Original Article

An important role of the hepcidin–ferroportin signaling in affecting tumor growth and metastasis

Wenli Guo¹, Shuping Zhang¹,², Yue Chen¹,³, Daoqiang Zhang⁴, Lin Yuan⁴, Haibo Cong⁴, and Sijin Liu¹,*

¹State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China, ²Institute for Medical Engineering and Science, Massachusetts Institute of Technology, Cambridge, MA 02139, USA, ³Department of Urology, The Second Hospital of Tianjin Medical University, Tianjin Institute of Urology, Tianjin 300211, China, and ⁴Weifang Medical College, Wenda Central Hospital, Weihai 264400, China

*Correspondence address. Tel/Fax: +86-10-62849330; E-mail: sjliu@rcees.ac.cn

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Abstract

Epidemiological and experimental studies have suggested that deregulated hepcidin–ferroportin (FPN) signaling is associated with the increased risk of cancers. However, the effects of deregulated hepcidin–FPN signaling on tumor behaviors such as metastasis and epithelial to mesenchymal transition (EMT) have not been closely investigated. In this study, LL/2 cancer cells were found to exhibit an impaired propensity to home into lungs, and a reduced ability to develop tumors was also demonstrated in lungs of Hamp1−/− mice. Moreover, hepatic hepcidin deficiency was found to considerably favor tumor-free survival in Hamp1−/− mice, compared with wild-type mice. These data thus underscored a contributive role of hepatic hepcidin in promoting lung cancer cell homing and fostering tumor progression. To explore the role of FPN in regulating tumor progression, we genetically engineered 4T1 cells with FPN over-expression upon induction by doxycycline. With this cell line, it was discovered that increased FPN expression reduced cell division and colony formation in vitro, without eliciting significant cell death. Analogously, FPN over-expression impeded tumor growth and metastasis to lung and liver in mice. At the molecular level, FPN over-expression was identified to undermine DNA synthesis and cell cycle progression. Importantly, FPN over-expression inhibited EMT, as reflected by the significant decrease of representative EMT markers, such as Snail1, Twist1, ZEB2, and vimentin. Additionally, there was also a reduction of lactate production in cells upon induction of FPN over-expression. Together, our results highlighted a crucial role of the hepcidin–FPN signaling in modulating tumor growth and metastasis, providing new evidence to understand the contribution of this signaling in cancers.

Key words: hepcidin, ferroportin, iron, cancer, metastasis, epithelial to mesenchymal transition (EMT)

Introduction

Systemic iron homeostasis is regulated by diverse signaling pathways, of which the hepcidin–ferroportin (FPN) axis plays a central role [1]. The iron-regulatory hepcidin is a small peptide with 25 amino acids, and is predominantly synthesized and secreted by hepatocytes [1]. Hepcidin systemically regulates iron flow through suppressing iron absorption from duodenum and iron egress from macrophages by controlling the membrane concentration of its receptor, FPN [1–3].
FPN is expressed in various types of cells with a high level of expression in macrophages and hepatocytes, and it is the only known iron exporter in mammalian cells thus far [1]. Hepcidin controls FPN concentration through binding and triggering its degradation in a ubiquitin-dependent manner [1–3]. Not limited to the cells described above, hepcidin and FPN are abnormally expressed in cancer cells with diagnostic significance, such as breast cancer cells [4–6]. Relative to adjacent tissues, the concentration of FPN is greatly diminished in human breast cancer cells [4,5]. Importantly, the expression level of FPN in breast cancer cells is predictive of prognosis for patients [4,5], as diminished FPN is associated with poor prognosis for the 10-year probability of distant metastasis for survival, in contrast to a better metastasis-free survival for patients with high FPN levels in a combined cohort with 504 patients [3]. These findings suggested a crucial role of FPN in governing breast cancer growth and progression. In contrast, although breast cancer cells are also found to express hepcidin [4,5], its expression level only reveals a marginal significance as a prognostic marker for the patient prognosis [3], suggesting that hepatic production is the predominant source of circulating and local hepcidin.

