Decreased Ferroportin Promotes Myeloma Cell Growth and Osteoclast Differentiation

Running title: Role of Ferroportin in Suppression of Myeloma

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Abstract

Iron homeostasis is disrupted in multiple myeloma (MM), a difficult-to-cure plasma cell malignancy with lytic bone lesions. Here we systematically analyzed iron gene expression signature and demonstrated that mRNA expression of iron exporter Ferroportin (FPN1) is significantly downregulated in myeloma cells and correlates negatively with clinic outcome. Restoring expression of FPN1 reduces intracellular liable iron pool, inhibits STAT3-MCL-1 signaling and suppresses myeloma cells growth. Furthermore, we demonstrated that mRNA of FPN1 is also downregulated at the initial stages of osteoclast differentiation and suppresses myeloma cell induced osteoclast differentiation through regulating iron regulator TFRC, NFκB and JNK pathways. Altogether, we demonstrated that downregulation of FPN1 plays critical roles in promoting myeloma cell growth and bone resorption in MM.
Introduction

Iron is an essential element for almost every type of cell; it is involved in various biological processes such as haem synthesis, mitochondria respiratory, DNA synthesis, cell cycle et al (1). Intracellular Iron homeostasis is strictly regulated in normal cells at the level of uptake, storage, export and even the microenvironment (2). Dysregulation of iron homeostasis results in change of cell fate and promotes diseases such as cancer (3, 4). Many types of cancer have alterations in iron metabolism resulting in increased intracellular iron levels which facilitate malignant cell growth and disease progression. For instance, iron enhances colorectal tumorigenesis in the presence of APC mutations by augmenting WNT signaling and downregulating E-cadherin (5). Reduced iron export is another way that cancer cells use to increase concentration of iron besides increased iron uptake and decreased iron storage. Unlike multiple ways of iron uptake by different proteins such as TFRC (6) and Lipocalin 2 (7), FPN1 is the only known exporter of iron in vertebrates (8). FPN1 encodes a multiple-transmembrane domain protein for the transfer of cellular iron to plasma, which regulates the exit of iron from enterocytes into the blood circulation (9). It is expressed in many iron-exporting cells, including placental syncytiotrophoblasts, duodenal enterocytes, hepatocytes, and reticuloendothelial macrophages (10). Mutations, resulting in loss-of-function of FPN1, cause hyperferritinemia and iron overload in macrophage or hepatocytes (11). Hepcidin, the physiological ligand of FPN1 (12), inhibits iron export by inducing internalization and proteasome degradation of FPN1 protein (13). Although regulation of iron by FPN1 has been well studied in iron homeostasis, the role of FPN1 in the context of cancer is just beginning to emerge. FPN1 is significantly down-regulated in breast cancer cells compared with their normal counterparts. Consistent with the low levels of FPN1 expression, the breast cancer cells showed a markedly higher labile iron pool than the non-malignant breast epithelial (14). Importantly, low expression of FPN1 was linked to poor prognosis in primary breast cancer samples using gene expression profiles (14).

Multiple myeloma (MM) is a difficult-to-cure plasma cell malignancy the growth, survival and drug resistance of which is intimately tied to interactions with stromal cells including osteoclasts, osteoblasts and extracellular matrix proteins in the bone microenvironment (15). The crosstalk between the MM cells and different cells of the BM microenvironment regulates survival and proliferation of MM cells and results in other pathological conditions such as bone destruction by osteoclasts (16). It is very common that patients with MM present with anemia due to systemic alteration in iron metabolism (17). The limited iron availability resulting in impaired erythropoiesis is probably the main reason for myeloma-associated anemia (18). Elevated IL-6 and bone morphogenetic proteins (BMPs) in the
Marrow of MM patients are the primary cytokines which induce high expression of hepcidin (19, 20). The myeloma cells also show increased requirement of iron due to a relatively high rate of proliferation and metabolism when compared to normal plasma cells (21). In addition to suppressing osteoblastogenesis by secreting the WNT signaling inhibitor DKK1(16), myeloma cells also promote osteoclastogenesis by eliciting stroma cells to produce RANKL which is the major inducer of osteoclast differentiation from its macrophage precursors (22). A recent study shows that activation of osteoclasts demands high iron uptake to facilitate mitochondrial biogenesis(23).

Here we explore the expression of FPN1 in different progression stages of MM and correlate FPN1 expression with patient outcome. We provide evidence that downregulation of FPN1 plays an important role in accelerating malignant cell growth and osteoclast differentiation. Our data indicate that therapeutics with ability of elevating expression of FPN1 might hold promising key of improving the outcome in MM.

