Am J Transl Res 2022;14(2):1234-1245
www.ajtr.org /ISSN:1943-8141/AJTR0129422

Original Article

Di(2-ethylhexyl)phthalate impairs erythropoiesis via inducing Klotho expression and not via bioenergetic reprogramming

Chang-Yi Tsai[1*], Te-Ping Fang[1*], Shuoh-Wen Chen[1*], Hsiao-Wen Chen[1], Eric Chang-Yi Lin[1], Ting-An Lin[1,2,4], Der-Cherng Tarng[1,3,5,6], Yuan-I Chang[1]

[1]*Department and Institute of Physiology,* [2]*Department of Internal Medicine,* [3]*Institute of Clinical Medicine, National Yang Ming Chiao Tung University, Taipei 112304, Taiwan;* [4]*Division of Hematology,* [5]*Division of Nephrology, Department of Medicine, Taipei Veterans General Hospital, Taipei 112201, Taiwan;* [6]*Center for Intelligent Drug Systems and Smart Bio-devices (IDS2B), Hsinchu 300093, Taiwan.* [*]*Equal contributors.*

Received January 1, 2021; Accepted December 26, 2021; Epub February 15, 2022; Published February 28, 2022

Abstract: Di(2-ethylhexyl)phthalate (DEHP) is the most widely used phthalate to manufacture various plastic products. However, the potential effects of DEHP on erythropoiesis have not been investigated comprehensively. Here, we aimed to investigate whether DEHP modulated the function of hematopoietic stem and progenitor cells (HSPCs) to influence erythropoiesis, and to explore the associated mechanisms. In the present study, human cell lines with a capacity to differentiate into erythroid cells and murine bone marrow cells were treated with DEHP. DEHP not only impaired HSPC function, but also suppressed erythroid differentiation in a dose-dependent manner. In addition, DEHP removal restored HSPC activity. To explore how DEHP interfered with erythroid differentiation, we focused on energy metabolism and Klotho expression. DEHP suppressed erythroid differentiation via upregulating Klotho expression, while it did not via modulating cellular bioenergetics. Therefore, our results provided a novel insight into the pathophysiological link between phthalates and dysregulated erythroid differentiation.

Keywords: DEHP, phthalates, erythroid differentiation, Klotho, bioenergetics

Introduction

Di(2-ethylhexyl)phthalate (DEHP) is a common phthalate, which is most frequently used as a plasticiser in plastic products, such as personal care products and medical devices. It is non-covalently bound to a polymer; hence, DEHP easily leaches, migrates, or evaporates into the atmosphere, foods, or directly into body fluids [1-3]. Thus, its ubiquity in the environment gives rise to health concerns regarding continuous DEHP exposure. To date, the adverse effects of DEHP in the liver, endocrine and reproductive systems, as well as in obesity and some types of cancers, have all been explored extensively [3, 4]. However, further investigations are required to determine whether DEHP exposure influences other physiological processes. According to a recent study, DEHP affects the function of human hematopoietic stem and progenitor cells (HSPCs), which are isolated from human umbilical cord blood [5]. DEHP also impairs the migration and colony-forming capacity of human CD34$^+$ HSPCs [6]. High-dosage and long-term dietary DEHP uptake causes chronic progressive nephropathy and significantly reduces hemoglobin (Hgb) production in rodents [7, 8]. However, DEHP has no effect in male cynomolgus monkeys that were treated with 500 mg/kg/day DEHP for 14 days [9]. These results arose concerns regarding whether DEHP exposure adversely affects erythropoiesis.

Previous studies have demonstrated that DEHP acts as an endocrine disruptor and reproductive toxicant via the inhibition of endocrine receptor signalling or the activation of peroxisome proliferator-activated receptors (PPARs) [3, 10]. In addition, DEHP causes epigenetic

changes, especially DNA methylation [3, 11], and impairs both insulin signalling and glucose metabolism [11-14]. In liver and muscle cells, DEHP exposure can influence the levels of glucose metabolites, including glucose-6-phosphate, fructose-6-phosphate, pyruvate, lactate, glucose-1-phosphate, and glycogen [13]. In hematopoietic systems, long-term hematopoietic stem cells (HSCs), which are at the top of the hematopoietic hierarchy, tend to generate energy via anaerobic glycolysis, whereas lineage-restricted progenitor cells generate adenosine triphosphate (ATP) primarily in the mitochondria via oxidative phosphorylation (OXPHOS) [15-18]. Erythroid progenitor cells also exhibit a similar rearrangement of glycolytic metabolism when they differentiate into mature erythroid cells [19]. In the present study, we investigated whether DEHP influenced erythroid differentiation through modulating glycolytic metabolism and mitochondrial respiration. In addition, we investigated the potential DEHP-mediated molecular mechanisms in erythropoiesis.

Materials and methods

Cell culture, differentiation and DEHP treatment

Human chronic myelogenous leukemia (CML) K562 and erythroleukemia HEL 92.1.7 (HEL) cell lines were obtained from the Bioresource Collection and Research Center (BCRC) (Hsinchu City, TW). K562 cells (4×10^4 cells/ml) were cultivated in Iscove's Modified Dulbecco's Medium (IMDM) (Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, USA), 100 IU/ml penicillin (Sigma-Aldrich, USA), and 100 IU/ml streptomycin (Sigma-Aldrich, USA), and were passaged every two days. HEL cells (1×10^5 cells/ml) were cultivated in Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher Scientific, USA) supplemented with 10% FBS, 1 mM sodium pyruvate (Sigma-Aldrich, USA), 100 IU/ml penicillin, and 100 IU/ml streptomycin, and were passaged every 2 to 3 days. For erythroid differentiation, K562 and HEL cells were treated with 2 mM sodium butyrate (NaB) (Sigma-Aldrich, USA) for K562 cells and at 0.1 mM NaB for HEL cells. Benzidine staining was performed to examine Hgb expression [20]. DEHP (Acros Organics, USA) was a kind gift from Dr. Tzong-Shyuan Lee (National Taiwan University, TW) [21, 22].

Transfection

The scramble shRNA (ASN0000000004) and human *KLOTHO* shRNAs, including A2 (TRCN0000161418), D2 (TRCN0000419459) and E2 (TRCN0000433013) were obtained from National RNAi Core Facility in Academia Sinica (Taipei City, TW). All plasmids were verified by DNA sequencing, which was carried out by Genomics (New Taipei City, TW). Transfection was performed using Lipofectamine™ 2000 reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

Analysis of gene expression

RNA isolation and first-strand cDNA synthesis were prepared by using the RNeasy Plus Mini Kit (Qiagen, Germany) and RevertAid First-Strand cDNA Synthesis Kit (Thermo Fisher, USA) following the manufacturer's protocols. The indicated transcripts in cDNA samples were quantified using Fast SYBR™ Green Master Mix (Applied Biosystems, USA) according to the manufacturer's protocol. Reaction and signal detection were measured by QuantStudio 3 Real-Time PCR System (Applied Biosystems, USA). The primer sets included: Human *KLOTHO*-F: 5'-GCCCACATACTGGATGGTATCAA-3'; Human *KLOTHO*-R: 5'-ACTGCACTCAGTACACACGGTGA-3'; Human *GAPDH*-F: 5'-TGGTTCACACCCATGACGAA-3'; Human *GAPDH*-R: 5'-GGAGTCCACTGGCGTCTTCA-3'; Mouse *Klotho*-F: 5'-CAAAGTCTTCGGCCTTGTTC-3'; Mouse *Klotho*-R: 5'-CTCCCCAAGCAAAGTCACA-3'; Mouse *Gapdh*-F: 5'-CATGGCCTTCCGTGTTCCTA-3'; Mouse *Gapdh*-R: 5'-GCGGCACGTCAGATCCA-3'. The Klotho expression level relative to GAPDH was calculated by using the difference in threshold cycle method for quantitative RT-PCR analysis.

