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Effects of Iron Deficiency and Overload on Angiogenesis and Oxidative Stress – A Potential Dual Role for Iron in Breast Cancer

Jinlong Jian¹, Qing Yang¹, Jisen Dai¹, Jonathan Eckard¹, Debrah Axelrod^{2,3}, Julia Smith^{3,4}, and Xi Huang^{1,3,*}

¹ Department of Environmental Medicine, NYU School of Medicine, New York, NY 10016;

² Department of Surgery, NYU School of Medicine, New York, NY 10016;

³ New York University (NYU) Cancer Institute, NYU School of Medicine, New York, NY 10016;

⁴ Department of Medicine, NYU School of Medicine, New York, NY 10016;

Abstract

Estrogen alone cannot explain the differences in breast cancer (BC) recurrence and incidence rates in pre- and postmenopausal women. In the present study, we have tested a hypothesis that, in addition to estrogen, both iron deficiency due to menstruation and iron accumulation as a result of menstrual stop play important roles in menopause-related BC outcomes. We first tested this hypothesis in cell culture models mimicking the high estrogen and low iron premenopausal condition and the low estrogen and high iron postmenopausal condition, respectively. Subsequently, we examined this hypothesis in mice that were fed iron deficient and iron overload diets. We have shown that estrogen only slightly up-regulates vascular endothelial growth factor (VEGF), an angiogenic factor known to be important in BC recurrence. It is, rather, iron deficiency that significantly promotes VEGF by stabilizing hypoxia inducible factor-1 α (HIF-1 α). Conversely, high iron levels increase oxidative stress and sustain mitogen-activated protein kinase (MAPK) activation, which are mechanisms of known significance in BC development. Taken together, our results suggest, for the first time, that an iron deficiency-mediated pro-angiogenic environment could contribute to the high recurrence of BC in young patients, and iron accumulation-associated pro-oxidant conditions could lead to the high incidence of BC in older women.

Keywords

Angiogenesis; breast cancer; estrogen; iron; menopause; oxidative stress

*To whom correspondence should be addressed: Department of Environmental Medicine and NYU Cancer Institute, NYU School of Medicine, HJD Room 1600, 550 First Avenue, New York, NY 10016 Fax: (212) 598-7604 xi.huang@nyumc.org.

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Introduction

Estrogen is the single most important risk factor for breast cancer (BC) [1]. Yet high estrogen levels in young premenopausal women cannot explain the high recurrence rate of BC with estrogen receptor negative (ER⁻) status and high tumor grades in young patients [2]. Paradoxically, when overall systemic estrogen levels are decreased by 90%, the BC incidence rate is increased several fold in older postmenopausal women with mostly low grade but estrogen receptor positive (ER⁺) tumors [3–5]. Specific risk factors that contribute to these discrepancies in BC outcomes between pre- and postmenopausal women have not been identified.

Young post-pubescent women in their reproductive years are exposed to high systemic estrogen levels that are endocrinologically regulated by the hypothalamus-pituitary-ovary axis [6]. Menstruation, which results from the detachment of the endometrial matrix prepared for egg fertilization, commonly causes iron deficiency among women of this age group [7,8]. The natural biological system in young women is high estrogen and low iron. As women pass through the menopausal transition, ovarian function diminishes and then ceases. Systemic levels of estrogen drop and the cessation of menstruation increases body iron levels in the form of ferritin by two to three fold. In older women, the system becomes low estrogen and high iron. Indeed, we have shown that a concomitant but inverse change occurs in estrogen and iron levels during menopausal transition [9].

We have previously hypothesized that in premenopausal women, an iron deficiency due to menstruation stabilizes hypoxia inducible factor-1 α , which increases vascular endothelial growth factor (VEGF) formation. This mechanism makes the premenopausal patients susceptible to angiogenesis and, consequently, leads to higher BC recurrence rates [10]. Conversely, increased iron levels in postmenopausal women due to menstrual period cessation contribute to higher BC incidence rates *via* the oxidative stress pathway. Therefore, an imbalance in iron levels could account for some important features of BC that are unexplained by estrogen.

