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Klotho Deficiency Disrupts Hematopoietic Stem Cell Development and Erythropoiesis

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Klotho deficiency is a characteristic feature of chronic kidney disease in which anemia and cardiovascular complications are prevalent. Disruption of the *Klotho* gene in mice results in hypervitaminosis D and a syndrome resembling accelerated aging that includes osteopenia and vascular calcifications. Given that the bone microenvironment and its cellular components considerably influence hematopoiesis, in the present study, we addressed the *in vivo* role of klotho in blood cell formation and differentiation. Herein, we report that genetic ablation of *Klotho* in mice results in a significant increase in erythropoiesis and a decrease in the hematopoietic stem cell pool size in the bone marrow, leading to impaired hematopoietic stem cell homing *in vivo*. Our data also suggest that high vitamin D levels are only partially responsible for these hematopoietic changes in *Klotho*$^{-/-}$ mice. Importantly, we found similar hematopoietic abnormalities in *Klotho*$^{-/-}$ fetal liver cells, suggesting that the effects of klotho in hematopoietic stem cell development are independent of the bone microenvironment. Finally, injection of klotho protein results in hematopoietic changes opposite to the ones observed in *Klotho*$^{-/-}$ mice. These observations unveil a novel role for the antiaging hormone klotho in the regulation of prenatal and postnatal hematopoiesis and provide new insights for the development of therapeutic strategies targeting klotho to treat hematopoietic disorders associated with aging. *(Am J Pathol 2014, 184: 827–841; http://dx.doi.org/10.1016/j.ajpath.2013.11.016)*

Hematopoiesis is a complex and tightly regulated process of blood cell formation that is hierarchically coordinated. During normal hematopoiesis, diverse blood cell types are produced by the bone marrow (BM) in a manner related to physiologic requirement. Certain conditions may trigger additional production of blood cells. When the oxygen content of body tissues is low, the kidneys produce and release erythropoietin (Epo), a hormone that stimulates the BM to produce more red blood cells (RBCs). Aging is associated with disruption of normal hematopoiesis, resulting in an increase in the prevalence of anemia, the emergence of hematopoietic malignancies, and the development of leukemias.[1,2] Deterioration of vital organ function, such as kidney and heart, is also associated with age-related changes, as seen in chronic kidney disease (CKD) and cardiovascular disease (CVD).

The antiaging hormone klotho, predominantly expressed in the kidneys, is emerging as a multifunctional protein regulating vital cellular functions.[3–5] *Klotho* was serendipitously discovered by Kuro-o et al[6] when they observed symptoms of accelerated aging associated with a mutation in a specific gene in mice. Klotho exists in a membrane-bound form expressed at high levels in the kidney and, to a lesser extent, in other tissues, whereas a soluble form of klotho is secreted into blood, urine, and cerebrospinal fluid after cleavage of the extracellular domain.[7–10] Earlier studies convincingly demonstrate that membrane-bound klotho (α-klotho) is indispensable for signaling of the phosphatonin fibroblast growth factor 23 and that secreted klotho functions as an endocrine hormone responsible for the multiple organ defects observed in *Klotho*$^{-/-}$ mice.[11–14]

Maintaining mineral ion homeostasis is critical and involves a delicate and concerted action between bone- and kidney-derived endocrine factors that operate through a complex feedback mechanism(s). Patients with CKD often present with bone diseases, such as osteopenia, osteoporosis, or osteomalacia, as a result of significant derangement

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of mineral metabolism.[15,16] In patients with CKD, failure of appropriate fibroblast growth factor 23/Klotho signaling results in hyperphosphatemia and vascular calcifications.[17] Klotho expression is decreased progressively with loss of renal function,[18] whereas blood levels of fibroblast growth factor 23 are elevated and are associated with increased CVD and mortality in these patients and in patients undergoing dialysis.[19–22] Moreover, abnormal blood cell production leading to severe anemia is a common complication in CKD and CVD and is caused by insufficient renal production of Epo.[23,24]

Disruption of the *Klotho* gene in mice due to mutations or inactivation (*Klotho*$^{-/-}$ mice) results in growth retardation and early demise, osteopenia, extensive vascular calcifications, and skin atrophy, coupled with phosphate retention and hypervitaminosis D.[6,13,25–27] Conversely, overexpression of *Klotho* has been shown to rescue the klotho-deficient phenotype and extend the life span in mice, suggesting that *Klotho* functions as an aging suppressor gene in mammals.[6,28] Loss of klotho is further known to cause endothelial dysfunction by promoting oxidative stress.[29] It has been well appreciated that aging and oxidative stress adversely affect hematopoiesis by altering the niche functions.[30,31] An earlier report has also highlighted that klotho deficiency in mice results in reduced B lymphopoiesis, suggesting changes in immune regulatory functions by klotho.[32] In addition, klotho expression at the mRNA level has been found to be significantly decreased in resting human CD4^{+} lymphocytes proportionally to advancing age.[33]

Signals emanating from the BM microenvironment and extrinsic soluble factors associated with the bone and marrow milieu are known to modulate hematopoietic stem cell (HSC) proliferation and differentiation.[34,35] Identifying the contributing factors involved in the regulation of hematopoiesis is an area of active research. Several lines of evidence highlight the role of bone-forming cells, the osteoblasts, in the HSC niche; postnatal depletion of osteoblasts negatively regulates the HSC pool size in the BM, whereas an increase in osteoblast number is associated with an augmentation in HSC number.[36–39] In addition, a series of advances indicate the importance of the bone-resorbing osteoclasts in regulation of the HSC microenvironment. Osteoclasts actively participate in HSC mobilization from the BM to the circulation and also promote formation of the HSC niche by controlling the maturation of osteoblasts.[40–44] Not only do bone cells participate in the regulation of hematopoiesis but the mineral content of the niche may also have a key function in localization of adult hematopoiesis, as reported in studies showing involvement of the calcium-sensing receptor and vitamin D signaling in this process.[45,46] Therefore, alterations in bone modeling and remodeling processes and/or mineralization seem to have a prominent effect on the modulation or formation of the hematopoietic niche. However, the regulation of mineral ion balance and hematopoiesis still remains largely a naive area.

Because the bone environment and its components and the process of aging are closely linked to the regulation of hematopoiesis, and klotho deficiency is associated with a marked defect in skeletal mineralization and premature aging-like features, we hypothesized that klotho is involved in the regulation of RBC production and differentiation. In the present study, we demonstrate that loss of klotho severely affects erythropoiesis and HSC number and function. More important, we show that klotho affects hematopoiesis independently of changes in the BM environment and that the absence of klotho results in aberrant hematopoiesis prenatally, providing evidence for a novel and direct role for klotho in hematopoietic development. Although the kidney is the adult hematopoietic organ in zebra fish equivalent to mammalian BM,[47–49] the present data demonstrate for the first time, to our knowledge, a link between the kidney-bone-hematopoiesis axes in the mammalian system and attest that klotho is a key factor in the process of hematopoiesis.

Materials and Methods

Mice

Klotho heterozygous mice (*Klotho*$^{+/-}$) were purchased from the Mutant Mouse Regional Resource Center (University of California, Davis, CA) and were interbred to obtain *Klotho*-null mice (*Klotho*$^{-/-}$). *1α(OH)ase* heterozygous mice were a gift from Dr. René St-Arnaud (Genetics Unit, Shriners Hospital, Montreal, QC, Canada). *Klotho* heterozygous and *1α(OH)ase* heterozygous mice were bred to obtain *Klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ double mutants. B6.SJL-Ptprc^{a}/BoyAiTac (CD45.1; Ly5.1) mice were purchased from Taconic Farms Inc. (New York, NY). All mice were kept on a light/dark (12 hours/12 hours) cycle at 23°C and received standard laboratory chow and water *ad libitum*. Genomic DNA was obtained from tail snips, and routine PCR was performed to identify the genotypes. PCR conditions were as follows: klotho—initial denaturation at 94°C for 5 minutes, 35 cycles of denaturation at 94°C for 1 minute, annealing at 63°C for 1 minute and extension at 72°C for 30 seconds, and final extension at 72°C for 10 minutes; 1α(OH)ase—94°C for 5 minutes, annealing at 56°C for 1 minute and extension at 72°C for 1 minute, followed by final extension at 72°C for 10 minutes. The following primers were used: KL0787-12, 5′-GATGGGGTCGACGTCA-3′; KL0787-13, 5′-TAAAGGAGGAAAGCCATTGTC-3′; KL0787-20, 5′-ATGCTCCAGACATTCTCAGC-3′; Neo3a, 5′-GCAGCGCATCGCCTTCTATC-3′; and 1α(OH)ase (forward and reverse), 5′-GCACCTGGCTCAGGTAGCTCTTC-3′ and 5′-GTCCCAGACAGAGACATCCGT-3′. All the animals were maintained in the New York University (NYU) College of Dentistry Animal Facility in accordance with the general guidelines of the NYU School of Medicine Division of Laboratory Animal Resources. All the animal studies were approved by the NYU Institutional Animal Care and Use Committee.

Blood Collection and Hematologic Analysis

Peripheral blood was collected after euthanasia from 6-week-old mice by cardiac puncture into EDTA-coated tubes

(BD Biosciences, San Jose, CA) to prevent clotting. Blood samples were then shipped overnight to Cornell University Animal Health Diagnostic Center (Ithica, NY) for automated complete blood cell count.

Tissue Collection

BM was isolated from dissected tibiae and femora from 6-week-old mice by flushing in Iscove's modified Dulbecco's medium (IMDM) (Sigma-Aldrich, St. Louis, MO) supplemented with 20% fetal bovine serum (20% IMDM) (HyClone; Thermo Scientific, Wilmington, DE) through a 27-gauge needle (Becton Dickinson Co., Franklin Lakes, NJ). Marrow cells were dispersed by manual agitation and then were filtered to remove foreign particles. Spleens from 6-week-old mice were surgically removed and were homogenized into a cell suspension in 20% IMDM. Timed pregnant *Klotho*$^{+/-}$ female mice were sacrificed at 15.5 days postcoitum, and fetal livers were collected by caesarean section and were homogenized into a cell suspension in 20% IMDM. Genomic DNA was obtained from tail snips, and routine PCR as described previously herein was used to identify the genotypes of the embryos.

