homozygous cells were leukopenic, lymphopenic, anemic, and thrombocytopenic as early as two weeks after transplantation (Fig. 3A–D, solid bars), whereas male mice engrafted and reconstituted cells normally, irrespective of the genotype of the cells received (Fig. 3E–H, solid bars). While the effect was more dramatic in certain female mice that succumbed to low blood counts by three months post-transplant, generally, the overall blood counts of the female mice stabilized and were equivalent to male mice two months after transplantation (Supplemental Fig. S3). We measured apoptotic potential and cell cycle profiles in cells isolated from bone marrows of mice receiving WT or *DNMT3B7* homozygous cells, and found that whereas there was no significant difference in the number of apoptotic cells in male versus female recipients, there were trends consistent with an increase in early-stage apoptotic cells in female recipients relative to males (Supplemental Fig. S4A). We also found a significant increase in cells in the S-phase in female mice receiving *DNMT3B7* homozygous receiving females compared to males or female mice receiving WT cells (Supplemental Fig. S4B–C). *DNMT3B7* was expressed both in fetal livers being transplanted into the mice (Supplemental Fig. S5A) and in spleens of mice that had been transplanted with *DNMT3B7* transgenic cells at four months post-transplantation (Supplemental Fig. S5B). We found that donor cell gender had no effect on the engraftment phenotype within our model (Supplemental Fig. S6). Complete blood count data was supported by the counts generated from peripheral blood smears (data not shown).

These observations suggested that hormonal microenvironment within the recipient mice influences gender-related differences. We hypothesized that within our mouse model, one or both of the following occurred: (i) female hormones repressed normal hematopoiesis and/or (ii) male hormones enhanced hematopoietic function. We used hormonally compromised females (oophorectomized females) and males (castrated males) to uncover the role played by the major female and male hormones on hematopoietic stem cell engraftment and repopulation. Oophorectomy reduces plasma estradiol levels to <5% of normal levels (34 pg/mL to 2 pg/mL) [27], and castration reduces circulating testosterone levels to <0.1 ng/nmL [28], allowing us to test the specific roles of female and male hormones on hematopoiesis. Oophorectomized mice receiving *DNMT3B7* transgenic cells had partially recovered white blood counts (WBC) (1,900±800/μL vs. 810±420/μL in hormonally intact

females) and lymphocyte numbers (760±410/μL vs. 470±360/μL in hormonally intact females) (Fig. 3A–B, hatched bars). Interestingly, oophorectomized mice that received wild-type cells reconstituted their WBC and lymphocyte number to a better extent than hormonally intact females that received wild-type cells, suggesting that the leukocyte phenotype is at least partially independent of *DNMT3B7* expression. There was complete recovery of hemoglobin levels in oophorectomized female mice receiving *DNMT3B7* homozygous cells (11.13±1.59 g/dL vs. 5.96±1.61 g/dL in hormonally intact females) (Fig. 3C, hatched bars). Platelet numbers in oophorectomized female mice were improved compared to hormonally intact females, although the variance in platelet number within this group of mice was high (Fig. 3D, hatched bars). Castrated male mice did not demonstrate any significant differences in engraftment and reconstitution of cells compared to the hormonally intact male mice, regardless of the genotype of cells they received (Fig. 3E–H, hatched bars), suggesting that male hormones did not influence hematopoiesis. These data suggest that the female hormones repress hematopoiesis, especially within the context of cells with aberrant DNA methylation. We also found that the genotype of the donor fetal liver stem cells, but not their gender, predicted total stem cell numbers (Fig. 1, Supplemental Table S2).

DNMT3B7 expression in HSPCs upregulates genes involved in hormone signaling

We hypothesize that expression changes in *DNMT3B7* transgenic HSPCs relative to WT cells drive the observed reduction in engraftment and repopulation within female recipients. To determine whether the introduction of *DNMT3B7* regulated the expression of specific genes that may alter hematopoietic function, we performed RNA-Sequencing of the LSK population isolated from wild-type, *DNMT3B7* hemizygous or *DNMT3B7* homozygous embryos at E14.5.

To distinguish genes that demonstrated altered expression with the introduction of *DNMT3B7*, we first identified genes that were uniquely expressed within LSK cells obtained from WT, *DNMT3B7* hemizygous, and *DNMT3B7* homozygous embryos (Fig. 4A). Further, to detect the overlap of the genes upregulated in WT, *DNMT3B7* hemizygous, and *DNMT3B7* homozygous LSK cells, we charted MA (M: log intensity ratios; A: log intensity averages) plots in a pair-wise fashion, and found that >60% of genes were virtually indistinguishable across all three genotypes (Fig. 4B). Using the gene ontology pathway analysis software on Webgestalt [26], we determined that homing-associated pathways were enriched in WT LSK cells (Supplemental Fig. S7A), whereas many receptor-ligand signaling pathways were enriched in the *DNMT3B7* hemizygous and *DNMT3B7* homozygous cells (Supplemental Fig. S7B–C). Furthermore, we performed gene ontology analysis on the list of genes that were up- and down-regulated in a dose-dependent manner with *DNMT3B7* expression (Fig. 4, C and D). Mostly, genes involved in metabolic pathways were enriched with both *DNMT3B7* up-and down-regulation. Genes that were downregulated with *DNMT3B7* expression were enriched in signaling pathways including the *Jak-STAT*, *Wnt*, and *ErbB* signaling pathways, all of which are known to be involved in the maintenance of normal hematopoietic function (Fig. 4C). Interestingly, genes that were upregulated with *DNMT3B7* expression were enriched in hormone-specific pathways, such

as progesterone mediated oocyte maturation and GnRH signaling (Fig. 4D), suggesting that gender-specific hormones may play a role in the observed hematopoietic defects.

Lower levels of DNA cytosine modifications within the CpG island of *Apolipoprotein E* in *DNMT3B7* transgenic fetal liver cells

Previously, we had observed that the expression of *DNMT3B7* in several types of cells was associated with global changes in DNA cytosine modifications [14, 15, 17]. Using liquid chromatography-tandem mass spectrometry (LC-MS/MS) we found no differences in the global levels of 5-mC and 5-hmC in WT and *DNMT3B7* transgenic fetal liver cells (Supplemental Fig. S8A–B). We tested the top twenty genes demonstrating dose-dependent alterations in gene expression with *DNMT3B7* introduction for regional cytosine modifications. We focused on genes that had CpG islands (in bold) and were known to be associated with hematopoiesis or were regulated hormonally (Table 1).