Western blot analysis

Cells were lysed with RIPA buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% Triton X-100; 0.1% SDS; 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride; 10 μg/ml aprotinin; 10 μg/ml leupeptin; 10 μg/ml pepstatin; 1% sodium deoxycholate; 1 mM sodium fluoride; 1 mM sodium orthovanadate; 25 mM β-glycerophosphate). All reagents, which were used for RIPA buffer

preparation, were purchased from Sigma-Aldrich. Western blot was performed with the following antibodies: anti-KLOTHO antibody (Cat. ab203576, Abcam, UK) and anti-GAPDH antibody (Cat. NB300-221, Novus Biologicals, USA). Detection was performed using Clarity Western ECL Substrate (Bio-Rad, USA).

Mouse bone marrow and spleen cell isolations

All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee (IACUC) of National Yang Ming Chiao Tung University (Taipei Yang Ming Campus) (IACUC number: 1081012). Pure C57BL/6 mice were purchased from the Laboratory Animal Center of National Yang Ming Chiao Tung University (Taipei Yang Ming Campus). Bone marrow (BM) cells were isolated carefully from the tibia and femur. For spleen (SP) cell preparation, a small piece of excised SP was pressed through the strainer using the plunger end of a syringe. Subsequently, BM and SP cells were washed through a strainer and collected in 50 ml tube [23, 24].

Colony formation assay

The methylcellulose-based media MethoCult™ M3334 and M3434 were obtained from StemCell Technologies (Canada). Colony formation assays were performed as previously described [24]. To detect burst-forming unit-erythroids (BFU-Es) and other types of colonies, including oligopotential progenitor colony-forming unit-granulocyte, erythrocyte, monocyte/macrophage, and megakaryocyte (CFU-GEMM), lineage-restricted progenitor CFU-granulocyte and monocyte/macrophage (CFU-GM) and the precursors of granulocytes (CFU-G) and monocytes/macrophages (CFU-M), 2×10^4 BM cells or 1×10^6 SP cells were plated in MethoCult™ GF M3434 methylcellulose medium according to the manufacturer's protocol. Colonies were counted after 10 days in culture. For re-plating analyses, 2×10^4 BM cells were plated in MethoCult™ GF M3434 methylcellulose medium. After 10 days, 2×10^4 colony cells were re-plated. To detect CFU-erythroid (CFU-E) colonies, 2×10^4 BM cells or 1×10^6 SP cells were plated in MethoCult™ M3334 methylcellulose medium according to the manufacturer's protocol. CFU-E colonies were counted after 2 days of culture.

Analysis of oxygen consumption rate and extracellular acidification rate

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using a Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience, USA). Oligomycin, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), rotenone, antimycin, glucose, and 2-deoxy-D-glucose (2-DG) were obtained from Seahorse Bioscience. Firstly, the Seahorse XF cell culture microplates were coated with 3.5 μg/cm^2 of Corning Cell-Tak™ adhesive reagent (Corning, USA). For OCR analysis, the constituents were prepared in non-buffered IMDM, which is sodium bicarbonate- and HEPES-free IMDM (Thermo Fisher Scientific, USA) with 2% FBS, 100 IU/ml penicillin, and 100 IU/ml streptomycin. 9×10^4 of K562 cells were resuspended in 100 μl non-buffered IMDM in one well of the Seahorse XF cell culture microplates, transferred into microplates, and then incubated at room temperature for 30 mins. Subsequently, 575 μl of non-buffered IMDM was added in each well. To measure OCR, oligomycin and FCCP were diluted to 1 μM, and rotenone and antimycin were diluted to 0.5 μM. For ECAR analysis, culture medium was changed to assay medium (XF base medium DMEM supplemented with 2 mM glutamine), and cells were incubated in a non-CO_2 incubator at 37°C for 1 h before ECAR assay. Glucose, oligomycin and 2-DG were diluted to 10 mM, 1 μM and 50 mM, respectively. The automatic measurement of OCR and ECAR in real time was performed according to the manufacturer's protocol to detect live-cell bioenergetic profiles.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 6.0 (GraphPad Software Inc., USA). Data were presented as means ± s.d. All experiments were performed at least three times. We compared the results of the treatment groups and the controls using Student's *t*-test or two-way analysis of variance (ANOVA) followed by the Tukey's multiple-comparison post-hoc test. Differences between groups were considered to be significant at a P value of <0.05.

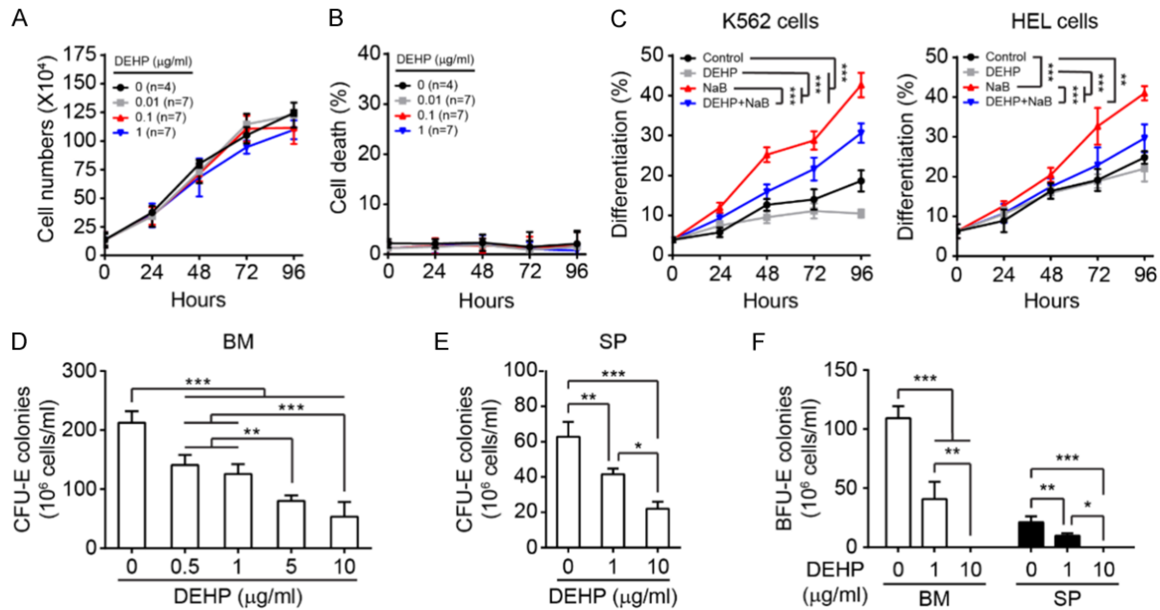

Figure 1. Exposure to DEHP suppresses erythroid differentiation. Human K562 cells were treated with different DEHP dosages. (A) Cell number and (B) cell death were analyzed by using trypan blue exclusion assay. (C) K562 and HEL cells were treated with NaB (K562: 2 mM; HEL: 0.1 mM) to induce erythroid differentiation in the presence of 1 μg/ml DEHP or not. The globin protein expression in cells was examined by using benzidine staining. (D) BM and (E) SP cells were cultured in methylcellulose-based media with EPO and different dosages of DEHP for 2 days. CFU-Es were counted by using phase-contrast microscopy. (F) BM and SP cells were cultured in methylcellulose-based media with other cytokines to maintain HSPC proliferation and differentiation in the presence of different DEHP dosages. The number of BFU-Es was counted by using phase-contrast microscopy. All experiments were repeated at least three times. The quantified data were presented as means ± s.d. Two-way ANOVA followed by the Tukey's multiple-comparison post-hoc test was used for statistical analysis. **P*<0.05; ***P*<0.01; ****P*<0.001.