Flow Cytometry Analysis

Tissues were dissected from 6-week-old or E15.5 embryo mice, and flow cytometry analysis for peripheral blood, BM, spleen, and fetal liver cells was performed in a BD FACSort flow cytometer equipped with 488 argon lasers (BD Biosciences). For immunostaining, cells were washed and then resuspended in 1× PBS containing 0.1% bovine serum albumin. Mouse Fc receptor was blocked before staining using CD16/32 antibody to reduce nonspecific binding. After the addition of antibodies, cells were incubated for 40 minutes on ice; for peripheral blood, RBCs were further lysed using BD FACS lysing solution (BD Biosciences). Labeled cells were then washed with 1× PBS and were analyzed by flow cytometry. Appropriate isotype controls were kept for each set. Forward and side scatter patterns were gated to exclude debris. A total of 50,000 events were collected and analyzed using FlowJo software version 7.6.5 (Tree Star Inc., Ashland, OR). Erythroid lineage was assessed using Ter119 APC/CD71 phosphatidylethanolamine (PE) markers combined with the forward scatter (FSC) properties.[50] CXCR4 expression was analyzed using PE-tagged CXCR4 antibody. Hematopoietic stem/progenitor cells were differentiated using SLAM markers (CD150 PE/CD48 APC), Sca1 fluorescein isothiocyanate (Ly6A-E), cKit Percp Cy5.5 (CD117), CD90 PE (Thy-1), and APC-tagged lineage cocktail composed of antibodies against CD3, B220 (CD45R), Ly6G and Ly6C (Gr1), CD11b (Mac1), and TER-119. CKit^{+}Sca1^{+} cells were gated on the lineage-negative fraction to analyze LSK (lin^{-}cKit^{+}Sca1^{+}). The LSK cells were then analyzed using a Thy-1^{low} gate to obtain the KTLS population (LSK Thy^{low}). CD45.1 PE and CD45.2 fluorescein isothiocyanate antibodies were used to differentiate donor and recipient populations after transplantation. All the antibodies except the SLAM markers were purchased from BD Pharmingen (San Jose, CA). SLAM markers CD150 and CD48 were purchased from eBioscience Inc. (San Diego, CA).

Gene Expression Analysis

Total RNA from bone, BM, spleen, kidney, adult liver, and fetal liver from 6-week-old or E15.5 wild-type (WT) and *Klotho*$^{-/-}$ mice was extracted using TRIzol reagent (Sigma-Aldrich) according to the manufacturer's protocol and was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific). RNA (1 μg) from each sample was reverse transcribed to cDNA in 10 μL of final volume using a high-capacity cDNA reverse transcription kit with random hexamers (Applied Biosystems, Foster City, CA). Real-time PCR analysis was performed using an Eppendorf Mastercycler ep gradient S *realplex*^{2} machine (Eppendorf, Hamburg, Germany) in a final reaction volume of 25 μL containing 1 μL of the prepared cDNA of each gene, 12.5 μL of PerfeCTa SYBR Green PCR SuperMix (Quanta BioSciences Inc., Gaithersburg, MD), and 1 μmol/L of primers amplifying the genes of interest. Thermal cycle conditions were as follows: 60°C for 2 minutes, 95°C for 10 minutes, and 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Analyses were performed in duplicate. Samples without reverse transcriptase were used as negative controls. The expression of klotho was measured in BM, spleen, kidney, and fetal liver; the expression of Epo, Hif-1α, and Hif-2α was analyzed in bone, BM, kidney, and liver; and the expression of transferrin, transferrin receptor, glucose transporter type 1, and phosphoglycerate kinase 1 was analyzed in BM and liver. All quantitative RT-PCR values were normalized to the housekeeping gene *HPRT*, and differences in gene expression between control (WT) mice and *Klotho*$^{-/-}$ mice were calculated based on the ΔC_T method. The primer sequences are shown in Table 1.

ELISA

Serum Epo and stromal-derived factor-1α (SDF-1α) levels were determined using Quantikine enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Serum samples were collected from 6-week-old WT and *Klotho*$^{-/-}$ mice and were run in duplicate for each assay.

Colony-Forming Unit Assay

A colony-forming unit assay was performed for BM, spleen, and fetal liver cells from 6-week-old or E15.5 WT and *Klotho*$^{-/-}$ mice. After isolation, the cells were incubated for 1 hour at 37°C and 5% CO_2 to allow for adherence to eliminate major stromal cell fractions. BM or fetal liver cells (1×10^4) and spleen cells (1×10^5) from the nonadherent fraction were plated in methyl cellulose medium (MethoCult M3231; STEMCELL Technologies Inc., Vancouver, BC, Canada). Murine recombinant growth factors, including IL-3

Table 1 Mouse Primer Sequences Used for Real-Time PCR Analysis

Gene	Forward sequence	Reverse sequence
Klotho	5′-ACTTGGCCTTTATTAGCCGGGTCT-3′	5′-AGATGGCCTCTTCCCTGTGTTCAA-3′
Erythropoietin (*Epo*)	5′-TCTACGTAGCCTCACTTCACT-3′	5′-ACCCGGAAGAGCTTGCAGAAA-3′
Hypoxia-inducible factor-1α (*HIF-1α*)	5′-TCTCGGCGAAGCAAAGAGTCT-3′	5′-TAGACCACCGGCATCCAGAAG-3′
Hypoxia-inducible factor-2α (*HIF-2α*)	5′-GGGAACACTACACCCAGTGC-3′	5′-TCTTCAAGGGATTCTCCAAGG-3′
Transferrin	5′-CCCTCTGTGACCTGTGTATTG-3′	5′-CTTTCTCAACGAGACACCTGAA-3′
Transferrin receptor	5′-TCCTGTCGCCCTATGTATCT-3′	5′-CGAAGCTTCAAGTTCTCCACTA-3′
Glucose transporter-1 (*Glut-1*)	5′-CCCAGGTGTTTGGCTTAGA-3′	5′-CAGAAGGGCAACAGGATACA-3′
Phosphoglycerate kinase-1 (*Pgk-1*)	5′-CACAGAAGGCTGGTGGATTT-3′	5′-CTTTAGCGCCTCCCAAGATAG-3′
HPRT	5′-AAGCCTAAGATGAGCGCAAG-3′	5′-TTACTAGGCAGATGGCCACA-3′

(4 ng/mL), stem cell factor (20 ng/mL), granulocyte macrophage colony-stimulating factor (2 ng/mL), and Epo (2 U/mL) (PeproTech, Rocky Hill, NJ), were added to the medium. The plates were incubated in humidified atmosphere at 37°C and 5% CO_2. After incubation (8 to 12 days), the colonies were scored based on morphology as burst-forming unit—erythroid) or granulocyte-erythroid-megakaryocyte-macrophage under a Nikon TMS inverted phase contrast microscope (Nikon Instruments, Melville, NY).

Transwell Migration Assay

Chemotactic responses of the WT and *Klotho*$^{-/-}$ BM cells to SDF-1α were assessed *in vitro* by Transwell migration assay. Briefly, 10^5 BM cells from 6-week-old WT and *Klotho*$^{-/-}$ mice were seeded to the upper chamber of the 8-μm BD Falcon 24-well cell culture insert (BD Biosciences). One hundred nanograms of SDF-1α (PeproTech) was added to the lower wells, containing 600 μL of medium. For each experiment, some wells were kept in both sets without SDF-1α to detect spontaneous migration. The cells were allowed to migrate for 3 hours at 37°C and 5% CO_2. The migrated cells were collected and manually counted. The percentage of migration was calculated as follows: (total number of input cells/number of cells migrated) × 100.

In Vivo Homing Experiments

BM cells (2×10^6 cells suspended in 1× PBS) from 6-week-old WT and *Klotho*$^{-/-}$ mice (CD45.2; Ly5.2) were isolated and injected into the tail vein of nonmyeloablated or myeloablated B6.SJL (CD45.1; Ly5.1) recipient mice ($n = 5$ per set for each experiment). Myeloablation was achieved by a lethal dose of irradiation (900 rads). Twenty hours after transplantation, peripheral blood, spleen, and BM were collected from recipient mice and were analyzed for the presence of CD45.2$^+$ donor cells by flow cytometry. A minimum of 50,000 events were acquired using a BD FACSort flow cytometer equipped with 488 argon lasers (BD Biosciences).

Klotho Protein Injections

Mouse recombinant klotho protein (10 μg/kg) (R&D Systems) was injected i.p. into 8-week-old C57/Bl/6 mice every other day for 3 days. Saline (1× PBS) was injected as vehicle. Peripheral blood, spleen, and BM cells were immunostained for erythroid and hematopoietic stem/progenitor markers and were analyzed by flow cytometry as described previously herein.

Statistical Analysis

Statistical differences between groups were analyzed by unpaired Student's *t*-test. More than six mice were analyzed for each set per experiment, and all the values are expressed as means ± SEM. Graphs were plotted using GraphPad Prism software version 5.0 (GraphPad Software Inc., San Diego, CA). A $P \leq 0.05$ was considered statistically significant.

Results

Klotho Expression in Hematopoietic Tissues

Klotho is predominantly expressed in the renal distal tubular epithelial cells and, to a lesser extent, in a variety of other tissues, such as the parathyroid gland, choroid plexus in the brain, placenta, and small intestine.[6,51–53] To dissect the role of klotho in hematopoiesis, we examined whether klotho transcripts are expressed in hematopoietic tissues. We analyzed klotho expression in total BM cells and splenocytes from 6-week-old WT mice and in fetal liver cells from E15.5 WT embryos by real-time PCR. Adult mouse kidney cells were used as a positive control. As expected, klotho was highly expressed in kidney, which is the primary source of klotho production. These results showed that klotho mRNA is also expressed in BM, spleen, and fetal liver cells, albeit at much lower levels than in kidney, suggesting a possible role for klotho signaling in hematopoiesis. The real-time data are expressed as fold change with respect to the housekeeping gene *HPRT* (Supplemental Figure S1A).