We first measured cytosine modifications in the CpG island of the selected genes in fetal liver gDNA from WT, *DNMT3B7* hemizygous, and *DNMT3B7* homozygous embryos at E14.5, using bisulfite sequencing. Hematopoietic genes, including *Runx2*, *Hist1h2al*, *Dek*, and *Atrx* did not demonstrate differences in cytosine modification between WT and *DNMT3B7* transgenic fetal livers (data not shown). *Apolipoprotein E* (*Apoe*) encodes a protein associated with plasma lipoproteins that is a ligand for cell surface receptors and promotes cellular cholesterol efflux and thus functions in removing cholesterol from the blood and cells and transporting it to the liver for processing. In addition, it has recently been found to repress proliferation and differentiation of hematopoietic stem cells in mouse models [16, 18]. *Apoe* had significantly lower levels of DNA cytosine modification in the *DNMT3B7* homozygous embryos compared to WT embryos (Fig. 5A). The *Apoe* CpG island is uniquely placed in exon 4 of *Apoe*, downstream of the transcriptional start site. Methylation at this island has been shown to regulate the transcription of *APOE* [19].

Since fetal liver gDNA was used for the cytosine modification analyses, the change in expression of *Apoe* observed in the RNA-Seq was validated in total fetal liver RNA, using real-time PCR (Fig. S9A, GEO submission GSE56170). As expected, WT fetal liver RNA had the lowest levels of *Apoe* mRNA and *DNMT3B7* homozygous fetal livers had very high levels of *Apoe* (Fig. S8A).

Apoe expression is regulated by levels of female hormones

We analyzed *Apoe* mRNA expression in the hematopoietic organs (spleen and bone marrow) of mice that had been transplanted with WT or *DNMT3B7* transgenic cells. By RT-PCR, we found that male mice receiving WT cells had the lowest levels of *Apoe*, and as *DNMT3B7* expression increased, there was a dose-dependent increase in *Apoe* levels (Fig. 5B, left solid bars). This dose-dependent increase in *Apoe* expression held true within each gender sub-group (Fig. 5B). Overall, female mice had significantly higher expression of *Apoe* than male mice (Fig. 5B, right solid bars). Strikingly, oophorectomized female mice had lower levels of *Apoe* than did hormonally intact females and exhibited trends very similar to the male mice (Fig. 5B, hatched bars), supporting the theory that *Apoe* levels are positively associated with the presence of female hormones. As indicated below the graph,

female hormone levels increase from left to right, and the increase in *Apoe* expression levels followed this dynamic trend. ApoE protein levels were also up-regulated in a similar genotype and gender-dependent manner: female mice expressing the *DNMT3B7* transgene had the highest levels of *Apoe* mRNA within the spleen, whereas male WT mice had the lowest levels (Supplemental Fig. S9B). We also observed a similar trend in *Apoe* mRNA expression within bone marrows isolated from transplanted male and female mice. Female mice consistently had higher levels of *Apoe* than male mice, and the expression of *DNMT3B7* led to *Apoe* up-regulation (Supplemental Fig. S9C), suggesting that the regulation of ApoE by female hormones influences HSPC function in all hematopoietic organs.

Apoe overexpression decreases the engraftment of HSPCs into male recipients

To test directly whether ApoE expression controls the engraftment and reconstitution of HSPCs within the bone marrow, competitive transplantation experiments were performed. Wild-type CD45.2+ E14.5 fetal liver cells were retrovirally transduced with *MigR1* or *MigR1-mApoe* and were mixed at a proportion of 4:1 with competitor CD45.1+ cells. Since transduction efficiency for both *MigR1* and *MigR1-mApoe* was ~30%, the final proportion of experimental GFP+ cells to competitor CD45.1+ cells was 1.2:1. The mixture of cells was transplanted into lethally irradiated CD45.1+/CD45.2+ male recipients. We found that the *MigR1*-tranduced cells engrafted normally, and even appeared to repopulate the hematopoietic system at a higher efficiency at the later time points of four and eight weeks. However, we found a significant deficit in the engraftment of *MigR1-mApoe* transduced cells in recipient male mice (Fig. 6A), with a parallel increase in the engraftment of competitor CD45.1 cells (Supplemental Fig. S10A). There was a 2-fold induction of ApoE expression in *MigR1-mApoe*-transduced cells relative to *MigR1*-transduced cells (Supplemental Fig. S10B), which was similar to the increase observed in mice transplanted with *DNMT3B7* homozygous cells (Fig. 5B). We also tested whether Apoe deficiency would affect engraftment in female mice. Bone marrow cells isolated from CD45.2+ WT or $Apoe^{-/-}$ males or females were mixed in equal quantities with competitor bone marrow cells from WT CD45.1+ female mice, and transplanted into irradiated CD45.1+/CD45.2+ WT female recipients (Supplemental Fig. S11A). Since equal quantities of CD45.1+ and CD45.2+ cells were transplanted into recipients, we would expect 1:1 engraftment if there were no differences in engraftment potential. We found that cells isolated from WT CD45.2+ mice engrafted at the expected 1:1 ratio at 2, 4, and 8 weeks after transplantation. However, *Apoe* deficiency enhanced donor repopulation of CD45.2+ cells by ~60–80% (Supplemental Fig. S11B). This was significantly different from the 1:1 ratio obtained from WT CD45.2+ cells (Supplemental Fig. S11B). Of note, donor gender had no effect on the engraftment of HSPCs (data not shown).

Discussion

Our study demonstrates for the first time that expression of *Apoe* contributes to gender-specific differences in blood counts. We find that the epigenetic changes intrinsic to the stem cells cooperate with the effect of female hormones that work in an extrinsic manner to regulate the efficiency of HSPC engraftment and reconstitution. Specifically, we

demonstrate that *DNMT3B7*-expressing HSPCs have fewer phenotypic stem and progenitor cells, and have a lower engraftment and repopulation potential compared to wild-type cells. While illustrating the molecular mechanism of this defect, we find that *DNMT3B7* expression decreases overall cytosine modification within the CpG island of *Apoe*, associated with an increase in *Apoe* mRNA expression. The increase in *Apoe* mRNA expression mediates defective engraftment in female recipients of *DNMT3B7* transgenic cells, given that overexpression of *Apoe* represses bone marrow engraftment of HSPCs in male mice.

The gender-mediated difference in engraftment is consistent with a recent study showing that oophorectomy, which diminishes the levels of female hormones, expanded the pool of short-term HSPCs and the eventual recovery after bone marrow transplantation via the CD40 ligand, a T-cell co-stimulatory molecule [29]. We also observed a short-term defect in stem cell reconstitution and engraftment, since female mice receiving *DNMT3B7* homozygous cells had stabilized their blood counts after two months post-transplantation (Fig. 3 and Fig. S3). Recent data has shown that proliferative multipotent progenitors are more prone to apoptosis, unlike LT-HSCs [30]. Likewise, it was shown previously that the proliferation of early B-lineage precursors is negatively regulated by estrogen treatment [2, 3, 31]. Contrary to our observations, however, another recent study demonstrated that estrogens promoted HSC self-renewal and the expansion of splenic HSCs and erythropoiesis during pregnancy via estrogen receptor signaling pathways [4]. Differences in experimental conditions likely led to these discrepancies. First, the majority of our experiments used fetal liver stem and progenitor cells, which contain a larger proportion of non-quiescent, cycling cells than do bone marrow stem cells. HSPCs derived from fetal livers function differently than do the HSPCs from bone marrow. For example, IL-7 signaling has been shown to be important in B-cell development in bone marrow, but not in fetal livers [32]. Furthermore, the *DNMT3B7* transgenic mice had disrupted epigenetic patterns resulting in the overexpression of *Apoe* and leading to the observed differences in engraftment. Nevertheless, together with our study, these observations substantiate the argument that gender-specific hormones play a key role in hematopoietic control by modulating hormone-mediated signaling pathways.