Materials and Methods

Cell culture, drug treatment and cell growth

Human myeloma cell lines (ARP1, OCI-MY5 and their derivative cell lines ARP1-FPN1, OCI-MY5-FPN1, OCI-FPN1-STAT3c and OCI-MY5-MCL-1, and their relative controls) and murine myeloma cell lines (STGM1 and STGM1 with inducible expression of FPN1) were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), penicillin (100 IU/mL), and streptomycin (100 µg/mL) in a humidified incubator at 37°C and 5% CO2/95% air. The murine macrophage cells RAW264.7 were cultured in DMEM medium (Invitrogen, Carlsbad, CA) with the same supplements. Primary bone marrow mononuclear cells collected from wild-type C57B mice were cultured in the macrophage medium (Cell Biologics, Chicago, IL) with M-CSF (PeproTech, Rocky Hill, NJ) for 3 days with medium change every day. The attached cells were considered as BMM for the following osteoclast differentiation experiments induced by myeloma conditioned medium (5%) or RANKL (50 ng/ml plus 10 ng/ml M-CSF, PeproTech, Rocky Hill, NJ). For experiments, human myeloma cells were incubated with FeCl3, DFO and FAC ( Sigma-Aldrich, St Louis, MO). Cell growth was counted by PestroBlue assay (Invitrogen, Carlsbad, CA) trypan-blue exclusion assay as reported(24).
Clonogenic assay

Clonogenic growth was performed as previous reported(25). Briefly, 10,000 myeloma cells were plated in 0.5 mL 0.33% agar/RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FBS. Cells were incubated (37°C, 5% CO2) and fed with the same medium twice a week. One colony was defined if more than 40 cells were observed. Plates were imaged and colonies were enumerated using Image J software.

Plasmids and virus production

Human FPN1 coding sequence was purchased from OriGene and subcloned into lentiviral vector pWPI using PacI and XhoI sites. An EGFP-N1 vector with mouse FPN1 was provided by Dr. Ivana De Domenico. The mouse FPN1 coding sequence was inserted to Age I and Mlu I sites of doxycycline inducible expression derivate from pTRIPZ from Dr. Dana Levasseur’s lab (University of Iowa, Iowa City, IA). The constitutively active form of STAT3 and MCL-1 expression vectors were purchased from Addgene. Lentivirus was produced in HEK293T cells using vsv-g and psPAX2 helper vectors (Addgene).

Mouse models

All mouse experiments were performed under protocols approved by the Institutional Animal Use and Care Committee of the University of Iowa. Human myeloma cells (1.5 X 10^6 cells in 100 ml PBS) were injected subcutaneously into the abdomen of NOD-Rag/null gamma mice. Tumor burdens were monitored by tumor volumes. In KaLwRij mouse model, STGM1 –FPN1 (1 X 10^6 cells in 100 ml PBS) were injected through tail vein. After 7 days, doxycycline (2 mg/ml, Sigma-Aldrich, St Louis, MO) was added to the drinking water (contains 5% sucrose) and dextran-iron (0.5 mg/kg, Sigma-Aldrich, St Louis, MO) was injected intraperitoneally twice a week. Mice were bled every week to harvest sera for detecting IgG2b by ELISA assay according to manufactory’s instruction (Bethyl Laboratories, Montgomery, TX).

Western blotting

Cell lysates were equally loaded onto 4-15% SDS-PAGE (Bio-Rad, Richmond, CA), electrophoresed, and transferred to enhanced chemiluminescence–nitrocellulose membranes (Bio-Rad, Richmond, CA). The membranes were stained with 0.2% Ponceau S red to ensure equal protein loading. After blocking with 5% nonfat milk in Tris-buffered saline, the membranes were incubated with antibodies against FPN1 (Abcam, Cambridge, MA), STAT3, p-STAT 3, MCL-1, ERK1/2, p-RRK, JNK, p-JNK, p38, p-p38, NfxB- pNFkB, AKT, p-AKT, BCL2, BCL-XL, BAX, BAK, BIM, NOXA, BID, PUMA (Cell Signaling Technology) overnight at 4°C,
followed by horseradish peroxidase (HRP)–linked secondary antibody (Cell Signaling Technology, Beverly, MA) for 1 hour at room temperature. Detection was developed by Immobilon Western ECL suberate (Millipore, Billerica, MA), according to the manufacturer's instruction. Beta-actin (Cell Signaling Technology, Beverly, MA) was used as an internal control.

**Quantitative real-time PCR**

For quantitative analysis of gene expression, total RNA was isolated by an RNeasy kit (Qiagen, Valencia, CA). Complementary DNA was synthesized using lScript reverse transcription kit according to the manufacturer's instruction (Bio-Rad, Richmond, CA). Real-time quantitative PCRs for mouse FPN1, IRF8, TFRC, PGC1α, NFATc1, CTSK, TRAP and β-actin were performed with SYBR Green Super Mixture Reagents (Bio-Rad, Richmond, CA) on the CFX connect real-time system (Bio-Rad, Richmond, CA). The specific primers used for detecting genes are listed in supplemental Table 2. PCR was initiated with 95°C for 3 minutes to hot-start the DNA polymerase and denature the template, and then 40 cycles consisted of denaturing at 95°C for 30 seconds, annealing and extension at 60°C for 30 seconds. Data were analyzed as previously reported(26).

**Liable iron pool (LIP) assay**

The cellular LIP was measured with fluorescent metallosensor calcein as described(14). The SIH (salicylaldehyde isonicotinoyl hydrazone) used in the assay was synthesized by Dr. Zhendong Jin lab (University of Iowa, Iowa City, IA).