Hematologic Phenotype of *Klotho*$^{-/-}$ Mice

To assess the effect of klotho in steady-state hematopoiesis, we compared the hematopoietic output of 6-week-old *Klotho*$^{-/-}$ mice with that of their WT and *Klotho*$^{+/-}$ (heterozygous) littermates. Complete blood cell count analysis

revealed a significant increase in RBC numbers, hemoglobin levels, and hematocrit values in *Klotho*$^{-/-}$ mice (Figure 1, A–C). Conversely, a significant decrease in RBC indices, such as mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH), was detected in peripheral blood of *Klotho*$^{-/-}$ mice (Figure 1, D and E). No differences were found in any of the examined hematologic parameters between WT and *Klotho*$^{+/-}$ (heterozygous) mice.

Loss of Klotho Results in Increased BM Erythropoiesis without Extramedullary Erythropoiesis

We further examined the effect of klotho deficiency in erythroid cell production and differentiation in BM as the origin of hematopoiesis in adult mice. Total BM cellularity was modestly decreased in *Klotho*$^{-/-}$ mice compared with WT littermates (data not shown). Successive stages of erythroid commitment were determined based on cell size (FSC) coupled with expression of Ter119 and the transferrin receptor CD71. We observed a significant increase in immature pro-erythroblasts (Pro-E) (Ter119^{med} CD71^{high}) in the BM of *Klotho*$^{-/-}$ mice (Figure 2A). Further analysis based on cell size showed that the absence of klotho resulted in a marked increase in the relatively mature erythroid C (Ery-C) fraction (Ter119^{high}CD71^{low}FSC^{low}) of erythroblasts in comparison to WT littermates (Figure 2B). However, there were no discernible changes in the intermediate Ery-A (Ter119^{high}CD71^{high} FSC^{high}) and Ery-B (Ter119-^{high}CD71^{med}FSC^{low}) populations (Supplemental Figure S1, B and C). In addition, an *in vitro* colony-forming unit assay showed that *Klotho*$^{-/-}$ BM cells generated more erythroid colonies (burst-forming unit–erythroid) than WT cells, indicating the presence of increased functional erythroid progenitors in *Klotho*$^{-/-}$ BM (Figure 2C).

Because the spleen is an important organ that supports erythropoiesis in stress and inflammation, we next analyzed splenic erythropoiesis. *Klotho* mutant mice developed postnatal growth retardation and early mortality, with most *Klotho*$^{-/-}$ mice dying by 9 weeks after birth.[6] K*lotho*$^{-/-}$ mice were significantly smaller than their control littermates, and their spleen size was also found to be markedly decreased. However, even when the spleen size was corrected to body weight, the ratio of *Klotho*$^{-/-}$ spleen weight to body weight was one-third that of their WT littermates (Supplemental Figure S1D). In addition, total spleen cellularity was also decreased in *Klotho*$^{-/-}$ mice compared with their WT littermates (Supplemental Figure S1E). Although there were no remarkable changes in primitive erythroid lineage cells in the spleens of *Klotho*$^{-/-}$ mice (data not shown), we observed a significant decrease in the mature Ery-C (Ter119^{high}CD71^{low}FSC^{low}) fraction of the erythroid population (Figure 2D) accompanied by a decrease in the number of burst-forming unit–erythroid colonies generated by *Klotho*$^{-/-}$ splenocytes (Figure 2E). These data suggested a decline in splenic erythropoiesis and rules out the possibility of extramedullary hematopoiesis in the absence of klotho.

Enhanced Erythropoiesis in *Klotho*$^{-/-}$ Mice Is Mediated through Epo and the HIF Signaling Pathway

BM erythropoiesis is under the tight control of Epo secreted from the kidneys. Therefore, we examined whether enhanced erythropoiesis in *Klotho*$^{-/-}$ mice is Epo mediated. Epo mRNA expression was significantly up-regulated in *Klotho*$^{-/-}$ kidney, BM, and liver cells (Figure 3, A–C). Similarly, serum Epo levels were significantly elevated in klotho$^{-/-}$ mice compared with their WT littermates, indicating enhanced Epo

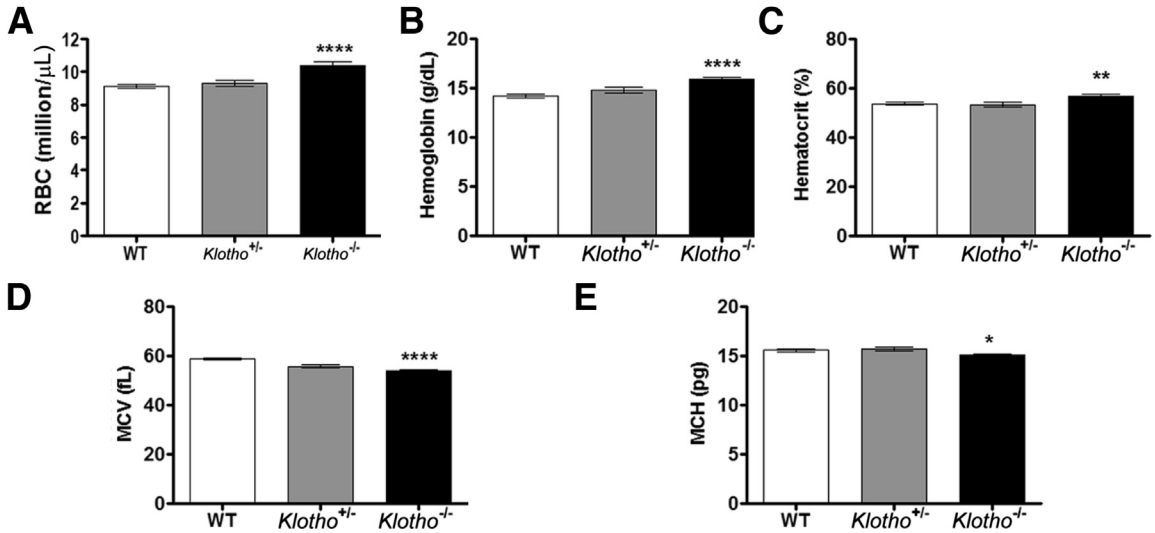

Figure 1 Complete blood cell count analysis of 6-week-old WT, heterozygous (*Klotho*$^{+/-}$), and *KlothoKlotho*-null (*Klotho*$^{-/-}$) mice. **A:** RBC counts (WT, $n = 23$; $+/-$, $n = 8$; $-/-$, $n = 18$). **B:** Hemoglobin levels (WT, $n = 19$; $+/-$, $n = 9$; $-/-$, $n = 16$). **C:** Hematocrit values (WT, $n = 19$; $+/-$, $n = 10$; $-/-$, $n = 16$). **D:** MCV (WT, $n = 21$; $+/-$, $n = 10$; $-/-$, $n = 20$). **E:** MCH (WT, $n = 16$; $+/-$, $n = 10$; $-/-$, $n = 16$). Data represent the means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{****}P < 0.0001$.

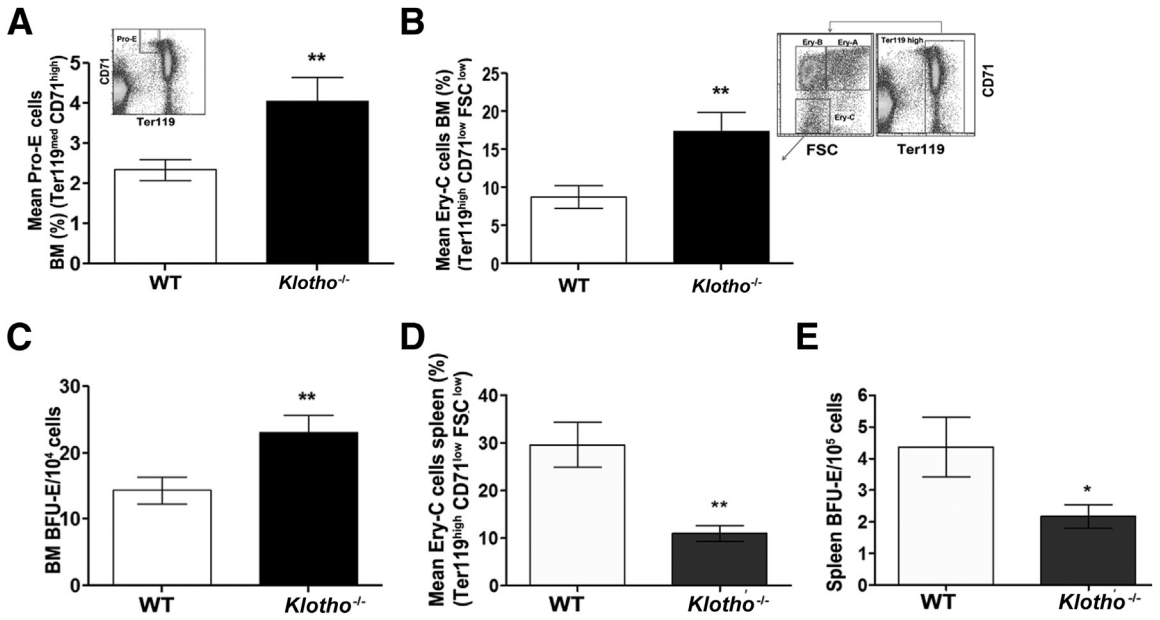

Figure 2 BM and splenic erythropoiesis in 6-week-old WT and *Klotho*$^{-/-}$ mice. **A:** Flow cytometry analysis shows an increase in primitive Pro-Es (Ter119^{med} CD71^{high}) in *Klotho*$^{-/-}$ mice (Pro-E: WT, $n = 12$; *Klotho*$^{-/-}$, $n = 9$); **inset** shows flow cytometry gating. **B:** Erythroid lineage commitment was analyzed using Ter119 and CD71 markers coupled with FSC measurements. A mature Ery-C (Ter119^{high} CD71^{low} FSC^{low}) population was observed in *Klotho*$^{-/-}$ compared with WT littermates (Ery-C: WT, $n = 9$; *Klotho*$^{-/-}$, $n = 7$); **inset** shows flow cytometry gating on the Ter119^{high} population. **C:** Colony-forming unit assay showing burst-forming unit—erythroid (BFU-E) colonies produced by *Klotho*$^{-/-}$ BM cells (WT, $n = 13$; *Klotho*$^{-/-}$, $n = 11$). Cells from each mouse were plated in triplicate, and the number of colonies was scored based on morphologic features. **D:** Graphic representation of flow cytometry analysis of the mature Ery-C population in *Klotho*$^{-/-}$ splenocytes (WT, $n = 9$; *Klotho*$^{-/-}$, $n = 8$). **E:** Colony-forming unit assay showing splenic BFU-E colonies in *Klotho*$^{-/-}$ mice (WT, $n = 4$; *Klotho*$^{-/-}$, $n = 4$). Cells from WT and *Klotho*$^{-/-}$ spleens were plated in triplicate. Data represent the means ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$.