That *Apoe* mRNA levels are highest in female mice receiving *DNMT3B7* transgenic cells in our model is reminiscent of historic studies that showed that adult females have higher *APOE* levels than age-matched males [33]. Estrogen has been shown to upregulate *Apoe* gene expression in C57Bl/6 mice by altering post-transcriptional mechanisms via the recruitment of the *Apoe* mRNA to the translating pool of polysomes [34], within most mouse strains tested, including C57Bl/6 [35]. Within an astrocytoma cell line, progesterone but not estrogen, was shown to induce APOE secretion [36], suggesting that cell-type specific differences in response to hormones may dictate the APOE-mediated control of HSPC function. Gender specific differences in *APOE* expression have been revealed within the context of Alzheimer's disease, in which the female gender and the *ε4* genotype of *APOE* increases risk for Alzheimer's disease up to four-fold [37].

Based on our observations, a potential molecular model involves the interplay between epigenetic modifications and female hormones to regulate hematopoietic stem cell function.

Apoe expression is upregulated in *DNMT3B7* transgenic fetal liver cells, likely as a result of cytosine modifications within its CpG island. This increase in Apoe makes the cells more sensitive to exposure to female hormones like estrogen, which further increases the *Apoe* levels in *DNMT3B7*-expressing HSPCs within the female microenvironment. In contrast to the physiological response in *Apoe* knockout mice [18, 38], the increase in *Apoe* levels in female mice transplanted with *DNMT3B7* transgenic cells leads to reduced proliferation of HSPCs and disrupted lineage-specific differentiation as a result of diminished growth and signaling pathways (Fig. 6C).

Our work emphasizes the importance of experimental design that includes both male and female investigative cohorts, an idea recommended by the NIH recently to make preclinical research more rigorous [39]. We could observe the striking difference in *Apoe* levels within the HSPC population in male and female bone marrows only by analyzing our transplantation data in gender-specific cohorts. This work also provides a basis for modifying the hormonal environment in patients experiencing bone marrow failure, or who have limited HSC populations, so rapid recovery of peripheral blood counts can be induced. Importantly, our work establishes a novel mechanism that includes hormonal action, epigenetic modifications, and cholesterol metabolism by which stem cell function is maintained, and provides several targets via which early stem cell function may be augmented.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Goldberg GL, Dudakov JA, Reiseger JJ, et al. Sex Steroid Ablation Enhances Immune Reconstitution Following Cytotoxic Antineoplastic Therapy in Young Mice. THE JOURNAL OF IMMUNOLOGY. 2010; 184(11):6014–6024. [PubMed: 20483779]
2. Medina KL, Kincade PW. Pregnancy-related steroids are potential negative regulators of B lymphopoiesis. PROC NATL ACAD SCI US A. 1994; 91(12):5382–5386.
3. Medina KL, Strasser A, Kincade PW. Estrogen influences the differentiation, proliferation, and survival of early B-lineage precursors. BLOOD. 2000; 95:2059–2067. [PubMed: 10706875]
4. Nakada D, Oguro H, Levi BP, et al. Oestrogen increases haematopoietic stem-cell self-renewal in females and during pregnancy. NATURE. 2014; 505(7484):555–558. [PubMed: 24451543]
5. Singer JW, Samuels AI, Adamson JW. Steroids and hematopoiesis. I. The effect of steroids on in vitro erythroid colony growth: structure/activity relationships. J CELL PHYSIOL. 1976; 88(2):127–134. [PubMed: 1270518]
6. Challen GA, Sun D, Jeong M, et al. Dnmt3a is essential for hematopoietic stem cell differentiation. NAT GENET. 2012; 44(1):23–31. [PubMed: 22138693]

7. Ley TJ, Ding L, Walter MJ, et al. DNMT3AMutations in Acute Myeloid Leukemia. N ENGL J MED. 2010; 363(25):2424–2433. [PubMed: 21067377]
8. Walter MJ, Ding L, Shen D, et al. Recurrent DNMT3A mutations in patients with myelodysplastic syndromes. LEUKEMIA. 2011; 25(7):1153–1158. [PubMed: 21415852]
9. Yamashita Y, Yuan J, Suetake I, et al. Array-based genomic resequencing of human leukemia. ONCOGENE. 2010; 29(25):3723–3731. [PubMed: 20400977]
10. Kim, SJ.; Zhao, H.; Hardikar, S., et al. A DNMT3A mutation common in AML exhibits dominant-negative effects in murine ES cells. 2013.
11. Bröske A-M, Vockentanz L, Kharazi S, et al. DNA methylation protects hematopoietic stem cell multipotency from myeloerythroid restriction. NAT GENET. 2009; 41(11):1207–1215. [PubMed: 19801979]
12. Trowbridge JJ, Snow JW, Kim J, et al. DNA Methyltransferase 1 Is Essential for and Uniquely Regulates Hematopoietic Stem and Progenitor Cells. CELL STEM CELL. 2009; 5(4):442–449. [PubMed: 19796624]
13. Madzo J, Liu H, Rodriguez A, et al. Hydroxymethylation at Gene Regulatory Regions Directs Stem/Early Progenitor Cell Commitment during Erythropoiesis. CELL REP. 2014; 6(1):231–244. [PubMed: 24373966]
14. Shah MY, Vasanthakumar A, Barnes NY, et al. DNMT3B7, a truncated DNMT3B isoform expressed in human tumors, disrupts embryonic development and accelerates lymphomagenesis. CANCER RES. 2010; 70(14):5840–5850. [PubMed: 20587527]
15. Vasanthakumar A, Lepore JB, Zegarek MH, et al. Dnmt3b is a haploinsufficient tumor suppressor gene in Myc-induced lymphomagenesis. BLOOD. 2013; 121(11):2059–2063. [PubMed: 23315164]
16. Okano M, Bell DW, Haber DA, et al. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. CELL. 1999
17. Ostler KR, Yang Q, Looney TJ, et al. Truncated DNMT3B isoform DNMT3B7 suppresses growth, induces differentiation, and alters DNA methylation in human neuroblastoma. CANCER RES. 2012; 72(18):4714–4723. [PubMed: 22815530]
18. Murphy AJ, Akhtari M, Tolani S, et al. ApoE regulates hematopoietic stem cell proliferation, monocytosis, and monocyte accumulation in atherosclerotic lesions in mice. J CLIN INVEST. 2011; 121(10):4138–4149. [PubMed: 21968112]
19. Yu C-E, Cudaback E, Foraker J, et al. Epigenetic signature and enhancer activity of the human APOE gene. HUM MOL GENET. 2013; 22(24):5036–5047. [PubMed: 23892237]
20. Sontag TJ, Krishack PA, Lukens JR, et al. Apolipoprotein A-I protection against atherosclerosis is dependent on genetic background. ARTERIOSCLER THROMB VASC BIOL. 2014; 34(2):262–269. [PubMed: 24334873]
21. Kiel MJ, Yilmaz ÖH, Iwashita T, et al. SLAM Family Receptors Distinguish Hematopoietic Stem and Progenitor Cells and Reveal Endothelial Niches for Stem Cells. CELL. 2005; 121(7):1109–1121. [PubMed: 15989959]
22. Trapnell C, Pachter L, Salzberg SL. TopHat: discovering splice junctions with RNA-Seq. BIOINFORMATICS. 2009; 25(9):1105–1111. [PubMed: 19289445]
23. Trapnell C, Salzberg SL. How to map billions of short reads onto genomes. NATURE BIOTECHNOLOGY. 2009
24. Trapnell C, Williams BA, Pertea G, et al. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. NATURE BIOTECHNOLOGY. 2010; 28(5):511–515.
25. Hulsen T, de Vlieg J, Alkema W. BioVenn - a web application for the comparison and visualization of biological lists using area-proportional Venn diagrams. BMC GENOMICS. 2008; 9:488. [PubMed: 18925949]
26. Zhang B, Kirov S, Snoddy J. WebGestalt: an integrated system for exploring gene sets in various biological contexts. NUCLEIC ACIDS RES. 2005; 33(Web Server issue):W741–8. [PubMed: 15980575]

