Di(2-ethylhexyl)phthalate Impairs Erythropoiesis via Inducing Klotho Expression and not via Bioenergetic Reprogramming

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Research

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Abstract

Background: Di(2-ethylhexyl)phthalate (DEHP) is one of the phthalates most widely used 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 modulate the function of haematopoietic stem and progenitor cell (HSPC) to influence erythropoiesis, and to explore the associated mechanism.

Results: 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 interferes with erythroid differentiation, we focused on energy metabolism and *Klotho* expression. DEHP suppressed erythroid differentiation via upregulation of *Klotho* expression in erythroblasts and not via cellular bioenergetic modulation.

Conclusion: Here, we have demonstrated a novel pathophysiological role of DEHP in erythroid differentiation. DEHP impaired erythroid differentiation by inducing *Klotho* expression and not by reprogramming cellular bioenergetic profiles during differentiation.

Background

Di(2-ethylhexyl)phthalate (DEHP) is one of the phthalates most frequently used as a plasticiser in plastic products such as personal care products and medical devices. It is non-covalently bound to the polymer; hence, DEHP easily leaches, migrates, or evaporates into the atmosphere, foods, or directly into body fluids [1–3]. Due to its ubiquity in the environment, concerns have been raised about the potential health impacts of continuous exposure to DEHP exposure. To date, the adverse effects of DEHP in the liver, the endocrine and reproductive systems, as well as in obesity and some types of cancers have been explored extensively [3, 4]. However, further investigations are required to determine if DEHP exposure influences other physiological processes. According to a recent study, DEHP affects the function of human haematopoietic stem and progenitor cells (HSPCs) 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 in rodents caused chronic progressive nephropathy and significantly reduced haemoglobin (Hgb) production [7, 8]. However, DEHP had no effect in male cynomolgus monkeys treated with 500 mg/kg/day DEHP for 14 days [9]. It remains unclear whether DEHP exposure adversely affects erythropoiesis until now.

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 haematopoietic systems, long-term haematopoietic stem cells (HSC), which are at the top of the haematopoietic 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 glycolytic metabolism rearrangement when they differentiate into mature erythroid cells [19]. In the present study, we investigated whether DEHP influences erythroid differentiation through the modulation of glycolytic metabolism and mitochondrial respiration. In addition, we investigated potential DEHP-mediated molecular mechanisms.

Results

DEHP suppresses erythropoiesis

To rapidly test whether DEHP influenced erythropoiesis, we used human chronic myelogenous leukaemia K562 and erythroleukemia HEL 92.1.7 (HEL) cell lines. The erythroid and megakaryocytic differentiation potentials of the two cell lines were comparable to those of human $CD34^+$ HSPCs [20, 21]. Sodium butyrate (NaB) was used to induce erythroid differentiation in both cell lines [21]. The addition of DEHP in the culture medium did not affect K562 cell proliferation (Fig. 1A) and survival (Fig. 1B). Based on previous reports on DEHP plasma concentrations in chronic kidney disease (CKD) patients undergoing haemodialysis [22, 23], 1 μg/ml of DEHP was selected for application in the 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 was resulted from increasing cell numbers, in both cell lines (Fig. 1C). Erythropoietin (EPO) is the primary cytokine that regulates erythropoiesis [24, 25]. An increase in DEHP concentration gradually and significantly suppressed the formation of EPO-induced colony-forming unit-erythroids (CFU-Es) in BM (Fig. 1D) and SP cells (Fig. 1E). CFU-Es are differentiated form the earlier erythroid progenitor cells, burst-forming unit-erythroids (BFU-Es) [26]. DEHP also suppressed BFU-E formation (Fig. 1F). The 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 removal of DEHP restores HSPC self-renewal activity

To examine the effect of DEHP in the proliferation and differentiation of HSPCs, BM and SP cells were cultured in a methylcellulose-based medium. Consequently, HSPCs proliferate and differentiate to form discrete colonies, including the oligopotential progenitor CFU-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). DEHP decreased the formation of CFU-GEMM, CFU-GM, CFU-G and CFU-M in both BM and SP cells in a dose-dependent manner (Fig. 2). The results clearly indicated that DEHP influenced HSPC differentiation.

HSPC self-renewal can be examined by re-plating cells in methylcellulose-based medium. HSPCs retained a similar capacity to generate haematopoietic cells in the second plating, while the potential decreased gradually in the third and fourth iterations (Fig. 3A), which is consistent with our previous observations [27]. The experimental design used to examine whether DEHP removal restored HSPC potential is presented in Fig. 3B. In the first plating, DEHP inhibited total colony number growth significantly. Subsequently, a cell number similar to the number of the first-generation colony cells (2×10^4 cells) was re-plated with or without DEHPs (1 μg/ml). Like normal HSPCs in BM cells retained similar ability to generate haematopoietic cells in the first and second plating, removal of DEHP in the second plating keep similar self-renewal capability. However, continuous exposure to DEHP significantly decreased HSPC function (Fig. 3C). Overall, the results suggested that DEHP interfered transiently with HSPC functions.

DEHP did 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]. Mammalian cells generate ATP by mitochondrial (oxidative phosphorylation) and non-mitochondrial (glycolysis) metabolism. Cellular oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) are key indicators of mitochondrial respiration and glycolysis, respectively. The indices could provide a systemic perspective of cellular metabolic function in live cells and can be evaluated using a Seahorse Extracellular Flux Analyzer [28, 29]. Here, we firstly 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. OCR is measured before and after the addition of various inhibitors to derive several mitochondrial respiration parameters. The cellular OCR baseline is measured before the addition of oligomycin, a complex V inhibitor. The ATP-linked respiration can be evaluated following treatment with oligomycin. Subsequently, FCCP, a protonophore, is added to collapse the inner membrane gradient, allowing the electron transport chain (ETC) to function at its maximum potential, and then maximal respiratory capacity is measured. Finally, antimycin A and rotenone, inhibitors of complex III and I, are added to shut down ETC function and reveal the non-mitochondrial respiration. Mitochondrial spare respiratory capacity is calculated by subtracting basal respiration from maximal respiratory capacity (Fig. 4A). DEHP treatment did not influence mitochondrial respiration parameters in undifferentiated K562 cells (Fig. 4B and 4C).