secretion in the absence of klotho (Figure 3D). Previous studies have demonstrated that the aging kidney is exposed to hypoxia, to which it responds by activation of HIF signaling and up-regulation of HIF-regulated genes.[54] Because *Klotho*$^{-/-}$ mice present with aging-like features and Epo is an HIF target gene, we next examined HIF-1α and HIF-2α expression in WT and *Klotho*$^{-/-}$ kidneys. Renal mRNA expression of HIF-1α (Supplemental Figure S1F) and HIF-2α (Figure 3E) was similarly up-regulated in *Klotho*$^{-/-}$ mice, consistent with the notion that activation of HIF by hypoxia results in increased Epo production. Although the kidney was the major Epo-producing organ, it has been reported that in some pathologic conditions, hepatic Epo production is increased.[55] Therefore, we studied the expression of important HIF target genes in the liver and BM of WT and *Klotho*$^{-/-}$ littermates. The expression of glucose transporter type 1, phosphoglycerate kinase 1, transferrin, and transferrin receptor was significantly up-regulated in the liver and BM in *Klotho*$^{-/-}$ mice (Figure 3, F–K), confirming activation of the HIF pathway and erythropoiesis. These findings suggest that enhanced erythropoiesis in *Klotho*$^{-/-}$ mice is Epo induced and is driven by augmented HIF signaling and may serve as a mechanism to respond to stress conditions, such as anemia and inflammation.

Absence of Klotho Results in Altered Localization of Hematopoietic Stem/Progenitor Cells

To investigate whether increased erythropoiesis in *Klotho*$^{-/-}$ mice is due to expansion of the HSC compartment and/or increased differentiation of progenitor cells, we analyzed by flow cytometry the frequency of HSCs in BM, peripheral blood, and spleen. These data show a significant decrease in the CD150^{+}CD48^{-} (SLAM) population highly enriched in HSCs and in the frequency of the Lin^{-}cKit^{+}Sca-1^{+} (LSK) and the more primitive cKit^{+} Thy-1^{low} Lin^{-} Sca1^{+} (KTLS) fraction in the BM of *Klotho*$^{-/-}$ mice compared with WT littermates (Figure 4, A–C). These findings suggest that loss of klotho results in an overall reduction in the HSC pool size in the BM possibly due to an increase in differentiation of HSCs to the erythroid lineage. We observed an increase in the circulating progenitors marked by SLAM (CD150^{+}CD48^{-}) markers in *Klotho*$^{-/-}$ mice (Figure 4D). Moreover, we observed an increase in the phenotypic HSCs in *Klotho*$^{-/-}$ spleens as detected by elevated SLAM (CD150^{+}CD48^{-}) (Figure 4E), LSK (Lin^{-}Sca-1^{+}cKit^{+}) (Figure 4F), and the most primitive KTLS (cKit^{+} Thy-1^{low} Lin^{-} Sca-1^{+}) (Figure 4G) hematopoietic stem/progenitor cells. The increase in circulating and splenic HSCs in *Klotho*$^{-/-}$ mice suggested a possible homing defect during development. Collectively, these observations indicate that the hematopoietic milieu of mice lacking *Klotho* is severely disturbed compared with control WT littermates.

Migratory Properties of BM Cells from *Klotho*$^{-/-}$ Mice Are Severely Impaired, Resulting in Disrupted Homing *in Vivo*

The present peripheral blood, BM, and spleen data demonstrated that loss of klotho results in highly perturbed

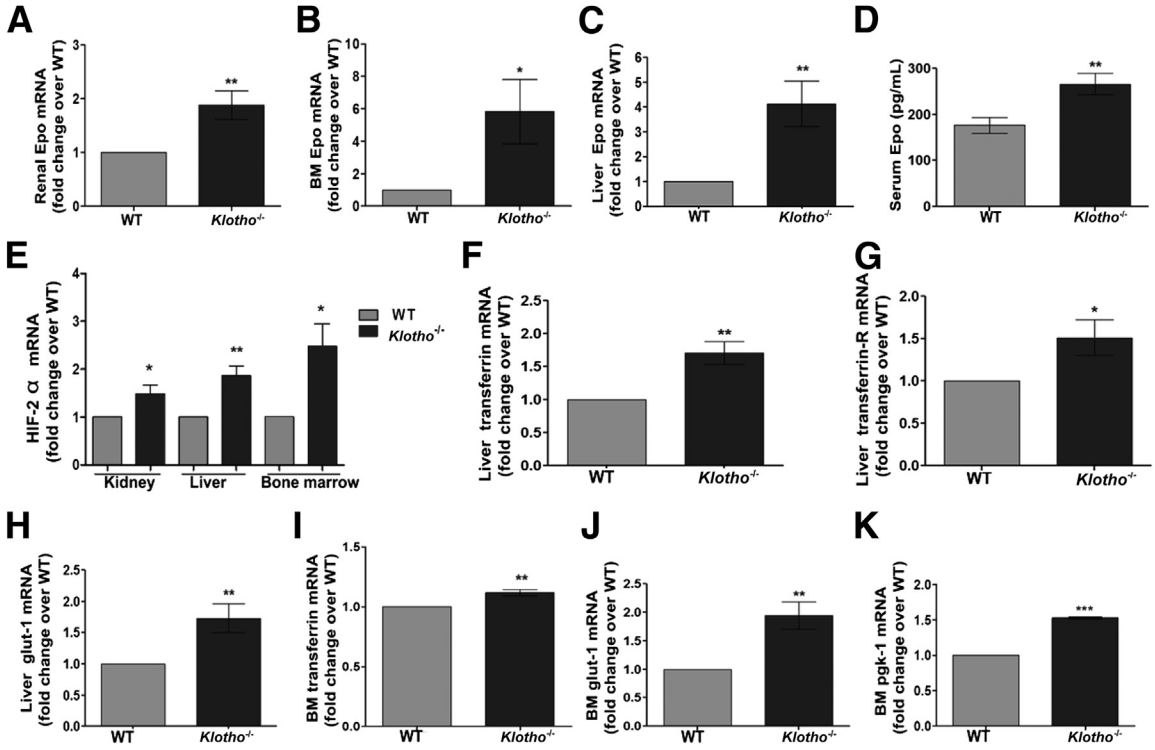

Figure 3 Increased erythropoiesis by activation of HIF signaling and up-regulation of HIF target genes in 6-week-old *klotho*$^{-/-}$ mice. Real-time quantitative PCR of renal Epo mRNA (WT, $n = 5$; *Klotho*$^{-/-}$, $n = 5$) (**A**), BM Epo mRNA (WT, $n = 14$; *Klotho*$^{-/-}$, $n = 6$) (**B**), and liver Epo mRNA (WT, $n = 9$; *Klotho*$^{-/-}$, $n = 7$) (**C**). **D:** Serum Epo levels (WT, $n = 14$; *Klotho*$^{-/-}$, $n = 14$). **E:** Real-time quantitative PCR of HIF-2α in kidney (WT, $n = 6$; *Klotho*$^{-/-}$, $n = 5$), liver (WT, $n = 5$; *Klotho*$^{-/-}$, $n = 4$), and BM (WT, $n = 6$; *Klotho*$^{-/-}$, $n = 4$). **F:** Liver transferrin (WT, $n = 5$; *Klotho*$^{-/-}$, $n = 6$). **G:** Liver transferrin receptor (WT, $n = 6$; *Klotho*$^{-/-}$, $n = 6$). **H:** Liver glucose transporter type 1 (glut-1) (WT, $n = 6$; *Klotho*$^{-/-}$, $n = 6$). **I:** BM transferrin (WT, $n = 6$; *Klotho*$^{-/-}$, $n = 4$). **J:** BM glut-1 (WT, $n = 6$; *Klotho*$^{-/-}$, $n = 4$). **K:** BM phosphoglycerate kinase 1 (pgk-1) (WT, $n = 6$; *Klotho*$^{-/-}$, $n = 4$). Data represent the means ± SEM, normalized to *HPRT* and plotted as fold change over WT (where WT = 1). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

erythropoiesis and suggest that this may be related to altered homing or retention of hematopoietic stem/progenitor cells in the *Klotho*-deficient BM. The homing mechanism is dependent on expression of the chemokine SDF-1α/CXCL-12 and other cell adhesion molecules and extracellular matrix proteins that guide the hematopoietic stem/progenitor cells to the niche. To address the hypothesis, we performed an *in vitro* Transwell migration assay that compared the migratory function of BM cells from WT and *Klotho*$^{-/-}$ mice toward SDF-1α. The findings showed that BM cells