Despite progressive advances in recognizing the contribution of hepcidin–FPN signaling to cancer growth and progression, a few questions still remain unanswered. First, although increased serum hepcidin levels were observed in various cancer types [4,7–11], the biological impacts of increased serum hepcidin on tumor behaviors remain elusive; and no study has been carried out to look at tumor development in the absence of hepcidin. Our recent study has demonstrated that the inhibition of hepatic hepcidin (namely circulating hepcidin) upon administration of heparin repressed breast tumor growth in mice [4]. However, there are some limitations yet to be addressed. For example, heparin itself incurs robust inhibition on tumors through diverse mechanisms such as anti-angiogenesis and suppression of extracellular-matrix protease heparanase [12]. Secondly, with respect to the abnormal expression of FPN in tumor cells, although previous in vitro and in vivo studies have demonstrated the involvement of FPN in regulating tumor growth, the insights into the impact of FPN on tumor progression are still limited. For instance, the possible effects of abnormal FPN on epithelial to mesenchymal transition (EMT) and metastasis have not been studied thus far.

In this study, we aimed to investigate the effects of deregulated hepcidin–FPN signaling on tumor progression with specific interest in metastasis and EMT. Our findings provide new insights into the mechanisms underlying the important role of deregulated hepcidin–FPN signaling in cancers, and pinpoint this signaling as a promising anti-cancer therapeutic target.

**Materials and Methods**

**Human tumor specimens**

All breast tumor specimens were collected from Weihai Wendeng Central Hospital, Shangdong Province, China, as described in our recent report [4]. This study with human tumor specimens was approved by the Institutional Ethics Committee, and complied with the relevant regulations established by the Weihai Wendeng Central Hospital.

**Cell culture**

Murine breast cancer cell line 4T1 tagged with luciferase gene was obtained from Caliper Lifesciences (Hopkinton, USA). Mouse Lewis lung cancer cell line LL/2 was purchased from the Shanghai Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The 4T1 cells and LL/2 cells were cultured in RPMI-1640 medium (Gibco, Grand Island, USA) and Dulbecco’s modified Eagle’s medium (Hyclone, Grand Island, USA), respectively. Cells were grown in culture medium supplemented with 10% fetal bovine serum (Hyclone) and 100 U/ml penicillin-streptomycin (Hyclone) at 37°C under 5% CO2, as previously described [4].

**Plasmid construction and viral packaging**

The FPN-GFP fusion construction was generously provided by Dr Tomas Ganz of University of California (Los Angeles, USA) [13]. The FPN-GFP fragment was then inserted into the pTRIPZ plasmid with two restriction sites, AgeI and MfeI. For the control vector, only GFP was inserted. There is a tet operator in the backbone of the pTRIPZ plasmid, which turns on FPN-GFP or GFP expression upon doxycycline (Dox) induction. Lentiviral packaging and cell infection were carried out using the method described in a previous study [14]. Stable transfectants were selected from single clones with strong GFP fluorescence using the limiting dilution method. After selection, the stable transfectants from 4T1 cells with over-expression of FPN-GFP construct (here termed FPN-GFP cells) or GFP-control construct (here called GFP-control cells) were obtained.

**Flow cytometry analysis**

To determine the fluorescent intensity, GFP signal was assessed by flow cytometry (FACS) analysis. FACS analysis was carried out for cells with or without Dox induction on the BD FACSCalibur™ platform (BD Biosciences, Franklin Lakes, USA), according to the protocols as previously described [15].

**DNA synthesis assay**

DNA synthesis was assayed by the BrdU incorporation assay using a BrdU Cell Proliferation Assay Kit (Roche, Mannheim, Germany). Briefly, cells were serum-starved overnight, and then re-seeded in 96-well plates at a concentration of 3.0 × 103 cells/well in the presence or absence of Dox (1 µg/ml). Cells were continually cultured for 72 or 96 h, and thereafter the BrdU incorporation was determined based on the standard protocol provided by the manufacturer.

**Colony formation assay**

Colony formation was performed according to established method, as described previously [16]. Briefly, cells were seeded in 6-well plates at densities of 400 or 200 cells per well, respectively. Eight days later, colonies in wells were visualized with crystal violet staining. The numbers of colonies were quantified.

