Estrogen contributes to regulating iron metabolism through governing ferroportin signaling via an estrogen response element

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ABSTRACT

Ferroportin (FPN) is the only known iron exporter in mammalian cells, and is universally expressed in most types of cells. FPN signaling plays a crucial role in maintaining iron homeostasis through governing the level of intracellular iron. Serum iron storage is conversely related with the estrogen level in the female bodies, and women in post-menopause are possibly subjected to iron retention. However, the potential effects of estrogen on iron metabolism are not clearly understood. Here, FPN mRNA transcription in all selected estrogen receptor positive (ER+) cells was significantly reduced upon 17 β -estradiol (E2) treatment; and this inhibitory effect could be attenuated by ER antagonist tamoxifen. Likewise, in murine bone marrow-derived macrophages (BMDMs), FPN reduction with elevated intracellular iron (reflected by increased ferritin) was observed in response to E2; however, ferritin level barely responded to E2 in FPN-null BMDMs. The observation of inhibition of FPN mRNA expression was not replicated in ER[−] cells upon E2. A functional estrogen response element (ERE) was identified within the promoter of FPN, and this ERE was responsible for the suppressive effect of E2 on FPN expression. Moreover, ovariectomized (OVX) and sham-operated (SHAM) mice were used to further confirm the *in vitro* finding. The expression of hepatic FPN was induced in OVX mice, compared to that in the SHAM mice. Taken together, our results demonstrated that estrogen is involved in regulating FPN expression through a functional ERE on its promoter, providing additional insights into a vital role of estrogen in iron metabolism.

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1. Introduction

Iron is an essential element to all forms of life. Iron is a necessary component for a large array of proteins involved in diverse functions including oxygen delivery, electron transfer, energy metabolism, transcriptional regulation and DNA replication [1–3]. It is considerably important to maintain a fine-tuned balance of iron metabolism. In fact, iron disorders in the form of either iron deficiency or overload cause detrimental impacts on cells [3–5]. Mammalian iron homeostasis is concertedly regulated through the hepcidin–ferroportin (FPN) axis that fundamentally governs iron absorption, transport, storage and utilization [3,6]. Iron needs the sole known iron exporter, FPN, to shuttle out of the cells.[7] As a transmembrane transporter, FPN is responsible for dietary iron absorption in enterocytes of small intestine, and also controls iron release from macrophages and other cells [8,9]. High levels of FPN lead to enforced dietary iron absorption and egress from storage

out of cells including macrophages and hepatocytes, which is associated with several types of diseases, such as hereditary hemochromatosis and β -thalassemia caused by iron overload [5]. Inversely, FPN deficiency or loss of iron-exporting function could also result in problematic iron metabolism, namely iron retention in enterocytes and macrophages coupled to low plasma iron concentration [3,10,11]. Patients with this disease are characterized by low plasma transferrin saturation and iron retention predominantly in macrophages [5].

In light of FPN regulation, FPN protein concentration is prominently modulated through a posttranscriptional mechanism by the iron responsive element/iron regulatory protein (IRE/IRP) signaling, as iron abundance enforces FPN translation and iron deficit represses FPN translation [12–15]. Additionally, FPN could also be regulated by hypoxia inducible factor 2 (HIF2 α), nuclear factor erythroid (Nrf2), and metal-regulatory transcription factor 1 (MTF-1) at the transcriptional level in macrophages [11,12,16]. However, little is known about the regulation of FPN under different physiological settings including natural alterations of endogenous steroid hormones, such as estrogen.

Estrogen is an indispensable hormone implicated in a wide spectrum of physiological functions for organs including breast, bone and

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skin, especially in females [17–19]. Within the life span of a woman, the endogenous level of estrogen greatly fluctuates, particularly after menopause [20]. The pronounced manifestation of menopause is the cessation of menstruation, accompanied by estrogen deficiency and reduced functions of the ovaries [17]. A few previous studies have suggested the consequential change of iron levels after menopause [20,21]. Considering the change of iron, several reasons should be taken into account. For example, reduction of monthly blood loss would certainly help to retain body iron [17]. Recent studies from our research group and another group have uncovered the regulation of hepcidin by estrogen, linking estrogen to iron homeostasis [22,23]. Nonetheless, the impact of estrogen on iron metabolism is still elusive, and specifically, no insight has been gained for the potential regulation of estrogen on FPN.