**Flow cytometry**

Cell apoptosis were measured by Annexin-V/PI kit (eBioscience, San Diego, CA) according to manufacturer’s instruction. To detect surface expression of TFRC, isolated BMM were firstly block with anti-CD16/32(eBioscience, San Diego, CA) and then stained with PE-conjugated TFRC (eBioscience, San Diego, CA) in PBS containing 2% FBS on ice for 20 minutes. Then cells washed with cold PBS, resuspended and detected by Flow cytometry (University of Iowa Flow Cytometry Core, Iowa City, IA).

**Prussian blue and TRAP staining**

Prussian blue and TRAP staining were performed according to manufacturer’s instruction (Sigma-Aldrich, St Louis, MO).

**Microarray data sets**
Bone marrow macrophages were harvested from both Fpn1+/+ wild type and Fpn1+/− mice. The Affymetrix Mouse Genome 430 2.0 Arrays were applied to identify fn1 related genes in BMMs in duplicate as reported previously (27, 28). We also used 3 publicly available datasets for human MM microarrays to compare normal plasma cells and MM cells (GSE2658, GSE9782, GSE19784). The supervised cluster analysis was performed in statistical software R (Version2.6.2).

Statistical analyses

Unpaired T Test was used to evaluate the difference between 2 different Groups. In correlation of FPN1 and MCL-1 expression with disease progression, event-free survival and overall survival were measured using the Kaplan-Meier method, and the log-rank test was used for group comparison as previously reported(29). A P value of less than 0.05 was considered statistically significant.

Results:

Low-expression of FPN1 is linked to poor survival in MM.

To evaluate iron regulation in multiple myeloma cells, we reanalyzed our previous gene expression profiling date containing 22 normal plasma cell samples (NPCs) and 351 newly diagnosed MMs from the Total Therapy 2 (TT2) cohort using the Affymetrix U133Plus 2 platform(30). Of the 61 signature genes related to iron metabolism (31) (131 probe sets), we identified 29 genes significantly dysregulated by comparison of NPC versus MM samples (Table S1; p < 0.005; ratio >= 1.5 fold). A supervised hierarchical cluster in Figure 1A showed clearly that a subset of MM patients have 18 genes down-regulated and 11 genes up-regulated in MM. These include iron importing genes, IREB2 and TFRC, and iron exporting gene, FPN1. We then correlated the expression of these 29 genes with patient outcome in the TT2 cohort, FPN1, the only vertebrate iron exporter, was found to be the top gene whose down-regulation in MM was associated with an inferior outcome (Table S.1, overall survival (OS): p = 0.0032). Since FPN1 had a dysregulated expression and was associated with poor survival, we explored its functional role in MM. We also evaluated the expression of FPN1 in sequential MM samples from the same patient, in the different genetic subgroups and in the different risk-related subtypes(27, 28). FPN1 expression was significantly lower in PCs derived from MM patients compared to PCs derived from patients with monoclonal gammopathy of undetermined significance (MGUS) and healthy donors (p < 0.0001, Figure
1B). We also found that expression of FPN1 had the lowest expression in the proliferation subgroup (PR), which is the subgroup with the poorest prognosis (p < 0.0001, Figure 1C), and was significantly lower in the high-risk group compared with the low-risk group based on our 70-gene model (p = 0.013, Figure 1D). We performed survival analyses in three different data sets. Consistent with the low FPN1 expression in the aggressive MM subgroups, decreased FPN1 in the 351 TT2 cohort showed that about 60% of such cases showed short event-free survival (EFS) (Figure 1E, p < 0.001) and also inferior overall survival (OS) (p < 0.001, Figure 1F). The correlation of low FPN1 with inferior patient outcome was further validated in two other independent cohorts, including 270 newly diagnosed MM enrolled in the HOVON-65 clinical trial (Figure 1G) and 264 relapsed myeloma patients enrolled in the APEX phase 3 clinical trial (Figure 1H) (32).

Restoring expression of FPN1 suppresses MM cell growth both in vitro and in vivo.

The altered iron gene expression signature strongly suggests that MM cells have abnormal iron metabolism and harbor intracellular iron to fuel the growth of the malignant cells. To test the requirement of iron for the survival of MM cells, two MM cell lines ARP1 and OCI-MY5 were treated with iron supplement FAC or FeCl₃, or the iron chelator DFO. Supplementation of iron in the range of 0 – 500 μM had minimal effect on cell growth (Figure 2A and 2B), possibly because MM cells were already fully saturated with intracellular iron. However, MM cell growth was significantly inhibited by DFO treatment starting at 50 μM, which is a commonly used concentration in vitro (Figure 2A, B and Figure S.1A), indicating intracellular iron is essential for MM cell growth.