Results

DEHP suppresses erythropoiesis

To rapidly test whether DEHP influenced erythropoiesis, we used sodium butyrate (NaB) to induce erythroid differentiation in human K562 and HEL cells [20]. The addition of DEHP in the culture medium did not affect K562 cell proliferation (**Figure 1A**) and survival (**Figure 1B**). Based on previous reports about the plasma concentration of DEHP in chronic kidney disease (CKD) patients undergoing hemodialysis [25, 26], 1 μg/ml of DEHP was selected for most experiments in the present study. DEHP treatment substantially decreased NaB-induced erythroid differentiation, which was evaluated based on Hgb production, in both K562 and HEL cells. Without NaB treatment, DEHP also decreased spontaneous erythroid differentiation, which resulted from increasing cell numbers (**Figure 1C**). Erythropoietin (EPO) is the primary cytokine that regulates erythropoiesis [27, 28]. The increment of DEHP concentration gradually and significantly suppressed the formation of EPO-induced CFU-Es in bone marrow (BM) (**Figure 1D**) and spleen (SP) cells (**Figure 1E**) in a dose-dependent manner. CFU-Es are differentiated form the earlier erythroid progenitor cells, BFU-Es [29]. DEHP also suppressed BFU-E formation (**Figure 1F**). Additionally, DEHP did not influence the morphology of CFU-Es (Figure S1), or BFU-Es (Figure S2). These results not only demonstrated that DEHP suppressed erythropoiesis but also indicated that the two human cell lines were comparable cell models that could be adopted in subsequent investigations.

DEHP suppresses the differentiation of HSPCs, whereas DEHP removal restores HSPC self-renewal activity

To examine the effect of DEHP in the proliferation and differentiation of HSPCs after DEHP treatment, BM and SP cells were cultured in a methylcellulose-based medium. DEHP decreased the formation of CFU-GEMM, CFU-GM,

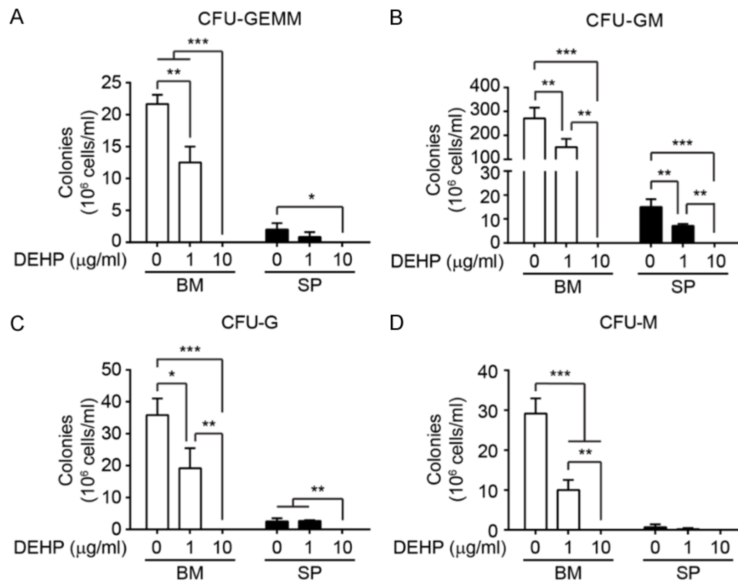

Figure 2. Exposure to DEHP impairs HSPC differentiation. In the presence or absence of DEHP (1 μg/ml), BM and SP cells were cultured in methylcellulose-based media with various cytokines to maintain HSPC proliferation and differentiation for 10 days. The numbers of (A) CFU-GEMM, (B) CFU-GM, (C) CFU-G, and (D) CFU-M in the cultures were counted under phase-contrast microscopy. All experiments were repeated at least three times. The quantified data were presented as means ± s.d. One-way ANOVA followed by the Tukey's multiple-comparison post-hoc test was used for statistical analysis. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

CFU-G and CFU-M in both BM and SP cells in a dose-dependent manner (**Figure 2**). DEHP treatment did not affect the morphology of different types of CFUs (Figure S2). HSPC self-renewal could be examined by re-plating cells in methylcellulose-based medium. HSPCs retained a similar capacity to generate hematopoietic cells in the second plating, while the potential decreased gradually in the third and fourth iterations (**Figure 3A**), which is consistent with our previous observations [24]. The experimental design used to examine whether DEHP removal restored HSPC potential was presented in **Figure 3B**. In the first plating, DEHP significantly inhibited the clonogenic growth. Subsequently, similar colony numbers, which were compared to the number of first-generation clonogenic formation in the DEHP-treated group, were observed in the re-plated experiments without DEHP. However, continuous exposure to DEHP in the second plating significantly decreased colony-forming capability (**Figure 3C**). Overall, DEHP interfered transiently with HSPC functions, because removal of DEHP in the second plating maintained similar self-renewal capability.

DEHP does not reprogram cellular bioenergetics during erythroid differentiation

Previous studies have indicated that DEHP treatment influences cellular glucose metabolism [13], and erythropoiesis is highly associated with reprogramming of glucose metabolism and energy consumption [19]. Firstly, we examined whether DEHP influenced energy metabolism in undifferentiated K562 cells. K562 cells were treated with 1 μg/ml DEHP for 24 h, and then collected for OCR and ECAR assays. Mitochondrial respiration reflected by OCR levels was detected (**Figure 4A**). DEHP treatment did not influence mitochondrial respiration parameters in undifferentiated K562 cells (**Figure 4B**). For the ECAR assay, cells were cultured in the assay buffer without glucose for 1 h. Then, glucose was used to elicit glycolytic activity (**Figure 4C**). Similar to the OCR analysis, DEHP treatment did not influence any glycolytic functions in undifferentiated K562 cells (**Figure 4D**). Subsequently, we analyzed whether DEHP reprogramed cellular bioenergetics in the course of erythroid differentiation.