27. Altavilla D, Saitta A, Galeano M, et al. The Phytoestrogen |[agr]|-Zearalenol Reverses Endothelial Dysfunction Induced by Oophorectomy in Rats. LABORATORY INVESTIGATION. 2001; 81(2):125–132. [PubMed: 11232633]
28. Christoffersen B, Raun K, Svendsen O, et al. Evalution of the castrated male Sprague–Dawley rat as a model of the metabolic syndrome and type 2 diabetes. INT J OBES RELAT METAB DISORD. 2006; 30(8):1288–1297.
29. Li JY, Adams J, Calvi LM, et al. Ovariectomy expands murine short-term hemopoietic stem cell function through T cell expressed CD40L and Wnt10B. BLOOD. 2013; 122(14):2346–2357. [PubMed: 23954891]
30. Sánchez-Aguilera A, Arranz L, Martín-Pérez D, et al. Estrogen signaling selectively induces apoptosis of hematopoietic progenitors and myeloid neoplasms without harming steady-state hematopoiesis. CELL STEM CELL. 2014; 15(6):791–804. [PubMed: 25479752]
31. Medina KL, Garrett KP, Thompson LF, et al. Identification of very early lymphoid precursors in bone marrow and their regulation by estrogen. NAT IMMUNOL. 2001; 2(8):718–724. [PubMed: 11477408]
32. Kondo M, Wagers AJ, Manz MG, et al. Biology of hematopoietic stem cells and progenitors: implications for clinical application. ANNU REV IMMUNOL. 2003; 21:759–806. [PubMed: 12615892]
33. Phillips NR, Havel RJ, Kane JP. Sex-related differences in the concentrations of apolipoprotein E in human blood plasma and plasma lipoproteins. J LIPID RES. 1983; 25:1525–1531. [PubMed: 6655368]
34. Srivastava RA, Srivastava N, Averna M, et al. Estrogen up-regulates apolipoprotein E (ApoE) gene expression by increasing ApoE mRNA in the translating pool via the estrogen receptor alpha-mediated pathway. J BIOL CHEM. 1997; 272(52):33360–33366. [PubMed: 9407129]
35. Srivastava RA, Krul ES, Lin RC, et al. Regulation of lipoprotein metabolism by estrogen in inbred strains of mice occurs primarily by posttranscriptional mechanisms. MOL CELL BIOCHEM. 1997; 173(1–2):161–168. [PubMed: 9278267]
36. Fan J, Shimizu Y, Chan J, et al. Hormonal modulators of glial ABCA1 and apoE levels. J LIPID RES. 2013; 54(11):3139–3150. [PubMed: 23999864]
37. Altmann A, Tian L, Henderson VW, et al. Sex modifies the APOE-related risk of developing Alzheimer's disease. ANN NEUROL. 2014
38. Westerterp M, Gourion-Arsiquaud S, Murphy AJ, et al. Regulation of hematopoietic stem and progenitor cell mobilization by cholesterol efflux pathways. CELL STEM CELL. 2012; 11(2): 195–206. [PubMed: 22862945]
39. Clayton JA, Collins FS. Policy: NIH to balance sex in cell and animal studies. NATURE. 2014; 509(7500):282–283. [PubMed: 24834516]

Significance Statement

Alteration of epigenetic patterns, specifically DNA methylation patterns, within blood forming stem cells led to diminished function and reduced blood counts in female transplantation recipients. Stem cells with epigenetically altered patterns expressed high levels of a protein involved in cholesterol metabolism, Apolipoprotein e (Apoe). Apoe was also highly expressed in bone marrow isolated from female mice, regardless of genotype. Introduction of Apoe-deficient stem cells into female mice rescued the defective function and recapitulated the effects seen in male mice. Our work has identified a novel mechanism whereby gender-specific hormones can modulate blood cell development.

