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Disordered signaling governing ferroportin transcription favors breast cancer growth

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ABSTRACT

Iron is a necessary chemical element needed by all organisms. Iron metabolism is finely tuned in mammals, and the hepcidin–ferroportin (FPN) axis is the central signaling in governing systemic iron homeostasis. Deregulation of this signaling would lead to iron disorders and likely other diseases including cancers. Reduced FPN was previously found to correlate with poor prognosis in breast cancer patients. Nonetheless, the biological effects of abnormal FPN expression in tumor cells remain largely unexplored, and the mechanisms underlying misregulated expression of FPN in cancers keep elusive. In the current study, we scrutinized the effects of abnormal FPN on tumor growth and the molecular mechanisms of diminished tumor FPN. Downregulation of FPN significantly promoted breast cancer growth, whereas FPN upregulation impeded tumor growth. We demonstrated that the transcription factors Nrf2 (nuclear factor erythroid 2-like 2) and MZF-1 (myeloid zinc finger-1) synergistically transactivated FPN expression in breast cancer cells. Moreover, CpG island methylation at the FPN promoter was the reason of attenuated FPN expression. Downregulation of Nrf2 and MZF-1 and hypermethylation of the FPN promoter were concurrently associated with decreased FPN concentration in breast tumors. Taken together, our study highlighted the contribution of disordered iron metabolism to breast cancer growth, and also signified an oncogenic effect of misregulated ferroportin in breast cancers. This work represents a promising starting point to the possibility of restraining breast cancer through targeting FPN or its upstream regulatory factors.

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1. Introduction

Iron is a mineral necessary for a myriad of biological processes, such as heme synthesis, DNA replication, and enzyme-mediated functions [1,2]. Systemic iron homeostasis is tightly governed under normal settings and disorders, and the hepcidin–ferroportin (FPN) axis is predominantly responsible for the systemic iron supply, utilization, recycling, and storage [3,4]. FPN is the sole iron exporter identified thus far in mammalian cells [5,6], and is highly expressed in duodenal enterocytes, macrophages and hepatocytes [7–9]. As a crucial node within the iron homeostasis network, FPN governs iron egress outside of cells. FPN mutations would lead to iron overload and consequently to diseases associated with either low or normal transferrin saturation [10,11]. Mounting evidence unravels that dysregulation of FPN may also contribute to cancer development including hepatocellular carcinoma and breast cancers [12–14]. For instance, reduced FPN expression was found to correlate with poor prognosis, whereas a better survival rate was associated with breast cancer patients with high FPN levels [13]. Additionally, we recently uncovered mechanisms responsible for an adapted hepcidin–ferroportin axis in breast cancer [15]. Nonetheless, the biological effects of abnormal FPN on tumor behaviors and the molecular mechanisms underlying the misregulation of FPN in cancer remain largely unexplored.

Previous studies manifest that the FPN concentration is modulated through 3 mechanisms, *i.e.* post-translational regulation, posttranscriptional regulation, and transcriptional regulation. The molecular mechanisms responsible for the former two regulations have been extensively investigated. Hepcidin, a small peptide hormone, mainly secreted by liver, directly binds to FPN and induces internalization, ubiquitination and degradation of FPN [5,16,17]. Thus, hepcidin-induced FPN degradation primarily accounts for the regulation of FPN at the post-translational level. On the other hand, FPN translation is tightly modulated by iron through the iron-regulatory protein (IRP)/iron-

Abbreviations: FPN, ferroportin; Nrf2, nuclear factor erythroid 2-like 2; MZF-1, myeloid zinc finger-1; IRP, iron-regulatory protein; IRE, iron-responsive element; UTR, untranslated region; PBS, phosphate-buffered saline; ChIP, chromatin immunoprecipitation; TSS, transcriptional starting site; MARE, Maf recognition elements; ARE, antioxidant response elements; HRE, hypoxia response element.

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responsive element (IRE) system [18,19]. FPN mRNA contains a stem loop structure called IRE within its 5′-untranslated region (UTR). Under iron depletion, IRPs inhibit the translation of FPN by occupying the IRE; in contrast, when iron is in excess, FPN translation is enforced by dissociation of IRPs upon iron binding [20]. However, our current understanding of the transcriptional regulation of FPN is still rather limited. Burgeoning studies have documented that HIF-2α, Nrf2 and MTF-1 transcriptionally modulate FPN expression in mouse macrophages and other non-tumor cells [21–23]. Other than these, the upstream signaling responsible for FPN's transcriptional regulation remains elusive, especially in tumor cells.

In the current study, we aimed at scrutinizing the molecular mechanisms underlying misregulated FPN in breast cancers. We demonstrate that the level of FPN crucially affects breast tumor growth through determining intracellular iron concentration. Mechanistic investigations unravel that misregulated transcription of ferroportin by MZF-1 and Nrf2, and hypermethylation of the ferroportin promoter, jointly favor breast cancer development.

2. Material and methods

2.1. Cell culture

Human breast cancer cell lines MDA-MB-231, human prostate cancer cell line PC3 and human embryonic kidney cell line HEK293T were from the Shanghai Cell Bank of Type Culture Collection of Chinese Academy of Sciences. Cell culture was carried out, as described previously [24,25].

