Fanconi Anemia Mesenchymal Stromal Cells-Derived Glycerophospholipids Skew Hematopoietic Stem Cell Differentiation Through Toll-Like Receptor Signaling

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ABSTRACT

Fanconi anemia (FA) patients develop bone marrow (BM) failure or leukemia. One standard care for these devastating complications is hematopoietic stem cell transplantation. We identified a group of mesenchymal stromal cells (MSCs)-derived metabolites, glycerophospholipids, and their endogenous inhibitor, 5-(tetradecyloxy)-2-furoic acid (TOFA), as regulators of donor hematopoietic stem and progenitor cells. We provided two pieces of evidence that TOFA could improve hematopoiesis-supporting function of FA MSCs: (a) limiting-dilution cobblestone area-forming cell assay revealed that TOFA significantly increased cobblestone colonies in Fanca−/− or Fancd2−/− cocultures compared to untreated cocultures. (b) Competitive repopulating assay using output cells collected from cocultures showed that TOFA greatly alleviated the abnormal expansion of the donor myeloid (CD45.2+Gr1+Mac1+) compartment in both peripheral blood and BM of recipient mice transplanted with cells from Fanca−/− or Fancd2−/− cocultures. Furthermore, mechanistic studies identified Tlr4 signaling as the responsible pathway mediating the effect of glycerophospholipids. Thus, targeting glycerophospholipid biosynthesis in FA MSCs could be a therapeutic strategy to improve hematopoiesis and stem cell transplantation.

SIGNIFICANCE STATEMENT

Elevated levels of Glycerophospholipids impairs hematopoietic supporting function of Fanconi Anemia Mesenchymal Stromal Cells. Inhibition of glycerophospholipid biosynthesis in Fanconi Anemia Mesenchymal Stromal Cells by 5-(Tetradecyloxy)-2-furoic acid treatment or Lipin1 knockdown suppresses myeloid expansion. Glycerophospholipids regulate Hematopoietic Stem Cell differentiation through Toll-like receptor 4 signaling.

INTRODUCTION

Fanconi anemia (FA) is an inherited disorder associated with hematopoietic aplasia and cancer predisposition [1–3]. FA is genetically heterogeneous and the clinical phenotypes associated with FA are the result of deficiency of any of the 16 FA genes (FANCA-Q) [4–7]. Although physical signs appear from birth and early childhood, bone marrow (BM) failure is typically seen between ages 5 and 15 and in later ages leading to myelodysplastic syndrome and acute myeloid leukemia [8–10]. One standard care for these devastating complications is hematopoietic stem cell transplantation (HSCT). However, little is known about the interaction between healthy donor HSCs and FA BM microenvironment (niche). Recent HSC-BM niche interaction studies have demonstrated that nestin-expressing mesenchymal stromal cells (MSCs) constitute an essential HSC niche component [11, 12]. Adipocytes, one of the niche compartments, act as predominantly negative regulators of HSCs [13]; while osteoblasts and chondroblasts are known to support HSCs [14]. Although the role of majority of these cellular constituents forming the niche in the BM is becoming clear, the metabolism of these cell types in the context of hematopoietic support during disease state is still unclear.

To address this question, we used an untargeted metabolomics approach that provides a comprehensive platform to identify metabolites whose levels are altered between wild-type (WT) and FA MSCs. Metabolomics...
has become a powerful technique for understanding the small-molecule basis of biological processes either in physiological or pathological conditions [15]. We show here that a group of MSCs-derived metabolites, glycerophospholipids, and their endogenous inhibitor, 5-(Tetradecyloxy)-2-furoic acid (TOFA), are aberrantly produced by FA MSCs. To investigate

Figure 1.
the effect of these metabolites on hematopoietic-supporting function, we have modeled FA HSCT using ex vivo coculture followed by cobblestone area-forming cell (CAFC) and BM transplantation (BMT) assays and demonstrated that suppression of glycerophospholipid biosynthesis by TOFA or Lipin1 knockdown rescued differentiation skew of donor HSC and progenitor cells (HSPCs).

**Materials and Methods**

**Mice**

Fanca<sup>+/−</sup> and Fanca<sup>2+/−</sup> mice (C57BL/6: B6, CD45.2<sup>+</sup>) were provided by Dr. Madeleine Carreau (Laval University, Quebec, Canada) and Dr. Markus Grompe (Oregon Health & Sciences University, Portland, OR), respectively [16, 17]. Tlr2<sup>−/−</sup>, Tlr4<sup>−/−</sup> [18], and MyD88<sup>−/−</sup> [19] mice on C57BL/6 background were kindly provided by Drs. Senad Divanovi, Khurana Hershey, and Kasper Hoebe, respectively, at CCHMC with the permission of Shizuo Akira at Osaka University, Osaka, Japan. All the animals including BoyJ (C57BL/6: B6, CD45.1<sup>+</sup>) recipient mice were maintained in the animal barrier facility at Cincinnati Children's Hospital Medical Center. Mice used for the experiments were 8–12 weeks old. All experimental procedures conducted in this study were approved by the Institutional Animal Care and Use Committee of Cincinnati Children’s Hospital Medical Center.