In the current study, in an effort to determine the contribution of estrogen to FPN regulation, we employed a panel of *in vitro* and *in vivo* strategies. Overall, we deciphered a novel mechanism underlying the regulation of FPN by estrogen. Estrogen (17 β -estradiol, a primary endogenous female sex hormone) could significantly inhibit the mRNA transcription of FPN through a functional estrogen response element (ERE) within its promoter. Cells with FPN deficiency (namely FPN knockout cells) were resistant to estrogen-induced iron alterations through FPN. Consistent with the *in vitro* findings, FPN expression was elevated upon estrogen deficiency in mice with ovariectomy. Our combined data highlighted a crucial role for estrogen in iron metabolism through repressing FPN expression via an ERE within its promoter.

2. Materials and methods

2.1. Chemicals and reagents

17 β -Estradiol and tamoxifen were purchased from Sigma-Aldrich Co. Dimethyl sulfoxide (DMSO) (Solarbio Inc., China) was used to dissolve 17 β -estradiol and tamoxifen. The final concentration of DMSO in culture medium was <0.1%.

2.2. Cell culture

Human acute monocytic leukemia cell line THP-1, human hepatic carcinoma cell lines SMMC-7721 and HepG2, and human breast cancer cell lines T47D and MDA-MB-231 were purchased from the Shanghai Cell Bank of Type Culture Collection of Chinese Academy of Science. All cells were cultured in medium with 5% charcoal-treated FBS (Hyclone) at 37 °C with 5% CO₂. To initiate differentiation, THP-1 cells were treated with 200 nM Phorbol-12-myristate-13-acetate (PMA) for 24 h, as previously described [24]. Thereafter, the medium containing PMA was replaced with fresh medium for further experiments. Murine bone marrow-derived macrophages (BMDMs) were isolated and cultured as previously described [25].

2.3. Animal experiments

All animal care and surgical procedures followed the guidelines of Animal Ethics Committee at the Research Center for Eco-environmental Sciences, Chinese Academy of Sciences. For animals, 8-week-old female C57BL/6 mice were purchased from the Vital River Laboratories (Beijing, China), and FPN1-floxed and LysM-Cre mice with the 129/SvEvTac background were provided by Dr. Fudi Wang [6,26–28]. LysM-Cre mice were mated with FPN1^{flox/flox} mice to create the strain in which FPN was inactivated in macrophages. Mice were housed under specific pathogen-free and sterile conditions.

C57BL/6 mice were randomly divided into 2 groups of seven (*n* = 7). One group was bilaterally ovariectomized (OVX), and the mice in the other group were sham-operated following a surgical protocol previously described [29]. After 8 weeks, these mice were sacrificed. The liver, spleen, uterus and blood specimens from each mouse were collected for further experiments.

2.4. Iron measurements

Serum iron was measured with a kit on the basis of the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute). Liver and spleen iron contents were determined as previously described [25].

2.5. Cell proliferation assay

Cell growth was measured by Alamar blue and BrdU incorporation assays (Roche). Briefly, PMA-differentiated THP-1 cells were starved in serum-free medium overnight and then seeded at a density of 1.0×10^4 cells/well in 96-well plates with or without 1×10^{-7} M E2 for 24 h. Thereafter, resazurin (Sigma) was added into each well at a final concentration of 10%, followed by incubation for additional 2 h at 37 °C. Finally, fluorescence intensity was recorded at an excitation wavelength of 530 nm with an emission wavelength of 590 nm. With respect to the BrdU assay, we followed the standard instructions provided by the manufacturer.