Because human studies are correlative in nature and appropriate animal models that recapitulate estrogen and iron conditions *in vivo* are lacking, this hypothesis was first tested in the *in vitro* menopausal systems. We developed two cell culture models mimicking the premenopausal high estrogen and low iron conditions and postmenopausal low estrogen and high iron conditions. Two distinct sets of biomarkers, linked to either BC recurrence or its onset, were measured. Further, to exclude estrogen as a confounding factor in iron's role, mice with intact ovaries but fed iron deficient and overload diets were used to validate our *in vitro* finding.

Materials and methods

Reagents and cells

Ferritin, apo-transferrin (Tf without iron), holo-transferrin (two binding sites of Tf are fully saturated with iron), 17 β -estradiol (E2) water soluble, and anti-tubulin antibody were purchased from Sigma Chemical Co., (St. Louis, MO, USA). Antibodies against phospho-ERK, JNK, and P38, as well as non-phosphorylated counterparts were purchased from Cell Signaling (Danvers, MA, USA). Human BC cell line MCF-7 was purchased from the American Type Culture Collection. Bovine capillary endothelial (BCE) cells were a kind gift of Dr. Paolo Mignatti (Department of Cell Biology, NYU School of Medicine). For initial MCF-7 cell culture conditions, iron-free α -MEM containing 10% fetal bovine serum was supplemented with L-glutamine and antibiotics. For treatment, serum-free α -MEM was supplemented with selenium (5 ng/ml) and insulin (5 μ g/ml) (Sigma).

Development of tissue culture models mimicking pre- and postmenopausal conditions

Based on the concurrent and inverse changes in E2 and Fe [9], the concentration of E2 was set at 500 pg/ml, equivalent to breast tissue levels under premenopausal conditions [11]. The level of ferritin under postmenopausal conditions was at 20 ng/ml, equal to tissue levels containing 10% serum, given that the physiologic upper limit of serum ferritin is 200 ng/ml [12]. Transferrin was added in its wholly unsaturated form (apo-Tf), or its fully 100% iron saturated form (holo-Tf), at 5 µg/mL, to the pre- and postmenopausal models, respectively.

Cell treatments and Western blot

MCF-7 cells were seeded in a 6-well plate containing 2 ml complete α -MEM. After 24 h incubation, culture media was replaced with freshly prepared premenopausal (high E2, low Fe) or postmenopausal (low E2, high Fe) media. Cells were then exposed to hypoxia (1% O₂) for 6 h or normoxia (see Figure 1 for details). For HIF-1 α Western blotting, cells lysates were probed with mouse anti-human HIF-1 α antibody (Novus Biologicals). Immunoblotting for phosphorylation of ERKs, JNKs, and p38 was performed using phosphospecific antibodies against phosphorylated sites of ERKs, JNKs, and p38, respectively. Non-phosphospecific antibodies against ERKs, JNKs, and p38 and β -tubulin were used to normalize the phosphorylation and to show equal protein loadings.

In vitro angiogenesis

BCE cells were seeded in a 6-well, gelatin-coated plate. After washing, BCE cells were incubated with culture media collected from MCF-7 grown under the two menopausal conditions and photographed at 24 h and 48 h, respectively.

In vivo validation of iron deficiency on angiogenesis and iron overload on oxidative stress

All animal experiments were performed according to the protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the New York University School of Medicine. Twelve 3-week old Balb/C mice were purchased from Jackson Laboratory (Bar Harbor, MA) and randomly divided into four groups. To exclude estrogen as a confounding factor, mice with intact ovaries were used so that E2 levels were comparable among groups. Mice were fed diets containing 3.5 ppm, 35 ppm, 350 ppm, and 3,500 ppm ferrous sulfate, respectively (Dyets Inc., Bethlehem, PA). After 23 weeks on the iron diets, the mice were sacrificed and blood was collected by heart puncture. Livers and kidneys were collected to isolate RNA and proteins. The very limited amount of mammary fat pads only allowed us to isolate RNA.

Serum iron status, including serum iron and transferrin saturation, was measured as previously described by our laboratory [13]. Body iron status was evaluated by mRNA levels of erythropoietin (EPO), a hormone promoting formation of red blood cells in the kidney, and hepcidin, a peptide hormone controlling iron uptake in the liver. In brief, total RNA from the kidney, liver, and mammary fat pads were isolated by Trizol (Invitrogen, Carlsbad, CA) and 1 µg RNA per sample was reverse-transcribed into cDNA by SuperScript® III (Invitrogen). The quantitative real-time PCR (qRT-PCR) was run in a 384-well plate (ABI 7900 series, Applied Biosystems, Foster City, CA). The mRNA expression levels of EPO in the kidney, hepcidin in the liver, and HIF-1 α and VEGF in the mammary fat pads were normalized to the geomean of three housekeeping genes, GAPDH, G6PD, and HPRT1 in the control group (35 ppm Fe). Data were expressed as fold changes over the control.