We then overexpressed FPN1 in two MM cell lines ARP1 and OCI-MY5, which expressed FPN1 at non-detectable level by western blotting, to evaluate the effect of FPN1 on MM cell growth using lentiviral delivery (Figure 2C). Overexpression of FPN1 significantly inhibited cell proliferation compared to cells transduced with empty vector (EV) in both cell lines at day 5 (Figure 2D). In a soft agar clonogenic assay which assesses the expansion of more primitive MM cells, overexpression of FPN1 greatly inhibited the clonogenic potential of MM cells in ARP1 and OCI-MY5 cells (Figure 2E and F). To further elucidate the tumor suppressing role of FPN1, the OCI-MY5 EV/FPN1 cells were subcutaneously injected into the flank immunocompromised NOD-SCID mice. Consistent with the in vitro study, FPN1 inhibited tumor growth in vivo at five weeks compared to its EV control (0.81 ± 0.59 cm³ vs 2.80 ± 0.18 cm³, p<0.0001). These data indicate that FPN1 is a tumor suppressor both in vitro and in vivo.

FPN1 regulates intracellular iron in vitro and in vivo in MM cells.
To test whether FPN1 regulates iron exportation in MM cells, the liable iron pool (LIP) was measured with fluorescent metallosensor calcein. ARP1 and OCI-MY5 cells overexpressing FPN1 had lower LIP compared to their EV counterparts (Figure 3A). We further employed 5TGM1- KaLwRij model to test the role of FPN1 on MM progression in vivo. Real-time PCR confirmed that 5TGM1 myeloma cells had much lower expression of FPN1 than normal bone marrow plasma cells in KaLwRij mice (Figure 3B). The coding region of FPN1 cDNA in a doxycycline inducible lentiviral construct was stably transduced into the 5TGM1 cells with lentivirus, in which the expression of FPN1 could be induced upon addition of doxycycline (Figure 3C). One week after transduced 5TGM1 cell injection, mice were administrated doxycycline and dextran-iron to increase systemic iron content in the mouse body as previously reported. In the absence of dextran-iron, over-expression of FPN1 (activated by administration of doxycycline) significantly delayed tumor progression evidenced by decreased tumor burden measured by mouse serum IgG2b level (Figure 3D; p=0.008) and prolonged survival (Figure 3E, p<0.001) compared to non-induced (no doxycycline) group. Addition of iron accelerated tumor progression of the 5TGM1 mice resulting in a shorter survival and higher tumor burden than those without iron in drink water (Figure 3E; p =0.009); the effect of iron administration on MM progression could be blocked by activation of FPN1 expression (Figure 3D and E).

**STAT3-MCL-1 mediates FPN1 effect on myeloma cell growth and survival.**

As shown in Figure 4A and Figure S.1B, overexpression of FPN1 predominantly suppressed tyrosine phosphorylation of STAT3 and decreased expression of MCL-1, while it had only a minimal effect on other survival factors such as BAK and BID. Fpn1<sup>+/−</sup> mice harbor a single copy of H32R mutation in Fpn1 gene which functions as dominate negative form (33) (Figure S.1C). Gene expression data from Fpn1<sup>+/−</sup> mice showed that MCL-1 expression was upregulated in bone marrow plasma cells and spleen B cells when compared to wild type mice (Figure S.1D). The expression pattern of MCL-1 was negatively correlated with that of FPN1 in NPC and myeloma patient samples. MCL-1 was increased in MM cells comparing to NPCs (Figure 4B) and the highest expression levels were observed in the proliferator group (Figure 4C) and in the high-risk group designated by the 70-gene model (Figure 4D). Consistent with the high MCL-1 expression in aggressive MM subgroups, high expression of MCL-1 was linked to an inferior outcome, significant for both event-free and overall survival in newly diagnosed MM patients (Figure 4E, EFS p=0.048; Figure 4F, OS p=0.006). Furthermore, by combining FPN1 and MCL-1, we
identified that MM patients with low expression of FPN1 and high expression of MCL-1 had the worst outcome (Figure 4G, EFS p<0.001; Figure 4H, OS p<0.001).

The above data suggest that STAT3 and MCL-1 are likely involved in FPN1 signaling in MM. To test whether STAT3 or MCL-1 can rescue cell growth inhibition induced by high FPN1, a constitutively active form of STAT3 (STAT3c) and MCL-1 were transduced into OCI-MY5-FPN1 cells (Figure S.1E and F). As shown in Figure 4I, overexpression of STAT3c almost completely reversed cell growth inhibition in the presence of ectopic expression of FPN1. Overexpression of MCL-1 only partially recovered cell growth, indicating that STAT3 also activates other pathways than just MCL-1. As MCL-1 is the major factor involved in the survival of bone marrow plasma cells, we tested whether the STAT3-MCL-1 pathway would have an effect on the cell survival in the presence of high FPN1 levels. As shown in Figure 4J, overexpression of FPN1 sensitized MM cell to apoptotic stimuli, which was abrogated by overexpression of STAT3c or MCL-1.