**Animal experiment**

All animal experimental protocols were approved by the Animal Ethics Committee at the Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences (Beijing, China). Wild-type (WT) mice were purchased from the Vital River Laboratories (Beijing, China). Hamp1−/− mice under the C57BL/6 background were generously provided by Dr Sophie Vaulont of Institut Cochin (Paris, France) [17] and Dr Tomas Ganz [18]. All mice were housed under the sterile and pathogen-free environment during the experiments. To prevent iron overload, Hamp1−/− mice were iron-depleted by being placed on a low-iron diet (4 ppm iron) for longer than 1 month before weaning and before tumor cell inoculation, following the procedure of a previous study [18]. Meanwhile, these mice were maintained on the low-iron diet after tumor cell inoculation. In the tumor cell homing experiment, LL/2 cells [1.0 × 105 cells in 0.2 ml phosphate-buffered saline
(PBS) were injected into the lateral tail vein of mice. Animal survival was continuously monitored for nearly 3 months. In the experiments with 4T1 cells, the experimental setup was similar to the methods described previously [4,19]. Briefly, 1.0 x 10^4 cells were orthotopically inoculated into the fourth mammary fat pad (MFP) of mice. To induce FPN expression, mice were administered with drinking water containing 2 mg/ml Dox and 4% sucrose. In contrast, mice were administered with drinking water containing 4% sucrose but no Dox to prevent FPN expression. All mice were imaged for luciferase activity every week using the IVIS Spectrum and Living Image Software (Caliper Life Sciences, Waltham, USA). The experiments were terminated when primary tumors reached a size of 1 cm³ or caused significant morbidity. In the luminescent imaging experiment, mice received intraperitoneal injections of 200 µl of 15 mg/ml luciferin (PerkinElmer, Waltham, USA). After 5 min, mice were anesthetized by isoflurane inhalation, followed by imaging. Various organs were also dissected and imaged after mice were sacrificed. Histological examination was performed after hematoxylin–eosin (H&E) staining.

Western blot analysis

After treatments, cells were harvested, and then lysed in the RIPA lysis buffer containing protease inhibitor cocktail (Roche). Protein concentrations were assayed using the Lowry method, and equal amounts of protein (80–120 µg) for each lysate sample were subject to SDS-PAGE. Western blot analyses were then carried out as described previously [4,19]. Antibodies used here were anti-GAPDH (1:1000; Santa Cruz, Dallas, USA), anti-GFP (1:500; Santa Cruz), and anti-caspase-3 (1:500; Cell Signaling Technology, Boston, USA). GAPDH was used as a housekeeping control. Chemiluminescent detection was carried out on the ChemiDoc™ XRS+ System with Image Lab™ Software (Bio-Rad, Hercules, USA).

Cell cycle analysis

Cell cycle progression was determined following a standard protocol described in our previous study [20]. Briefly, after treatments, cells were washed with PBS, and then fixed in 70% ethanol overnight. Then, cells were re-suspended in 0.5 ml of PBS with 200 µg/ml RNase and 50 µg/ml propidium iodide and incubated for 30 min at 37°C. Finally, 2.0 x 10^4 cells per sample were collected for analysis on the BD FACSCalibur™ platform, and data were analyzed with the software ModFit LT (Verity Software House, Topsham, USA).

Quantitative reverse transcription-polymerase chain reaction analysis

Total RNAs were isolated from cells using Trizol reagent (Invitrogen, Grand Island, USA) following the manufacturer’s instructions. For tumor and liver specimens, tissues were first pulverized in liquid nitrogen. Thereafter, total RNAs were isolated with Trizol reagent. Total RNAs (3 µg) were reverse transcribed into complementary DNA. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis was done with SYBR Green qPCR Master Mix (Promega, Madison, USA) on a qPCR system (Bio-Rad). GAPDH, beta-actin (ACTB), or beta-2-microglobulin (B2M) was used as the internal control. Primer sequences used in PCR analysis were shown in Supplementary Table S1.

Determination of iron content

Serum iron concentration was determined with the serum iron assay kit (Jian Cheng Bioengineering Institute, Nanjing, China) following the manufacturer’s instructions, as previously reported [4,21].

Inductively coupled plasma mass spectrometry analysis

Total iron in tumors was assayed through the inductively coupled plasma mass spectrometry (ICP-MS) analysis. Sample preparation was carried out as previously described [22]. Iron concentration was determined using Agilent 8800 ICP-MS (Agilent Technologies, Santa Clara, USA), following a protocol described previously [22].

Cellular lactate concentration detection

After treatments, cells were collected, washed with PBS, and then lysed in lysis buffer containing 2% Triton X-100, followed by incubation in ice for 40 min. Supernatants were collected for the detection of lactate concentration using the Lactic acid assay kit (Jian Cheng Bioengineering Institute) according to the manufacturer’s instructions. Protein concentrations were used for normalization of each sample.