Measurements of VEGF and oxidants in cell and animal tissues

Levels of VEGF in cell culture media and tissue extracts were measured using a commercially available kit (R & D Systems). To detect intracellular oxidant formation, 2,7-dichlorofluorescein diacetate (Invitrogen), a fluorescent probe sensitive to oxidants, was employed [14]. Lipid peroxidation levels were determined by measuring fluorescence intensities of thiobarbituric acid-reactive substances at excitation of 515 nm and emission of 553 nm [15]. MCF-7 cells were lysed with M-PER solutions (Pierce) and liver tissues were homogenized in a 1 ml ice-cold RIPA lysis buffer before measurements. The results were normalized by protein concentrations.

Statistics

All values were expressed as means + SD. Student's *t* tests were used for comparison between experimental groups. Two levels of significance were used at P value of less than 0.05 or 0.01.

Results

MCF-7 cells grown under high E2 and low Fe are pro-angiogenic

We chose VEGF as a target gene for this study because VEGF has been associated with a poor prognosis in BC. Patients with early stage BC whose tumors have elevated levels of VEGF have a higher likelihood of recurrence and death than patients with tumors that have low-angiogenic levels [16]. Figure 1A shows that MCF-7 cells grown in pre-menopausal high E2 and low Fe conditions produced high levels of VEGF following a 6-h hypoxic exposure. The increased VEGF levels were further potentiated and persisted when culture conditions were shifted to normoxia. These same cells, grown in conditions mimicking the postmenopausal state of low E2 and high Fe, yielded lower levels of VEGF and were less responsive to hypoxic treatment as compared to the premenopausal condition (Figure 1A) or the control without E2 and Fe (data not shown). When BCE cells were incubated with culture media collected from MCF-7 grown in either pre- or post-menopausal conditions, the high E2 and low Fe levels promoted the elongation and extension of BCE cells as well as capillary development, a morphological change towards angiogenesis (pictures not shown). In contrast, BCE cells treated with low E2 and high Fe media appeared atrophied with cell shortening. HIF-1 α is a major transcription factor controlling VEGF expression, and the cascade of HIF and VEGF plays a key role in tumor angiogenesis, metastasis, and recurrence [17]. Figure 1B shows that background levels of HIF-1 α under normoxia as well as under hypoxia were higher in cells grown under premenopausal conditions than in the same cells grown under postmenopausal conditions. HIF-1 α was up-regulated by hypoxia in the untreated control and Ni-treated positive control. These results suggest that the differences in VEGF levels were likely due to differences in HIF-1 α stabilization.

Strong inhibition of VEGF by ferritin *in vitro*

E2, transferrin, and ferritin were analyzed separately in MCF-7 cells to determine their relative contributions to the alterations in VEGF levels. Figure 2A demonstrates a slight but significant increase in VEGF formation with the addition of E2 from physiological to pharmacological levels (10 and 100 nM). Importantly, however, the addition of ferritin starting at a concentration of 1 ng/ml significantly decreases VEGF in a dose-dependent manner (Figure 2B). Neither iron-free apo-transferrin at various concentrations (0–160 μ g/ml) nor the same concentration of transferrin (5 μ g/ml) at different iron saturations (0–100% iron saturation) had significant effects on VEGF formation (Figure 2C and 2D).