**MSC Culture and Treatment**

BM cells isolated from WT, Fanca<sup>+/−</sup> and Fanca<sup>2+/−</sup> mice were gently flushed out of tibias and femurs using DPBS + 10% Fetal Bovine Serum. RBCs were lysed using red blood cell lysis buffer. Cells obtained from two tibias and two femurs were plated in 100 mm culture dish (BD Falcon, San Jose, CA) in 10 ml of MSCS media. MSCS media were prepared with Iscove’s Modified Dulbecco’s Medium (Invitrogen # 12440-053, Grand Island, NY), 20% bovine calf serum (Hyclone # SH30072.03, Logan, UT), epidermal growth factor (rmEGF—10 ng/ml; R&D Systems # 2028-EG-200, Minneapolis, MN), platelet-derived growth factor (rhPDGF—200 ng/μl; R&D Systems # 220-BB-010, Minneapolis, MN), 1% Penicillin-Streptomycin (Life Tech # 15140-122, Grand Island, NY), and 10−4 mol/l 2-mercaptoethanol (Life Tech # 21985-023, Grand Island, NY). Plastic adherent cells were passaged three times and were analyzed for MSCS purity using flow cytometry with cell surface markers positive for CD90 and negative for CD45 and CD34. Cells were further stained with antibodies Osteopontin, Fabp4, and Collagen II to identify osteoblasts, adipocytes, chondroblasts, respectively [20], using the Mouse MSC Functional Identification Kit (R&D Systems # SC010, Minneapolis, MN). At least 98% MSC purity was obtained with this culture method. MSCs at passages three were plated to obtain 95% confluence. Cells were pretreated with 2 mM TOFA (Sigma # T6575, St. Louis, MO) for 48 hours, followed by coculture in fresh media with WT Lin−/Sca1<sup>+</sup> cKit<sup>+</sup> (LSK) cells. For experiments with 1,2-dipalmitoyl-sn-glycerol-3-phosphoethanolamine (PE), WT LSK cells were treated with 0.5 mM PE (Sigma # P1348, St. Louis, MO) for 24 hours.

**CAFC Assay**

Confluent WT, Fanca<sup>−/−</sup> and Fanca<sup>2−/−</sup> MSCS in 35 mm culture dish (BD Falcon, San Jose, CA) were overlaid with WT BMMCs to allow the precursor cells forming hematopoietic clones under the stromal layers. The cells were cocultured at 37°C, 5% CO2, and were fed weekly by changing half of the medium. Phase-dark hematopoietic clone was imaged under phase-contrast images were taken at ×20 objective and the area was analyzed with image J software.

**Limited Dilution Assay**

Limiting dilution assay (LDA) of a LSK cells included the use of five dilutions (0, 10, 30, 90, 270, and 810) differing with a factor of 3, and 10 wells per cell concentration. Three different LDA experiments were performed with independently derived MSCS from WT, Fanca<sup>−/−</sup>, and Fanca<sup>2−/−</sup> mice. A well was scored as “positive” if contained one or more cobblestone areas and “negative” if contained no cobblestone areas. Cobblestone area is at least six cells (in proximity of each other) growing underneath the stroma. Although cobblestone-like cells appear as phase dark, these cells appear as nonrefractile in 96-well plates because of the deflection of light. Only dilutions with both negative and positive wells are informative for frequency analysis.

**BM Transplantation**

In the competitive repopulation study, 1×10<sup>5</sup> output cells (CD45.2) collected from cocultures were mixed with 3×10<sup>5</sup> competitor cells (CD45.1), and injected into lethally irradiated (split dose of 700Rad + 475Rad with 3 hours apart) Boy J mice (CD45.1). After 16 weeks, the recipient mice were sacrificed, and nucleated cells from peripheral blood and the BM were analyzed were stained with CD45.2 and CD45.1 for chimeras and Gr1, Mac1, B220, and CD3e for lineage.