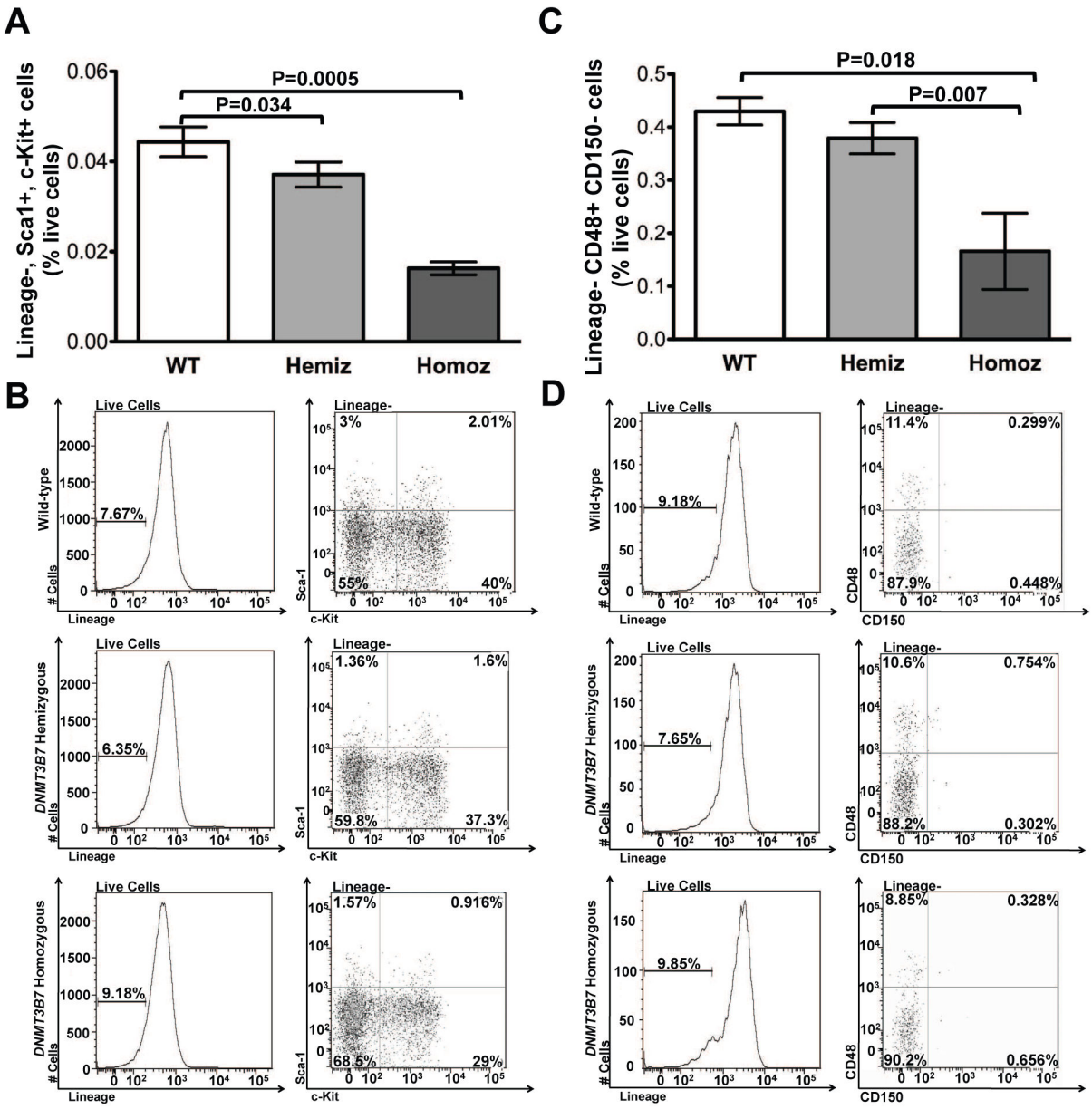

Figure 1. *DNMT3B7* homozygous E14.5 embryos have fewer numbers of HSPCs

(A) Lineage (FITC)-, Sca-1 (PE)+, c-Kit (APC)+ cells were stained in fetal livers isolated from E14.5 embryos that were Wild-type (WT: white), hemizygous *DNMT3B7* transgenic (Hemiz: light grey) and homozygous *DNMT3B7* transgenic (Homoz: dark grey). Average percentages from n ≥ 5 per group ± SEM are plotted. Two-tailed Student's *t*-test was used to determine statistical significance. (B) Representative plots analyzed using FlowJo to quantify the relative levels of the analyzed populations. (C) Lineage (FITC)-, CD48 (PE)+, CD150 (APC)+ cells were stained in fetal livers isolated from E14.5 embryos that were Wild-type (WT: white), hemizygous *DNMT3B7* transgenic (Hemiz.: light grey) and homozygous *DNMT3B7* transgenic (Homoz.: dark grey). Average percentages from n ≥ 4 per group ± SEM are plotted. Two-tailed Student's *t*-test was used to determine statistical

significance. (D) Representative plots analyzed using FlowJo to quantify the relative levels of the analyzed populations.

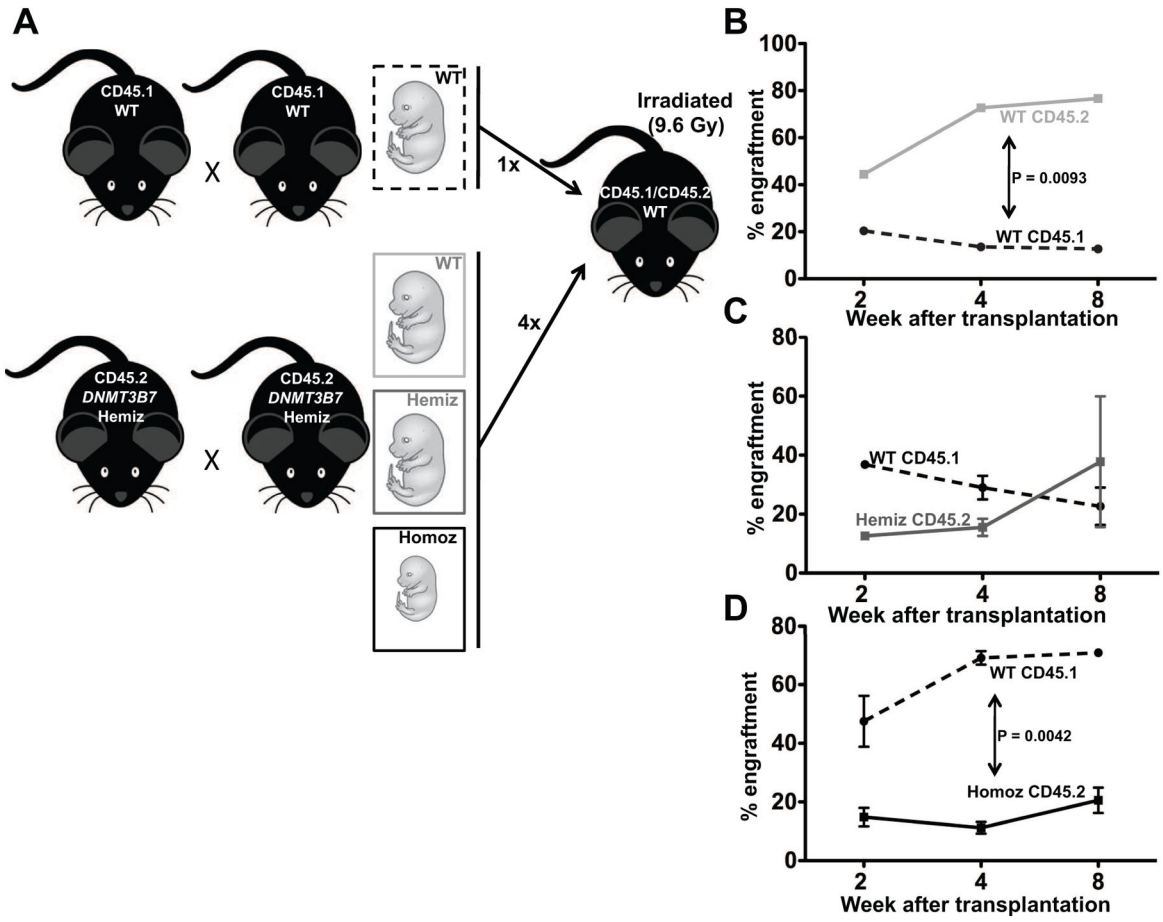

Figure 2. *DNMT3B7* homozygous cells are functionally incapable of repopulating the recipient bone marrow