MCF-7 cells grown under low E2 and high Fe are pro-oxidant

Iron is known to catalyze Fenton, Haber-Weiss, and iron autoxidation ($\text{Fe}^{2+}\text{-O}_2$) reactions, leading to oxidant formation [18,19]. We measured another set of biomarkers to investigate whether increased iron after menopause forms oxidants and alters oxidant-responsive signaling pathways. Figure 3A demonstrates that MCF-7 cells grown in postmenopausal conditions of low E2 and high Fe were oxidatively damaged as measured by lipid peroxidation. Initial starting levels of lipid peroxidation were higher in MCF-7 cells grown in conditions imitating postmenopause as compared to premenopause. Interestingly, in the presence of H_2O_2 (10 μM H_2O_2 for 4 h), MCF-7 cells grown in postmenopausal conditions induced significant lipid peroxidation, but the same cells grown in premenopausal conditions were protected from H_2O_2 -induced lipid peroxidation. Addition of the fluorescent oxidant probe, 2',7'-dichlorofluorescein diacetate, to MCF-7 cells grown under post-menopausal conditions resulted in an increase in fluorescence intensity (data not shown). Furthermore, phosphorylations of ERKs and p38 MAPK were all activated by 10 μM H_2O_2 at 10 min but were sustained at 1 h only in cells grown under postmenopausal conditions (Figure 3B).

Iron status in mice fed different iron diets

To further validate our *in vitro* findings, mice fed four different levels of iron in their diets were used. Consistent with previous literature reporting that iron levels in diets ranging from 35 to 350 ppm were considered normal [20,21], these two dietary iron levels did not lead to significant differences in serum iron and transferrin saturation rates (Figure 4A). Interestingly, an iron level at 3.5 ppm in the diet caused an iron deficiency in mice, with a transferrin saturation rate at approximately 20%, and an iron level at 3500 ppm triggered an iron overload. Although the serum iron status showed no difference between normal low (35 ppm) and normal high (350 ppm) iron levels, EPO levels in the kidney showed a dose-dependent decrease (Figure 4B), indicating that iron deficiency and anemia increase EPO and promote the formation of red blood cells [22]. On the other hand, hepcidin levels in the liver demonstrated a dose-dependent increase (Figure 4C), revealing that iron overload increases hepcidin and inhibits further iron uptake from the diet [23]. These results also suggest that EPO and hepcidin are more sensitive iron markers than serum iron and transferrin saturation.

Levels of HIF-1 α , ferritin, VEGF, and lipid peroxidation in mice

Figure 5 shows that there were no significant differences in HIF-1 α , ferritin, and VEGF protein levels in the liver tissue samples between mice fed normal low (35 ppm Fe) and normal high (350 ppm Fe) iron diets. However, when comparing iron deficiency to iron overload, HIF-1 α was approximately 50% higher under iron deficient conditions (3.5 ppm Fe) than under iron overload conditions (3500 ppm Fe) (Figure 5A). Conversely, ferritin levels in mice fed iron overload diets were much greater than in those fed iron deficient diets. The absence of ferritin protein in the livers of mice fed 3.5 ppm iron suggests that iron storage in the liver was deprived. Liver VEGF was higher in mice fed iron deficient diets, approaching statistical significance, than mice fed iron overload diets (Figure 5B).

It is noteworthy that mammary fat pad in mice is a relevant organ to breast in humans. Because of the small amounts collected, we were not able to carry a Western blot. However, qRT-PCR showed that mRNA levels of HIF-1 α and VEGF in the mammary were approximately 15- and 7-folds higher as compared to the other three groups, respectively (Figure 6A and 6B). On the other hand, levels of lipid peroxidation in the livers of mice fed iron overload diets were at least 4.5 times higher than in the other three groups (Figure 6C).

Discussion

Estrogen and iron are two important growth factors in a female's body development. Estrogen influences the growth, differentiation, and function of tissues of the female reproductive system, *i.e.*, the uterus, ovary, and breast [24]. Iron is essential for oxygen transport, DNA synthesis, and energy metabolism [25]. In the present study, our *in vitro* and *in vivo* data suggest a pro-angiogenic environment in young premenopausal women with high systemic estrogen but low iron levels (Figures 1, 5, and 6). This is consistent with previous reports showing that levels of VEGF in normal breasts as well as in breast tumors were significantly higher in pre- than in postmenopausal subjects [26,27].

Separate testing of each individual component in our model systems demonstrated that E2 only slightly up-regulated VEGF (Figure 2A), which is in agreement with a previously published report [28]. Most importantly, we have found that ferritin was a major inhibitor of VEGF (Figure 2B) and, thus, lack of ferritin or iron deficiency stabilizes it. Hypoxia, an *in vitro* condition simulating anemia *in vivo*, dramatically increased VEGF. These results suggest that iron deficiency and anemia rather than high estrogen level is probably the driving force for increased VEGF levels in young women.