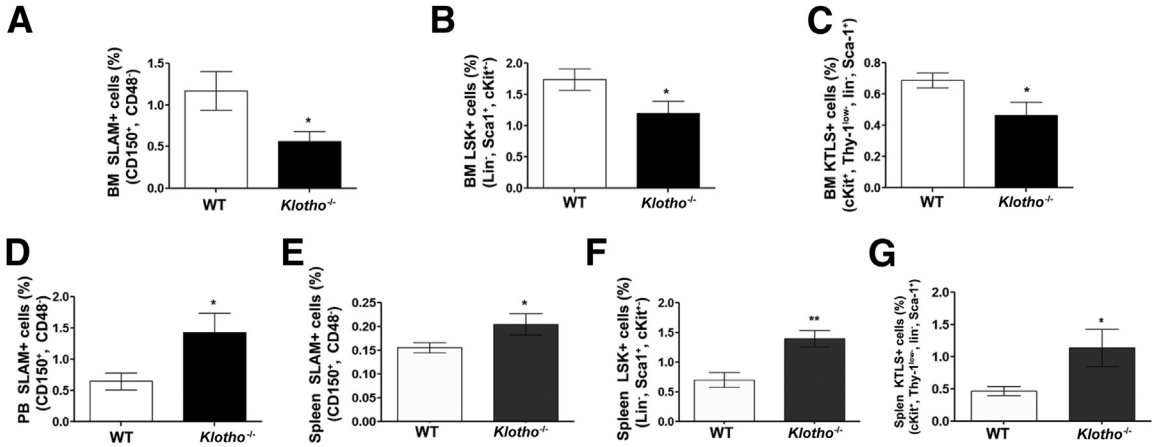

Figure 4 Altered localization of hematopoietic stem/progenitor cells in *Klotho*$^{-/-}$ mice. Frequency of the HSC population stained for SLAM (CD150^{+} CD48^{-}) (WT, $n = 14$; *Klotho*$^{-/-}$, $n = 14$) (**A**), the Lin^{-} Sca-1^{+} (**C**) Kit^{+} (LSK) HSC population (WT, $n = 9$; *Klotho*$^{-/-}$, $n = 7$) (**B**), and the primitive c-Kit^{+}Thy1^{+}Lin^{-}Sca 1^{+} (KTLS) HSC population (WT, $n = 7$; *Klotho*$^{-/-}$, $n = 7$) (**C**) in the *Klotho*$^{-/-}$ BM compartment. **D:** Circulating SLAM (CD150^{+} CD48^{-}) population in peripheral blood (PB) (WT, $n = 7$; *Klotho*$^{-/-}$, $n = 6$). Retention of phenotypic SLAM (WT, $n = 11$; *Klotho*$^{-/-}$, $n = 8$) (**E**), Lin^{-} Sca-1^{+} c-Kit^{+} (LSK) (WT, $n = 9$; *Klotho*$^{-/-}$, $n = 9$) (**F**), and primitive c-Kit^{+}Thy1^{+}Lin^{-}Sca 1^{+} (KTLS) population (WT, $n = 10$; *Klotho*$^{-/-}$, $n = 9$) (**G**) in splenocytes from *Klotho*$^{-/-}$ mice compared with their WT littermates. Data represent the means ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$.

from *Klotho*-deficient mice exhibited a twofold decrease in chemotaxis toward an SDF-1α gradient compared with WT mice (Figure 5A). Spontaneous migration in the absence of SDF-1α was also assessed simultaneously and was found to be low, with no difference between WT and *Klotho*$^{-/-}$ mice. Furthermore, we analyzed the expression of CXCR4, the putative receptor for SDF-1α, and found that the reduced chemotactic response of *Klotho*$^{-/-}$ BM cells was associated with a decrease in overall CXCR4^{+} population in *Klotho*$^{-/-}$ BM (Figure 5B). These findings were confirmed by quantifying serum levels of SDF-1α in WT and *Klotho*$^{-/-}$ mice. SDF-1α levels were found to be significantly reduced in mice lacking *Klotho*, indicating that trafficking of stem/progenitor cells between peripheral blood and the BM compartment is impaired (Figure 5C). These results were further confirmed by an *in vivo* assay in which we compared the direct and competitive steady-state homing potential of BM cells from WT and *Klotho*$^{-/-}$ mice. Studies have shown that lethal irradiation creates a noncompetitive host HSC pool that can be easily replaced by donor HSCs, whereas a nonmyeloablated environment may better portray natural trafficking patterns of HSCs *in vivo* and allow greater recovery and better survival of donor cells.[56–58] Therefore, we transplanted *Klotho*$^{-/-}$ or WT BM cells into nonmyeloablated and myeloablated B6.SJL WT recipient mice and assessed their direct homing efficiency 20 hours after transplantation. We found a marked reduction in the ability of *Klotho*$^{-/-}$ cells to *lodge* in a normal BM microenvironment (Figure 5, D and G, and Supplemental Figure S2, C and D) compared with transplanted WT BM cells. Furthermore, these experiments showed that most transplanted *Klotho*$^{-/-}$ BM cells were localized in the peripheral blood (Figure 5, D and E, and Supplemental Figure S2, A and D). However, localization of *Klotho*$^{-/-}$ and WT transplanted BM cells was similar in the spleens of nonmyeloablated and myeloablated recipient mice (Figure 5, D and F, and Supplemental Figure S2, B and D). Together, these results confirm a defect in the homing ability of *Klotho*$^{-/-}$ BM cells and clearly demonstrate that lack of klotho results in impaired lodgment and retention of BM cells in a normal microenvironment owing to a defect in their migratory function.

Increased Vitamin D Levels Are Only Partially Responsible for the Hematopoietic Changes in *Klotho*$^{-/-}$ Mice

Because *Klotho*$^{-/-}$ mice exhibit highly elevated vitamin D levels and vitamin D is known to regulate physiologic functions of klotho,[59] we investigated whether elimination of vitamin D activity can rescue the hematopoietic changes observed in *Klotho*$^{-/-}$ mice. We generated compound mutant mice lacking both *Klotho* and *1α(OH)ase*, the enzyme responsible for converting vitamin D to its active form. A complete blood cell count analysis was performed in WT, *Klotho*$^{-/-}$*/1α(OH)ase*$^{+/+}$, *Klotho*$^{-/-}$*/1α(OH)ase*$^{-/-}$, *and Klotho*$^{+/+}$*/1α(OH)ase*$^{-/-}$ littermates. As shown in Figure 6A, although *Klotho*$^{-/-}$*/1α(OH)ase*$^{-/-}$ double-mutant mice still displayed an increased number of circulating RBCs, genetic ablation of *1α(OH)ase* from *Klotho*$^{-/-}$ mice restored the MCV and MCH to normal levels in the double mutants (Figure 6, B and C).

However, flow cytometry analysis of erythroid populations in the BM showed that the frequency of immature erythroid population (Pro-E; pro-erythroid) and the more mature Ery-C fraction was similarly increased in *Klotho*$^{-/-}$ and *Klotho*$^{-/-}$*/1α(OH)ase*$^{-/-}$ double-mutant mice but not in *1α(OH)ase*$^{-/-}$ mice (Figure 6, D and E). Similarly, circulating Epo levels were highly elevated in *Klotho*$^{-/-}$ and *Klotho*$^{-/-}$*/1α(OH)ase*$^{-/-}$ mutant mice compared with WT and *1α(OH)ase*$^{-/-}$

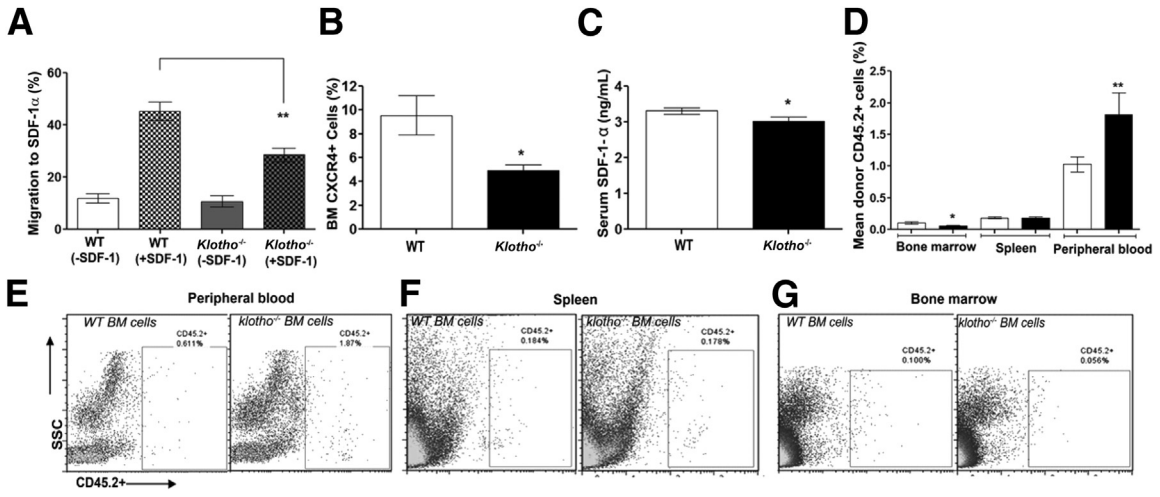

Figure 5 The perturbed *in vitro* and *in vivo* homing ability of *Klotho*$^{-/-}$ BM cells. **A:** *In vitro* migration experiment of the migratory capacity of *Klotho*$^{-/-}$ BM cells toward an SDF-1α gradient (WT, $n = 5$; *Klotho*$^{-/-}$, $n = 5$). **B:** Flow cytometry of CXCR4 in *Klotho*$^{-/-}$ BM compared with WT mice (WT, $n = 11$; *Klotho*$^{-/-}$, $n = 8$). **C:** SDF-1α concentration measured by ELISA in the serum of WT ($n = 10$) and *Klotho*$^{-/-}$ ($n = 10$) mice. Samples were measured in duplicate. **D:** Graphic representation of flow cytometry analysis of the BM, spleen, and peripheral blood after transplantation of WT ($n = 8$) or *Klotho*$^{-/-}$ ($n = 8$) BM cells in myeloablated B6.SJL recipient mice. **E**–**G:** Flow cytometry profiles of the peripheral blood, spleen, and BM after transplantation. SSC, side scatter. Data represent the means ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$.