For the ECAR assay, cells were cultured in the assay buffer without glucose for 1 h. Glucose is used to elicit glycolytic activity. Afterward, oligomycin injection blocks mitochondrial respiration to allow glycolysis to function at its maximum rate. Finally, the addition of 2-DG, a glucose analogue, inhibits glycolysis, and, therefore, provides a baseline ECAR measurement. Glycolytic reserve capacity is

calculated by subtracting basal ECAR from maximal ECAR (Fig. 4D). Similar to the OCR analysis case, DEHP treatment did not influence any glycolysis parameter in undifferentiated K562 cells (Fig. 4E and 4F). Subsequently, we analysed whether DEHP reprogramed cellular bioenergetics in the course of erythroid differentiation.

K562 cells were treated with NaB with or without DEHP (1 μg/ml) for 24 h, and then subjected to OCR and ECAR assays. Induction of erythroid differentiation increased basal and maximal mitochondrial respiration dramatically, in addition to ATP production (Fig. 5A and 5B). In addition, erythroid differentiation induction decreased glycolytic activity significantly (Fig. 5C and 5D). The 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 differentiated cells did not influence cellular bioenergetics further (Fig. 5). The results indicated that the effects of DEHP on erythroid differentiation are not via modulating cellular bioenergetics.

DEHP induces *Klotho* expression, which affects erythroid differentiation

Previous studies have demonstrated that loss of *Klotho*, which was initially considered an anti-ageing gene, disrupted HSPC homing, and increased erythropoiesis [30]. Therefore, *Klotho* expression was analysed in K562 cells under different treatments. Compared to the wild-type (WT) K562 cells (Control, C), the expression of *Klotho* was decreased in NaB-treated K562 cells. DEHP addition in NaB-treated K562 cells induced *Klotho* expression markedly (Fig. 6A). The association between DEHP and *Klotho* expression was further examined in CFU-E cells. DEHP exposure induced *Klotho* expression in CFU-E cells significantly (Fig. 6B). To confirm the influence of Klotho in DEHP-mediated suppression of erythropoiesis, the knockdown approach was used. Three *Klotho* short-hair (shRNA) were expressed transiently in K562 cells for 3 days, and then endogenous Klotho protein expression was analysed using Western blot analysis. The C2 and E2 shRNAs exhibited better knockdown efficiency (Fig. 6C). Afterward, C2 and E2 shRNA-expressed K562 cells were treated with NaB with or without DEHP. Knockdown of *Klotho* increased erythroid differentiation significantly, which was consistent with the findings of a previous report [30]. Notably, *Klotho* knockdown abolished DEHP-mediated suppression of erythroid differentiation (Fig. 6D). Overall, the results indicated that DEHP downregulated erythroid differentiation via *Klotho* expression induction.

Discussion

In the present study, our results demonstrated an unprecedented link between DEHP and *Klotho* in erythroid differentiation. DEHP seemed to transiently impair HSPC function and erythroid differentiation. We further revealed that DEHP suppressed erythroid differentiation through the upregulation of *Klotho* expression in haematopoietic cells and not via modulation of cellular bioenergetics (Fig. 7). Although our results indicated that DEHP does not interfere with cellular bioenergetics in haematopoietic cells,

numerous studies have demonstrated that DEHP influences mitochondrial function, energy metabolism, and insulin resistance in other cell types [31, 32, 33, 34, 35].

Klotho proteins, Klotho and β-Klotho, are required for high-affinity binding of fibroblast growth factors (FGFs) including FGF19, FGF21, and FGF23 to their cognate FGF receptors (FGFR) [36]. In the present study, we focused on Klotho, because *β-Klotho* is expressed abundantly in metabolic tissues [37]. *Klotho*$^{-/-}$ mice developed multiple phenotypes observed commonly in naturally aged WT mice in an accelerated manner, such as decreased spontaneous activity, hypogonadism, impaired bone mineralisation, thymic involution, B lymphocytopenia [38]. In contrast, *β-Klotho* loss protected against obesity by increasing energy expenditure, which is due to crosstalk among liver, microbiota, and brown adipose tissue [39]. Loss of *Klotho* not only interfered with HSC function but also impaired erythropoiesis [30]. Our results further demonstrated the critical role of Klotho in DEHP-mediated suppression of erythropoiesis.

In patients with CKD, increased DEHP concentrations were observed when they underwent haemodialysis [22, 23, 40]. Anaemia is mainly due to impaired EPO production in the kidney [41], and is highly associated with morbidity and mortality in CKD patients [41]. EPO is the primary cytokine regulating erythropoiesis; therefore, anaemia correction using recombinant human EPO or erythropoiesis-stimulating agents (ESAs) markedly improves the quality of life of CKD patients [42, 43]. Nevertheless, other factors could interfere with responses to EPO or ESAs [43, 44]. Unfortunately, some CKD patients still exhibit poor responses to EPO or ESAs, even when the therapies are combined with other therapeutic strategies to minimise inflammation, improve vitamin D status, and decrease hyperparathyroidism [44]. Conversely, increasing evidence indicates that Klotho, which is mainly expressed in the kidney, is correlated significantly with the development and progression of CKD and its complications [36, 45]. Here, our results suggest that DEHP is an uncharacterised factor involved in the progression of anaemia, or in the development of peripheral resistance or decreased response to EPO or ESAs in CKD patients. However, further studies are required to validate the findings above.