Our finding is clinically relevant because iron deficiency and iron deficiency anemia (IDA) affect 20% of non-pregnant women aged 16 to 49 in industrialized countries and over 40% of all women in developing countries [8]. Our results indicate that at least two distinguished mechanisms exist in iron-mediated HIF regulation and VEGF formation. First, iron deficiency and IDA at the systemic level induce HIF-1 α because fewer red blood cells transport oxygen in the bodies of young women, resulting in a hypoxic condition in the breasts [29]. Intratumoral hypoxia and HIF are hallmarks of BC malignancy and aggressiveness [29–31]. To support our view on iron deficiency and hypoxia, an association between low iron levels, measured as hemoglobin, and increased serum VEGF has been reported in cancer patients [32]. A correlation was also established between anemia and intratumoral hypoxia [29,33].

Second, iron deficiency and IDA stabilize HIF-1 α by lowering HIF-degrading prolyl hydroxylases because iron is a co-factor of the enzymes [34,35]. Stability of HIF-1 α is predominately regulated at the protein levels by iron- and oxygen-dependent prolyl hydroxylation, which lead to rapid pVHL (von Hippel-Lindau protein)-mediated ubiquitination and proteasomal degradation [34,36]. In support of the notion that iron deficiency favors HIF-1 α stability, cells grown under the premenopausal condition of high estrogen and low iron lead to a stronger HIF-1 α band than the same cells grown under a postmenopausal condition of low estrogen and high iron (Figure 1C). Mice fed iron deficient diets showed increased levels of HIF-1 α and VEGF as compared to mice fed iron overload diets. Moreover, iron chelation significantly induced and stabilized HIF-1 α [37,38].

Taken together, considering that iron deficiency and anemia are highly prevalent in young women, these ailments may increase HIF-1 α induction and stabilization as well as production of VEGF through the activated HIF-1 α pathway. An increase in VEGF levels could subsequently promote tumor angiogenesis and metastasis and, consequently, higher tumor grades and greater recurrence rates in young BC patients [10].

In the present study, we have also shown that increased iron as a result of menopause provides a pro-oxidant environment (Figures 3 and 6), which could make post-menopausal women more susceptible to oxidative stress. Indeed, investigations of normal and cancerous tissues have revealed a substantial increase in base lesions in the DNA of invasive ductal carcinoma of the female breast [39–41]. Elevated levels of ferritin are detected in BC tissue

samples [42] and hemochromatosis patients with iron overload condition are at increased risk of BC [43].

As mentioned earlier, the majority of serum E2 in premenopausal women is derived from ovarian secretion and subsequently distributed through the bloodstream in an endocrine fashion. In postmenopausal women, almost all of the circulating E2 is from extragonadal conversion of C19 steroid precursors [44]. The conversion occurs locally in breast tissues and E2 acts in a paracrine fashion [45]. Therefore, we must bear in mind that the breast tissues in postmenopausal women are exposed not only to high iron as a result of the cessation of menstruation [9,10], but also to high estrogen, which is produced locally by aromatase CYP19A1 [44,46]. Both estrogen and iron are considered cancer promoters. We have previously shown that high E2 and high Fe synergistically enhance cell proliferation in ER⁺ cells [47].

Moreover, high estrogen levels could result in redox cycling of estrogen metabolites and the formation of superoxide anions [48]. These superoxide anions are capable of reducing ferritin-bound Fe³⁺ to Fe²⁺, causing further oxidative DNA damage [49]. Dietary iron intake also significantly increases estrogen-induced kidney tumors in Syrian hamsters [50]. Thus, our results indicate that increased iron levels after menopause, along with high estrogen levels at the local breast tissues, may play important roles in increased BC incidence, mostly of low grade but ER⁺ status, in post-menopausal women [10].

To date, most research involving iron in cancer has been mainly centered on iron overload causing oxidant-mediated cancer promotion [18,51–53]. The present study investigated two ends of the same iron spectrum, namely deficiency and over-sufficiency, and examined how changes in iron levels affect the body system or the host. Based on the “seed and soil” hypothesis [54], the pathogenesis of tumor angiogenesis, metastasis, and recurrence is dependent on both intrinsic properties of tumor cells (seed) and the response of the host (soil). Our data indicate that iron deficiency and anemia in young women may provide a host factor that favors the poor prognosis observed in this young age group of BC patients by increasing HIF-1 α and VEGF. Conversely, increased iron levels in postmenopausal women may contribute to breast cancer initiation, promotion, and high BC incidence rates *via* the oxidative stress pathway. For the first time, our data suggests that iron could be a risk factor affecting BC outcomes before and after menopause. Future studies are needed to test our concepts that tumor cells disseminate greatly in iron deficient mice but spontaneous tumors develop in high estrogen and high iron mice.