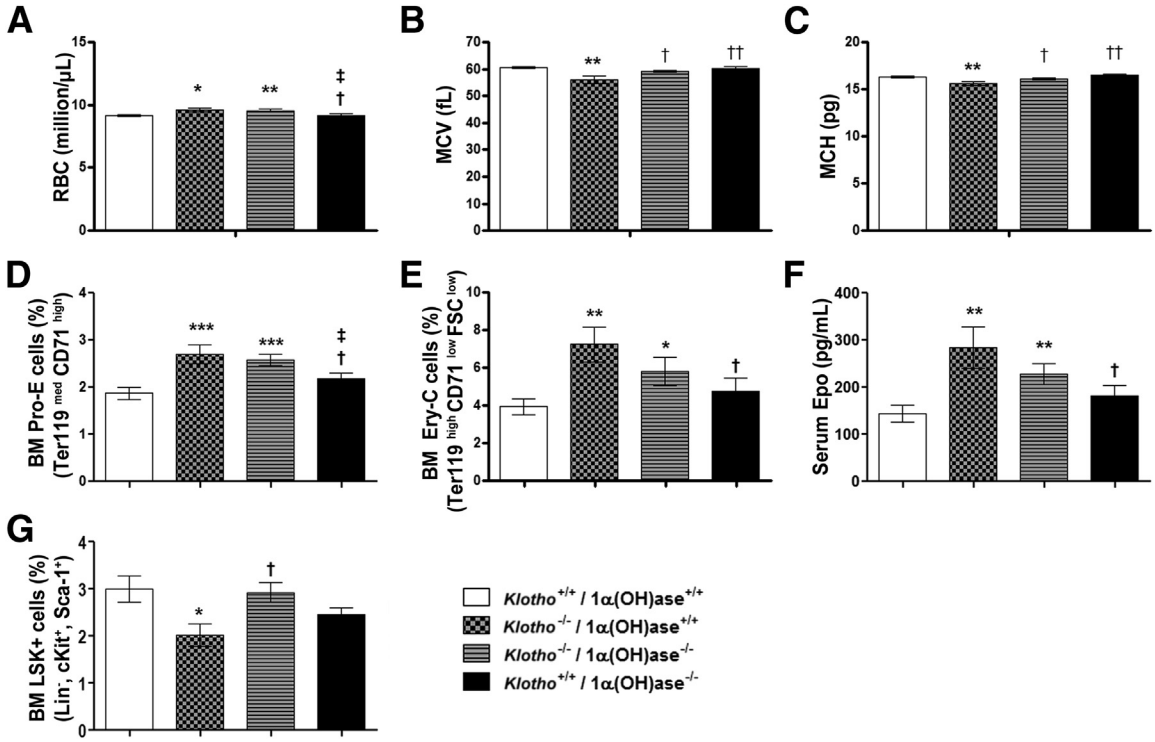

Figure 6 Genetic ablation of *1α(OH)ase* partially rescued hematopoietic changes in *Klotho*$^{-/-}$ mice. **A**–**C:** Complete blood cell count analysis of WT, *Klotho*$^{-/-}$/*1α(OH)ase*$^{+/+}$, *Klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$, and *Klotho*$^{+/+}$/*1α(OH)ase*$^{-/-}$ mice. RBC counts [WT, $n = 14$; *Klotho*$^{-/-}$/*1α(OH)ase*$^{+/+}$, $n = 11$; *Klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$, $n = 16$; *Klotho*$^{+/+}$/*1α(OH)ase*$^{-/-}$, $n = 11$] (**A**), MCV [WT, $n = 12$; *Klotho*$^{-/-}$/*1α(OH)ase*$^{+/+}$, $n = 13$; *Klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$, $n = 15$; *Klotho*$^{+/+}$/*1α(OH)ase*$^{-/-}$, $n = 15$] (**B**), and MCH [WT, $n = 17$, klotho$^{-/-}$/1α(OH)ase$^{+/+}$, $n = 12$; *Klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$, $n = 17$; *Klotho*$^{+/+}$/*1α(OH)ase*$^{-/-}$, $n = 17$] (**C**). **D**–**F:** Flow cytometry analysis of BM cells from all four genotypes. Percentage of the Pro-E population [WT, $n = 16$; *Klotho*$^{-/-}$/*1α(OH)ase*$^{+/+}$, $n = 14$; *Klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$, $n = 12$; *Klotho*$^{+/+}$/*1α(OH)ase*$^{-/-}$, $n = 13$] (**D**) and the Ery-C population [WT, $n = 13$; *Klotho*$^{-/-}$/*1α(OH)ase*$^{+/+}$, $n = 10$; *Klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$, $n = 9$; *Klotho*$^{+/+}$/*1α(OH)ase*$^{-/-}$, $n = 12$] (**E**) and serum Epo levels [WT, $n = 9$, *Klotho*$^{-/-}$/*1α(OH)ase*$^{+/+}$, $n = 6$; *Klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$, $n = 9$, *Klotho*$^{+/+}$/*1α(OH)ase*$^{-/-}$, $n = 7$] (**F**). **G:** Percentage of LSK progenitors [WT, $n = 7$; *Klotho*$^{-/-}$/*1α(OH)ase*$^{+/+}$, $n = 7$; *Klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$, $n = 7$; *Klotho*$^{+/+}$/*1α(OH)ase*$^{-/-}$, $n = 7$]. Data represent the means ± SEM. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus WT; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ versus *Klotho*$^{-/-}$/*1α(OH)ase*$^{+/+}$; $^{\ddagger}P < 0.05$ versus *Klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$.

mice (Figure 6F). Erythropoiesis seemed to be unaffected in *1α(OH)ase*$^{-/-}$ mice, as indicated by the examined parameters. Therefore, these data suggest that the increase in erythropoiesis in the absence of klotho is independent of vitamin D levels and is most likely associated with loss of klotho function. There was no difference in the frequency of hematopoietic progenitor cells (LSK; Lin^{-}Sca-1^{+}cKit^{+}) among WT, *Klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ double mutants, and *1α(OH)ase*$^{-/-}$ mice, in contrast to decreased LSK frequency in *Klotho*$^{-/-}$ mice (Figure 6G), suggesting that excess vitamin D negatively affects hematopoietic progenitor numbers. Taken together, these findings suggest that elimination of vitamin D could not completely rescue the hematopoietic abnormalities observed in *Klotho*$^{-/-}$ mice, signifying the importance of klotho in the regulation of hematopoiesis.

Klotho Protein Administration Influences Steady-State Hematopoiesis *in Vivo*

These findings so far clearly reveal that lack of klotho adversely affects hematopoiesis. To determine whether the effect of klotho is direct, we investigated whether exogenous administration of klotho protein can modulate steady-state hematopoiesis. Intraperitoneal injection of klotho protein in 8-week-old WT mice resulted in differential regulation of HSCs in various tissues analyzed. Flow cytometry analysis showed a decrease in erythroid progenitors in peripheral blood and BM (Figure 7, A–D), followed by augmented splenic erythropoiesis (Figure 7, E and F) in klotho-injected mice compared with vehicle-injected mice. Moreover, administration of klotho protein resulted in higher LSK (Lin^{-}Sca-1^{+}cKit^{+}) hematopoietic stem/progenitor cell levels (Figure 7G) in BM and more progenitor cell (granulocyte-erythroid-megakaryocyte–macrophage) colonies *in vitro* (Figure 7H). These data show that klotho administration results in hematopoietic changes opposite to the ones observed in klotho-deficient mice. Taken together, these results clearly demonstrate a novel role for klotho in the regulation of steady-state hematopoiesis. However, the exact mechanism of the differential modulation of hematopoiesis requires further investigation.

Klotho Deficiency Results in Disrupted Hematopoiesis Independent of the BM Microenvironment

The BM hematopoietic changes observed postnatally in *Klotho*-deficient mice could be associated with the dramatic