Confocal laser scanning microscopy

FPN-GFP cells were induced by Dox for 48 h, and then treated with 10 µg/ml human hepcidin peptide or 1.4 µg/ml mouse hepcidin peptide (Peptide Institute, Osaka, Japan) for 3.5 h. Afterwards, confocal laser scanning microscopy was carried out as previously described [23].

Statistical analysis

Two-tailed Student’s t-test was used to analyze the experimental data and determine statistical significance. Statistical significance was determined with a P-value <0.05.

Results

Repressed homing of cancer cells in Hamp1−/− mice

Although there is increasing evidence suggesting that elevated hepatic hepcidin likely plays a contributive role in tumor initiation and progression, a direct demonstration is still missing. Hence, we tried to address this question with Hamp1−/− mice. First, the knockout mice were characterized following the method from the original study [24]. Hepcidin mRNA expression was determined in liver specimens by RT-PCR. As shown in Fig. 1A, hepcidin (encoded by gene Hamp1) was completely removed in Hamp1−/− mice, consistent with the original study where Hamp1−/− mice were generated [24]. In contrast, the Hamp2 gene, with no reported role in iron metabolism [25,26], was intact in both WT and Hamp1−/− mice. Owing to hepcidin deficiency, there are increased iron dietary absorption and iron egress from macrophages, leading to severe iron overload in various organs (such as liver, heart, brain, and pancreas) in Hamp1−/− mice [17,18]. This will give rise to elevated serum iron. However, enriched iron in circulation will surely increase iron supply to tumor cells, which is associated with increased tumorigenic effect of iron [27]. Therefore, to avoid iron overload (in other words, to rule out, to the largest extent, a confounding variable of iron overload in the knockout mice, and to study the role of hepcidin as the only variable without the implication of simultaneous iron overload), Hamp1−/− mice in the current study were first iron-depleted. This regime actually would make the Hamp1−/− mice to have a comparable serum iron content with the WT mice, so that we could exclude a confounding variable of increased serum iron in the Hamp1−/− mice. As shown in Fig. 1B,C, the serum iron concentrations in the iron-depleted Hamp1−/− mice showed similar pattern from tumor cell injection to the end of the experiments, in comparison with the WT mice. Despite the promising data from our previous research with breast cancer [4], different...
To circumvent this issue, Lewis lung cancer LL/2 cells with the same genetic background were used.

As shown in Fig. 2A, the Kaplan–Meier survival curve showed that Hamp1−/− mice displayed much better survival rate after tumor cell
injection than the WT mice (P < 0.05). From day 40, some mice started to die, and finally 8 WT mice (8 of 17) died during the 81-day experiment. In contrast, only 1 mouse (1 of 18) died of tumors during the 81-day experiment in Hamp1−/− mice. Tumors in lung and other organs of the dead mice were examined. After 81 days, all the survived mice were sacrificed, and tumors in lung and other organs were also examined. Results showed that only the dead mice (one in the Hamp1−/− mice, but eight in the WT mice) were found with tumors in lung. The total incidence of lung tumors was 47% in WT mice and 6% in Hamp1−/− mice (Fig. 2B). Moreover, massive immersion of tumors was observed in lungs of WT mice, as the histological examination showed massive tumor growth in tumor-burdened WT mice (Fig. 2C). Additionally, no tumor occurrence was observed in other organs of both Hamp1−/− mice and WT mice. Owing to hepcidin deficiency, a significant decline of total iron content (by more than 50%) was demonstrated in the tumor from one of the dead WT mice, when compared with the dead Hamp1−/− mouse (Supplementary Fig. S1), indicating the role of hepcidin in controlling cellular iron concentration.

To address whether cancer cells could promote the induction of hepatic hepcidin, we thus measured the hepatic hepcidin level in WT mice that developed tumors as well as that did not develop tumors, after LL/2 cell injection. As shown in Fig. 2D, the expression of hepcidin was not altered in mice with tumors compared with that in mice without tumors, suggesting that tumors derived from LL/2 cells appeared to have no impact on hepatic hepcidin expression. It was demonstrated that hepatic hepcidin was indispensable for lung cancer cell seeding and the consequential tumor development in lungs, as remarkably reduced seeding propensity and inhibited tumor progression of LL/2 lung cancer cells were characterized in Hamp1−/− mice. These data thus implied that targeting hepatic hepcidin would represent a promising strategy for cancer therapeutics.