K562 cells were treated with either NaB or NaB+DEHP for 24 h, and then subjected to OCR and ECAR assays. Compared to control cells, induction of erythroid differentiation not only increased basal and maximal mitochondrial respiration, but also promoted ATP production (**Figure 5A** and **5B**). In addition, NaB treatment induced the decrease of glycolytic activities (**Figure 5C** and **5D**). These results were consistent with previous findings on the rearrangement of glucose metabolism and energy consumption during erythropoiesis in chicken T2EC cells [19]. However, the addition of DEHP into NaB-treated cells did not further influence cellular bioenergetics, compared to cells treated with NaB only (**Figure 5**). These results indicated that the effects of DEHP on erythroid differ-

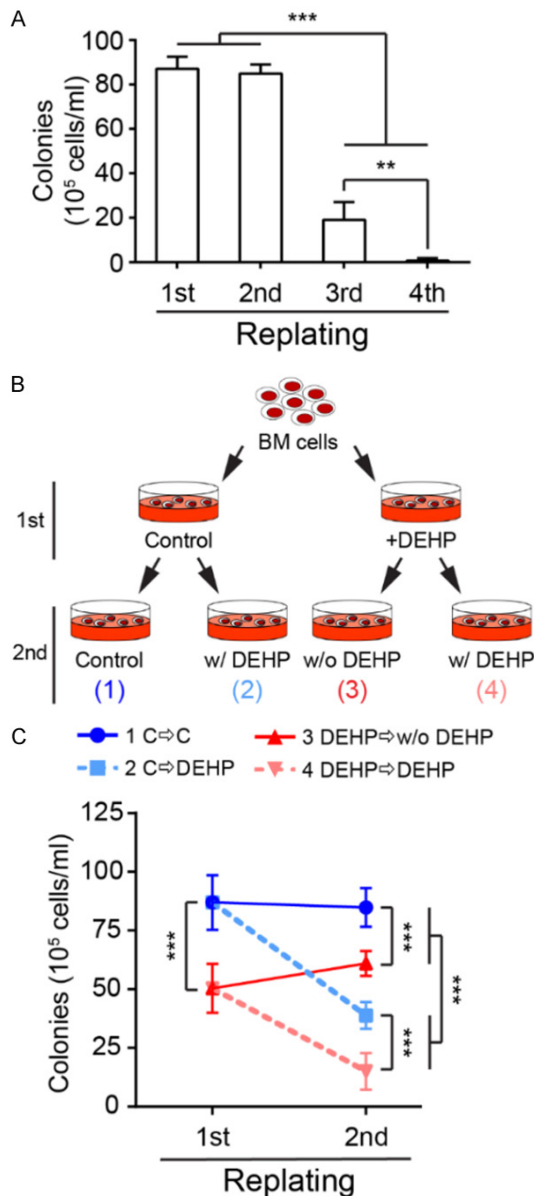

Figure 3. DEHP removal restores the self-renewal capacity of HSPCs. A. BM cells were cultured in methylcellulose-based media with various cytokines to maintain HSPC proliferation and differentiation. After 10 days, the total colony number in the culture was counted under phase-contrast microscopy. Subsequently, 2×10^4 colony cells were re-plated. B. A schematic of the examination of DEHP effects on HSPC function by using re-plating approach. C. BM cells were cultured in methylcellulose-based media with or without DEHP (1 μg/ml), and the results of the first plating and re-plating assay were shown. All experiments were repeated at least three times. The quantified data were presented as means ± s.d. Two-way ANOVA followed by the Tukey's multiple-comparison post-hoc test was used for statistical analysis. $^{**}P<0.01$; $^{***}P<0.001$.

entiation do not occur via modulation of cellular bioenergetics.

DEHP induces Klotho expression which affects erythroid differentiation

Previous studies have demonstrated that loss of *Klotho*, which was initially considered an anti-aging gene, disrupted HSPC homing, and increased erythropoiesis [30]. Here, we found that the addition of DEHP in NaB-treated K562 cells markedly upregulated *KLOTHO* expression, while NaB treatment alone downregulated *KLOTHO* expression (**Figure 6A**). Furthermore, DEHP exposure induced *Klotho* expression in mouse CFU-E cells significantly (**Figure 6B**). To confirm the influence of Klotho in DEHP-mediated suppression of erythropoiesis, the knockdown approach was used. Three human *KLOTHO* shRNAs were expressed transiently in K562 cells for 3 days, and *KLOTHO*-C2 and -E2 shRNAs exhibited better knockdown efficiency (**Figure 6C**). Afterward, *KLOTHO*-C2 and -E2 shRNA-expressed K562 cells were treated with either NaB or NaB+DEHP. Knockdown of *KLOTHO* significantly increased erythroid differentiation, which was consistent with a previous report [30]. Notably, *KLOTHO* knockdown abolished DEHP-mediated suppression of erythroid differentiation (**Figure 6D**). Overall, the results indicated that DEHP downregulated erythroid differentiation via induction of Klotho expression.

Discussion

In the present study, our results demonstrated an unprecedented link between DEHP and Klotho in erythroid differentiation. DEHP seemed to transiently impair HSPC functions and erythroid differentiation. We further revealed that DEHP suppressed erythroid differentiation through the upregulation of Klotho expression in hematopoietic cells, and not via modulation of cellular bioenergetics (**Figure 7**). Our results indicated that DEHP did not interfere with cellular bioenergetics in hematopoietic cells, while numerous studies have demonstrated that DEHP influences mitochondrial function and energy metabolism in other cell types [31-35]. Previous studies have demonstrated that DEHP affects HSPC functions [5, 6]. Our results further explored the fact that