**Figure 1.** Fanca<sup>−/−</sup> and Fanca<sup>2−/−</sup> MSCS impair WT hematopoietic stem cell and progenitor cell (HSPC) self-renewal and induce myeloid expansion. (A): Schematic representation of the ex vivo coculture experiments. WT LSK cells isolated by fluorescence-activated cell sorting were cultured on confluent stromal layers of WT, Fanca<sup>−/−</sup>, or Fanca<sup>2−/−</sup> MSCS followed by in CAFC or BM transplantation (BMT). (B): Limited dilution analysis of CAFC assay. Assay was conducted in a flat bottom 96-well plate with confluent MSCS before plating the sorted LSK cells. Cultures were maintained in 40% methyl cellulose medium for 2 weeks and the colonies were counted on weeks 1 and 2. Group of at least six phase dim cells were counted as one colony. (C): Abnormal myeloid expansion of WT HSPCs cocultured on Fanca<sup>−/−</sup> or Fanca<sup>2−/−</sup> MSCS in peripheral blood of irradiated recipient mice. 1×10<sup>5</sup> WT output cells (CD45.2<sup>+</sup>) collected after coculturing on WT, Fanca<sup>−/−</sup>, or Fanca<sup>2−/−</sup> MSCS for 5 days, along with 3×10<sup>5</sup> recipient BM cells (CD45.1<sup>+</sup>), were injected into lethally irradiated recipient mouse. Donor chimerism and lineage reconstitution in peripheral blood of the recipients were examined at 4 months post-transplantation. Representative flow plots (Left) and quantifications (Right) are shown. Results are means plus or minus SD of three independent experiments (n = 9 per group). *p < .05, **p < .01; ns: not significant. Error bars represent mean ± SD. Abbreviations: BM, bone marrow; CAFC, cobblestone area-forming cell; LSK, Lin−/Sca1+cKit<sup>+</sup>; MSCS, mesenchymal stromal cells; WT, wild type.
Figure 2. Metabolome profile of Fanca\(^{-/-}\) and Fancd2\(^{-/-}\) mesenchymal stromal cells (MSCs) reveals abnormal glycerophospholipid biosynthesis. (A): Cloud plot presentation of metabolite features of Fanca\(^{-/-}\) MSCs versus WT MSCs and Fancd2\(^{-/-}\) MSCs versus WT MSCs with fold change ≥3 and \(p\) value ≤.01. The statistical significance of the fold change was calculated by a Welch t test with unequal variances. Upregulated features (features that have a positive fold change) are graphed above the x-axis in green while downregulated features (features that have a negative fold change) are graphed below the x-axis in red. The x-axis represents retention time. The y-axis represents mass-to-charge (m/z) ratio. Features with higher fold change have larger radii. Features with lower \(p\) value have higher color intensity. (B): Venn diagram demonstrating the separate and overlapping metabolite features in Fanca\(^{-/-}\) and Fancd2\(^{-/-}\) MSCs compared to WT MSCs showing both upregulated and downregulated with fold change ≥3 and \(p\) value ≤.01. (C): Summary plot for metabolite set enrichment analysis where metabolic pathways are ranked according to log 2-fold change with the cut off \(p\)-value ≤.01. (D): Heat map of significantly altered metabolite features upregulated and downregulated in both Fanca\(^{-/-}\) and Fancd2\(^{-/-}\) MSCs plotted against log 2-fold change (metabolite extraction for the metabolome profile was done from independently derived MSCs). (E) Immunofluorescence of MSCs. WT, Fanca\(^{-/-}\), and Fancd2\(^{-/-}\) MSCs were stained with BODIPY-PE and DAPI. The images were the Z-stack images captured with Nikon C2+ confocal microscope. Abbreviation: WT, wild type.
Metabolome Profiling

Metabolites were extracted from three independently derived, ≥98% pure Fanca−/−, Fancd2−/−, and WT MSCs. MSCs were washed with ice cold Dulbecco’s Phosphate Buffered Saline (DPBS) twice to remove any culture media. Cells were collected into 300 μl LC/MS-grade H2O containing 1 mM HEPES and 1 mM EDTA (pH 7.2). Samples were vortexed for 30 seconds, and incubated 1–2 minutes in boiling water and subsequently in LN2 for 1 minute. Samples were then thawed on ice and normalized based on the protein content. Two milliliters of −20°C metabolite extraction solution containing Methanol, Acetonitrile, and H2O at a ratio of 2:2:1 was added to each sample and vortexed for 1 minute. Samples were then incubated at 4°C for 30 minutes. Samples were centrifuged at 1,500g for 10 minutes. The supernatants (~2 ml total) were pooled in an HPLC vial (Sigma# 27115-U, St. Louis, MO) and dried under forced N2 at room temp before reconstituted for LC-Q-TOF-MS analysis. All pure standards were

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STEM CELLS
purchased from Sigma Aldrich. Samples were resuspended in 50 μl of 50:50 water/acetonitrile solutions for mass spectrometry analysis. Untargeted metabolomics was performed on the MSCs extract to identify metabolites whose levels are altered in Fanca−/− and Fancd2−/− compared to WT. Samples were analyzed at Scripps Center for Metabolomics and Mass Spectrometry, La Jolla, CA. Using liquid chromatography quadrupole time-of-flight mass spectrometry (LC-Q-TOF-MS), hundreds of peaks with a unique m/z ratio and retention time were detected in Fanca−/−, Fancd2−/−, and WT MSCs. Each peak, termed a metabolomic feature, is characterized on the basis of its accurate mass, retention time, and tandem mass spectral fragmentation pattern using the METLIN metabolite database. The data were then analyzed with the bioinformatics program XCMS Online [21], widely used XCMS software that is freely available at https://xcmsonline.scripps.edu.