**Decreased FPN expression in tumors with nodal metastasis**

Next, we asked whether deregulated FPN is involved in the regulation of tumor growth and metastasis. To address this question, we first looked into the difference of FPN expression level between primary tumors with metastasis and primary tumors without metastasis from our breast cancer patients. As shown in Supplementary Fig. S2, the level of FPN mRNA was greatly reduced by more than 70% in breast tumors with nodal metastasis, compared with tumors without nodal metastasis. These results implied a crucial role of FPN in controlling tumor progression including metastasis.

**Selection of 4T1 cells with inducible FPN expression**

To recognize the role of FPN in tumor progression, we deliberately established a construct with forced expression of FPN (Supplementary Fig. S3). As described in the ‘Materials and Methods’ section, the stable transfectants with inducible over-expression were named as FPN-GFP cells, and those with GFP-control construct were termed as GFP-control cells (Supplementary Fig. S3). As evidenced by the FACS analysis, FPN-GFP or GFP expression could be substantially induced by Dox, with nearly all GFP-positive cells, relative to uninduced cells (Fig. 3A). Dox induction enhanced the expression of FPN mRNA by more than 12 folds (Fig. 3B, P < 0.001). The over-expression of FPN was also reflected by the remarkable appearance of FPN-GFP fusion protein in FPN-GFP cells upon Dox induction, demonstrated by the western blotting using anti-GFP antibody (Fig. 3C).

Moreover, confocal laser scanning microscopy showed sharp green fluorescence on the plasma membrane (Fig. 3D), which is the expected location of the FPN-GFP fusion protein. To access the function of the FPN-GFP fusion protein, we examined its cellular localization in response to hepcidin. FPN is the only iron efflux pump in mammalian cells, and its concentration is posttranslationally regulated by its ligand hepcidin [3,28–30]. Hepcidin binds to FPN to induce its internalization into cytoplasm, and then promotes its degradation [3,30]. As shown in Fig. 3D, considerable induction of FPN internalization was recorded for the FPN-GFP fusion protein upon either human hepcidin or mouse hepcidin, demonstrated by the fluorescence signal relocated from plasma membrane into cytoplasm. These observations revealed that the FPN-GFP fusion protein responded to the treatment of hepcidin, and also confirmed the transduction of the hepcidin–FPN signaling.

**Reduced tumor occurrence and growth for 4T1 cells with FPN over-expression**

To understand the potential involvement of FPN in tumor progression, we evaluated tumor growth and metastasis in a mouse model, as described previously [15,31]. Upon FPN over-expression, cell morphology was remarkably changed, as shown in Fig. 4A. The GFP-control cells and FPN-GFP cells without Dox induction showed typical epithelial morphology, while the FPN-GFP cells with Dox induction lost, at least partially, the epithelial morphology and gained new morphological features, such as smaller size and rounder shape (Fig. 4A).

After these cells were orthotopically inoculated into MMF of mice, tumor growth and metastasis were closely monitored. At the end of the animal experiments, primary tumors were dissected and weighed, and metastatic tumors were also detected through bioluminescence assessment. The percentage of tumorigenesis was drastically decreased for FPN-GFP cells with Dox induction, compared with other groups (Fig. 4B, 60% vs. 100%), indicating reduced tumor occurrence for breast tumor cells in vivo. With regard to the tumor growth, as shown in Fig. 4C, the final size of tumors derived from GFP-control cells was not changed upon the Dox induction (compared with that without Dox induction), suggesting that Dox had little effects on tumor growth. In contrast, the tumor size was dramatically reduced for tumors derived from FPN-GFP cells upon Dox induction, and the average tumor weight was decreased by over 80%, compared with the tumors developed from FPN-GFP cells without Dox induction (Fig. 4C, P < 0.05). Similar results were obtained in an independent replication of this experiment (Fig. 4D). Representative histological images of primary tumors are shown in Fig. 4E,F. Additionally, as shown in Supplementary Fig. S4, the tumor iron content in the FPN-GFP + DOX group was nearly 70% less than that in the FPN-GFP – DOX group, in parallel to the role of FPN in governing cellular iron egress. The above findings suggested that the level of FPN was crucial for tumor growth. Consistent with this finding, elevated expression of FPN also greatly restrained the growth of MDA-MB-231 breast cancer cells [19].