**FPN1 is downregulated in the initial phases of myeloma cell-induced osteoclast differentiation.**

Osteolytic bone disease is a frequent complication of multiple myeloma affecting up to 80% of patients (34). It is widely known that FPN1 is highly expressed in normal macrophages which are the precursors of osteoclast (OCL) in bone marrow, we hypothesized that FPN1 had a major role in MM cell-induced OCL differentiation. The murine macrophage cells RAW264.7 and primary bone marrow macrophages (BMMs) from wild-type C57BL6 mice were cultured in the conditioned media collected from ARP1, OCI-MY5 and STGM1 MM cells to induce OCL differentiation (Figure S.2A and B). FPN1 expression was dramatically downregulated by qRT-PCR in RAW264.7 and primary BMMs cultured with conditioned media (Figure 5A and B). To validate this observation using in vivo model(s), BMMs were isolated from both wild type KaLwRij mice and KaLwRij mice bearing STGM1 MM cells. FPN1 showed significantly lower expression in tumor associated macrophages (TAM) than in macrophages derived from wild type mice (Figure 5C). We also examined FPN1 expression in human OCLs in MM disease. Monocytes were isolated from peripheral blood of 8 myeloma patients and induced to differentiate in vitro to osteoclast by the addition of M-CSF and RANKL. These OCLs were co-cultured with MM cells for 2 weeks and collected for GEP analysis. FPN1 expression in OCLs was markedly down-regulated in 7 of 8 samples co-cultured with MM cells (Figure 5D). To clarify whether the downregulation of FPN1 in OCL precursors was the cause or the consequence of osteoclastogenesis, expression of FPN1 mRNA was monitored by real-time PCR in a time course of osteoclast differentiation induced by M-CSF plus RANKL (Figure S.2C). The decreased expression of FPN1 was observed as early as 6 hours after addition of RANKL and M-CSF.
(Figure 5E), which was very similar to the previously reported osteoclastogenesis inhibitory factor IRF8 (35) (Figure 5E). Previous reports show that iron uptake coordinates with mitochondrial biogenesis orchestrated by PGC-1α to induce osteoclastogenesis (23). Here we observed that significant upregulation of TFRC and PGC-1α occurred at 48 hours after induction (Figure 5E), indicating the downregulation of FPN1 preceded the changes of most critical driving factors (TFRC, PGC-1α and NFATc1) (23, 36, 37) or markers (CTSK and TRAP of) osteoclastogenesis (38), is a critical event of osteoclast differentiation (Figure 5E).

High FPN1 suppresses myeloma cell-induced osteoclast differentiation.

The early down-regulation of FPN1 in OCL precursors by MM cells or RANKL/M-CSF induced osteoclast differentiation suggests that high FPN1 in OCL precursors may block osteoclastogenesis. The RAW264.7 cells were transiently transduced with Fpn1-cDNA and the expression of FPN1 was confirmed by qRT-PCR (Figure S.3A). These cells were then cultured in the presence of conditioned media collected from MM cell culture for 3 days, TRAP staining showed that overexpression of FPN1 significantly decreased osteoclast differentiation compared to control RAW264.7 cells transfected with empty vector (Figure 6A and B). We further repeated this study on the primary OCL precursors by transducing FPN1 into mouse BMMs (Figure S.3B). As shown in Figure 6C and 6D, TRAP positive OCLs were much less frequent in Fpn1-high BMMs than in the control BMMs. The real-time PCR verified that high FPN1 in BMMs down-regulated the expression of NFATc1 and CTSK, reliable makers for osteoclastogenesis (Figure 6E). In contrast, the BMMs derived from Fpn1+/- mice increased OCL formation (Figure 6F and G) and had higher expression of NFATc1 and CTSK after RANKL /M-CSF induction compared to those derived from Fpn1+/- mice (Figure 6H).

To probe the mechanism of FPN1 in osteoclast differentiation, gene array analysis was performed in BMMs from both Fpn1+/+ and Fpn1+/- mice. Of the iron gene signature, only TFRC was up-regulated gene in Fpn1+/- BMMs (Figure 6I and Figure S.3C). Because TFRC mediates iron uptake, the main consequence of low FPN1 and high TFRC in BMMs and osteoclast is increasing intracellular iron. The 5TGM1 cells in KaLwRij mouse model were used to confirm this observation. PBS or dextran-iron was administrated to KaLwRij mice one week after injection of 5TGM1 cells. Prussian blue staining indicated an abundance of positive macrophages in mice administrated dextran-iron, but not in those control mice (Figure S. 3D). TRAP positive osteoclasts were significantly increased in the dextran iron treated mice compared to controls (Figure S. 4D), demonstrating that iron overload increases osteoclastogenesis resulting in bone loss. We screened several typical pathways involved in osteoclast differentiation by western blot,
Fpn1<sup>−/−</sup> BMMs showed significantly increased phosphorylation of NFκB and JNK compared to Fpn1<sup>+/+</sup> BMMs (Figure 6J). Thus, downregulation of FPN1 promotes osteoclast differentiation probably by activating NFκB and JNK, the major two pathways related to increased osteoclastogenesis (39). The model of our working hypothesis is summarized in the Figure 6K.