2.6. Real-time RT-PCR analysis

Total RNAs from cells or liver tissues were purified with Trizol reagent following the manufacturer's instructions (Invitrogen). Liver tissues were first grinded in liquid nitrogen before total RNAs were extracted. Gene expression was measured using SYBR Green qPCR master mix (Promega) on a qRT-PCR machine (Bio-Rad). Primer sequences were as follows. Human FPN: forward, 5'-CTACTTGGGGAGATCGGA TGT-3', reverse, 5'-CTGGGCCACTTTAAGTCTAGC-3'; human CXCR4, forward, 5'-TCATCAGCTTCCCTTCTGG-3', reverse, 5'-CATGGACTGCCTTG CATAGGA-3'; human IL-6, forward 5'-CCAGAGCTGTGCAGATGAGT-3', reverse, 5'-CTGCAGCCACTGGTCTGT-3'; human IL-1 β , forward, 5'-CTGTCC TCGGTGTTGAAAGA-3', reverse, 5'-TTGGGTAATTTTGGGATCTACA-3'; human GAPDH, forward 5'-GAAGGTGAAGGTCCGAGT-3', reverse, 5'-GAAGATGGTGATGGGATTTC-3'; mouse FPN, forward, 5'-GTCGGCCAGA TTATGACATTG-3', reverse, 5'-ATTCCAACCGAAATAAACCA-3'; mouse GAPDH, forward, 5'-AAGGTCATCCCAGAGCTG-3', reverse, 5'-GCCATGAGGTCCACCACCT-3'. GAPDH was used as a loading control.

2.7. Western blotting

Cells were washed with ice cold PBS and then lysed in RIPA lysis buffer (Solarbio, China) supplemented with protease inhibitor cocktail (Roche), as previously described [30]. An equal amount of each lysate (80–100 μ g/sample) was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes afterwards. Antibodies were against GAPDH (1:1000, Santa Cruz Biotechnology), FPN (1:500, Sigma), and ferritin light chain (1:1000, Abcam).

2.8. Vector construction of FPN promoter with predicted ERE

We identified a potential ERE (5'-TGTCAGTCTGGCC-3', –376/–388 bp) in the promoter of the human FPN gene using Dragon ERE Finder version 3.0 [31]. Two DNA fragments of the FPN promoter, one with the predicted ERE (–376/–388 bp) and the other with deletion of the ERE, were inserted into the pGL3-promoter luciferase reporter vector (Promega). The vector with the predicted ERE was named as Pro-ERE, and the one without was named as Pro-ERE Δ . These two plasmids were confirmed by DNA sequencing (Invitrogen).

2.9. Luciferase assays

Cells were seeded at a concentration of 1×10^5 cells/ml in 24-well plates. Cells were then co-transfected with 0.8 μ g target plasmids (Pro-ERE or Pro-ERE Δ) and 80 ng Renilla luciferase plasmid by

Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. After 4 h incubation, the culture medium was replaced with phenol red-free medium with 5% charcoal-treated FBS, and thereafter 1×10^{-7} M E2 was added for 24 h. Cells were then lysed with 100 μ l lysis buffer after washing with PBS, and cellular extracts were subsequently examined for luciferase activity using the dual-luciferase reporter assay system (Promega). The relative luciferase activities were normalized to those of Renilla luciferase.

2.10. Image quantification of Western blots

The intensity of autoradiogram in Western blotting was quantified using the software ImageJ (NIH, <http://rsbweb.nih.gov>). Quantified results for each protein were normalized to those of the loading control.

2.11. Statistical analysis

Independent *t* test or One-Way ANOVA test was employed to analyze the experimental data. Data were shown in mean $\pm$ standard error. Statistical significance was determined with *P* value less than 0.05.

3. Results

3.1. E2 repressed the expression of FPN in PMA-differentiated THP-1 cells

The ER α -positive THP-1 cells are widely used as an ideal model in studying functions of macrophages, such as inflammation [32]. THP-1 cells were also used to study iron metabolism in macrophages [3,33]. Here, to determine the potential role of E2 in regulating the expression of FPN, we first measured the mRNA and protein levels of FPN upon 1×10^{-7} M E2 treatment in PMA-differentiated THP-1 cells for 24 h. E2 treatment per se did not undermine the cell viability, as evidenced by the viability assay and the BrdU incorporation assay (Fig. 1A & B). The FPN mRNA level was reduced by approximately 30% in response to E2 (Fig. 2A, *P* < 0.05), in analogy to the alteration of the established estrogen down-regulated gene chemokine receptor type 4 (CXCR4) [34] upon E2 (Fig. 2A). Consistently, E2 also reduced FPN concentration by around 30% at the protein level (Fig. 2B), suggesting that E2 could significantly repress FPN expression. Moreover, upon pre-treatment with 40 μ g/ml cycloheximide (CHX), an inhibitor of protein biosynthesis in eukaryotic organisms, the FPN mRNA level was still reduced by 25% in response to E2 (Fig. 2C), suggesting that the suppressive effect of E2 on FPN was through a transcriptional mechanism, rather than a translational mechanism. Additionally, tamoxifen, which acts as an antagonist of E2 action by binding to ER, [35] notably reversed this inhibitory effect at the concentration of 1 μ M (Fig. 2D), further indicating that FPN could be modulated through E2 signaling pathway.