**Impaired metastasis for FPN-GFP cells upon Dox induction**

To further delineate the influence of elevated FPN on tumor progression, the metastasis in various organs of mice implanted with FPN-GFP or GFP-control cells was studied in the presence or absence of Dox induction. As shown in the bioluminescent images, metastasis was found in livers and/or lungs of all mice implanted with GFP-control cells with or without Dox induction or FPN-GFP cells with no Dox induction (Fig. 5A–C). In contrast to the significant metastasis in the other three groups of mice, no metastasis was detected in mice implanted with FPN-GFP cells in the presence of Dox induction.
To confirm the occurrence of metastasis, histological examination was performed in liver specimens. Consistent with the results of bioluminescence detection, numerous metastatic tumors were found in the livers of mice implanted with GFP-control cells with or without Dox induction or FPN-GFP cells with no Dox induction. However, no tumor cells could be found in liver sections from mice implanted with FPN-GFP cells upon Dox induction (Fig. 5E,F). The number of micrometastatic tumors per field was quantified (Fig. 5G). These results together demonstrated that increased FPN expression significantly impeded the metastatic propensity of breast tumor cells.

FPN over-expression leads to cell cycle arrest and reduction of colony formation

Then, we endeavored to investigate the molecular mechanisms responsible for FPN-mediated regulations of tumor behaviors. First, DNA synthesis and cell division were assessed upon FPN over-expression. The BrdU assay is a desirable method to measure the efficacy of DNA synthesis [32,33]. The BrdU incorporation assay indicated that DNA synthesis was repressed by ∼20% for FPN-GFP cells upon Dox induction for 72 h, compared with uninduced FPN-GFP cells (Fig. 6A, P < 0.05). DNA synthesis was further inhibited by ∼30% for FPN-GFP cells after 96 h Dox induction, compared with untreated cells (Fig. 6A, P < 0.05). Meanwhile, cell cycle was analyzed for these cells in response to FPN over-expression. As shown in Fig. 6B, the percentage of G0/G1 phase was increased for FPN-GFP cells after 48 h Dox induction, compared with untreated FPN-GFP cells. As a result, there was a concomitant reduction of the percentage of S phase and G2/M phase in Dox-treated FPN-GFP cells, when compared with the uninduced cells (Supplementary Fig. S5). This was indicative of cell cycle arrest upon FPN over-expression. The capability of colony formation was determined by counting the number of colonies. The number of colonies derived from FPN-GFP cells with Dox induction was markedly reduced, compared with that of colonies from FPN-GFP cells without Dox induction (Fig. 6C). Similar results were obtained from experiments with two different initial cell concentration, 400 or 200 cells seeded in each well of 60 mm plates (Fig. 6C). The number of colonies showed more than 60% reduction when 400 cells were initially seeded per well (Fig. 6D, P < 0.001). These data were consistent with the above results in mice.

Furthermore, cell death was also determined for cells upon FPN over-expression. We looked into the activation of caspase-3, the central executor in triggering apoptosis [34], upon Dox treatment. As demonstrated by western blot analysis, no activation of caspase-3 was found

Figure 3. Evaluation of FPN over-expression in 4T1 cells upon Dox induction

(A) FACS analysis of GFP fluorescence in FPN-GFP and GFP-control cells upon Dox induction for 48 h. (B) Relative changes of FPN mRNA upon Dox induction for 96 h. (C) Western blot analysis of FPN-GFP fusion protein in FPN-GFP cells upon Dox induction for 48 h. (D) The confocal images reflecting the fluorescence changes between plasma membrane and cytoplasm in Dox-induced FPN-GFP cells (after 48 h induction) upon human or mouse hepcidin peptide for 3.5 h. ***P < 0.001.
Figure 4. FPN over-expression represses tumor occurrence and growth