**Discussion**

Iron is mainly absorbed in the duodenum by duodenal cytochrome b (DCYTB) and exits enterocytes by the iron efflux pump FPN1. The high demand of iron by red blood cells (RBCs) to synthesize hemoglobin makes the bone marrow the main site of iron utilization. Macrophages recycle iron by catabolizing RBCs and export iron to blood stream through FPN1. Excessive iron is primarily stored in the liver and regulated by systemic iron levels (40). Many of these iron metabolic steps are altered in cancer, resulting in iron-related clinic symptoms such as anemia (1). In addition, the cancer cell itself also has dramatic changes in iron metabolism. The storage of intracellular iron into ferritin is often disrupted by oncogenes such as MYC or RAS, leading to increased availability of iron which promotes DNA synthesis and cell proliferation (41, 42). Decreased iron export is relatively less well studied in the context of cancer cells.

In the present study, we systemically investigated, for the first time, the expression of iron gene signature using gene expression profiling in MM. We observed that iron metabolism in MM is strongly dysregulated by applying an iron signature to a clinical data set from a previously studied cohort of patients (30). When compared to NPCs, MM cells are prone to up-regulate signals that maintain a high intracellular iron level. A marked reduction of the only known iron exporter FPN1 was observed in MM cells compared to NPCs. The lowest FPN1 levels were observed in the most aggressive subgroups of myeloma, which is consistent with previous reports that FPN1 is a strong and independent predictor of prognosis in breast cancer (14).

Previous studies show that high hepcidin is responsible for myeloma associated anemia and is correlated with MM progression (43). Unlike breast cancer cells, hepcidin expression is significantly decreased in MM cells compared to normal plasma cells (Table S.1), suggesting MM is unlikely to have an autocrine way to regulate iron by depletion of FPN1 protein through hepcidin. However, the abundance of IL-6 and elevated expression of Bone morphogenetic proteins (BMPs) may induce high
production of hepcidin in liver in MM patients (19, 20). Our data show that the decrease of FPN1 in MM cells happens at the transcription level. Therefore we think that hepcidin has only a minor effect on the regulation of FPN1 in MM cells. Elevated hepcidin may have more predominant role on other cells like in hepatocytes, duodenal enterocytes, RBCs and macrophages, which regulate systematic iron metabolism.

We found that FPN1 downregulates STAT3-MCL-1 signaling, one of the critical pathways of plasma cell growth and survival (44, 45). Although JAK-STAT3 signaling is not required for hepcidin induced proteasome degradation of FPN1 (46), overexpression of FPN1 indeed suppresses STAT3 signaling. One possible mechanism is that downregulation of FPN1 promotes accumulation of intracellular LIP and reactive oxygen species (ROS), which might in turn, activate STAT3-MCL-1 signaling (47). FPN1 regulated LIP and STAT3-MCL-1 signaling are likely related rather than independent events. The detailed mechanism of how FPN1 regulates STAT3-MCL-1 signaling needs further study.

Accumulation of MM cells in bone marrow induces bone resorption, which is a hallmark of MM. Enhanced osteoclastogenesis and suppressed osteoblastogenesis induced by MM cells account for the lytic bone lesions. The macrophages, osteoclast precursors in the bone marrow, are the main cells to recycle iron, a process regulated by FPN1. Osteoclast differentiation requires high rates of biogenesis through respiratory mechanisms in mitochondria and demands iron (23). The importance of downregulation of FPN1 in MM cells lead us to uncover the role of FPN1 in MM cell-induced bone resorption. Compared to other key regulators in osteoclast differentiation such as PGC1β, TFRC and NFATc, downregulation of FPN1 is induced earlier (around 6 hours) after incubation with RANKL plus M-CSF. The pattern of downregulation is similar to that of IRF8, a critical inhibitory factor of osteoclast differentiation (35). The experiments of ectopic expression of FPN1 or loss of function mutation of Fpn1 demonstrate that FPN1 has a predominant role in suppressing osteoclast differentiation through regulation of iron deposit, JNK and NFκB signaling. Thus, downregulation of FPN1 in bone marrow macrophages induced by MM cells plays an important role in bone resorption in MM. The fact that we did not observe consistent result of bone resorption in Fpn1−/− mice is possibly due to some activity of the wild type allele of Fpn1 gene. It will be very interesting to test the physiological role of fpn1 in bone homeostasis using conditional knockout mice model in future.

In summary, downregulation of FPN1 both in MM cells and macrophage-osteoclast cells favors the expansion of the malignant clone and osteoclastogenesis, which both have a negative effect on the outcome of MM. Exploring of agents capable to upregulate FPN1 expression could be a novel direction
in MM treatment which may result in a strategy that targets both the myeloma tumor cells and their microenvironment.

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**Disclosure of Potential Conflicts Interest**

No potential conflicts of interest were disclosed.