Conclusion

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 erythroid differentiation. We also demonstrated that DEHP suppresses erythroid differentiation through the upregulation of *Klotho* expression in haematopoietic 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.

Methods

Materials

DEHP (Acros Organics) was a kind gift from Dr. Tzong-Shyuan Lee (National Taiwan University, Taiwan), and was prepared using dimethylsulfoxide (DMSO) as the solvent at a concentration of 1 or 10 mg/ml [46, 47]. The reference sequence for *Klotho* shRNA design is NM 004795. The scramble shRNA (ASN0000000004) and *Klotho* shRNAs, including A2 (TRCN0000161418), D2 (TRCN0000419459), and E2 (TRCN0000433013), were obtained from National RNAi Core Facility (Academia Sinica, Taipei City, Taiwan). All plasmids were verified by DNA sequencing, which was carried out by Genomics (New Taipei City, Taiwan).

Cell culture, Differentiation, and Transfection

Human K562 and HEL cells were obtained from the Bioresource Collection and Research, Hsinchu City, Taiwan. K562 cells (4×10^4 cells/ml) were cultivated in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% foetal bovine serum (FBS), 100 IU/ml penicillin, and 100 IU/ml streptomycin, and were passaged every two days. HEL cells (1×10^5 cells/ml) were cultivated in Roswell Park Memorial Institute 1640 medium supplemented with 10% FBS, 1 mM sodium pyruvate, 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 NaB for K562 cells and at 0.1 mM NaB for HEL cells. Benzidine staining was performed to examine Hgb expression [21]. Transfection was performed using Lipofectamine™ 2000 reagent (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. The K562 cells, which were used to express *Klotho* shRNAs, were cultured with 0.5 μg/ml puromycin for at least 12 days.

Mice and cell isolations

Pure C57BL/6 mice were purchased from the Laboratory Animal Center of National Yang-Ming University. BM cells were isolated carefully from tibia and femur. For SP cell preparation, a small piece of excised spleen was pressed through the strainer using the plunger end of a syringe. Subsequently, BM and SP cells were washed through the strainer and collected [27, 48].

Colony forming assay

HSPCs can differentiate into different types of colony-forming unit cells. The methylcellulose-based media MethoCult™ M3334 and M3434 were obtained from StemCell Technologies (Vancouver, Canada). Colony forming assays were performed as previously described [27]. To detect BFU-E and other types of colonies (CFU-G, CFU-M, CFU-GM, and CFU-GEMM), 2×10^4 BM cells or 1×10^5 SP cells were plated in duplicate 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 duplicate in MethoCult™ GF M3434 methylcellulose medium. After 10 days, 2×10^4 colony cells were re-plated. To detect CFU-E colonies, 2×10^4 BM cells or 1×10^5 SP cells were plated in duplicate in MethoCult™ M3334 methylcellulose medium according to the manufacturer's protocol. Colonies were counted after 2 days of culture.

Analysis of OCR and ECAR

OCR and ECAR were measured using a Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA). Firstly, the Seahorse XF cell culture microplates were coated with 3.5 μg/cm^2 of Corning Cell-Tak™ adhesive reagent (Corning, NY, USA). For OCR, the constituents were prepared in sodium bicarbonate- and HEPES-free IMDM (Gibco™, Thermo Fisher Scientific) with 2% FBS (Gibco™, Thermo Fisher Scientific), 100 IU/ml penicillin (Sigma-Aldrich), and 100 IU/ml streptomycin (Sigma-Aldrich) (non-buffered IMDM). Ninety-thousand K562 cells were resuspended in 100 μl non-buffered IMDM for one well of the Seahorse XF cell culture microplates, transferred into microplates, and then incubated at room temperature for 30 min. Subsequently, 575 μl of non-buffered IMDM was added in each well. Oligomycin, FCCP, rotenone, antimycin, glucose, and 2-DG were obtained from Seahorse Bioscience (North Billerica, MA, US). To measure OCR, oligomycin and FCCP were diluted to 1 μM, and otenone and antimycin were diluted to 0.5 μM. For ECAR, 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 profile.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA). Data are presented as means ± s.d. All experiments were performed at least three times. We compared the result 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 significant at $P < 0.05$.

Abbreviations

2-DG: 2-deoxyglucose; ATP: adenosine triphosphate; ANOVA: Analysis of variance; BFU-E: Burst-forming unit erythroid; BM: Bone marrow; CFU-E: Colony forming unit erythroid; CFU-GEMM: Colony forming unit-granulocyte, erythrocyte, monocyte/macrophage, and megakaryocyte; CFU-GM: Colony forming unit-granulocyte and monocyte/macrophage; CFU-G: Colony forming unit-granulocyte; CFU-M: Colony forming unit-monocyte/macrophage; CKD: Chronic kidney disease; ; DEHP: Di(2-ethylhexyl)phthalate; DMSO: Dimethylsulfoxide; ECAR: Extracellular acidification rate; EPO: Erythropoietin; ESA: Erythropoiesis-stimulating agent; ETC: electron transport chain; FBS: Foetal bovine serum; FCCP: Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone; Fibroblast growth factor (FGF); Hgb: Haemoglobin; HSC: Haematopoietic stem cells; HSPC: Haematopoietic stem and progenitor cell; IACUC: Institutional Animal Care and Use Committee; IMDM: Iscove's Modified Dulbecco's Medium; NaB: Sodium butyrate; OCR: Oxygen consumption rate; OXPHOS: Oxidative phosphorylation; PPAR: Peroxisome proliferator-activated receptor; Short-hair RNA: shRNA; SP: Splenocytes; SCF: Stem cell factor; WT: Wild-type.