(A) The cellular morphological changes of FPN-GFP cells after 48 h Dox induction. (B) The occurrence of primary tumors derived from FPN-GFP or GFP-control cells with or without Dox administration. Mice were sacrificed 25 days after tumor cell inoculation. (C) The representative image of tumors from the first experiment. (D) The representative image of tumors from the second experiment. (E,F) The representative images of H&E staining of tumor sections from primary tumors derived from FPN-GFP cells without (E) or with (F) Dox administration. Original magnification, ×200.
Figure 5. FPN over-expression diminishes metastasis. (A–D) The representative bioluminescent images indicate metastasis to organs. (E,F) The representative images of H&E staining of liver sections from mice inoculated with FPN-GFP cells without (E) or with (F) Dox administration. The yellow arrow denotes a micrometastatic tumor. Original magnification, ×200. (G) Quantification of the number of micrometastatic tumors in liver sections. Micrometastatic tumors were counted in each field for three randomly selected fields in every liver section (n=5), and an average was calculated for each sample (n=3×5).
after Dox induction for 48, 72, and 96 h (Fig. 7A). In contrast, staurosporine (STA), a positive control to initiate caspase-3 activation [34], greatly induced the activation of caspase-3, evidenced by the pronounced induction of cleaved caspase-3 (Fig. 7A). To substantiate this finding, we further used TUNEL staining to recognize apoptotic cells. Consistent with the results of western blotting, no positive staining was found in Dox-treated cells and untreated cells (Fig. 7B). Positive staining could be detected in STA-treated cells (Fig. 7B). These results thus precluded the implication of cell death in FPN over-expression-mediated mechanisms in tumor cells.

FPN over-expression changes the EMT properties and reduces glycolysis

Since metastasis was greatly inhibited for tumors with FPN over-expression, we hypothesized that metastasis-related properties (such...
as EMT) could also be altered. To test this hypothesis, we investigated the changes of typical EMT markers in 4T1 cells upon FPN over-expression. A few representative EMT markers were selected in this study. The expression level of Snail1, a crucial transcription factor in EMT mediation [35], was reduced by more than 20% in Dox-induced FPN-GFP cells, compared with the uninduced cells (Fig. 8A, P < 0.05). Another crucial transcriptional factor in EMT [36], Twist1, was diminished by over 40% in Dox-treated FPN-GFP cells in comparison with uninduced cells (Fig. 8A, P < 0.05). With a similar role as Twist1 in repressing E-cadherin expression [36], ZEB2 also transcriptionally suppresses E-cadherin [37]. As shown in Fig. 8A, the level of ZEB2 was repressed by ∼20% upon FPN over-expression. Moreover, FPN over-expression also repressed the level of the mesenchymal marker vimentin by more than 40% in Dox-induced FNP-GFP cells in comparison with the control cells (Fig. 8A, P < 0.05).

To confirm the qRT-PCR results based on GAPDH for normalization, we used two additional sets of housekeeping genes, i.e. ACTB and B2M, for normalization. Similar results were obtained with three different internal controls for normalization, as shown in Supplementary Table S2. These results together demonstrated that EMT was greatly compromised in FPN-GFP cells upon FPN over-expression.

Cancer cells with enhanced metastatic propensity tend to have more stem cell-like properties, including the energy mode transition from oxidative phosphorylation to glycolysis, coupled to increased products, such as lactate [38–40]. To evaluate this aspect, the lactate concentration was measured in cells over-expressing FPN. As shown in Fig. 8B, the lactate concentration was significantly reduced by ∼20% in FPN-GFP cells treated with Dox for 72 or 96 h (P < 0.05), compared with the uninduced control cells, indicating inhibited glycolysis in these cells upon FPN over-expression. These results were also in agreement with the changes of EMT markers, as described earlier (Fig. 8A).

Discussion

Despite a great deal of inspiring progresses in the past years, cancer remains to be one of most common causes of death worldwide [41,42]. Primary tumor causes significant morbidity and mortality, whereas metastasis to distant organs often accounts for more deaths [43,44]. The demand for iron is greatly enhanced in cancer cells [4,27,45,46]. Accumulating clinical and experimental evidence suggests the involvement of the hepcidin–FPN signaling in tumor growth, as deregulation of this signaling was reported in tumors coupled with disordered systemic and/or tumor iron metabolism [4,5,10,27]. Previous studies have revealed that circulating hepcidin secreted from liver is elevated in patients with various cancers, such as myeloma, renal cell carcinoma, prostate cancer, breast cancer, and lung cancer [4,7–11]. Moreover, reduced FPN was found in tumors relative to adjacent tissues, and its expression level was associated with prognostic significance in cancer patients [5,7,8,10,47]. However, the biological effects of hepcidin–FPN signaling on cancer behaviors have not been fully understood, and many questions remain to be answered, e.g. its contribution to tumor metastasis and EMT process. In the current study, our findings identified the important role of the hepcidin–FPN signaling in affecting the tumor behaviors beyond tumor growth.