**RESULTS**

**FA MSCs Impair WT HSPC Self-Renewal and Induce Myeloid Expansion**

We first established an ex vivo coculture to examine the effect of Fanca−/− or Fancd2−/− MSCs on WT HSPCs...
Limiting-dilution CAFC assay [22] with graded numbers of flow sorted WT LSK cells shows that the frequency of CAFC, both at week 1 and week 2, was significantly reduced in cocultures on Fanca−/− or Fancd2−/− MSCs compared to those on WT MSCs (Fig. 1B and Supporting Information Fig. S1A, S1B), indicating that Fanca−/− and Fancd2−/− MSCs compromise HSPC self-renewal capacity. To evaluate the repopulating ability of the cocultured HSPCs in vivo, we performed competitive repopulation assay using 1 \times 10^5 output cells that had been cocultured for 1 week on WT, Fanca−/−, or Fancd2−/− MSCs, along with 3 \times 10^5 fresh BM competitor cells. At 16 weeks post-transplantation, the frequency of donor-derived cells, presumably the progenies of the output HSPCs, in the peripheral blood (PB) of mice that received cells cocultured on Fanca−/− or Fancd2−/− MSCs was significantly increased compared with mice that received cells cocultured on WT MSCs (p = .0003 for Fanca−/− vs. WT and for p = .0001 Fancd2−/− vs. WT; Fig. 1C). Remarkably, we observed a dramatic expansion of donor myeloid lineage in PB of mice transplanted with cells cocultured on Fanca−/− or Fancd2−/− MSCs compared to WT controls (25.88%, 28.83% and 11.93% for Fanca−/−, Fancd2−/−, and WT, respectively; Fig. 1C). Similar increase in both total donor engraftment (49.4%, 51.88%, and 27.8% for Fanca−/−, Fancd2−/−, and WT, respectively) and myeloid expansion (65.18%, 72.15%, and 37.05% for Fanca−/−, Fancd2−/−, and WT, respectively) was also observed in the BM of recipients transplanted with cells cocultured on Fanca−/− or Fancd2−/− MSCs compared with mice that received cells cocultured on WT MSCs (Fig. 1D). Consistently, colony-forming unit assay using donor-derived (CD45.2+) BM cells from 16-week post-transplant mice revealed a marked expansion of myeloid progenitor populations in mice transplanted with Fanca−/− or Fancd2−/− MSCs (Supporting Information Fig. S1C). Collectively, these results indicate that Fanca−/− and Fancd2−/− MSCs exert a dramatic effect on the self-renewal and differentiation of ex vivo cocultured WT HSPCs.

Metabolome Profile Reveals Abnormal Glycerophospholipid Biosynthesis in Fanca−/− and Fancd2−/− MSCs

Since metabolites produced by MSCs in the BM niche are vital to HSC function, we performed untargeted metabolic profile
to screen the entire metabolome of MSCs by LC-Q-TOF-MS [15]. This global metabolic platform identified 200–600 metabolites upregulated and 60–150 metabolites downregulated in Fanca−/− or Fancd2−/− MSCs compared to WT MSCs (Fig. 2A, 2B). Significantly, metabolites in the glycerophospholipid pathway showed the highest upregulated fold change (150-fold in Fanca−/− and 120-fold in Fancd2−/− MSCs) (Fig. 2C, 2D). Furthermore, immunofluorescence staining of MSCs showed elevated level of PE (1,2-dipalmitoyl-sn-glycerol-3-phosphoethanolamine), one of the major metabolites in the lipids.
glycerophospholipid pathway in Fanca−/− or Fancd2−/− MSCs compared to WT MSCs (Fig. 2E). Very little is known about the effect of this group of phospholipids on hematopoiesis [23]; however, a recent report demonstrates that lysophosphatidic acid, a pleiotropic phospholipid induces myeloid but not lymphoid differentiation in CD34+ human hematopoietic progenitors [24].