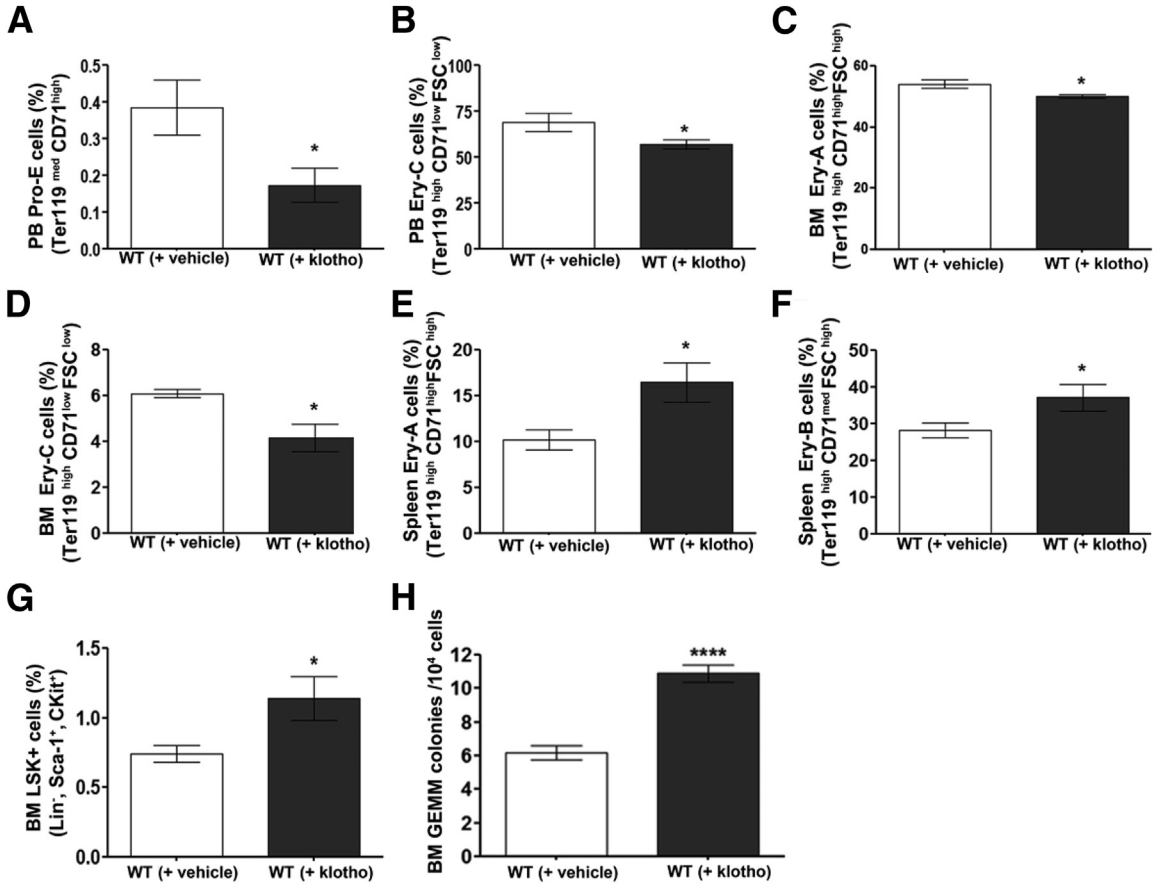

Figure 7 Klotho protein injection influenced hematopoiesis in WT mice. **A**–**G:** Flow cytometry analysis of erythroid populations in peripheral blood (PB) and BM and of erythroid cells in spleen. Peripheral blood: Percentage of the Pro-E population [WT (+ vehicle), $n = 3$; WT (+ Klotho), $n = 10$] (**A**) and the Ery-C population [WT (+ vehicle), $n = 6$; WT (+ klotho), $n = 7$] (**B**). BM: Percentage of the Ery-A population [WT (+ vehicle), $n = 5$; WT (+ klotho), $n = 9$] (**C**) and the Ery-C population [WT (+ vehicle), $n = 5$; WT (+ klotho), $n = 8$] (**D**). Spleen: Percentage of the Ery-A population [WT (+ vehicle), $n = 7$; WT (+ klotho), $n = 8$] (**E**) and the Ery-B population [WT (+ vehicle), $n = 7$; WT (+ klotho), $n = 7$] (**F**). **G:** Percentage of LSK (lin^- Sca-1^+ c-Kit^+) progenitors in the BM of injected mice [WT (+ vehicle), $n = 8$; WT (+ klotho), $n = 6$]. **H:** Colony-forming unit assay of BM granulocyte-erythroid-megakaryocyte-macrophage (GEMM) mix colonies in klotho-injected mice [WT (+ vehicle), $n = 8$; WT (+ klotho), $n = 10$]. Data represent the means ± SEM. $^*P < 0.05$, $^{****}P < 0.0001$.

reduction in osteoblast and osteoclast numbers and bone mineral density in *Klotho*$^{-/-}$ bones. However, the decrease in HSCs in *Klotho*$^{-/-}$ BM and their retention in the spleen along with the defect in their homing ability (as seen *in vitro* and *in vivo*) suggest that klotho deficiency impairs hematopoiesis regardless of the BM microenvironment. To address this hypothesis, we studied prenatal hematopoiesis in *Klotho*$^{-/-}$ mice. Because embryonic hematopoiesis in fetal liver declines after E15.5, we isolated fetal liver cells from time pregnant female mice on E15.5 and studied changes in fetal liver hematopoiesis. The yield of fetal liver cells from *Klotho*$^{-/-}$ mice was significantly lower compared with WT littermates (Supplemental Figure S2E). We found that fetal liver hematopoiesis in *Klotho*$^{-/-}$ mice was similarly affected, with changes similar to those detected in the BM compartment of adult *Klotho*$^{-/-}$ mice. Analysis of the erythroid population showed an increase in early erythroid cells (Pro-E; Ter119^{med}CD71^{high}) (Figure 8A) and in the more mature Ery-C fraction (Ter119^{high}CD71^{low}FSC^{low}) (Figure 8B) of erythroblasts in *Klotho*$^{-/-}$ fetal liver cells, a finding supported by enhanced erythroid colony-forming ability *in vitro* (Figure 8C). More importantly, we observed a marked reduction in Lin$^-$Sca1$^+$cKit$^+$ (LSK) and cKit$^+$ Thy-1^{low} Lin$^-$ Sca-1$^+$ (KTLS) HSCs in *Klotho*$^{-/-}$ fetal liver cells compared with WT fetal livers (Figure 8, D and E). This was concomitant with a significant decrease in the ability of *Klotho*$^{-/-}$ fetal liver cells to form granulocyte-erythroid-megakaryocyte–macrophage colonies *in vitro* (Figure 8F). These data indicate a global defect in the HSC turnover in the absence of klotho. This novel observation, that klotho deficiency affects prenatal hematopoiesis, clearly demonstrates a potential role for the antiaging hormone klotho in hematopoietic development.

Discussion

Several lines of evidence suggest that aging has a major effect on HSC function and localization.[60–66] *Klotho* has previously been identified as an aging-suppressor gene that has an active role in the renal control of calcium, phosphate, and vitamin D metabolism, and *Klotho* gene deficiency is

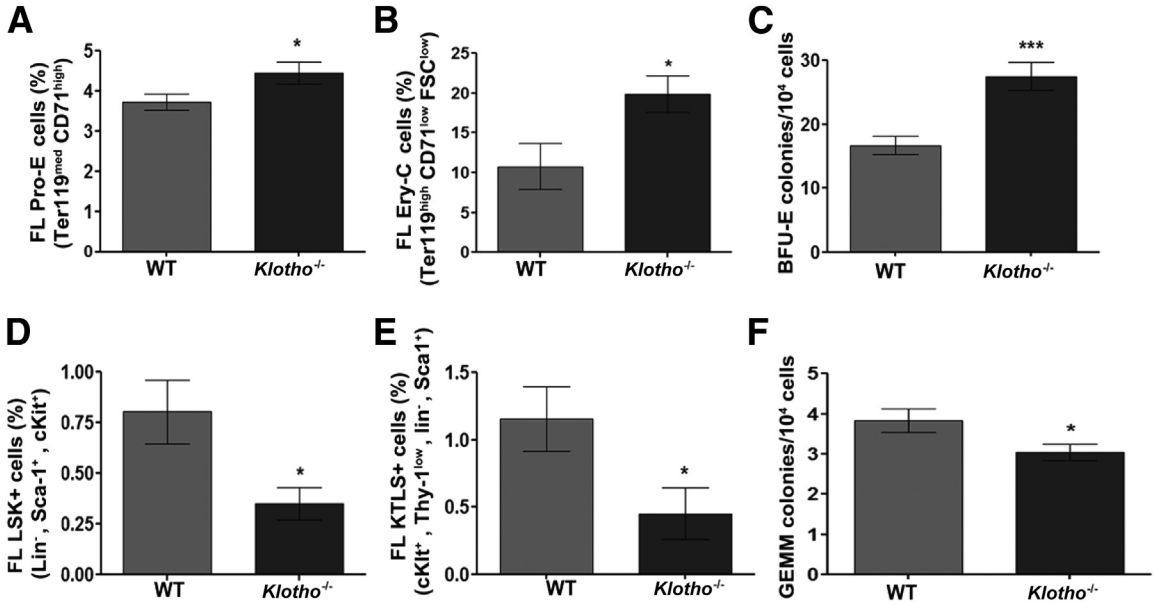

Figure 8 Klotho deficiency affected fetal liver (FL) hematopoietic milieu. Flow cytometry analysis of E15.5 FL cells. Immature erythroid cells (Pro-E; $Ter119^{med}CD71^{high}$) (WT, $n = 9$; $Klotho^{-/-}$, $n = 10$) (**A**) and Ery-C fraction ($Ter119^{high}$ $CD71^{low}$ FSC^{low}) (WT, $n = 8$; $Klotho^{-/-}$, $n = 6$) (**B**) in $Klotho^{-/-}$ FLs. **C:** Colony-forming unit assay of burst-forming unit—erythroid (BFU-E) colonies produced by $Klotho^{-/-}$ FL cells (WT, $n = 7$; $Klotho^{-/-}$, $n = 9$). Cells from each mouse were plated in triplicate, and the number of colonies in each plate was counted. Frequency of Lin^- $Sca\text{-}1^+$ $c\text{-}Kit^+$ (LSK) (WT, $n = 10$; $Klotho^{-/-}$, $n = 8$) (**D**) and $c\text{-}Kit^+Thy1^+Lin^-Sca\text{-}1^+$ (KTLS) (WT, $n = 7$; $Klotho^{-/-}$, $n = 8$) (**E**) HSC populations in $Klotho^{-/-}$ FLs. **F:** Colony-forming unit assay of primitive granulocyte-erythroid-megakaryocyte-macrophage (GEMM) colonies in $Klotho^{-/-}$ FL cells (WT, $n = 7$; $Klotho^{-/-}$, $n = 9$). Cells from each mouse were plated in triplicate, and the number of colonies in each plate was counted. Data represent the means ± SEM. $^*P < 0.05$, $^{***}P < 0.001$.

associated with renal and skeletal abnormalities, including low-bone-turnover osteopenia characterized by a decreased bone formation rate, reduced osteoblast and osteoclast numbers, and severely impaired bone mineral density.[26,67–69] Defects in bone ossification and altered mineral ion metabolism have adverse effects in the BM microenvironment that houses the hematopoietic stem and progenitor cells.[38,70] Several studies have reported that decreased osteoblast and osteoclast numbers and bone mineral content result in reduced B-lymphocyte and HSC numbers.[37–39,44,45,71–74] In addition, defective endochondral ossification causes aberrant B lymphopoiesis in mice.[75,76] The present study is the first to demonstrate that normal function of klotho is required for balanced erythropoiesis and to establish a novel role for klotho in the regulation of RBC formation. We show that mice deficient in *Klotho* exhibit perturbed prenatal and postnatal hematopoiesis and, hence, are incapable of sustaining a balanced hematopoietic milieu.