It has been recognized that hepcidin is predominantly secreted by hepatocytes, and it primarily governs iron metabolism through regulating dietary iron absorption and iron efflux from cells (mostly macrophages under normal settings) [2]. Hepcidin hinders iron egress out of cells by binding and inducing the degradation of its receptor FPN, resulting in intracellular iron retention [2]. Mounting evidence suggests that increased hepcidin contributes to tumor development [4,5,27], and limiting hepatic hepcidin production may be a prospective strategy to restrain tumor growth [4,21]. We here have furthered
the understanding by demonstrating that the absence of hepcidin greatly repressed homing of LL2 cells into lungs, and promoted tumor-free survival of mice. Compromised development of tumors could be due to a smaller number of seeding tumor cells and repressed tumor growth in lungs of Hamp1−/− mice, compared with the WT mice. The current study represented the first line of direct evidence identifying the crucial role of circulating hepcidin in promoting lung tumor cell homing and consequential tumor development in lungs of mice. This study thus may represent a promising approach for the treatment of cancers through targeting hepatic hepcidin or its downstream signaling molecules.

As the only known iron exporter in mammals, FPN is universally expressed in most types of cells including tumor cells [27], and reduced FPN expression was verified to enforce tumor growth due to ineffective iron efflux, coupled to increased intracellular iron retention in tumor cells [4,5,19,48]. Furthermore, we here identified an important function of FPN in repressing metastasis and EMT. FPN over-expression greatly inhibited lung and liver metastasis of 4T1 cells through altering the metastasis-relevant properties including EMT, as evidenced by a reduction of typical EMT markers (such as Snail1, Twist1, ZEB2, and vimentin). As a member of the Snail superfamily, Snail1 plays a fundamental role in modulating EMT [49]. As a zinc-finger transcription factor, Snail1 regulates a wide network of targets implicated in the regulation of cell adhesion/invasion, immune resistance, and stem cell properties [35]. Another crucial transcriptional factor associated with EMT is Twist1 that is an important member of the big family of helix-loop-helix (HLH) transcription factors responsible for EMT mediation [36]. Twist1 promotes EMT by regulating a number of targets including E-cadherin and noncoding RNAs [36]. As a member of the ZEB family, ZEB2 also transcriptionally suppresses the expression of E-cadherin transcription, and the ratio of ZEB2 to E-cadherin was identified to be positively correlated with poor prognosis for breast cancer patients [37]. Vimentin, the major intermediate filament, functions to maintain cellular integrity and supports resistance against outside stresses. It is normally expressed in mesenchymal cells. However, vimentin is often highly expressed in a variety of

![Figure 8. Alterations of EMT markers and lactate concentrations in response to FPN over-expression](image-url)

(A) Relative changes of EMT markers in FPN-GFP cells in response to Dox for 48 h. (B) The concentrations of cellular lactate in FPN-GFP cells upon Dox treatment for 72 h and 96 h. *P < 0.05.
epithelial cancers including breast and lung cancers [50,51]. Thus, vimentin is recognized as an EMT marker for these cancers [50,51]. EMT is a process of transition from epithelial cells into migratory and invasive mesenchymal cells. EMT is closely associated with metastasis, whereas inhibition of EMT could suppress metastasis [52,53]. Targeting the process of EMT appears to be a promising strategy to restrain cancer progression [54,55]. For instance, quite a few approaches have been developed to attenuate the TGF-β signaling, a key player of EMT [54,55]. In the meantime, energy metabolism is also closely associated with EMT, as energy reprogramming during the EMT is necessary for cancer cells to overcome metastatic inefficiency [38,39]. We here demonstrated that FPN over-expression repressed metastasis, whereas inhibition of EMT could suppress metastasis [52,53].

Figure 9. A schematic diagram depicting the important role of the hepcidin–FPN signaling in modulating tumor growth and progression

and interacting under the setting of FPN over-expression. Their cross-talk needs to be explored by further detailed investigation in the future.

Supplementary Data
Supplementary data is available at ABBSS online.

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