**Author’s Contributions**

**Conception and design:** F. Zhan, Z. Gu

**Development of Methodology:** Z. Gu, Y. Yang, H. Wang, J. Xia

**Acquisition of data (provided patients or reagents):** G. Tricot, Z. Jin, I. De Domenico
Analysis and interpretation of data: F. Zhan, Z. Gu, H. Wang, J. Shi, J. Xia

Writing, review, and/or revision of the manuscripts: F. Zhan, Z. Gu, G. Tricot, I. De Domenico

Administrative, technical, or material support: H. Xu

References


Figure Legends

Figure 1. Identification of FPN1 as a poor prognostic marker in MM. (A) Supervised cluster analysis of iron signature genes in normal plasma cells and multiple myeloma cells. (B) Scatter plots depict the Affymetrix Signal of FPN1 in normal plasma cells (NPC), MGUS, newly diagnosed MM (TT2 cohort), and MM cell lines (MMCL). One-way ANOVA was performed and identified the p < 0.0001 among these 4
groups. The p value presented in the Figure was obtained by comparison between NPC and indicated group respectively. (C) The expression of FPN1 among 8 MM subgroups is highly variable (p < 0.0001 by One-way ANOVA). Indicated p value was obtained among 8 subgroups by One-way ANOVA. (D) Expression of FPN1 between low- and high-risk subgroups in MM. The difference was compared by the Student t test between these 2 groups. (E ~ H) Survival analyses were performed based on FPN1 expression in different cohorts. The event-free survival (E) and overall survival (F) was performed in the TT2 cohort and overall survival was also analyzed in the HOVON65 (G) and APEX (H) clinical trials.

**Figure 2. Ferroportin plays a role of tumor suppression in MM cell growth both in vitro and in vivo.** (A, B) MM cells are sensitive to iron chelator. ARP1 (A) and OCI-MY5 (B) cells were treated with DFO, FeCl3 and FAC at indicated concentration for 48 hours. Cell proliferation was measured by Prestoblue assay and data was collected by measuring emission at 610 nm, p value indicated significant difference between treatment and non-treatment. (C) Confirmation of FPN1 expression in FPN1 overexpressing MM cells by western blot. FPN1 cDNA was transduced into MM cell lines using lentiviral delivery, the expression of FPN1 was examined by western blot, β-actin served as internal control. (D) Overexpression of FPN1 inhibits MM cell growth. The cell growth between FPN1 and EV cells from ARP1 and OCI-MY5 cell lines were counted by trypan blue assay from day 0 to day 5. (E and F) Overexpression FPN1 inhibits colony formation. ARP1 and OCI-MY5 MM cells overexpressed FPN1 and their controls were plated in methylcellulose to evaluate their clonogenic potential assay for 7 days, the difference between FPN1 and EV cells was compared by the Student t test. (G and H) FPN1 inhibits MM cell growth in vivo. Indicated cells were subcutaneously injected to NOD-SCID mice (n=5) for five weeks. Xenograft tumor was circled (G). Tumor volume was measured from week 3, 4 and 5 and graphed; p value indicated a significant difference between EV and FPN1 overexpression xenograft tumors at indicated time points (H).

**Figure 3. FPN1 regulates MM cell intracellular iron in vitro and in vivo.** (A) High FPN1 decreases intracellular LIP. The intracellular LIP was measured in indicated cells using fluorescent metallosensor calcein. (B) FPN1 expression is significantly lower in mouse MM cells than in normal mouse plasma cells. Expression of FPN1 mRNA in normal bone marrow plasma cells from wild type KaLwRij mice and 5TGM1 cells was measured by qRT-PCR and compared by the Student t test. (C) Confirmation of FPN1 expression in an inducible mouse MM cell line. Inducible expression of FPN1 in 5TGM1 cells was detected by western blot. (D and F) High FPN1 antagonizes iron induced cell growth in vivo. 5TGM1-FPN1 KaLwRij mice were administrated with or without doxycycline and dextran-iron as indicated one
week after cell injection. Kaplan-Meier showed the survival curves, and p value was analyzed by log-rank test (D) tumor burden was measured mouse serum IgG2b by the ELISA assay, and One-way ANOVA analyzed the significance (p<0.0001).

**Figure 4 FPN1 down-regulates STAT3-MCL-1 signaling in myeloma cells.** (A) Phosphor-STAT3 (Tyr705) and MCL-1 expression were detected by western blot in indicated cell lines. (B) Expression of MCL-1 was compared among NPC, MGUS, MM, and MMCL by GEP (p < 0.0001 by One-way ANOVA). (C) Expression of MCL-1 was compared in myeloma subgroups. Note: the PR and MS are the most aggressive subtypes (p < 0.0001 by One-way ANOVA). (D) Expression of MCL-1 was compared between the low-risk and high-risk subgroups (p = xx by t test). (E ~ H) Survival analyses were performed on TT2 cohort based on MCL-1 and FPN1 expression. Kaplan-Meier showed event free survival (E) and overall survival (F) curves according to MCL-1 expression, and event free survival (G) and overall survival (H) based on combination of MCL-1 and FPN1 expression. The log-rank test was used to analyze the p values. (I) Determine STAT3-MCL-1 function in FPN1 induced cell growth inhibition. Cell number of indicated cells was counted by trypan blue on day 5 after plating. (J) Flow cytometry showed cell apoptosis in FPN1 signaling. Indicated cells were washed and serum starved overnight, apoptosis was measured by annexin-V/PI assay as described in Material and methods. Annexin-V+ cells were counted as apoptotic cells.