Due to the reduction of FPN concentration, ferritin, an iron storage protein, was increased by more than 50% (Fig. 2B). Previous studies have suggested that the increased cellular iron was highly associated with reduced production of inflammatory cytokines from macrophages, including interleukin-6 (IL-6) and IL-1 β [36–38]. Therefore, we hypothesized that E2-induced reduction of FPN could likely in turn undermine the inflammatory responses of THP-1 cells. To test this hypothesis, we detected the mRNA levels of IL-6 and IL-1 β upon treatment of E2 or ferric nitrilotriacetate (Fe-NTA at 100 μ M) as a positive control. As a result, the expression levels of IL-6 and IL-1 β were impaired coupled to elevated intracellular iron content, due to the reduction of FPN concentration (Fig. 3A & B), analogous to a previous study [36]. These data suggested that E2 could remarkably down-regulate FPN at the transcriptional level in human macrophages, leading to elevated intracellular iron levels and weakened inflammatory responses.

3.2. Confirmation of E2-mediated transcriptional regulation of FPN

To validate the regulation of FPN expression by estrogen, we further examined this mechanism in different types of cells. Hepatocytes have relatively high level of FPN on the plasma membrane [39]. Representative cell models of hepatocytes, such as HepG2 and SMMC-7721 cells, were conventionally used in estrogen-related studies [22,23]. Thus, we looked into the alteration of FPN expression upon E2 in HepG2 and SMMC-7721 cells. In parallel to the alterations in THP-1 cells upon E2 (Fig. 2A), the expression of FPN was also reduced in both HepG2 and SMMC-7721 cells by 30% and 40% at the mRNA level, respectively (Fig. 4A & B, *P* < 0.05). Similar alterations of FPN at the protein level were demonstrated (Fig. 4A & B). In support of the regulation of FPN by estrogen, CXCR4 expression was similarly restrained in both HepG2 and SMMC-7721 cells by approximately 50% (Fig. 4A & B, *P* < 0.05).

In an attempt to confirm the finding of E2 in governing FPN expression, we additionally chose two human breast cancer cell lines: T47D and MDA-MB-231 cells, which were ER α -positive and ER α -negative, respectively [40,41]. As illustrated in Fig. 4C, both the mRNA and protein levels of FPN were reduced by over 20% in T47D cells in the presence of E2. However, FPN expression did not respond to E2 in MDA-MB-231 cells (Fig. 4D). Therefore, these results together indicated that E2 played a role in repressing FPN expression in ER-positive cells.

3.3. E2 inhibited FPN transcription in BMDMs

Next, we sought to investigate whether E2 induced similar influence on murine macrophages. Thus, primary wild type (WT) BMDMs from mice with the 129/SvEvTac background were cultured, and then treated with or without 1×10^{-7} M E2 for 24 h. Consistent with the results in human cell lines, WT BMDMs revealed nearly 50% reduction of FPN at both mRNA and protein levels upon E2 (Fig. 5A, *P* < 0.05). As a result of FPN reduction, the iron storage protein ferritin was elevated by 30%

Fig. 1. Cell viability of differentiated THP-1 cells upon E2. PMA-differentiated THP-1 cells were treated with 1×10^{-7} M E2 for 24 h, followed by (A) the Alamar blue assay (*n* = 6) and (B) the BrdU assay (*n* = 6).

Fig. 2. FPN expression in differentiated THP-1 cells upon E2. PMA-differentiated THP-1 cells were treated with 1×10^{-7} M E2 or 1 μ M tamoxifen for 24 h, and then total RNAs and proteins were extracted for further analyses. (A) The mRNA levels of FPN and CXCR4 were determined by qRT-PCR analysis ($n = 4$). (B) FPN and ferritin protein concentrations were evaluated by Western blotting. (C) The mRNA levels of FPN were assessed by qRT-PCR upon E2 and 40 μ g/ml CHX treatments ($n = 4$). (D) Relative mRNA levels of FPN were determined by qRT-PCR upon exposure to E2 and/or 1 μ M tamoxifen ($n = 4$).