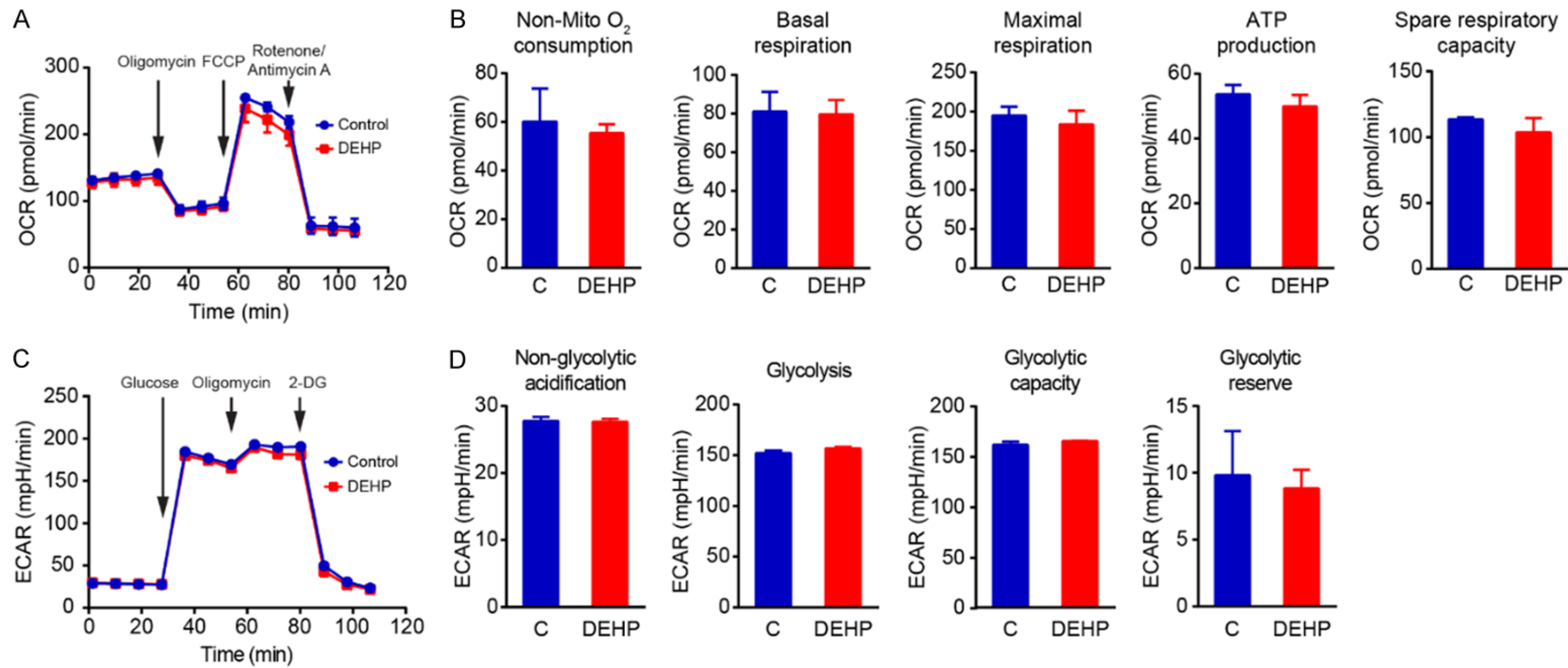

Figure 4. DEHP does not affect mitochondrial respiration and glycolytic function in undifferentiated K562 cells. A. The OCR profile of K562 cells treated with (red line) or without 1 μg/ml of DEHP (blue line). B. The non-mitochondrial oxygen consumption, basal and maximal respiration, ATP production and spare respiration capacity were determined by calculating average values for each phase in the K562 cells. C. The ECAR profile of K562 cells treated with (red line) or without 1 μg/ml of DEHP (blue line). D. Non-glycolytic acidification, glycolysis, glycolytic capacity, and glycolytic reserve were determined by calculating the average values for each phase in K562 cells. All experiments were repeated at least three times. The quantified data were presented as means ± s.d. The Student's *t*-test was used for statistical analysis.

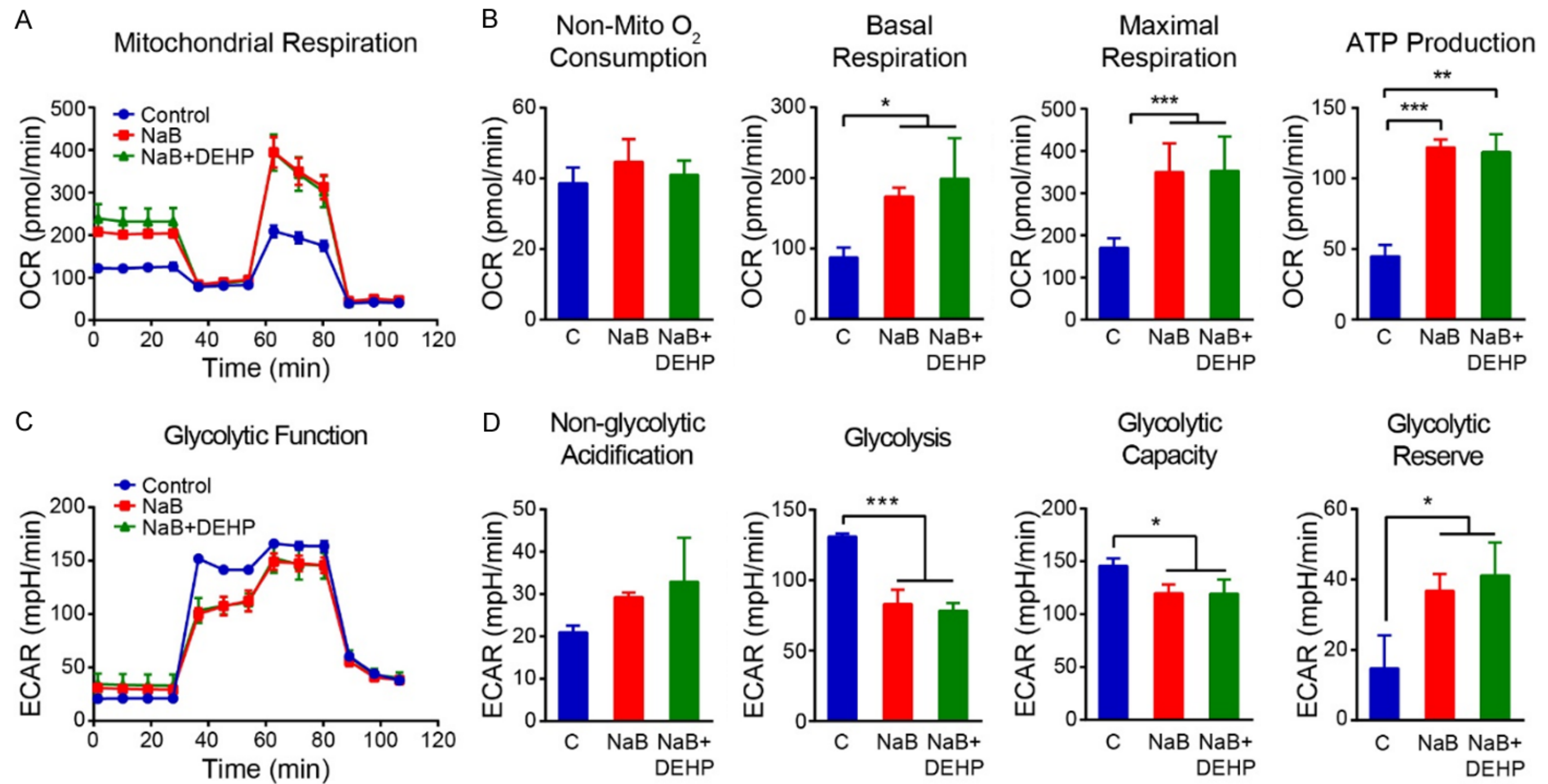

Figure 5. DEHP does not reprogram cellular bioenergetics during erythroid differentiation. K562 cells were treated with or without DEHP (1 μg/ml) in the presence or absence of NaB (2 mM). A. The OCR profile of K562 cells with different treatments. B. Non-mitochondrial oxygen consumption, basal and maximal respiration, and ATP production were determined by calculating average values for each phase in K562 cells. C. The ECAR profile of K562 cells with different treatments. D. Non-glycolytic acidification, glycolysis, glycolytic capacity, and glycolytic reserve were determined by calculating average values for each phase in K562 cells. All experiments were repeated at least three times. The quantified data were presented as means ± s.d. Two-way ANOVA followed by Tukey's multiple-comparison post-hoc test was used for statistical analysis. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