Declarations

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Author's contributions

CYT performed the experiments about DEHP in survival and differentiation in cell lines and identifying molecular mechanism, data analysis, and manuscript writing. TPF performed the analysis of OCR and ECAR and the identification of molecular mechanism, data analysis, and manuscript writing. YIC performed colony forming assay. SWC, HWC, ECYL and TAL participated in parts of experiments and supervised the animal harvest and care. TAL, DCT and YIC conceived and designed the study. YIC supervised the project, edited the manuscript and gave approval to its final version. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All animal experiments were conducted in accordance with Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee (IACUC) of National Yang-Ming University (IACUC number: 1081012). No human data were used for this study, and thus consent was not required.

Consent for publication

No human data were used for this study, and thus consent was not required.

Competing interests

The authors declare that they have no competing interests.

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Figures

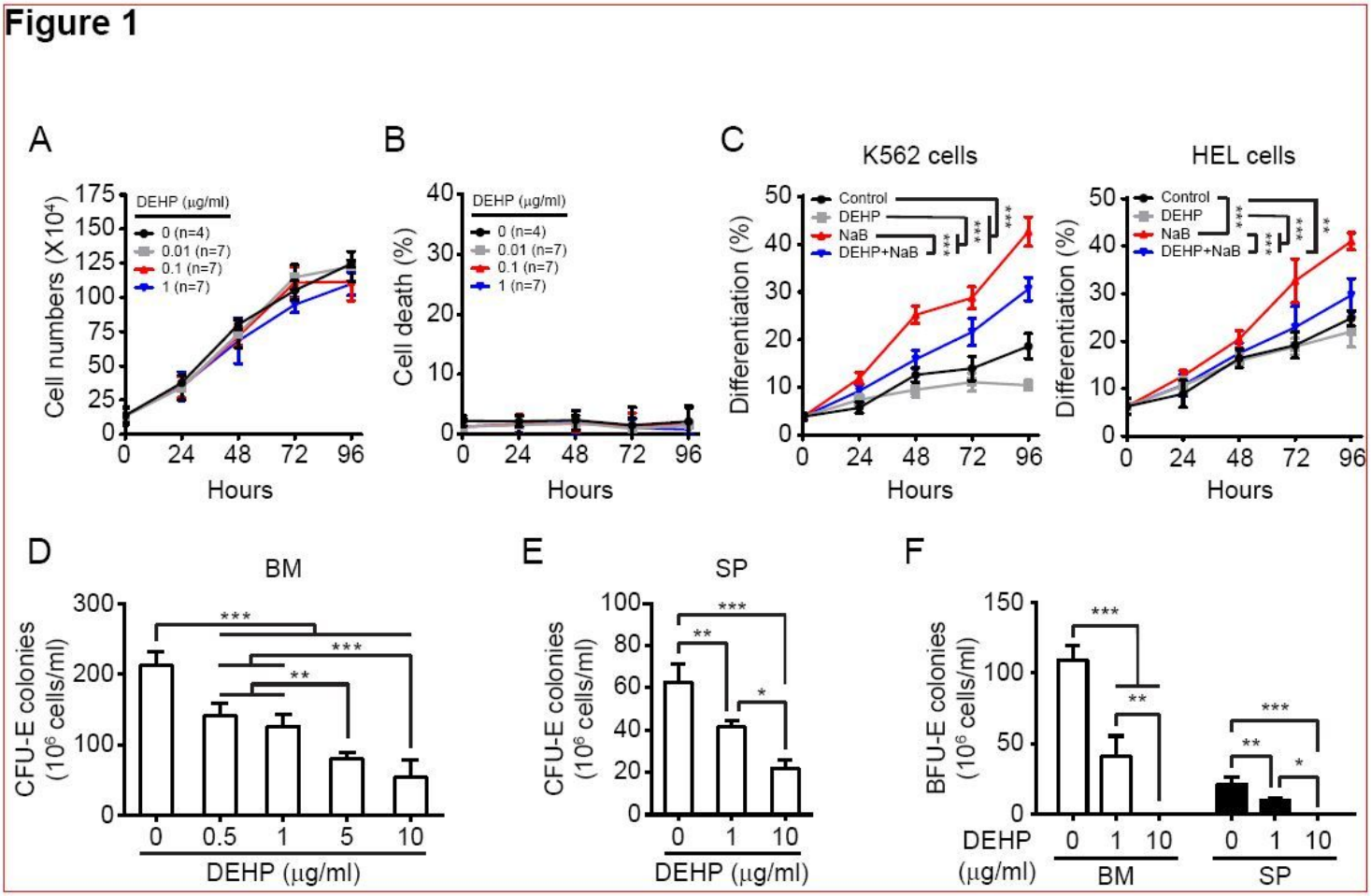

Figure 1

Exposure to DEHP suppresses erythroid differentiation. Human K562 cells were treated with different DEHP dosages. a Cell number and b cell death were analysed 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 without DEHP. The globin gene expression in cells was examined using Benzidine staining. d-e BM cells and splenocytes were cultured in methylcellulose-based media with EPO and other cytokines to maintain HSPC proliferation and differentiation. Different DEHP dosages were added. The numbers of CFU-E and BFU-E were counted 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.