(A) Schematic of competitive transplantation experiments. Briefly, CD45.2-bearing *DNMT3B7* hemizygous mice were timed-mated and embryos were isolated at E14.5. Simultaneously, CD45.1-bearing wild-type (WT) mice were timed-mated and embryos isolated at E14.5. Fetal liver cells were made into single-cell suspensions, and the CD45.1-bearing competitor WT cells were mixed with the CD45.2-bearing experimental cells at 1:4, and injected into lethally irradiated (at 960 rads) CD45.1/CD45.2-bearing WT female mice. Mice were monitored for two months and assessed for the engraftment of CD45.2+ cells. (B) Percent engraftment of CD45.1-bearing WT competitor cells (dashed line) and CD45.2-bearing WT experimental cells (light grey solid line). (C) Percent engraftment of CD45.1-bearing WT competitor cells (dashed line) and CD45.2-bearing *DNMT3B7*-hemizygous (Hemiz) experimental cells (dark grey solid line). (D) Percent engraftment of CD45.1-bearing WT competitor cells (dashed line) and CD45.2-bearing *DNMT3B7*-homozygous (Homoz) experimental cells (black solid line). In panels B–D, average percentages from n ≥ 4 per group ± SEM are plotted.

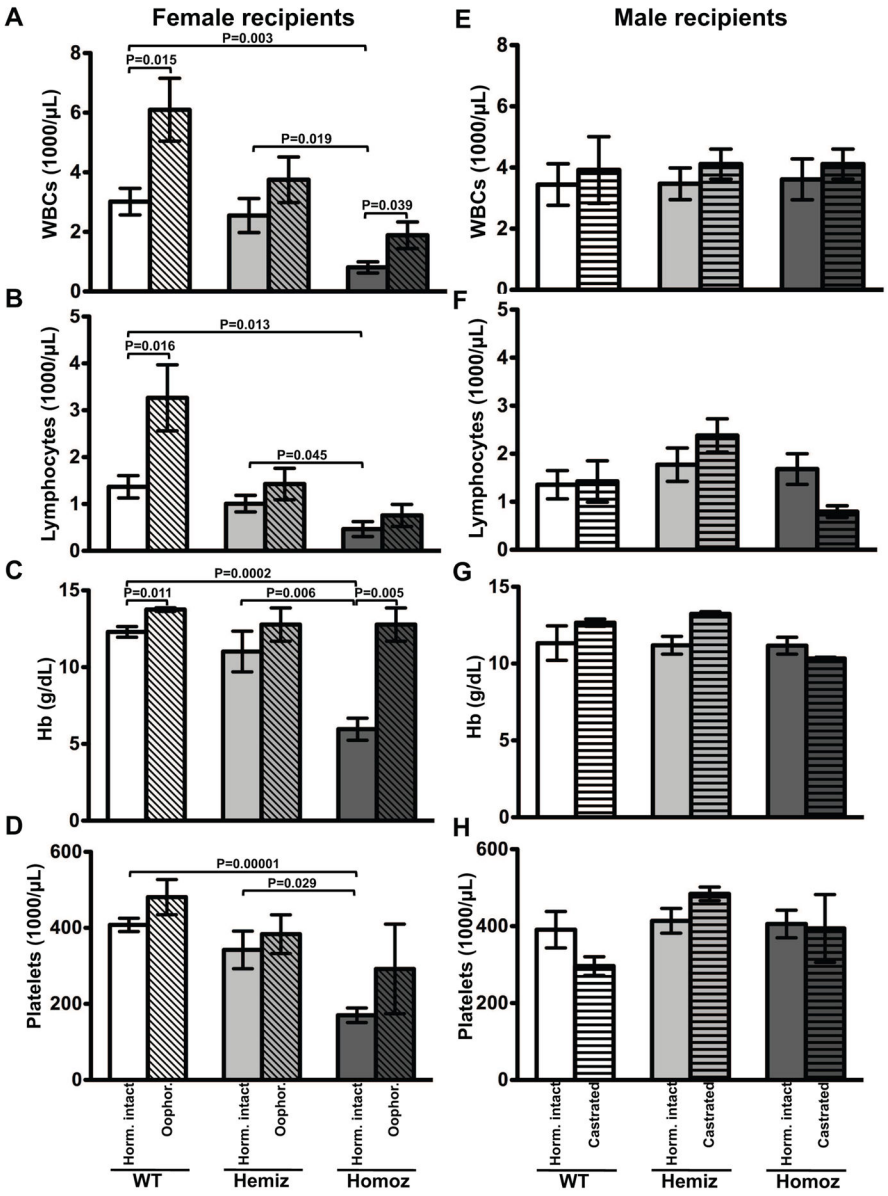

Figure 3. *DNMT3B7* transgenic HSPCs reconstitute normal blood counts in male, but not in female recipients

(A–D) Female and (E–H) Male recipients of wild-type (WT: white), hemizygous *DNMT3B7* transgenic (Hemiz: light grey) and homozygous *DNMT3B7* transgenic (Homoz: dark grey) cells at two weeks post-transplantation. Ten million cells isolated from fetal livers of E14.5 embryos of the three genotypes were transplanted into hormonally intact (solid bars) or hormonally compromised (hatched bars) females (A–D) or males (E–H) that had been lethally irradiated at 960 rads. (A), (E) Average WBC number (x1000/μL); (B), (F) lymphocyte number (x1000/μL); (C), (G) Hb concentration (g/dL); and (D), (H) platelet number (x1000/μL) from n ≥ 4 mice per genotype per gender are plotted ± SEM. Two-tailed Student's *t*-test was used to determine statistical significance.