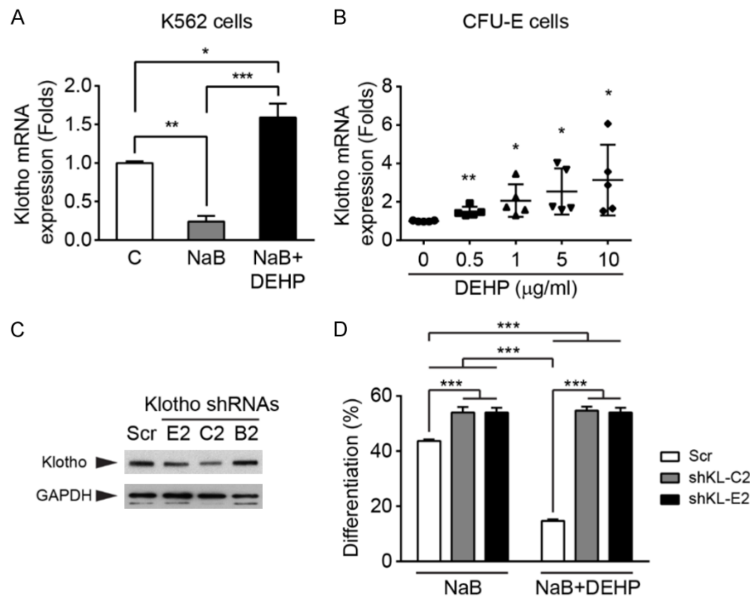

Figure 6. DEHP induces Klotho expression to impair erythroid differentiation. A. K562 cells were treated with or without DEHP (1 μg/ml) in the presence or absence of NaB (2 mM). After 24 h, cells were collected, and then subjected to RNA extraction. Human *KLOTHO* expression was measured using quantitative reverse transcription polymerase chain reaction (RT-qPCR). B. Mouse BM cells were cultured in methylcellulose-based media with EPO for 2 days to generate CFU-E cells under different DEHP doses. The CFU-E cells were collected, and then subjected to RNA extraction. Mouse *Klotho* expression was measured using RT-qPCR. C. Scrambled shRNA (Scr) and three human *KLOTHO* shRNA were transiently expressed in K562 cells. The KLOTHO proteins were detected by using western blot analysis. GAPDH protein levels were used as loading controls. D. The transfected K562 cells were treated with or without DEHP (1 μg/ml) in the presence of NaB (2 mM) for 3 days. The globin protein expression was examined by using benzidine staining. All experiments were repeated at least three times. The quantified data were presented as means ± s.d. Two-way ANOVA followed by Tukey's multiple-comparison post-test was used for statistical analysis. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

DEHP transiently impairs HSPC function and suppresses erythroid differentiation. In addition, DEHP induces cell cycle arrest and increases apoptosis in human H9 embryonic stem cell line via the PPARg/PTEN/AKT pathway [36]. Taken together, this evidence revealed a new health concern about DEHP in stem cell function and viability.

Klotho proteins are required for high-affinity binding of fibroblast growth factors (FGFs) including FGF19, FGF21, and FGF23 to their cognate FGF receptors (FGFR) [37]. While the role of FGF19 in erythropoiesis remains unclear, loss of FGF23 or injection of FGF23 blocking peptides in mice increases EPO expression and erythropoiesis [38, 39]. In contrast, knockdown of FGF21 in zebrafish embryos decreases the expression of key genes that are involved in erythropoiesis, and reduces the production of erythroid cells [40]. Taken together, these results suggest that Klotho might collaborate with FGF23 to participate in DEHP-mediated suppressive effect in erythropoiesis.

Although our results demonstrated that DEHP did not interfere with cellular bioenergetics during erythropoiesis, a recent report indicated that DEHP and other plasticizers, which are used to make plastic bags for whole blood collection, offer different influences in cellular metabolism and ATP production in stored RBCs [41]. RBCs are typically kept in plastic bags for transfusions. During storage, DEHP leaches from the bags, and intercalates into RBC membranes, which results in the reduction of morphological lesions and hemolysis [42], and improvement of RBC survival [43]. In addition, DEHP has an active effect on RBC shape by increasing the flip-flop of lipids between leaflets of plasma membranes, instead of just preventing the storage-related shape changes [44]. This RBC membrane stabilization is usually associated with vesiculation, osmotic stress, and Hgb loss. DEHP and other plasticizers exhibit different effects in membrane stabilization [45]. The RBC vesiculation is associated with the shedding of extracellular vesicles (EVs) from RBC surface. EVs loaded with proteins, lipids, and microRNAs might be vital for cell-cell communication. RBCs release EVs during erythropoiesis, aging and disease conditions. Current evidences indicate that these RBC-secreted EVs could regulate NO and O_2 homeostasis, redox balance and immunomodulation. The well-known RBC-EV-driven disease sequelae are its procoagulant effects [46]. Due to how DEHP influences RBC membrane stabilization when RBCs are stored for transfusion, whether DEHP affects EV biogenesis under blood banking conditions and the consequen-

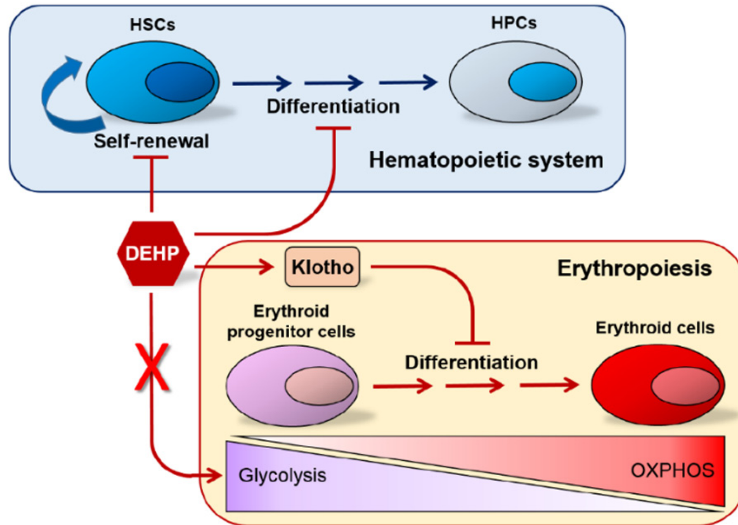

Figure 7. Schematic illustration of erythroid differentiation impaired by DEHP via modulating Klotho expression and not via reprogramming cellular bioenergetics.

tial outcomes after transfusion are another health issue.

Conclusions

DEHP, the most common phthalate, leaches into the environment or directly into human tissue easily. Previous studies have demonstrated that DEHP interferes with some HSPC functions. In the present study, we demonstrated that exposure to DEHP transiently impairs HSPC function and suppresses erythroid differentiation. We also demonstrated that DEHP downregulates erythroid differentiation through upregulation of Klotho expression in hematopoietic cells and not via modulation of cellular bioenergetics. Consequently, the present study provides a novel link between DEHP and Klotho in erythroid differentiation. In addition, our results could facilitate the assessment of novel DEHP-mediated effects in the environment and in human tissues.

Acknowledgements

We are grateful to Dr. Tzong-Shyuan Lee (National Taiwan University, Taiwan) for providing DEHP. This research was supported by funding from Ministry of Science and Technology (MOST 105-2628-B-010-010-MY3 and MOST 108-2320-B-010-043-MY3) (Y.I.C.), a grant from Yen Tjing Ling Medical Foundation (CI-109-6) (Y.I.C.). This work was particularly supported by "Yin Yen-Liang Foundation Development and Construction Plan" of the School of Medicine, National Yang Ming Chiao Tung University (Y.I.C.). We thank the Instrumentation Research Center of National Yang Ming Chiao Tung University for use of the shared services to complete this research. We also thank the National RNAi Core Facility at Academia Sinica in Taiwan for providing shRNA reagents and related services.

Disclosure of conflict of interest

None.