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Abbreviations

A.U	arbitrary units
BC	breast cancer
E2	17 β -estradiol
EPO	erythropoietin
ER	estrogen receptor
HIF-1α	hypoxia-inducible factor-1 α

IDA	iron deficiency anemia
MAPK	mitogen-activated protein kinases
VEGF	vascular endothelial growth factor

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Figure 1. Effects of high E2 and low Fe versus low E2 and high Fe on VEGF formation, *in vitro* angiogenesis, and HIF-1 α stabilization

(A) MCF-7 cells grown under pre- (high E2 and low Fe) or postmenopausal conditions (low E2 and high Fe) were exposed to 1% O₂ for 6 h, followed by overnight culture under normoxia (hypoxia + culture). (B) MCF-7 grown under the two conditions were exposed to normoxia or hypoxia (1% O₂) for 6 h and then lysed for HIF-1 α blotting. A representative gel from three independent experiments was displayed. Bar graph below shows quantitation by densitometry after normalizing to the housekeeping gene and then to the control under normoxia. Ni was used as a positive control for HIF-1 α induction and β -tubulin as a loading control of proteins. Results are reported as the mean $\pm$ SD. *: Significantly different among the groups compared by Student's *t* test (n=6).

Figure 2. Effects of E2, ferritin, and transferrin on VEGF formation

MCF-7 cells were grown in α -MEM media in the presence of various concentrations of (A) E2, (B) ferritin, (C) apo-transferrin (no iron), and (D) the same amounts of transferrin (5 μ g/ml) but with different iron saturations. After 24 h treatment, cell culture media were collected for VEGF measurements. E2: 17 β -estradiol; Ftn: ferritin, Tf sat: transferrin saturation. Significantly different among the groups compared (n=4).

(A) MCF-7 cells were grown under pre- and postmenopausal conditions. After overnight culture, cells were collected or further exposed to 10 μ M H₂O₂ for 4 h before lipid peroxidation measurements. (B) MCF-7 cells grown under the two conditions were treated with or without 10 μ M H₂O₂ for various times. Cells were lysed and probed for phosphorylated ERK, p38, and JNK (shown) and non-phosphorylated ERK, p38, and JNK (data not shown). A representative gel from three independent experiments was displayed. β -tubulin was used to show an equal loading of proteins. Ratios indicate quantitation by densitometry after normalizing to the β -tubulin and then to the untreated control. a.u.: Arbitrary units. Significantly different among the groups compared (n=6).

Figure 4. Body iron status in mice fed four different levels of iron diets

(A) Serum iron and transferrin saturation rate in mice fed 3.5 ppm iron diet (iron deficient), 35 ppm and 350 ppm iron diets (normal low and normal high iron levels), and 3500 ppm iron diet (iron overload). (B) mRNA levels of EPO by qRT-PCR in kidneys of mice fed different levels of iron diets. (C) mRNA levels of hepcidin by qRT-PCR in livers of mice fed different levels of iron diets. Significantly different among groups compared (n=3) per group.

Figure 5. Effects of iron deficiency and overload on liver HIF-1 α stabilization and VEGF formation

(A) Protein expressions of HIF-1 α and ferritin heavy chain in livers of mice fed four different levels of iron diets. Bar graphs on the right represent average level of HIF-1 α and heavy-chain (H)-ferritin from three mice per group. The band intensities were first quantitated by densitometry, normalized to the loading control, and then the control group fed 350 ppm iron diet. (B) Levels of liver VEGF in the mice fed four different levels of iron diets (n=3).

Figure 6. Effects of iron deficiency on mammary mRNA levels of HIF-1 α and VEGF and iron overload on liver lipid peroxidation

(A) Levels of mRNAs of HIF-1 α (A) and VEGF (B) by qRT-PCR in mammary fat pads of mice fed different levels of iron diets. (C) Levels of lipid peroxidation from the liver samples of the same mice and data were expressed as a.u. per mg protein (n=3). Significant different among the groups compared (n=3).