TOFA Suppresses Lipid Biosynthesis in Fanca−/− and Fancd2−/− MSCs

To examine the effect of the elevated glycerophospholipids on HSC function, we hypothesized that targeted reduction of these lipids might improve hematopoietic-supporting function of FA MSCs. In searching for utility to reduce glycerophospholipids in FA MSCs, we identified TOFA, one of downregulated metabolites in both Fanca−/− and Fancd2−/− MSCs (12-fold in Fanca−/− and 10-fold in Fancd2−/− MSCs) (Fig. 2D). TOFA inhibits the activity of acetyl-CoA carboxylase (ACC) [25, 26], a rate-limiting enzyme in lipid synthesis that catalyzes the conversion of acetyl-CoA to malonyl-CoA. We performed five independent assays to confirm the effectiveness of TOFA in suppressing lipid biosynthesis in Fanca−/− and Fancd2−/− MSCs: (a) TOFA suppressed ACC activity by enzyme assay (Fig. 3A); (b) TOFA inhibited the expression of genes involved in glycerophospholipid biosynthesis by q-PCR (Fig. 3B); (c) TOFA reduced the levels of Fasn but not Acsl1, Fasn is the major enzyme involved in glycerophospholipid biosynthesis by Western blot (Fig. 3C); (d) TOFA repressed the biosynthesis of total lipids by oil red O staining (Fig. 3D); and (e) TOFA decreased the levels of Fasn and Phosphoethanolamine by immunofluorescence staining (Fig. 3E).

TOFA Partially Corrects the Effects of FA MSC Cells on Self-Renewal and Differentiation

We next determined whether TOFA could restore or improve the hematopoiesis-supporting function of FA MSCs. We conducted two sets of experiments to examine the effect of TOFA on MSC-dependent HSC function. First, we used limiting-dilution CAFC assay to evaluate the self-renewal capacity of the cocultured LSK cells on WT, Fanca−/−, or Fancd2−/− MSCs that had been treated with or without TOFA for 48 hours. TOFA increased the number of cobblestone colonies in Fanca−/− and Fancd2−/− cocultures to WT levels (Fig. 4A). Second, we performed competitive repopulating assay using approximately 1 × 10^5 output cells collected from cocultures treated with or without TOFA. Four months later, we analyzed donor engraftment in both peripheral blood and the BM. The cells from Fanca−/− and Fancd2−/− cocultures produced approximately two- to threefold more donor chimerism in peripheral blood compared to WT cocultures, and pretreatment of the Fanca−/− and Fancd2−/− MSCs with TOFA resulted in 30%–50% reduction of donor chimerism (Fig. 4B, 4C). Therefore, reduction of donor chimerism by TOFA was likely due to its inhibitory effect on the expansion of the myeloid cells rather than on self-renewal capacity of the donor HSCs. Moreover, TOFA greatly alleviated the abnormal expansion of the donor myeloid (Gr1+Mac1+) compartment in both the peripheral blood and BM of recipient mice transplanted with cells from Fanca−/− and Fancd2−/− cocultures (Fig. 4B, 4C). Consistently, TOFA significantly inhibited the proliferation of donor-derived (CD45.2) total (Fig. 4D) and myeloid (Fig. 4E) progenitors isolated from the BM of recipient mice. These data show that the endogenous ACC inhibitor TOFA corrects the defect of the Fanca−/− and Fancd2−/− MSCs and support the notion that the aberrant myeloid explosion is resulted from elevated levels of lipid metabolites, including Phosphoethanolamine, in FA MSCs.

To determine whether overexpression of glycerophospholipids in normal MSCs could cause similar defect in hematopoiesis as with FA MSCs, we performed experiments in which WT MSCs were treated with or without adipogetic supplement to induce Fabp (fatty acid binding protein), which is known to activate PPARγ leading to increased fatty acid metabolism and overproduction of glycerophospholipids [27–29] (Supporting Information Fig. S2). The expression levels of Fabp in adipogetic supplement-treated WT MSCs were compared to those in Fanca−/− and Fancd2−/− MSCs without treatment by immunostaining using an antibody against the Fabp protein. The immunofluorescence levels of Fabp show that adipogetic supplement effectively induced Fabp expression in WT MSCs to a level that was comparable to untreated FA MSCs (Supporting Information Fig. S3A). Induction of Fabp in treated WT MSCs led to elevated production of glycerophospholipids, as determined by BODIPY-PE immunofluorescence staining (Supporting Information Fig. S3B), and decreased frequency of CAFC of cocultured LSK cells, as analyzed by the CAFC assay (Supporting Information Fig. S3C). These results indicate that overproduction of glycerophospholipids impairs hematopoiesis-supporting function of WT MSCs, mimic of the phenotype observed in Fanca−/− or Fancd2−/− MSCs.