Klotho deficiency is a characteristic feature of CKD in which anemia and vascular calcifications are prevalent. Moreover, kidney disease is associated with inflammation and aging. Direct involvement of klotho in vascular calcifications was demonstrated in recent studies.[17,18] Therefore, understanding the extrarenal functions of klotho is of great clinical significance for therapeutic intervention in pathologic conditions associated with CKD and CVD. This study shows that loss of klotho in mice results in reduced MCV and MCH in circulating RBCs. Erythropoiesis is tightly regulated between the kidney and BM microenvironment by the production of Epo, a hormone secreted by the kidney and acting on the erythroid progenitors in the BM. BM erythropoiesis is a complex and dynamic process that involves proliferation of immature erythroblasts and maturation through successive stages, leading to enucleation of mature erythrocytes. As a response to inherited or acquired anemia, the kidneys produce Epo to increase RBC production and oxygenation. Hypoxia acts as a physiologic stimulus to induce Epo transcription.[77,78] Studies have shown that administration of recombinant Epo can induce klotho expression in a rat nephropathy model, suggesting that these kidney-derived proteins can interact *in vivo*.[79] Herein we demonstrate that despite the low MCV and MCH, klotho deficiency in mice results in increased erythropoiesis through activation of the HIF signaling pathway and subsequent increased Epo secretion and up-regulation of renal Epo mRNA. Although the kidney is the primary site for Epo production in adult animals, other tissues also produce Epo. It has been recently reported that osteoblasts can also produce Epo through activation of the HIF pathway under physiologic and pathophysiologic conditions.[80] In the present studies, we found that the expression of HIF-1α and HIF-2α was significantly attenuated in $Klotho^{-/-}$ bone, resulting in local suppression of Epo (unpublished data, S.V.M., L.M.C., C.C., D.S.). These findings suggest that reduced osteoblast numbers and osteopenia in $Klotho^{-/-}$ mice may be responsible for the inhibition of HIF and Epo in bone and further support the hypothesis for tissue-specific hypoxic induction of Epo, leading to increased erythrocyte production.

We found that klotho deficiency results in decreased frequency of $Lin^-cKit^+Sca1^+$ (LSK) and $cKit^+$ $Thy\text{-}1^{low}$

Lin^- $Sca\text{-}1^+$ (KTLS) HSCs in the BM and fetal liver. However, HSC numbers were increased in the blood and spleen of *Klotho*$^{-/-}$ mice, suggesting that in the absence of klotho, primitive HSC populations reside in sites other than the BM. These observations raised the possibility that the decrease in BM HSC numbers may be due to altered homing or retention of the cells in the correct microenvironment. Retention and homing of HSCs to the BM depends on the presence of chemokines, extracellular matrix proteins, and osteoblasts. The present data show that *Klotho* deletion results in a decreased response of BM cells to a gradient of SDF-1α, low serum SDF-1α, and reduced $CXCR4^+$ cells in the BM, suggesting that the *Klotho*$^{-/-}$ BM stroma does not support HSC retention, and that the migratory function of *Klotho*$^{-/-}$ BM cells is impaired. Furthermore, *in vivo* homing assays confirmed these observations and demonstrated that transplanted *Klotho*$^{-/-}$ BM cells were unable to competently lodge in a normal BM microenvironment regardless of whether there was competition (nonmyeloablation) or not (myeloablation). Also, trafficking from the marrow to the periphery was increased in klotho deficiency. The present findings, together with the current understanding that loss of osteoblasts negatively regulates the size of the HSC niche and results in defective HSC localization,[37–39,45,81] suggest that i) impaired bone mineralization and loss of osteoblasts and osteoclasts in *Klotho*$^{-/-}$ mice may alter the BM microenvironment, rendering it nonconducive to supporting normal hematopoiesis, ii) the decrease in HSC numbers in *Klotho*$^{-/-}$ mice may be due to an accelerated differentiation of these cells toward the erythroid lineage, and/or iii) the abnormalities found in *Klotho*$^{-/-}$ HSCs may be due to a cell-autonomous defect in their ability to engage the niche *in vivo*. Preliminary data from the Despina Sitara Laboratory (Department of Basic Science and Craniofacial Biology New York University College of Dentistry, and Department of Medicine New York University School of Medicine, NY) show that after long-term competitive repopulation transplantations, *Klotho*$^{-/-}$ BM cells do not engraft as competently as WT BM cells to the BM environment of lethally irradiated WT mice (unpublished data, S.V.M., L.M.C., C.C., D.S.). These findings suggest that *Klotho* deletion results in an intrinsic abnormality of the HSCs to efficiently engraft to a normal BM niche and that HSCs do not depend on the *Klotho*$^{-/-}$ host BM environment. More transplantation studies are currently under way to confirm these observations.

Previously published data show that *Klotho*-null mice exhibit B lymphopenia and low levels of IL-7, which is essential for early B-cell development in the BM, and they attribute these changes to aging.[32] Klotho mRNA expression has also been found to be significantly decreased in resting human $CD4^+$ lymphocytes proportionally to advancing age.[33] Additionally, a shift toward myeloid lineage commitment and reduced B lymphopoiesis are well-documented phenomena in both murine and human aging.[65,82,83] Taken together, the effect of klotho deficiency in lymphopoiesis and myelopoiesis and the mechanisms mediating these effects require further investigation.

Lack of klotho disturbs mineral ion homeostasis and vitamin D balance, leading to hyperphosphatemia, hypercalcemia, and hypervitaminosis D. Previous studies convincingly demonstrated that genetic ablation of vitamin D can reverse phosphate and calcium excess and rescue aging and growth retardation in *Klotho*$^{-/-}$ mice.[59,84] In addition, the vitamin D receptor is known to play an important role in hematopoiesis.[46] Therefore, we investigated whether the mechanism of altered hematopoiesis in *Klotho*$^{-/-}$ mice is vitamin D mediated. We observed that the altered erythropoiesis in *Klotho*$^{-/-}$ mice could not be completely rescued in *Klotho*$^{-/-}$*/1α(OH)ase*$^{-/-}$ double-mutant mice, suggesting that the effect of klotho on erythroid cell production is independent of vitamin D levels. However, the low mean cell hemoglobin content and microcytosis were normalized in the double-mutant mice, indicating a possible involvement of vitamin D in maintaining normal RBC size. To our knowledge, there are no data available directly linking vitamin D to RBC mass, and the role of 1,25-dihydroxyvitamin D_3 in the proliferation of erythroid precursors has been controversial.[85,86] Moreover, studies have shown that increased intracellular calcium levels in circulating RBCs result in a decrease in RBC volume.[87] This is caused by activation of calcium channels present in RBCs, leading to RBC membrane deformation and a subsequent increase in Ca^{2+} permeability, inducing significant cell dehydration and cell shrinkage.[87] Therefore, it is possible that the effect on MCV in *Klotho*$^{-/-}$ mice is mediated by the hypercalcemia that these mice exhibit. Further studies are needed to determine whether calcium plays a role in the effects of klotho on erythropoiesis. In addition, genetic ablation of 1α(OH)ase resulted in normal numbers of BM hematopoietic progenitor cells (LSK; $Lin^-Sca\text{-}1^+cKit^+$) compared with *Klotho*$^{-/-}$ mice, suggesting that high vitamin D levels associated with klotho deficiency may be responsible for the decline in HSC numbers.

The prevalence of anemia in patients with CKD is mainly attributed to reduced renal Epo production. Klotho expression is also reduced with kidney disease progression.[18] However, we found that klotho deficiency in mice is associated with up-regulation in renal Epo production. It has been shown that Epo levels are increased with aging, as a compensation for increased erythrocyte turnover.[88,89] With advanced aging, however, this compensatory mechanism eventually becomes inadequate, leading to the development of anemia.[89] Moreover, previous studies have demonstrated that in the aging kidney, HIF signaling is activated by hypoxia, resulting in the coordinated induction of HIF-regulated genes, such as *Epo* and *Glut-1*.[54] Therefore, the aging-related changes in *Klotho*-null mice may be partially responsible for the elevated Epo production and increased erythropoiesis in these mice. Furthermore, patients with CKD exhibit low serum levels of vitamin D,[90] whereas *Klotho*-null mice have significantly elevated vitamin D levels.[59] It has been shown that 1,25-dihydroxyvitamin D_3

induces up-regulation of Epo-R expression followed by stimulation of the growth and proliferation of erythroid precursor cells.[91] In addition, it has been previously reported that the hematon fraction of normal BM, a low-density floating layer that constitutes the most productive erythropoietic compartment, contains significantly high active vitamin D content.[92] Therefore, it would be tempting to speculate that excess vitamin D in *Klotho*$^{-/-}$ mice may have also induced erythroid activity. However, the present finding that increased erythropoiesis in *Klotho*$^{-/-}$ mice was not affected by genetic ablation of *1α(OH)ase* suggests that it is most likely a vitamin D–independent process. Further studies are required to address the exact mechanism of enhanced erythropoiesis associated with klotho deficiency.

In addition, exogenous administration of klotho resulted in hematopoietic changes opposite to the ones observed in *Klotho*-deficient mice. Based on these observations, the present study demonstrates for the first time that klotho directly regulates HSC differentiation and erythroid cell generation and maturation. Moreover, we observed that klotho deficiency affected the prenatal fetal liver erythroid compartment in a similar manner, clearly suggesting a novel function for klotho in the regulation of hematopoiesis beyond the skeletal abnormalities and senescence features ascribed to its deficiency. Embryonic hematopoiesis begins in the yolk sac and moves to the aorta-gonad-mesonephros region, followed by the fetal liver, which becomes the primary site of fetal hematopoiesis.[93,94] The present findings that klotho expression is detected in fetal liver cells from WT mice and that loss of klotho results in hematopoietic changes in fetal liver similar to the ones observed in adult mice are crucial. These data strongly suggest that aberrant production or function of HSCs in *Klotho*-null mice occurs before translocation of HSCs from fetal liver to BM, linking klotho directly to the development of hematopoiesis. Given that fetal liver does not generate HSCs but rather serves as a remarkable organ for HSC expansion, the changes we confront may be due to events upstream of the fetal liver, suggesting an exciting possibility of klotho having a function in embryonic hematopoietic development. Additional studies are required to determine the role of klotho in migration and colonization of the fetal liver and the mechanisms of action of klotho in prenatal hematopoiesis.

In summary, the present observations show that klotho deficiency not only plays a role in aging and aging-related conditions, such as CKD, CVD, and osteopenia, but also severely affects hematopoietic development, suggesting that the therapeutic manipulation of klotho has considerable clinical implications. Taken as a whole, this study identifies klotho as a potent regulator of prenatal and postnatal hematopoiesis and provides clear evidence that it plays an essential role in hematopoietic development. The findings herein extend our knowledge of the processes participating in establishment of the HSC niche and provide a better understanding of the connections among renal function, bone development, hematopoiesis, and aging. Moreover, therapeutic targeting of klotho and its associated signaling components may serve as a mechanism to boost immune functions and treat blood cell disorders associated with aging or kidney disorders.

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Supplemental Data

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