**Figure 5. FPN1 is downregulated at initial phase of myeloma cell-induced osteoclast differentiation.** (A and B) Determine if MM cells regulates FPN1 expression in OCL precursors. RAW264.7 cells were cultured in the conditioned media from indicated myeloma cells for 72 hours, FPN1 mRNA was detected by real-time PCR (A). Primary bone marrow monocytes from wild type KaLwRij mice were differentiated to macrophage by M-CSF and then cultured in the conditioned media from indicated myeloma cells for 72 hours, FPN1 mRNA was detected by real-time PCR (B). (C) Compare the FPN1 expression between normal pacrophages and myeloma-associated-macrophages. Primary BMMs (F4/8+ CD11b+) were isolated from wild type KaLwRij mice and KaLwRij mice bearing 5TGM1-induced MM, FPN1 mRNA was detected by real-time PCR. (D) Evaluate if MM cells regulate FPN1 expression in coculture OCLs. Monocytes were separated from peripheral blood of 8 myeloma patients differentiated to osteoclast by addition of RANKL and M-CSF in vitro. The differentiated osteoclasts were then co-cultured with MM cells for 2 weeks. Expression of FPN1 was compared in OCLs before and after coculture with MM cells by GEP. (E) qRT-PCR examines expression of genes related to osteoclastogenesis. Primary bone marrow monocytes were differentiated to macrophage by M-CSF and then stimulated by RANKL for indicated time. Expression of indicated genes was detected by real-time PCR.
Figure 6. High FPN1 suppresses myeloma cell-induced osteoclast differentiation. (A and B) RAW264.7 cells transfected with EV or FPN1 were cultured in the conditioned media collected from indicated myeloma cells for 72 hours. OCLs were detected by TRAP staining (A) and quantified (B). (C ~ E) Primary bone marrow monocytes from wild type KaLwRij mice were transduced with EV or FPN1 and induced by M-CSF plus RANKL for 7 days. OCLs were detected by TRAP staining (C) and quantified (D), the expression of genes related to osteoclastogenesis was examined by real-time PCR. (F ~ H) Primary bone marrow monocytes were isolated from wild type Fpn1\(^{+/+}\) and Fpn1\(^{+/−}\) mice and induced by M-CSF plus RANKL for 4 days. OCLs were detected by TRAP staining (G) and quantified (G), the expression of genes related to osteoclastogenesis was examined by real-time PCR. (I) BMMs were isolated and stained with TFRC-PE antibody and surface expression of TFRC was examined by flow cytometry. (J) Indicated proteins were detected by western blot in BMMs from Fpn1\(^{+/+}\) and Fpn1\(^{+/−}\) mice. (K) An illustration of role of decreased expression FPN1 in MM progression. Briefly, downregulation of FPN1 in MM cells upregulates STAT3-MCL-1 signaling which promotes the survival of MM cells; decreased expression of FPN1 in BMMs increases expression of TFRC, NFκB and p-JNK resulting in osteoclast differentiation. The prolonged MM cell survival and enhanced osteoclast differentiation accelerate MM disease progression.
Figure 1

(A) Heatmap showing gene expression levels for various genes.

(B) scatter plot showing gene expression levels for FPN1

(C) scatter plot showing gene expression levels for FPN1

(D) scatter plot showing gene expression levels for FPN1

(E) Kaplan-Meier curve for TT2 dataset showing high and low FPN1 expression

(F) Kaplan-Meier curve for TT2 dataset showing high and low FPN1 expression

(G) Kaplan-Meier curve for HOVON65 dataset showing high and low FPN1 expression

(H) Kaplan-Meier curve for APEX dataset showing high and low FPN1 expression
**Figure 2**

Panel A: Graph showing the emission at 610 nm as a function of drug concentration for ARP1 and OCI-MY5 cells treated with DFO, FeCl₃, and FAC. The p-values indicate a significant difference for each treatment group.

Panel B: Similar graph for OCI-MY5 cells, showing a significant difference in emission for each treatment.

Panel C: Western blot analysis showing the expression levels of FPN1 and β-actin in ARP1 and OCI-MY5 cells treated with EV and FPN1. Significantly increased expression of FPN1 is observed in FPN1-treated cells compared to EV-treated cells.

Panel D: Growth curve showing the proliferation of ARP1-EV, ARP1-FPN1, OCI-MY5-EV, and OCI-MY5-FPN1 cells over 6 days. The FPN1 treatment results in significantly reduced cell numbers compared to EV treatment.

Panel E: Phase contrast images showing the morphology of ARP1 and OCI-MY5 cells treated with EV and FPN1. FPN1 treatment results in a reduced number of colonies per well compared to EV treatment.

Panel F: Bar graph comparing the number of colonies per well between EV and FPN1-treated cells. FPN1 treatment significantly reduces the number of colonies.

Panel G: Images showing the xenograft tumor model in nude mice. OCI-MY5-EV and OCI-MY5-FPN1 tumors. FPN1 treatment results in significantly smaller tumors.

Panel H: Tumor growth curve showing the volume of OCI-MY5-EV and OCI-MY5-FPN1 tumors over 5 weeks. FPN1 treatment results in significantly reduced tumor volume growth compared to EV treatment.
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