Figure 4. TOFA suppresses abnormal differentiation of hematopoietic stem cells into myeloid cells. (A): TOFA rescues stemness of cocultured WT hematopoietic stem cell and progenitor cells. Confluent WT, Fanca−/−, or Fancd2−/− MSCs were pretreated with TOFA (8 μM) for 48 hours, and graded numbers of flow sorted WT LSK were plated on confluent stromal layers of WT, Fanca−/−, or Fancd2−/− MSCs. Scoring of Cobblestone area as endpoint was determined after 7 days. (B): TOFA prevents abnormal expansion of donor myeloid cells in peripheral blood of irradiated recipient mice. Confluent WT, Fanca−/−, or Fancd2−/− MSCs were pretreated with TOFA (8 μM) for 48 hours, and flow sorted WT LSK were added to the cultures. Five days later, 1 × 10^5 WT output cells (CD45.2+) were collected and, along with 3 × 10^5 recipient BM cells (CD45.1+), injected into each lethally irradiated recipient mouse. Donor chimerism and lineage reconstitution in peripheral blood of the recipients were examined at 4 months post-transplantation. Representative flow plots (Left) and quantifications (Right) are shown. Results are means plus or minus SD of three independent experiments (n = 9 per group). (C): TOFA prevents abnormal expansion of donor myeloid cells in the BM of irradiated recipient mice. Flow analysis of donor chimerism and lineage reconstitution in the BM of the recipients, described in (A), at 4 months post-BMT. Representative flow plots (Left) and quantifications (Right) are shown. Results are means plus or minus SD of three independent experiments (n = 9 per group). (D): CFU of donor-derived (CD45.2+) bone marrow cells. 2 × 10^6 BMMS isolated from transplant recipients, described in (A), at 4 months post-BMT were plated in triplicates (n = 3–5 recipient mice). CFU is the total count of BFU-E, CFU-M, CFU-G, CFU-GM, CFU-GEMM, and Pre-B colonies. (E): CFU of progenitor lineages having significant difference. *, p < .05; **, p < .01; ***, p < .001, ns: not significant. Error bars represent mean ± SD. Abbreviations: BM, bone marrow; CAFC, cobblestone area-forming cell; LSK, Lin−Sca1+Kit+; MSC, mesenchymal stromal cell; TOFA, 5-(tetradecyloxy)furoic acid; WT, wild type.
Lipin 1 Knockdown Prevents Myeloid Expansion by Correcting the Defects of FA MSCs

To genetically demonstrate the role of glycerophospholipids in MSC function, we depleted Lipin1, a proximal enzyme that converts Diacyl glycerol to Phosphoethanolamine and other glycerophospholipids [30] (Fig. 5A), using lentiviral shRNA. Baseline Lipin1 was higher in both Fanca−/− and Fancd2−/− MSCs, and the Lipin1 shRNA effectively reduced the levels.
of Lipin1 proteins, as analyzed by immunofluorescence (Fig. S5B) and Western blotting (Fig. S5C). Like TOFA treatment, Lipin1 knockdown in Fanca−/− and Fancd2−/− MSCs also reduced Phosphoethanolamine biosynthesis to WT levels (data not shown). We next determined whether Lipin1 knockdown was capable of recapitating the effect of TOFA on cocultured HSPCs. Indeed, Lipin1 knockdown effectively prevented myeloid expansion in transplant recipients of Fanca−/− or Fancd2−/− MSC-supporting cells (Fig. 5D). These genetic data thus corroborate the notion that elevated lipid biosynthesis in Fanca−/− and Fancd2−/− MSCs is associated with myeloid expansion observed in cocultured cells.

**Tlr4 Signaling Mediates the Effect of Glycerophospholipids on HSPC Function**

To understand the mechanism involved in myeloid skewing of HSCs induced by MSC-derived glycerophospholipids, we analyzed our microarray data obtained with freshly isolated phenotypic HSC (CD150+CD48− LSK; SLAM) cells from WT and Fanca−/− mice (accession number GSE64215 at http://www.ncbi.nlm.nih.gov/geo/). We used significance analysis of microarrays with the criteria of at least a 1.5-fold change in expression to identify genes as being upregulated or downregulated in Fanca−/− SLAM population. Gene set enrichment analysis identified significant enhancement of Toll-like receptor (TLR) signaling in Fanca−/− SLAM cells compared with WT cells (Fig. 6A). To evaluate the effect of glycerophospholipids on HSPC function, we focused on one of glycerophospholipids, Phosphoethanolamine, because it was one of the highly produced metabolites in FA MSCs (Fig. 2D, 2E). We first treated sorted LSK cells with or without 1 mM PE for 24 hours and performed qRT-PCR for major genes in the TLR signaling pathway. Enhancement of Tlr4 signaling and its downstream targets was evident in HSCs after PE treatment (Fig. 6B). In addition, Tlr2 and MyD88 were also upregulated in HSCs upon PE treatment. To genetically validate the involvement of TLR signaling pathway, we treated mice deficient for Tlr2, Tlr4, or MyD88 with PE. Peripheral blood was collected for analysis at 16 weeks post-bone marrow (BM) transplantation. Competitive repopulating assay was done with 105 WT outbred BM cells from Boy J mice (CD45.1) either treated or untreated with 1 mM PE for 24 hours. Equal numbers of CD45.2 and untreated BM cells from Boy J mice (CD45.1) were mixed and transplanted into lethally irradiated Boy J mice. Analysis of lineage reconstitution at 8 weeks post-transplant showed that PE phenocopied the effect of Fanca−/− or Fancd2−/− MSCs on WT and Tlr2−/− donor cells but not on Tlr4−/− or MyD88−/− cells. That is, PE significantly increased myeloid lineage repopulation in recipient mice transplanted with WT and Tlr2−/− donor cells compared to those transplanted with Tlr4−/− or MyD88−/− cells (Fig. 6D). These data indicate that Tlr4 signaling contributes to the glycerophospholipid-mediated myeloid skew of HSC differentiation (Fig. 7).