2.2. Clinical specimens

All tumor and serum specimens were provided by Weihai Wendeng Central Hospital, China, with consent according to the guidelines of the Institutional Ethics Committee. Tumor specimens were collected from all patients with no other diseases. All sera and tumor specimens were stored at −80 °C.

2.3. Animal experiments

All animal experiments were performed with the approval of the Animal Ethics Committee at the Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences. All SCID/beige mice were purchased from the Vital River Laboratories, China. Mice were housed under a sterile and pathogen-free condition. Cancer cell inoculation was operated according to the methods described previously [24,25]. The tumor size was measured every 2 days and calculated according to the formula $\pi/6 \times L \times W^2$. When tumor size reached 1.0 cm^3 or mice significantly suffered from tumors, the animals were sacrificed.

2.4. RNA extraction and qRT-PCR analysis

Total RNAs were extracted from cells using Trizol (Invitrogen) according to the instruction provided by the manufacturer. Total RNAs from tissue samples were isolated with TriPure (Roche) following the manufacturer's instructions after the samples were pulverized in liquid nitrogen. mRNA expression was quantified by qRT-PCR analysis using SYBR Green qPCR master mix (Qiagen). GAPDH was used as an internal control and the primer sequences are presented in S Table 1.

2.5. Western blotting

Cells after treatments were collected and washed twice with phosphate-buffered saline (PBS). Tissue samples were pestled on ice. Total proteins were extracted with ice-cold RIPA lysis buffer (Solarbio, China) with protease inhibitor cocktail (Roche). Equal amounts of protein lysates were subjected to immunoblot according to the method described previously [26], using the following antibodies, GAPDH (1:1000, Santa Cruz Biotechnology), MZF-1 (1:200, Santa Cruz Biotechnology), Nrf2 (1:200, Santa Cruz Biotechnology), ferritin (1:500, Abcam) and FPN (1:500, Sigma-Aldrich).

2.6. Cell proliferation and colony formation assays

Cell growth was determined by cell counting. Briefly, cells were starved in serum-free medium overnight and then seeded in 24-well plates with different treatments. Cell growth was assessed at different time points. For colony formation assay, cell suspension at a density of 5 cells/ml was prepared, and 100 μl of complete medium per well was inoculated into 96-well plates. After 4 h, all 96 wells were examined under a microscope and the wells containing one cell were selected. Cells were checked for up to 12 days. Wells containing colonies were recorded at day 12, and the percentage of colony formation was calculated.

2.7. Microarray analysis

MDA-MB-231 cells were cultured in serum-free medium for 24 h with or without the addition of 10% human sera from our breast cancer patients. Thereafter, total RNAs were extracted for microarray analysis, which was performed at the CapitalBio, Inc., China. The 35 k human Genome Array microchips, containing 35,035 probes, representing 25,100 genes and 39,600 transcripts, were used. The experimental procedure was similar to the one previously described [27]. Significance Analysis of Microarrays (SAM, version 3.02) was used to determine the significantly expressed genes. Differentially expressed genes were defined by changes of gene expression of either 2-fold greater or lower than 0.5-fold in serum-treated cells compared to the untreated control with FDR <0.01. Gene expression data are available in NCBI GEO database, accession no. GSE40108.

2.8. Prediction of transcription factor binding sites and ChIP assay

A 3000-bp fragment from the human FPN promoter region was analyzed for potential transcription factor binding sites using the web-based software TFSEARCH. One Nrf2 binding site (−2656/−2647) and three MZF-1 binding sites were identified and for further validation. ChIP assays were performed using the ChIP assay Kit (Millipore) with minor modifications. Briefly, cells were fixed with 1% formaldehyde for 10 min at 37 °C, and washed with cold PBS supplemented with 1 mM PMSF. Fixed cells were resuspended in lysis buffer, and chromatin was sonicated into sizes ranging from 200 to 1000 bp. After sonication, the supernatant containing chromatin was diluted in ChIP dilution buffer. At this step, a small aliquot of chromatin was saved as input control. Seventy microliters of chromatin was used to incubate with 2 μg anti-Nrf2 Ab, anti-MZF-1 Ab, or normal IgG. The immunoprecipitated DNA was purified and then analyzed by PCR. Primer sequences are listed in Table 2. The PCR conditions were as follows: 94 °C 3 min, then 94 °C 20 s, 63 °C 20 s, and 72 °C 20 s for 32 cycles. PCR products were subjected to agarose gel electrophoresis, and visualized by ethidium bromide staining.

2.9. Luciferase reporter assays

DNA fragments bearing full- or deleted-binding motifs for Nrf2 and MZF-1 were introduced into a luciferase reporter system. Briefly, DNA fragments containing complete binding motifs for Nrf2, MZF-1 Site A, MZF-1 Site B and MZF-1 Site C were cloned from human genomic DNA, and then subcloned into the pGL3-Promoter luciferase reporter vector (Promega). Deletion of core binding motifs for Nrf2 and MZF-1 was carried out with a site-directed mutagenesis kit (Biomed, China). All constructs were validated by DNA sequencing (Invitrogen). Cells were co-transfected with 0.8 μg of the target firefly plasmid and 80 ng

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