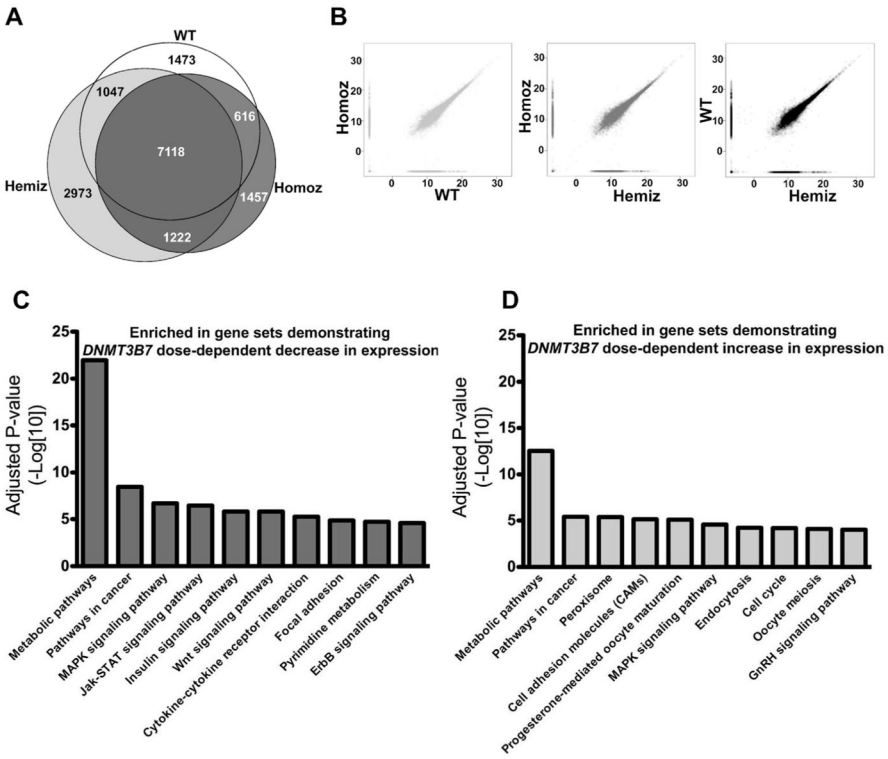

Figure 4. Enrichment of genes associated with hormone signaling pathways in LSK cells isolated from *DNMT3B7* transgenic fetal livers from E14.5 embryos

(A) Venn diagram depicting the number of unique genes expressed in WT (white), in *DNMT3B7* hemizygous (light grey), and in *DNMT3B7* homozygous (dark grey) LSK cells. Number of genes that overlap in all the three genotypes is in the center, and overlap of each pair of genotypes is shown at the intersection of the respective colors. (B) Pairwise M-A plots to visualize the extent of overlap between each pair of samples (WT vs. *DNMT3B7* Homoz; *DNMT3B7* Hemiz vs. *DNMT3B7* Homoz; *DNMT3B7* Hemiz vs. WT). Genes ordered by *DNMT3B7* dosage effects were input into gene ontology pathway analyses, and enriched pathways are shown in (C) genes showing *DNMT3B7* dose-dependent decrease and (D) genes showing *DNMT3B7* dose-dependent increase in expression.

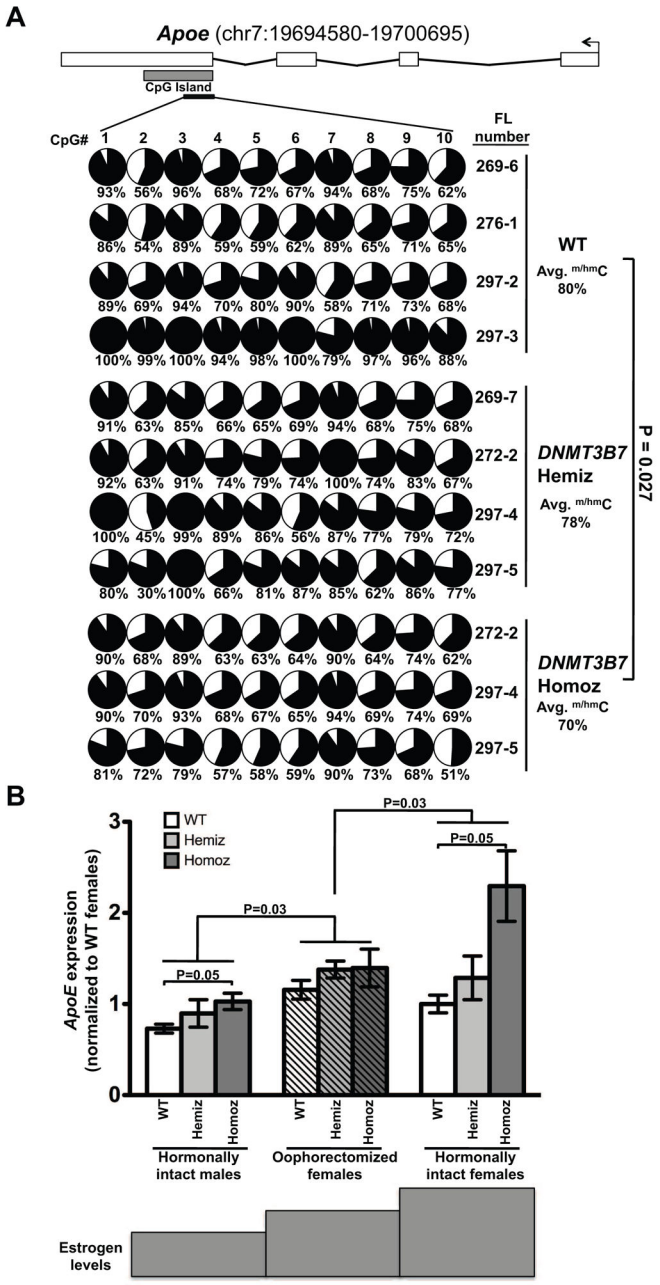

Figure 5. Reduction in DNA cytosine modifications in the CpG island of *Apoe* leads to increased *Apoe* expression in female mice transplanted with *DNMT3B7* transgenic cells

(A) DNA cytosine modifications in WT, *DNMT3B7* hemizygous, or *DNMT3B7* homozygous fetal liver cells measured by bisulfite sequencing around the CpG island of mouse *Apoe* (located within exon 4). Schematic diagram for *Apoe* is shown with exons represented by vertical rectangles, and the location of the CpG island shown with a horizontal shaded rectangle. The black arrow indicates the transcriptional start site (TSS). The smaller, black horizontal rectangle indicates the location of CpGs analyzed for changes in DNA cytosine modifications. Each row represents DNA methylation in a single E14.5 fetal liver, identified by the number on the right. Numbers across the top indicate specific

CpG dinucleotides in a region of the CpG island. Changes in DNA cytosine modification are indicated by shaded circles, with the shading indicating average amount of DNA cytosine modification at each CpG, and numbers below represent percent modified cytosine. Average percent modification is indicated to the right, and P-values calculated using the two-tailed Student's *t*-test. (B) *Apoe* expression in spleens isolated from hormonally intact males (left set of bars), oophorectomized females (middle set of bars), and hormonally intact females (right set of bars) receiving WT (white), *DNMT3B7* hemizygous (light grey) or *DNMT3B7* homozygous (dark grey) fetal liver cells. Relative expression was measured by real-time PCR and with *β-Actin* serving as the internal control and plotted normalized to WT hormonally intact females. Two-way ANOVA was used for comparison of the three groups, using genotype and gender as the two factors.