(Fig. 5B), indicating that estrogen could substantially alter iron status in WT BMDMs through down-regulating FPN expression. In an effort to further confirm the finding of FPN reduction induced by E2 on murine macrophages, FPN deficient BMDMs were employed according to the strategy described by Zhang et al. [28] FPN mRNA expression was decreased by nearly 90% in FPN-null BMDMs (Fig. 6A), compared to the WT cells. Moreover, the protein level of FPN was almost undetectable (Fig. 6B), suggesting that FPN was mostly ablated in these BMDMs. Under the setting of FPN deficiency, we demonstrated that the ferritin protein level was no longer responding to E2 levels (Fig. 6C). These data collectively demonstrated that E2 acted to restrain the transcription of FPN, and in turn caused intracellular iron retention in mouse macrophages.

3.4. FPN transcription was regulated by E2 through a functional ERE within the FPN promoter

Furthermore, we strived to decipher the molecular mechanisms responsible for the inhibition of FPN transcription upon E2. Hou and colleagues from our research group previously identified an ERE within the promoter of hepcidin, and this ERE was responsible for E2-induced suppression of hepcidin expression in hepatocytes [23]. Therefore, we assumed that the promoter of FPN might also harbor a similar estrogen response site. It has been recognized that the conserved sequence of canonical ERE consists of palindromic half-sites interspaced by three nucleotides, namely, 5'-GGTCA-NNN-TGACC-3' [42]. However, other non-canonical EREs possibly have one or more base aberrations from

Fig. 3. Synthesis of inflammatory cytokines upon E2 treatment. PMA-differentiated THP-1 cells were treated with 1×10^{-7} M E2 for 24 h. The mRNA levels of (A) IL-6 and (B) IL-1β were assayed ($n = 4$). Fe-NTA at 100 μM was used here as a control to mimic excess iron.

Fig. 4. FPN regulation by E2 in hepatocytes and breast cancer cells. (A) SMMC-7721, (B) HepG2, (C) T47D and (D) MDA-MB-231 cells were exposed to 1×10^{-7} M E2 for 24 h. The mRNA levels of FPN and CXCR4 ($n = 4$), and the FPN protein concentrations were then determined, respectively.

Fig. 5. E2 repressed the expression of FPN in WT BMDMs. WT BMDMs were exposed to 1×10^{-7} M E2 for 24 h. Afterwards, (A) the mRNA level of FPN in WT BMDMs was assayed ($n = 4$) and (B) FPN and ferritin protein levels were determined in WT BMDMs.

this sequence [43]. We found a putative ERE (5'-TGTCAGTCTGGCC-3', -376/-388 bp) in the promoter region of the FPN gene with the software Dragon ERE Finder version 3.0, as previously reported [31] (Fig. 7A). In order to define the function of this predicted ERE, we constructed two reporter plasmids, i.e. one with intact ERE (Pro-ERE) and one with ERE deletion (Pro-EREΔ). As shown in Fig. 7B, the transcriptional activity of Pro-ERE was reduced by 20% upon E2 treatment, whereas the response to E2 was thoroughly lost in Pro-EREΔ transfected cells, similar to the mechanism of E2-mediated repression of hepcidin, in our previous study [23]. These findings therefore suggested that the putative ERE (-376/-388 bp) functionally responded to E2. In other words, the transcription of FPN was inhibited through the action of E2 on the ERE in the FPN promoter region.