**DISCUSSION**

In this study, we used integrated metabolome, genetic, and functional approaches to identify and a group of FA MSCs- derived metabolites, glycerophospholipids, and their endogenous inhibitor as regulators of donor HSPCs in an experimental transplant model. FA is a major inherited BM failure syndrome with extremely high risk of developing acute myeloid leukemia. The only curable treatment for this devastating disease is stem cell and gene therapies through HSCT. However, the effects of metabolic alterations of transplant recipient BM niche on donor HSCs have been underestimated, and it remains unclear whether the metabolites released by the recipient niche into the BM are responsible for signaling directly to the mechanisms driving donor HSCs into abnormal differentiation and/or leukemia initiation. This study is aimed at identifying critical donor HSC-niche interaction regulators in a significant health-care setting, and thus would lead to an improved mechanistic understanding of donor HSC maintenance in the context of HSCT.

It has been shown in several studies that the stromal feeder layer can be used to support HSC expansion and maintain quiescence both in vivo and in vitro [31–33]. To understand the hematopoiesis-supporting role of FA stromal cells, we modeled FA HSCT using ex vivo coculture followed by CAFC and BM transplantation assays. Although it is speculated that the environment beneath and/or niche atmosphere created by healthy MSC layer can keep HSCs in an immature state, it was demonstrated that human CD34+CD38− HSCs prefer to migrate through the MSC layer [33]. We hypothesized that the HSC-MSC interaction in the ex vivo coculture model has an impact on HSC differentiation. Indeed, the true cobblestone formation (phase-dim cells) was reduced by MSCs derived from the Fanca−/− or Fancd2−/− BM, indicating the loss of the...
The stemness of the WT HSCs when cocultured with the FA niche. Furthermore, transplantation of the WT HSCs cocultured on FA MSCs into lethally irradiated WT recipient mice showed skew differentiation of these cocultured HSCs toward myeloid lineage, suggesting potential myeloid transformation induced by the FA niche. Significantly, with TOFA treatment and lipin1
knockdown in FA niche this myeloid-skewing phenotype was reversed, indicating a crucial role of phospholipids produced by FA MSCs in affecting the function of healthy HSCs. Although phospholipids have been traditionally considered as membrane lipids and their roles in cell signaling are yet to be discovered, our findings and emerging data [24] implicate a role of phospholipids in hematologic malignancies making the glycerophospholipid biosynthesis pathway potentially a novel therapeutic target in blood cancer that can be manipulated. In addition, we showed that genetic knockdown of Lipin1 could also ameliorate the myeloid-skewing phenotype induced by elevated glycerophospholipids. Lipin1 is a key enzyme having dual role in glycerophospholipid biosynthesis and adipocyte maturation and maintenance by modulating the C/EBPz (CCAAT/enhancer-binding protein z) and PPARg (peroxisome-proliferator-activated receptor g) network [34, 35].

Our mechanistic study suggests the involvement of Toll-like receptor signaling in mediating the effect of FA MSC-derived glycerophospholipids on HSC differentiation. It has been reported that although the activation of Tlr signaling pathway did not affect the overall health of the mice, HSCs from the BM were unable to maintain quiescence and myeloid skewed upon BM transplantation [36]. TLR2 and TLR4 use TIRAP and MyD88 as adaptor proteins to engage in transducing the signal to downstream molecules and activate the NF-kB pathway [37, 38]. Genome-wide chromatin immunoprecipitation-Seq analysis of H3K4me3 in BM CD34+ cells derived from Myelodysplastic syndrome (MDS) patients identified a large majority of pathogenic genes involved in TLR-mediated innate immunity signaling and NF-kB activation [39]. Expression of many of TLRs (TLR1, 2, 6, 7, 9, 10, and RP105) in B cells has been identified that mediate proliferation, plasma cell differentiation, and anti-apoptotic effects in B cells but only TLR4 and 8 were noted as a possibility [40]. TLR4-mediated signaling has been implicated in a variety of cancers responsible for tumor cell invasion, survival, and metastasis. Studies involving loss of TLR4 suggest several beneficial roles that could inhibit proliferation and survival of breast cancer cells [41], play a protective role in radiation-induced thymic lymphoma [42], and reduce the risk of acute Graft versus host disease (GVHD) [43]. TLR4 and TLR7/8 induced overproduction of p38 mitogen-activated protein kinase-dependent tumor necrosis factor α (TNFα) was also linked to certain extent to BM failure in FA [44, 45]. We postulate that FA MSCs overproduce a group of glycerophospholipids including phosphocholine, phosphoethanolamine, and phosphoserine, which activates Tlr4 in HSCs. Tlr4 in turn signals through MyD88 to activate an NF-kB transcriptional program that leads to upregulation of myeloid-specific gene expression and consequently abnormal myeloid differentiation. Abbreviations: ACC, acetyl-CoA carboxylase; HSC, hematopoietic stem cell; MSC, mesenchymal stromal cell; WT, wild type.