Address correspondence to: Yuan-I Chang, Department and Institute of Physiology, National Yang Ming Chiao Tung University (Taipei Yang Ming Campus), No. 155, Sec. 2, Linong Street, Beitou District, Taipei 112304, Taiwan. Tel: +886-2-2826-7312; Fax: +886-2-2826-4049; E-mail: ychang@nycu.edu.tw

References

[1] Serrano SE, Braun J, Trasande L, Dills R and Sathyanarayana S. Phthalates and diet: a review of the food monitoring and epidemiology data. Environ Health 2014; 13: 43.
[2] Buckley JP, Kim H, Wong E and Rebholz CM. Ultra-processed food consumption and exposure to phthalates and bisphenols in the US national health and nutrition examination survey, 2013-2014. Environ Int 2019; 131: 105057.
[3] Yang O, Kim HL, Weon JI and Seo YR. Endocrine-disrupting chemicals: review of toxicological mechanisms using molecular pathway analysis. J Cancer Prev 2015; 20: 12-24.
[4] Rowdhwal SSS and Chen J. Toxic effects of di-2-ethylhexyl phthalate: an overview. Biomed Res Int 2018; 2018: 1750368.
[5] Gutierrez-Garcia AK, Flores-Kelly JM, Ortiz-Rodriguez T, Kalixto-Sanchez MA and De Leon-Rodriguez A. Phthalates affect the in vitro expansion of human hematopoietic stem cell. Cytotechnology 2019; 71: 553-561.
[6] Manz P, Cadeddu RP, Wilk M, Fischer JC, Fritz B, Haas R and Wenzel F. Influence of di(2-ethylhexyl)phthalate on migration rate and differentiation of human hematopoietic stem and progenitor cells (CD34+). Clin Hemorheol Microcirc 2015; 61: 111-118.

[7] David RM, Moore MR, Finney DC and Guest D. Chronic toxicity of di(2-ethylhexyl)phthalate in rats. Toxicol Sci 2000; 55: 433-443.

[8] David RM, Moore MR, Finney DC and Guest D. Chronic toxicity of di(2-ethylhexyl)phthalate in mice. Toxicol Sci 2000; 58: 377-385.

[9] Pugh G Jr, Isenberg JS, Kamendulis LM, Ackley DC, Clare LJ, Brown R, Lington AW, Smith JH and Klaunig JE. Effects of di-isononyl phthalate, di-2-ethylhexyl phthalate, and clofibrate in cynomolgus monkeys. Toxicol Sci 2000; 56: 181-188.

[10] Desvergne B, Feige JN and Casals-Casas C. PPAR-mediated activity of phthalates: a link to the obesity epidemic? Mol Cell Endocrinol 2009; 304: 43-48.

[11] Rajesh P and Balasubramanian K. Phthalate exposure in utero causes epigenetic changes and impairs insulin signalling. J Endocrinol 2014; 223: 47-66.

[12] Mushtaq M, Srivastava SP and Seth PK. Effect of di-2-ethylhexyl phthalate (DEHP) on glycogen metabolism in rat liver. Toxicology 1980; 16: 153-161.

[13] Martinelli MI, Mocchiutti NO and Bernal CA. Dietary di(2-ethylhexyl)phthalate-impaired glucose metabolism in experimental animals. Hum Exp Toxicol 2006; 25: 531-538.

[14] Xu J, Zhou L, Wang S, Zhu J, Liu T, Jia Y, Sun D, Chen H, Wang Q, Xu F, Zhang Y, Liu H, Zhang T and Ye L. Di-(2-ethylhexyl)-phthalate induces glucose metabolic disorder in adolescent rats. Environ Sci Pollut Res Int 2018; 25: 3596-3607.

[15] Vannini N, Girotra M, Naveiras O, Nikitin G, Campos V, Giger S, Roch A, Auwerx J and Lutolf MP. Specification of haematopoietic stem cell fate via modulation of mitochondrial activity. Nat Commun 2016; 7: 13125.

[16] Suda T, Takubo K and Semenza GL. Metabolic regulation of hematopoietic stem cells in the hypoxic niche. Cell Stem Cell 2011; 9: 298-310.

[17] Takubo K, Nagamatsu G, Kobayashi CI, Nakamura-Ishizu A, Kobayashi H, Ikeda E, Goda N, Rahimi Y, Johnson RS, Soga T, Hirao A, Suematsu M and Suda T. Regulation of glycolysis by Pdk functions as a metabolic checkpoint for cell cycle quiescence in hematopoietic stem cells. Cell Stem Cell 2013; 12: 49-61.

[18] Papa L, Djedaini M and Hoffman R. Mitochondrial role in stemness and differentiation of hematopoietic stem cells. Stem Cells Int 2019; 2019: 4067162.

[19] Richard A, Vallin E, Romestaing C, Roussel D, Gandrillon O and Gonin-Giraud S. Erythroid differentiation displays a peak of energy consumption concomitant with glycolytic metabolism rearrangements. PLoS One 2019; 14: e0221472.

[20] Hua WK, Chang YI, Yao CL, Hwang SM, Chang CY and Lin WJ. Protein arginine methyltransferase 1 interacts with and activates p38alpha to facilitate erythroid differentiation. PLoS One 2013; 8: e56715.

[21] Zhao JF, Hsiao SH, Hsu MH, Pao KC, Kou YR, Shyue SK and Lee TS. Di-(2-ethylhexyl) phthalate accelerates atherosclerosis in apolipoprotein E-deficient mice. Arch Toxicol 2016; 90: 181-190.

[22] Lee KI, Chiang CW, Lin HC, Zhao JF, Li CT, Shyue SK and Lee TS. Maternal exposure to di-(2-ethylhexyl) phthalate exposure deregulates blood pressure, adiposity, cholesterol metabolism and social interaction in mouse offspring. Arch Toxicol 2016; 90: 1211-1224.

[23] Chang YI, Kong G, Ranheim EA, Tu PS, Yu YS and Zhang J. Dnmt3a haploinsufficiency cooperates with oncogenic Kras to promote an early-onset T-cell acute lymphoblastic leukemia. Am J Transl Res 2017; 9: 1326-1334.

[24] Chang YI, You X, Kong G, Ranheim EA, Wang J, Du J, Liu Y, Zhou Y, Ryu MJ and Zhang J. Loss of Dnmt3a and endogenous Kras cooperate to regulate hematopoietic stem and progenitor cell functions in leukemogenesis. Leukemia 2015; 29: 1847-1856.

[25] Mettang T, Thomas S, Kiefer T, Fischer FP, Kuhlmann U, Wodarz R and Rettenmeier AW. Uraemic pruritus and exposure to di(2-ethylhexyl) phthalate (DEHP) in haemodialysis patients. Nephrol Dial Transplant 1996; 11: 2439-2443.

[26] Faouzi MA, Dine T, Gressier B, Kambia K, Luyckx M, Pagniez D, Brunet C, Cazin M, Belabed A and Cazin JC. Exposure of hemodialysis patients to di-2-ethylhexyl phthalate. Int J Pharm 1999; 180: 113-121.

[27] Grover A, Mancini E, Moore S, Mead AJ, Atkinson D, Rasmussen KD, O'Carroll D, Jacobsen SE and Nerlov C. Erythropoietin guides multipotent hematopoietic progenitor cells toward an erythroid fate. J Exp Med 2014; 211: 181-188.

[28] Wojchowski DM, Menon MP, Sathyanarayana P, Fang J, Karur V, Houde E, Kapelle W and Bogachev O. Erythropoietin-dependent erythropoiesis: new insights and questions. Blood Cells Mol Dis 2006; 36: 232-238.

[29] Lodish H, Flygare J and Chou S. From stem cell to erythroblast: regulation of red cell production at multiple levels by multiple hormones. IUBMB Life 2010; 62: 492-496.

[30] Vadakke Madathil S, Coe LM, Casu C and Sitara D. Klotho deficiency disrupts hematopoietic stem cell development and erythropoiesis. Am J Pathol 2014; 184: 827-841.