3.5. FPN expression was elevated in estrogen-deficient mice

In an attempt to elucidate the biological impact of FPN repression caused by estrogen in animals, OVX mice were chosen as an experimental model. First, uterus alteration of the mice with or without ovariectomy was investigated. A significant decline in the uterus weight (by 60%) was observed in the OVX group after 8 weeks, compared to the SHAM control group (Fig. 8A, $P < 0.05$), suggesting that estrogen was successfully suppressed in OVX mice. Meanwhile, the mRNA level of hepatic FPN was measured. An increase by approximately 50% was demonstrated in the OVX mice relative to the SHAM mice (Fig. 8B, $P < 0.05$), further supporting the regulation of FPN by estrogen. Additionally, serum iron was slightly reduced associated with mildly increase of hepatic and

splenic iron (Fig. 8C). As our previous study showed that hepcidin could also be down-regulated by E2 [23], we subsequently inspected the hepcidin expression. In agreement with the previous finding, the absence of estrogen greatly enhanced the mRNA level of hepcidin by nearly 2 fold (Fig. 8D, $P < 0.05$), suggesting that hepcidin was elevated to a greater extent than FPN. The induction of hepcidin counteracted the increase of FPN in response to estrogen deficiency, leading to the compensation of iron status. Thus, the iron mass in serum, spleen and liver was not dramatically changed when hepcidin and FPN were simultaneously altered upon estrogen loss in the OVX mice. Nonetheless, these data verified the regulation of FPN expression by estrogen in animals.

4. Discussion

Iron is an essential bio-metal for almost all types of organisms, as it is a component of iron-sulfur cluster-containing proteins, including DNA helicase enzymes involved in DNA replication and other factors implicated in electron transport and oxidative phosphorylation [44–46]. Thus, iron is necessary for cellular growth and other biological functions that closely depend on energy metabolism, DNA replication, and heme synthesis [47]. Systemic iron homeostasis is strictly fine-tuned through various mechanisms, of which the hepcidin-FPN axis fundamentally governs the global iron absorption, distribution, utilization, and egress [48]. However, disordered iron metabolism would likely give rise to a large array of dysfunctions, morbidities and even diseases, such as cancers [5,49–53]. In fact, a myriad of epidemiological studies have documented a considerable contribution of elevated body iron to cancer

Fig. 6. The transcription of FPN was resistant to E2 in FPN-null BMDMs. Determination of FPN (A) mRNA and (B) protein level between WT BMDMs and FPN-deficient BMDMs. (C) FPN-deficient BMDMs were treated with 1×10^{-7} M E2 for 24 h, and then the protein concentration of ferritin was assessed through Western blotting.

Fig. 7. E2 regulated FPN expression through a functional ERE in its promoter. (A) The schematic for the construction of the FPN-promoter luciferase reporter plasmid. The ERE in FPN promoter was identified with the web-based software, the Dragon ERE Finder version 3.0. The schematic also shows the deletion of the core sequence for the predicted ERE. (B) Relative luciferase activities. The pGL3-based plasmids containing with ERE (Pro-ERE, upper panel) or without ERE (Pro-EREΔ, lower panel) were co-transfected with Renilla luciferase plasmid into HepG2 cells. The relative firefly luciferase activities were normalized to those of the Renilla luciferase.

risk of occurrence and prognosis [52–57]. In stark contrast to normal cells, iron demand is remarkably enforced in cancer cells to meet the enhanced cellular activities including DNA synthesis and energy generation [58,59]. The reasons for elevated body iron burden are poorly understood in cancer patients. In light of iron metabolism in women, menopause and endogenous estrogen alteration have been postulated to physiologically account for the change of body iron mass [17,60].

Estrogen is a hormone that physiologically dictates the integrity and functions for a variety of cells. The endogenous estrogen level fluctuates before menopause, and drops drastically post menopause. Reduced estrogen in post-menopausal women is associated with a wide spectrum of symptoms, such as bone loss, sleep disorders, and weight gain and

depression, resulting in increased risk of osteoporosis and other diseases. Elevated body iron has been documented in postmenopausal women for unclear reasons [20,52,61]. For example, a previous study identified a negative correlation between serum ferritin and estrogen level in women [17]. The mechanisms underlying the correlation between estrogen and iron metabolism is still elusive thus far. Here in the current study, we offered a line of direct evidence demonstrating the contribution of estrogen to the regulation of the iron exporter FPN concentration. We overall recognized the transcriptional repression of FPN expression by estrogen through a functional ERE in the promoter region of FPN gene. The current findings would be beneficial to explain increased body iron mass in post-menopausal women.