**Figure 6.** Glycerophospholipids alters hematopoietic stem cell differentiation through Tlr4 signaling. (A): Heat map presentation of the toll-like receptor signaling pathway from microarray analysis of Lin−Sca1+cKit+ (LSK)−SLAM cells from WT and Fanca−/− mice (accession number GSE64215 at http://www.ncbi.nlm.nih.gov/geo/). (B): Relative gene expression of genes involved in response to PE treatment. Total mRNA was collected from WT LSKs either treated with or without 1 mM PE for 24 hours. mRNA expression levels of the genes involved in the response to PE as ligand were normalized to the housekeeping gene GAPDH. (C): Peripheral blood analysis from WT, Tlr2−/−, Tlr4−/−, and Myd88−/− mice intraperitoneally injected with either 1 mM PE or phosphate-buffered saline alone and analyzed for Gr1+ and Mac1+ cells by flow cytometry at 2 and 4 weeks postinjections. Representative flow plots (Left) and quantifications (Right) are shown. Results are means plus or minus SD of three independent experiments (n = 9 per group). (D): Abnormal myeloid expansion of WT and Tlr2−/− but not Tlr4−/−, and Myd88−/− hematopoietic stem cell and progenitor cells treated with 1 mM PE in peripheral blood of irradiated recipient mice. 1 × 106 WT, Tlr2−/−, Tlr4−/−, and Myd88−/− (CD45.2) LSK cells pretreated with or without (control) 1,2-dipalmitoyl-sn-glycerol-3-phosphoethanolamine (PE; 1 mM) for 24 hours, along with 3 × 105 recipient BM cells (CD45.1), were injected into each lethally irradiated recipient mouse. Donor chimerism and lineage reconstitution in peripheral blood of the recipients were examined at 8 weeks post-transplantation after stable hematopoietic reconstitution was established. Representative flow plots (Left) and quantifications (Right) are shown. Gr1+Mac1+ cells were analyzed from CD45.2 donor-derived cells as shown in the inset. Results are means plus or minus SD of three independent experiments (n = 9 per group). ***, p < .01; ***, p < .001. Error bars represent mean ± SD. Abbreviation: WT, wild type.

**Figure 7.** Model of glycerophospholipids-activated Tlr4-MyD88 signaling in HSC differentiation. Overproduced glycerophospholipids, including phosphocholine, phosphoethanolamine, and phosphoserine, in Fanca−/− and Fancd2−/− MSCs may act as ligands to activate Tlr4 receptor in cocultured WT HSC. Tlr4 in turn signals through MyD88 to activate an NF-kB transcriptional program that leads to upregulation of myeloid-specific gene expression and consequently abnormal myeloid differentiation. Abbreviations: ACe, acetyl-CoA carboxylase; HSC, hematopoietic stem cell; MSC, mesenchymal stromal cell; WT, wild type.
strategy of clinical trials [48–51]. Whether this constitutive activation of Tlr4 in HSCs is caused by a direct binding or by indirect effect of the FA MSC-derived glycerophospholipids requires further investigation.

CONCLUSIONS

In summary, our results show that the endogenous ACC inhibitor TOFA partially corrects the defects of FA MSCs and indicates that elevated levels of lipid metabolites produced by FA MSCs, including glycerophospholipids, are associated with aberrant myeloid expansion. Our studies suggest that targeting glycerophospholipid biosynthesis either by TOFA or modulating Lipin1 in FA MSCs could be a therapeutic strategy to improve hematopoiesis and stem cell transplantation for FA patients.

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AUTHOR CONTRIBUTIONS

S.A.: designed and performed research, analyzed data, and wrote the article; M.S., A.W., and X.L.: performed research and analyzed data; Q.P.: designed research, contributed vital new reagents, analyzed data, and wrote the article.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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