[31] Chiang HC, Wang CH, Yeh SC, Lin YH, Kuo YT, Liao CW, Tsai FY, Lin WY, Chuang WH and Tsou TC. Comparative microarray analyses of

mono(2-ethylhexyl)phthalate impacts on fat cell bioenergetics and adipokine network. Cell Biol Toxicol 2017; 33: 511-526.
[32] Inouye B, Ogino Y, Ishida T, Ogata M and Utsumi K. Effects of phthalate esters on mitochondrial oxidative phosphorylation in the rat. Toxicol Appl Pharmacol 1978; 43: 189-198.
[33] Kora S, Sado M and Terada H. Effect of the plasticizer di-(2-ethylhexyl)phthalate on oxidative phosphorylation in rat liver mitochondria: modification of the function of the adenine nucleotide translocator. J Pharmacobiodyn 1988; 11: 773-778.
[34] Dales RE, Kauri LM and Cakmak S. The associations between phthalate exposure and insulin resistance, beta-cell function and blood glucose control in a population-based sample. Sci Total Environ 2018; 612: 1287-1292.
[35] Kataria A, Levine D, Wertenteil S, Vento S, Xue J, Rajendiran K, Kannan K, Thurman JM, Morrison D, Brody R, Urbina E, Attina T, Trasande L and Trachtman H. Exposure to bisphenols and phthalates and association with oxidant stress, insulin resistance, and endothelial dysfunction in children. Pediatr Res 2017; 81: 857-864.
[36] Fang H, Fang W, Cao H, Luo S, Cong J, Liu S, Pan F and Jia X. Di-(2-ethylhexyl)-phthalate induces apoptosis via the PPARγ/PTEN/AKT pathway in differentiated human embryonic stem cells. Food Chem Toxicol 2019; 131: 110552.
[37] Kuro OM. The Klotho proteins in health and disease. Nat Rev Nephrol 2019; 15: 27-44.
[38] Coe LM, Madathil SV, Casu C, Lanske B, Rivella S and Sitara D. FGF-23 is a negative regulator of prenatal and postnatal erythropoiesis. J Biol Chem 2014; 289: 9795-9810.
[39] Agoro R, Montagna A, Goetz R, Aligbe O, Singh G, Coe LM, Mohammadi M, Rivella S and Sitara D. Inhibition of fibroblast growth factor 23 (FGF23) signaling rescues renal anemia. FASEB J 2018; 32: 3752-3764.
[40] Yamauchi H, Hotta Y, Konishi M, Miyake A, Kawahara A and Itoh N. Fgf21 is essential for haematopoiesis in zebrafish. EMBO Rep 2006; 7: 649-654.
[41] Serrano K, Levin E, Chen D, Hansen A, Turner TR, Kurach J, Reidel A, Boecker WF, Acker JP and Devine DV. An investigation of red blood cell concentrate quality during storage in paediatric-sized polyvinylchloride bags plasticized with alternatives to di-2-ethylhexyl phthalate (DEHP). Vox Sang 2016; 110: 227-235.
[42] AuBuchon JP, Estep TN and Davey RJ. The effect of the plasticizer di-2-ethylhexyl phthalate on the survival of stored RBCs. Blood 1988; 71: 448-452.
[43] Myhre BA, Johnson D, Marcus CS, Demaniew S, Carmen R and Nelson E. Survival of red cells stored for 21 and 35 days in a non-di-(2-ethylhexyl)phthalate plastic container. Vox Sang 1987; 53: 199-202.
[44] Melzak KA, Uhlig S, Kirschhöfer F, Brenner-Weiss G and Bieback K. The blood bag plasticizer di-2-ethylhexylphthalate causes red blood cells to form stomatocytes, possibly by Inducing lipid flip-flop. Transfus Med Hemother 2018; 45: 413-422.
[45] Bicalho B, Serrano K, Dos Santos Pereira A, Devine DV and Acker JP. Blood bag plasticizers influence red blood cell vesiculation rate without altering the lipid composition of the vesicles. Transfus Med Hemother 2016; 43: 19-26.
[46] Thangaraju K, Neerukonda SN, Katneni U and Buehler PW. Extracellular vesicles from red blood cells and their evolving roles in health, coagulopathy and therapy. Int J Mol Sci 2020; 22: 153.

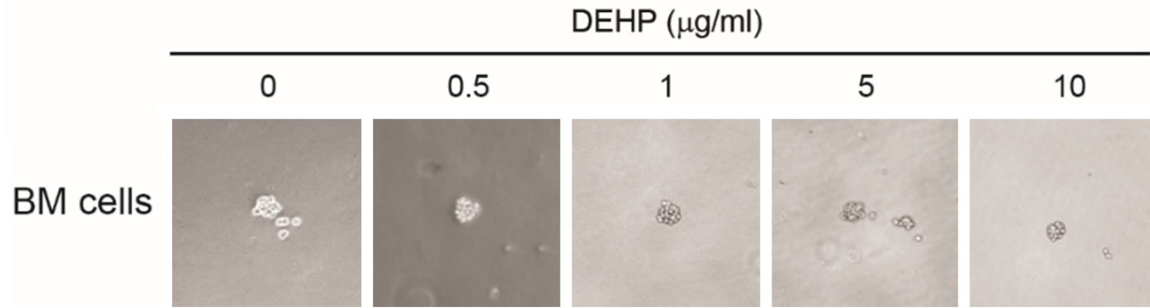

Figure S1. The morphology of BM-derived CFU-Es with different dosages of DEHP. 2×10^4 BM cells were plated in MethoCult™ M3334 methylcellulose medium for 2 days. The morphology of CFU-E colonies was taken by using phase-contrast microscope.

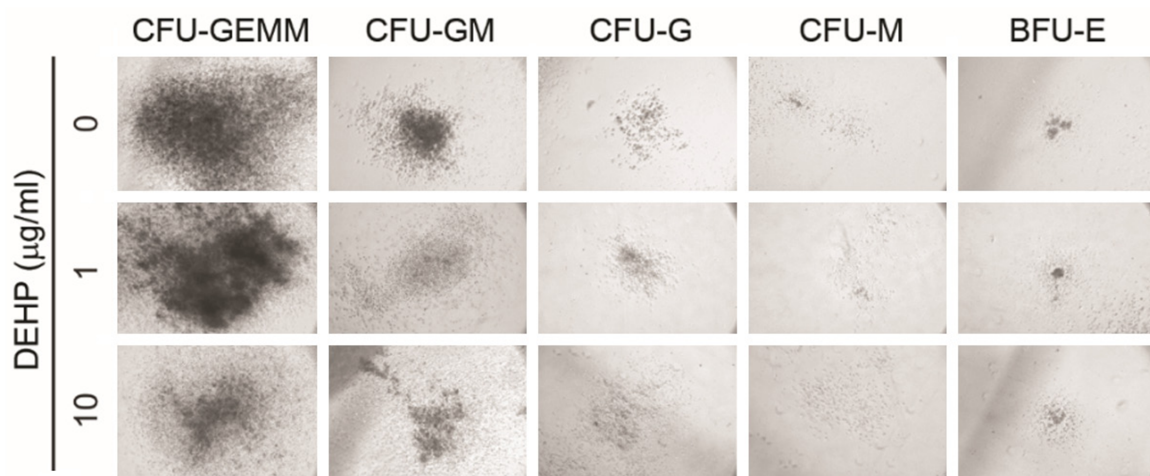

Figure S2. The morphology of BM-derived CFU-GEMM, -GM, -G, -M and BFU-E in different dosages of DEHP. 2×10^4 BM cells were plated in MethoCult™ M3434 methylcellulose medium for 10 days. The morphology of different types of colonies was taken by using phase-contrast microscope.