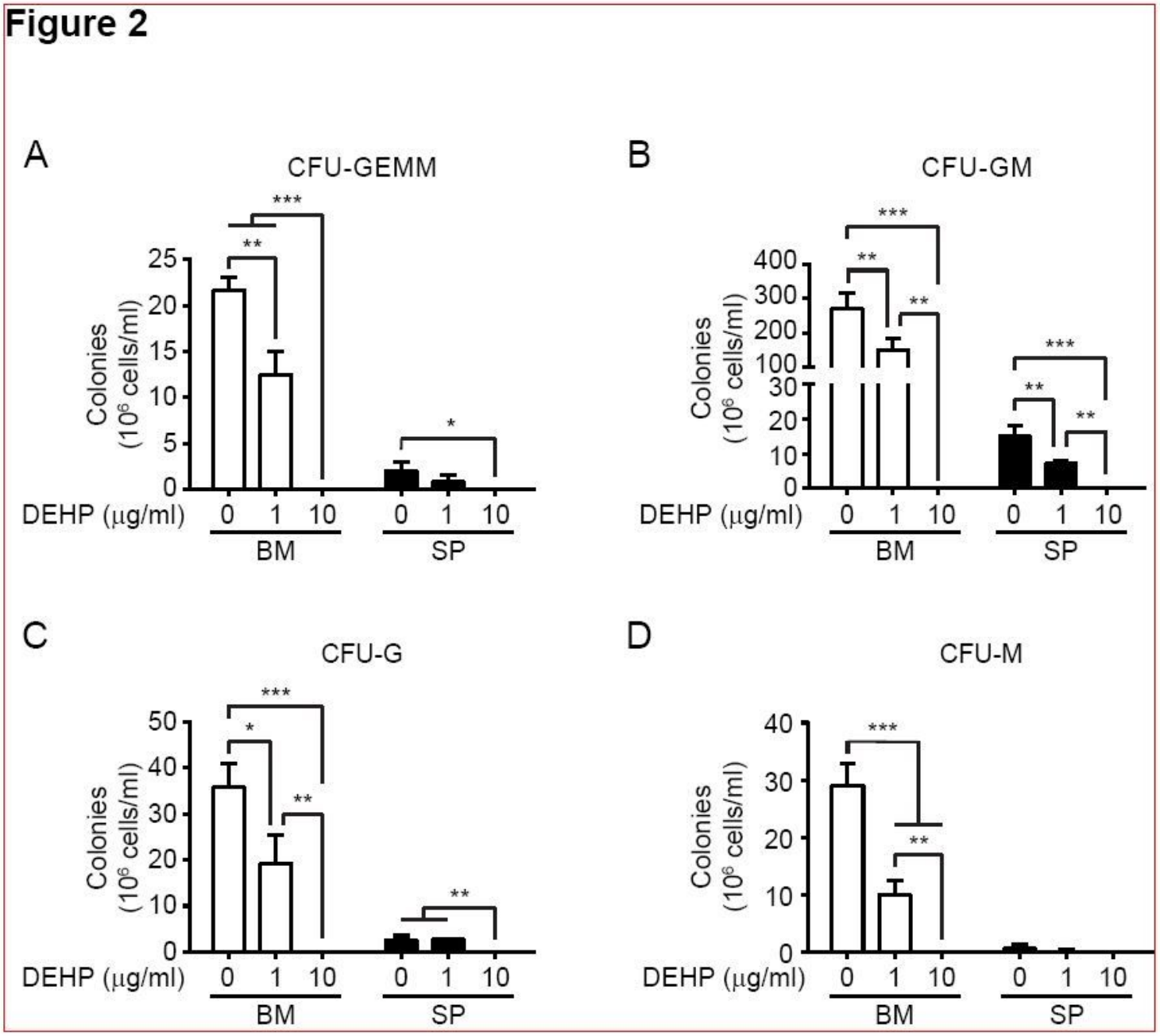

Figure 2

Exposure to DEHP impairs HSPC differentiation. a–d BM cells and splenocytes were cultured in methylcellulose-based media with various cytokines to maintain HSPC proliferation and differentiation. Different DEHP dosages were added. The numbers of CFU–GEMM, –GM, –G, and –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.

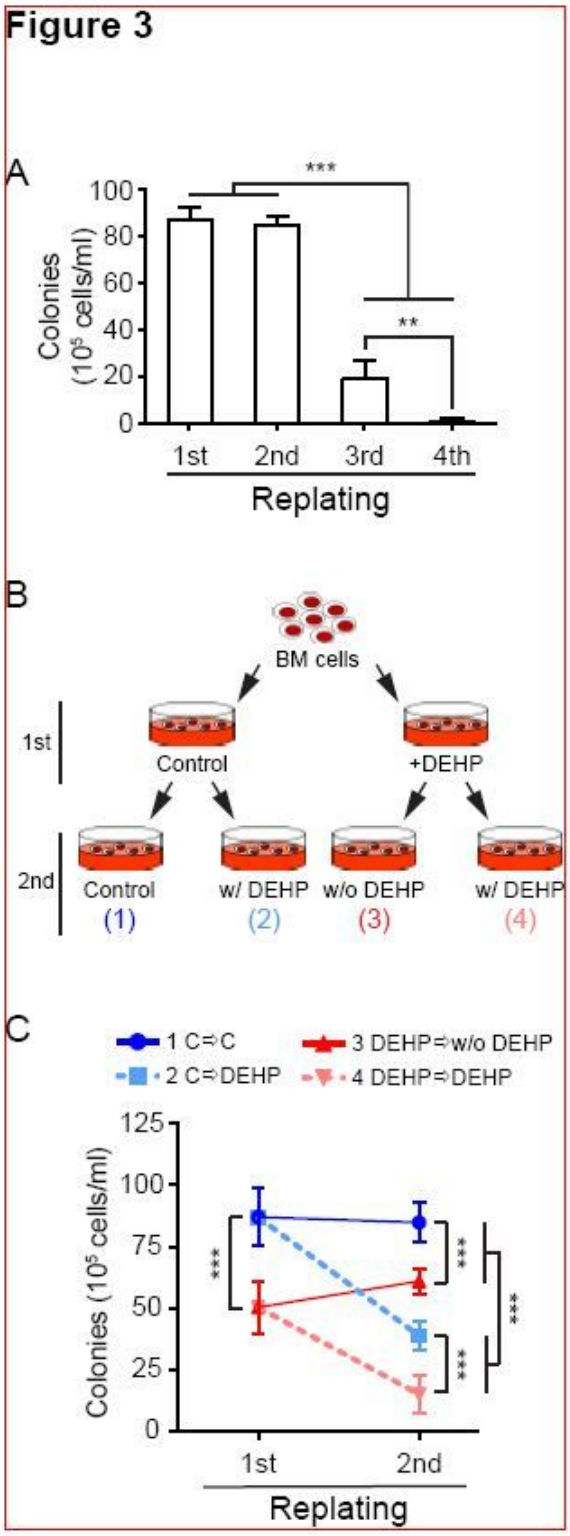

Figure 3

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 x 10^4 colony cells were re-plated. b A schematic of the examination of DEHP effects on HSPC function 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 are 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<0.001.