Fig. 8. Effects of estrogen deficiency on iron homeostasis in OVX mice. Female mice were ovariectomized for 8 weeks. (A) The average weight of uteri from OVX mice and SHAM mice. (B) Hepatic FPN mRNA level. (C) Iron mass in the serum, liver and spleen. (D) Hepatic hepcidin mRNA level for both OVX and SHAM groups (n = 7).

Fig. 9. A schematic delineating the role of estrogen in maintaining iron metabolism through regulating FPN transcription.

Our research group and another group have identified the regulation of hepcidin expression by estrogen [22,23]. Our previous data characterized an inhibitory effect of estrogen on hepcidin expression via a functional ERE within its promoter [23]. However, the ligand of hepcidin, FPN, has not been investigated in response to estrogen to date. FPN plays a crucial role in maintaining the concerted iron homeostasis through controlling iron egress, and dysregulation of FPN would give rise to disordered iron metabolism, [10] likely resulting in FPN diseases, such as hereditary hemochromatosis and β -thalassemia [5]. Additionally, we also recently found reduced level of FPN in breast cancers, associated with poor prognosis in breast cancer patients [11,30]. To keep the balance of systemic iron metabolism, FPN is predominantly regulated by hepcidin through binding and inducing ubiquitin-dependent proteasomal degradation of FPN protein [6]. FPN concentration is also subjected to the regulation by the iron responsive element (IRE)/iron responsive protein (IRP) system at the post-transcriptional level [15, 62,63]. Moreover, emerging evidence suggests that FPN expression is also transcriptionally regulated. For example, we recently demonstrated that myeloid zinc-finger 1 (MZF-1) could directly transactivate FPN expression and then enhance the FPN-mediated iron export, resulting in a significant suppression of prostate tumor growth [11]. In the current study, we first demonstrated that estrogen treatment significantly reduced the expression of FPN in multiple human cell lines including macrophages, hepatocytes and ER positive breast cancer cells, coupled to augmented intracellular iron. Moreover, the repression of FPN expression by estrogen was not replicated in ER-negative cells. Tamoxifen, an ER antagonist, could reverse this repression. These data together suggested that estrogen-regulated FPN expression was dependent on ER. Analogously, the expression of FPN in WT BMDMs was greatly inhibited upon E2 activation, leading to elevated ferritin levels; however, FPN-deficient BMDMs were resistant to the repressive effect by estrogen. Moreover, we defined the molecular mechanism underlying estrogen-conducted inhibition of FPN expression. Namely, a functional ERE within the promoter of FPN was responsible for the suppressive effect induced by E2. Our results offered an additional explanation for enhanced body iron storage in postmenopausal women [22]. In support of this statement, our results revealed that the expression of hepatic FPN was elevated in OVX mice, compared to SHAM mice. Meanwhile, hepatic hepcidin expression was also enforced in OVX mice, relative to

SHAM mice, indicating restrained iron absorption and iron egress in response to hepcidin-induced FPN degradation. In parallel to the findings from the current animal study, recent clinical investigations also identified increased serum hepcidin concentrations in postmenopausal women, relative to premenopausal women [64,65]. To this end, the induction of FPN was compensated by the increase of hepcidin in the absence of estrogen in mice. Thus, only mild alterations of serum iron and splenic and hepatic iron mass were observed in OVX mice, relative to SHAM mice. However, the relative contribution of estrogen-regulated FPN and hepcidin to the net effects on systemic iron homeostasis is still not clear, and therefore, further detailed investigations are warranted.

5. Conclusion

To summarize, we deciphered a novel mechanism for estrogen in contributing to maintaining iron metabolism through regulating FPN signaling (Fig. 9). Estrogen exerts the transcriptional inhibitory effect on FPN expression via a functional ERE within its promoter. This study provides additional explanation for body iron changes in women upon estrogen alterations, such as a dramatic decline in the estrogen level in postmenopausal women. Since increased serum iron and tissue iron retention were observed in postmenopausal women, [30,66] excess iron would likely incur in detrimental effects on health. Therefore, in addition to its pronounced role in ameliorating menopausal symptoms, administration of a proper dose of estrogen (namely estrogen replacement therapy) might help to orchestrate systemic iron homeostasis.

Competing interests

No potential conflicts of interest were disclosed by the authors. There are no financial competing interests to declare in relation to this manuscript.

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