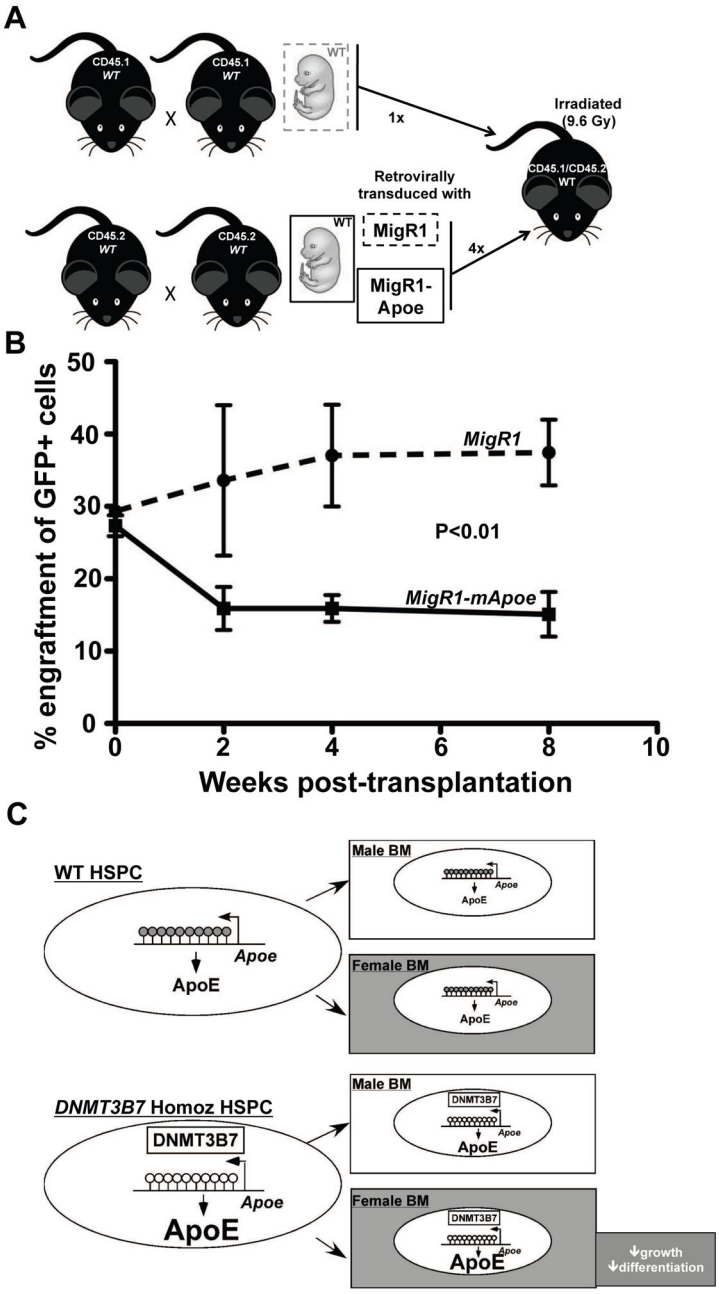

Figure 6. *Apoe* overexpression decreases HSPC engraftment potential in male mice

(A) Schematic of HSPC engraftment experiment. Retroviral vectors *MigR1* or *MigR1-mApoe* were transduced into CD45.2 wild-type E14.5 fetal liver cells. These cells were mixed with CD45.1-bearing competitor WT fetal liver cells at 4:1, and injected into lethally irradiated CD45.1/CD45.2 bearing WT female mice. Mice were monitored for two months and assessed for engraftment of GFP+ CD45.2-bearing cells. (B) Percent engraftment of cells transduced with *MigR1* (dashed line) or *MigR1-mApoe* (solid line) at two, four, and eight weeks post-transplantation. N= 5 for *MigR1* and N= 6 for *MigR1-mApoe* recipient mice; 2-way ANOVA was used to calculate biological significance (C) Proposed model for

the genotype and hormone-dependent differences observed in female mice. Expression of *DNMT3B7* alters DNA cytosine modifications within the *Apoe* gene and enhances ApoE protein expression. Whereas there is no further significant increase in ApoE in the male bone marrow microenviroment, within the female hormonal environment, with exposure to estrogen/progesterone, there is an increase in ApoE that is associated with *DNMT3B7* expression, which leads to downstream signaling of the IL-3 pathway and reduced growth and differentiation of HSPCs. Male bone marrow is depicted in white rectangular boxes and female bone marrow in light grey boxes.

Table 1

Top genes demonstrating *DNMT3B7* dose-dependent (i) decrease and (ii) increase in expression.

(i) Gene Name	WT FPKM >	HEMIZ FPKM >	HOMOZ FPKM	CpG Island
Mir669m-1	1,290,000.00	611,390.00	0.01	No
Mir3471-2	538,646.00	509,252.00	0.01	No
n-R5s89	493,694.00	466,753.00	0.01	No
Mir1949	416,091.00	262,257.00	0.01	No
n-R5s24	296,423.00	280,248.00	0.01	No
Lpo	35,307.00	15,335.10	0.01	No
Rps25-ps1	20,768.30	11,389.00	0.01	No
Trav6d-5	19,503.20	18,438.90	0.01	No
Fam111a	17,283.50	0.01	0.01	No
Fbxo4	**14,219.60**	**8,131.64**	**0.01**	**Yes**
Hist1h2aI	**12,046.40**	**5,694.52**	**0.01**	**Yes**
Marcksl1-ps4	11,428.50	10804.9	0.01	No
Dnajc5b	10,455.30	4,942.37	0.01	Yes
Rbfox3	**10,423.30**	**9,854.53**	**0.01**	**No**
Lsm10	10,217.00	6,568.01	0.01	No
Trav15d-1-	9,852.60	9,314.95	0.01	Yes
AC158135.1	**9,582.96**	**1,670.09**	**0.01**	**Yes**
Mnf1	**8,979.29**	**4,468.84**	**0.01**	**Yes**
Ppp1r3g	**8,545.86**	**8,079.52**	**0.01**	**Yes**
Dek	**7,949.11**	**1,475.83**	**0.01**	**Yes**

(ii) Gene Name	WT FPKM <	HEMIZ FPKM	HOMOZ FPKM	CpG Island
Traj31	0.01	2,980,000.00	3,300,000.00	No
n-R5s58	0.01	260,995.00	577,825.00	No
n-R5s169	0.01	252,029.00	278,987.00	No
Galp	0.01	44,542.80	117,116.00	No
Snora74a	0.01	38,130.10	42,208.70	No
Pcca	0.01	6,097.24	36,947.60	No
Blcap	**0.01**	**520.83**	**28,806.00**	**Yes**
Apoc2	0.01	17,140.30	19,495.80	No
Rpl18-ps2	0.01	2,367.30	13,606.70	No
Smt3h2-ps	0.01	11,451.90	12,676.80	No
Ptprv	0.01	167.96	11,960.60	No
Sirt4	0.01	8,527.06	10,355.40	No
Lepre1	**0.01**	**778.97**	**778.97**	**Yes**
Ldlrad2	0.01	8,818.54	9,761.81	No
Tnp2	0.01	2,875.13	9,547.98	No

(ii) Gene Name	WT FPKM <	HEMIZ FPKM	HOMOZ FPKM	CpG Island
Adarb1	**0.01**	**4,016.42**	**8,892.06**	**Yes**
Apoe	**0.01**	**3,936.69**	**8,715.54**	**Yes**
Clstn3	0.01	1,765.51	8679.54	No
Wrap53	0.01	5073.23	8433.84	No
Chn2	**0.01**	**4238.58**	**8175.30**	**Yes**