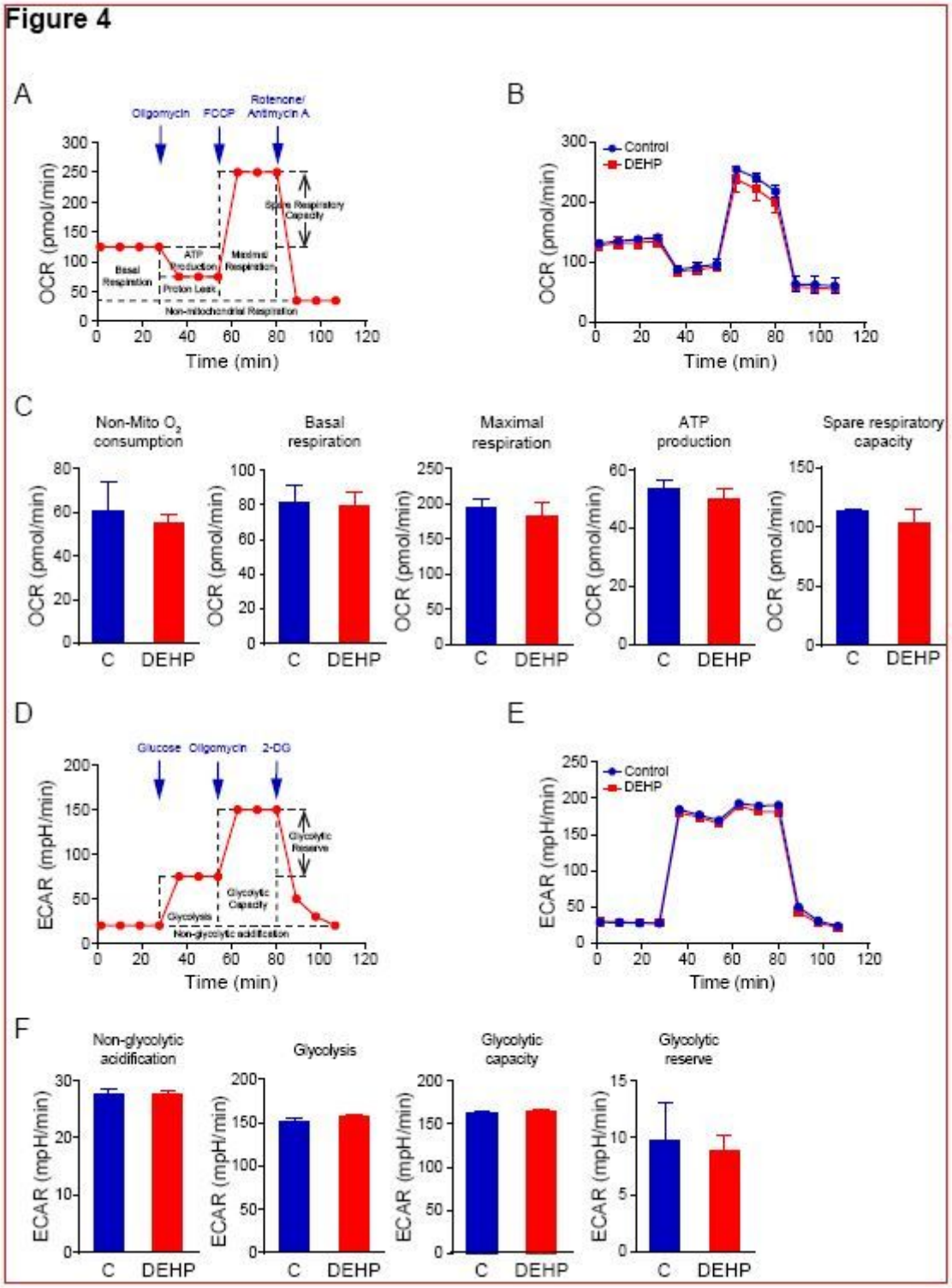

Figure 4

DEHP does not affect mitochondrial respiration and glycolytic function in undifferentiated K562 cells. a Schematic of OCR examination using Seahorse Cell Mito Stress assay. OCR is measured before and after

inhibitor addition to derive several parameters of mitochondrial respiration, which are used to determine the bioenergetic profile. b The OCR profile plot of K562 cells treated with (red line) or without DEHP (blue line) (1 μg/ml). c The non-mitochondrial oxygen consumption, basal and maximal respiration, and ATP production and spare respiration capacity were determined by calculating average values for each phase in the K562 cells. d Schematic of ECAR examination using a Seahorse Glycolysis Stress assay. e ECAR profile plot of K562 cells treated with (red line) or without DEHP (blue line) (1 μg/ml). f 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 are presented as means ± s.d. The Student's t-test was used for statistical analysis.

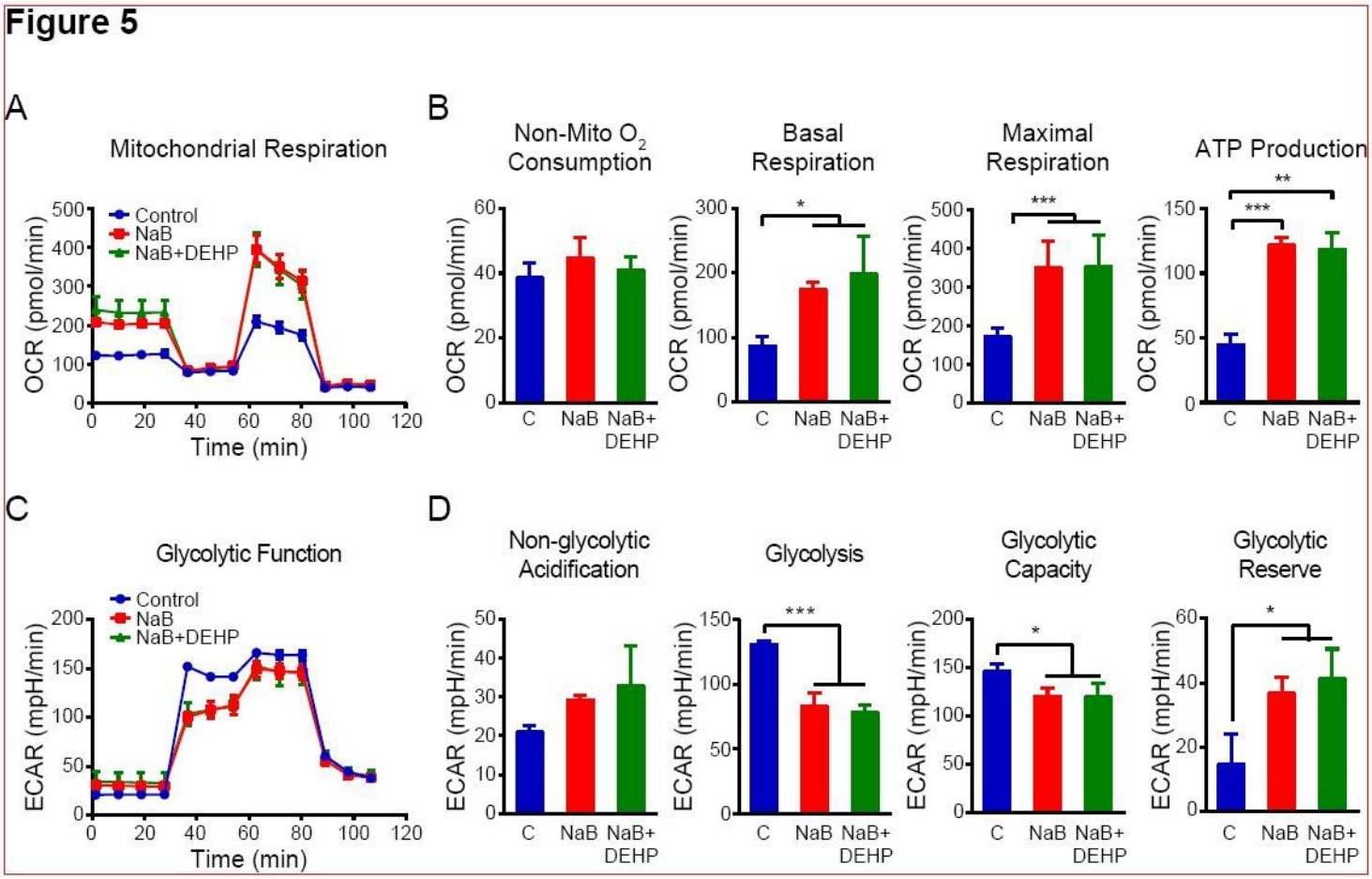

Figure 5

DEHP did 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 plot of K562 cells underwent 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 ECAR profile plot of K562 cells under 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 are

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.

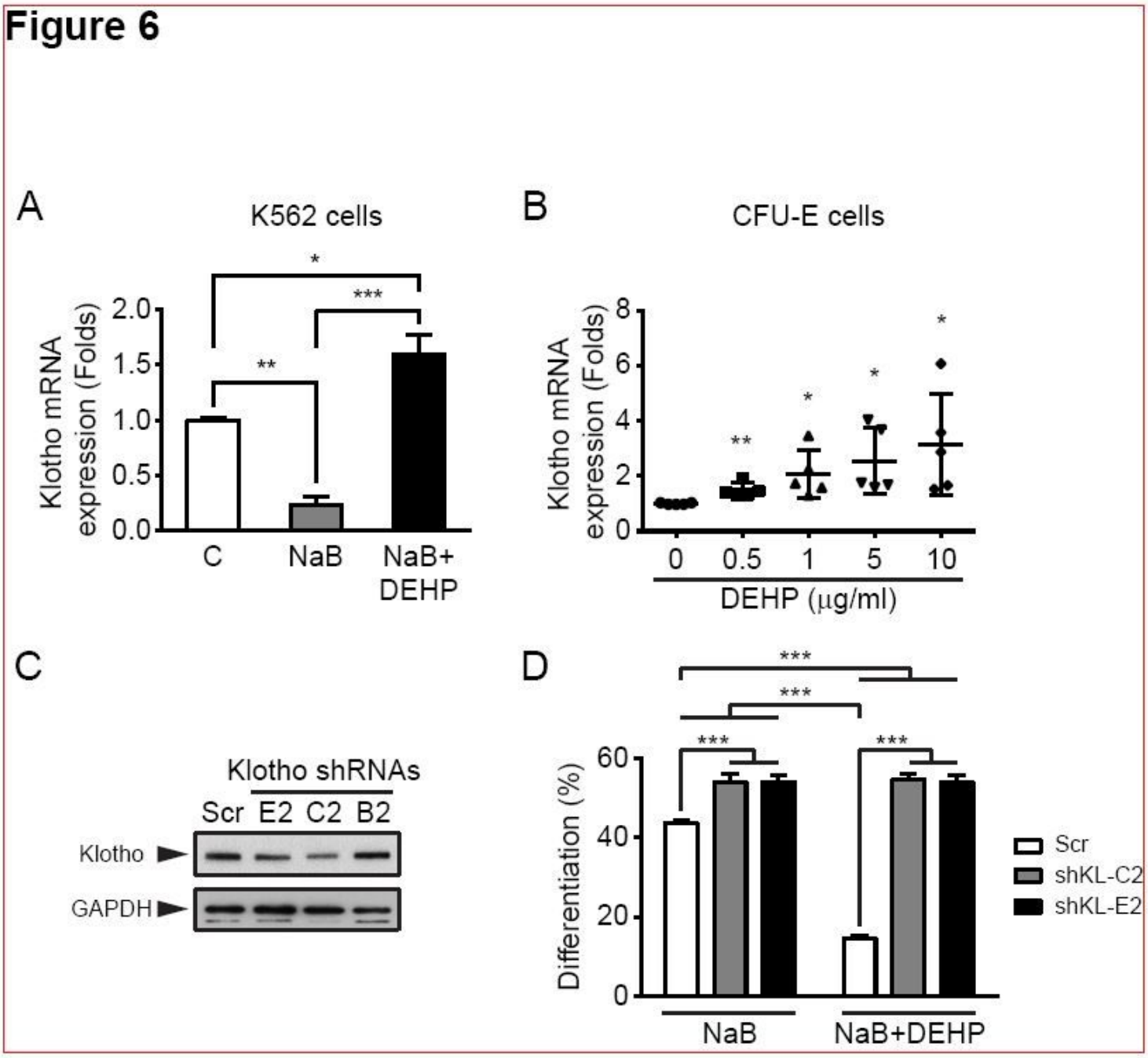

Figure 6

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. Klotho expression was measured using quantitative reverse transcription polymerase chain reaction (RT-qPCR). b 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. Klotho expression was measured using RT-qPCR. c Scrambled shRNA (Scr) and three Klotho shRNA were transiently expressed in K562 cells. The Klotho

proteins were detected 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 gene expression in cells was examined using Benzidine staining. All experiments were repeated at least three times. The quantified data are 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.

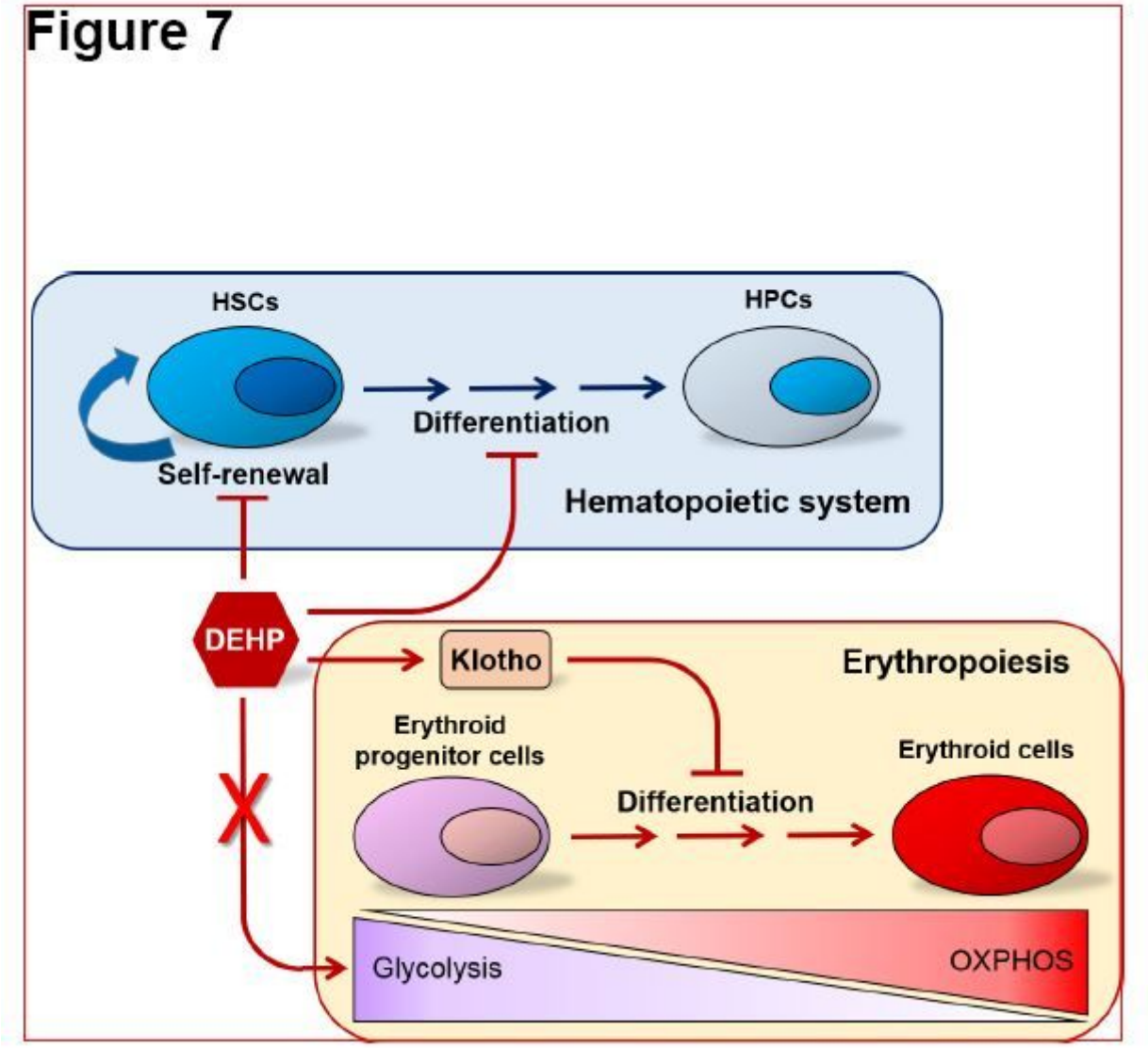

Figure 7

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