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Research Article

Fibroblast Growth Factor 23 Contributes to Regulation of Hepcidin/Ferroportin Axis

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Abstract

Background: Fibroblast Growth Factor 23 (*FGF-23*) is a recently discovered regulator of phosphate and mineral metabolism and has been associated with both the progression of Chronic Kidney Disease (CKD) and mortality in dialysis patients. Serum levels of *FGF23* are extremely elevated, as high as > 1 ng/ml in patients with Hemodialysis (HD) but the mechanisms are poorly understood.

Methods: Here, to confirm the direct implication of *FGF23* in iron homeostasis, we examined the mRNA of hepcidin, ferroportin, and Hypoxia Inducible Factors (*HIFs*) in HepG2 cells using quantitative real-time reverse transcription-Polymerase Chain Reaction (qPCR).

Results: A significant down-regulation of ferroportin and *HIFs* was observed in HepG2 cells after *FGF23* treatment. Conversely, hepcidin was regulated by *FGF23* in a dose-dependent manner. Low concentrations of *FGF23* increased hepcidin expression, and high concentrations of *FGF23* revealed a change-over to down-regulation of hepcidin expression.

Conclusion: Both hepcidin and ferroportin are well known to be the main regulators in iron homeostasis. This study, thus, demonstrated that the *FGF23* implicated directly in iron homeostasis as a clinical concentration iron is compatible with serum levels in long-term patients with HD.

Keywords: Fibroblast Growth Factor 23 (*FGF-23*); Hypoxia Inducible Factors (*HIFs*); Hepcidin; Ferroportin; Hemodialysis

Abbreviations

FGF23: Fibroblast Growth Factor; CKD: Chronic Kidney Disease; *HIFs*: Hypoxia Inducible Factors; HD: Hemodialysis; qPCR: quantitative real-time reverse transcription-Polymerase Chain Reaction; FPN: Ferroportin; BMP: Bone Morphogenetic Protein; *IL-6*: Interleukin-6

Introduction

Along with poor production of Erythropoietin (EPO), iron homeostasis disorder is the main pathophysiology of anemia in patients with chronic kidney disease, especially in those undergoing regular Hemodialysis (HD). The key molecule in iron homeostasis is hepcidin, which is produced predominantly in the hepatocyte and regulated negatively by an expression of membrane iron exporter, i.e., Ferroportin (FPN) in the hepatocyte, enterocyte, and macrophage [1,2]. Hepcidin binds to FPN and internalizes to degrade FPN in the proteasome [3]. In patients with HDs, circulating levels of hepcidin have been reportedly increased [4]. Contrariwise, in basic studies, an expression of liver hepcidin mRNA was reported to be remarkably enhanced by interleukin-6 (*IL-6*), bone morphogenetic protein-2 and -6 (*BMP-2*, and *BMP-6*) and Agoro et al. [5] have also reported an increase of liver hepcidin mRNA levels in CKD rats. In contrast, Peyssonnaux et al. [6] had demonstrated a drastic suppression of hepcidin mRNA expression by activation of the hypoxic transcription factor *HIF1α*, i.e., a critical sensor for hypoxia and iron deficiency [6]. Also, a definite decrease of hepcidin mRNA had also been reported in iron-deficient rats [7]. Currently, several clinical studies indicated a possible implication of *FGF23* in iron homeostasis in patients with HD [8,9]. However, the mechanism by which *FGF23* implicates in iron homeostasis remains unclear. Incidentally, serum levels of *FGF23* in patients with HDs were extremely high [10]; thus, we investigated the direct effect of recombinant human *FGF23* on the expression of *HIF1α*, HIF2α, FPN, and hepcidin in HepG2 cells, because the liver is a major organ for iron homeostasis.

Methods

Materials

Recombinant *FGF23* and *IL-6* were purchased from ProSpec (Ness-Ziona, Israel), whereas recombinant *BMP-6* was purchased from PeproTech (Rocky Hill, NJ, USA). Deferoxamine was purchased from Wako Pure Chemical Corp. (Osaka, Japan). Rabbit polyclonal anti-FPN was obtained from Novus Biologicals (Centennial, Co, USA). Rabbit polyclonal anti-hepcidin-25, goat anti-rabbit IgG, and mouse monoclonal anti-tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cells

HepG2 cells were purchased from Dainippon Pharm Co., Ltd. (Osaka, Japan) and cultured in Eagle's minimum essential medium (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% of fetal bovine serum, 1x non-essential amino acids, 1 mM sodium pyruvate, penicillin (100 U/ml), and streptomycin (100 U/ml) [11].

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Isolation of Total RNA and Quantitative Real-Time PCR

Total RNA was extracted with NucleoSpin RNA Plus (Takara Bio, Shiga, Japan). Quantitative real-time reverse transcription-Polymerase Chain Reaction (qPCR) was performed using Assay-on-Demand and TaqMan 5 fluorogenic nuclease chemistry (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. IDs of primers for hepcidin, ferroportin, *HIF1α*, HIF2α, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and 18S rRNA genes were Hs01057160_g1, Hs00205888_m1, Hs00153153_m1, Hs01026149_m1, Hs99999905_m1, and Hs4352655, respectively [12].

Western Blot Analysis

The cells were washed, homogenized by protein RIPA lysis buffer, and then sonicated as described previously [13]. After centrifugation at 13000 rpm for 15 minutes at 4°C, the supernatant was collected, and protein content was determined using the bicinchoninic acid protein Assay kit. Aliquots of the extract containing about 20 μg of protein were loaded and run on a single track of 10% SDS-PAGE under reducing conditions, and subsequently transferred to a polyvinylidene difluoride membrane (Invitrogen). The membrane was placed in a blocking solution (Nacalai Tesque) for 1 hour, and for an additional 1 hour, with the primary antibodies: rabbit polyclonal anti-FPN (1:1000) or rabbit polyclonal anti-hepcidin-25 (1:1000). After being washed three times, the blots were incubated with goat anti-rabbit IgG (1:5000) for 2 hours at room temperature. Bound IgG was visualized by ECL Plus (GE Healthcare, Buckinghamshire, UK) and analyzed with Ez-Capture II (ATTO Corporation, Tokyo, Japan). To ensure even loading of the samples, the same membrane was probed with mouse monoclonal anti-tubulin at a 1:10,000 dilution.

Statistical Analysis

All values were presented as mean ± standard deviation. A Student's t-test or one-way analysis of variance followed by Tukey's HSD test or Dunnett's test was performed for statistical comparisons; a p-value < 0.05 was considered significant.

Results

FGF23 Increases Hepcidin Expression and Down-Regulates Ferroportin

FGF23 is an established biomarker of adverse outcomes in patients with CKD [14]. High serum *FGF23* levels were associated with an increased risk of anemia in patients with non-dialysis CKD [8,9]. Therefore, we first investigated the effect of *FGF23* in regulating hepcidin and ferroportin in HepG2 cells. Incubation of HepG2 cells with different concentrations of *FGF23* (10 ng/mL and 50 ng/mL) resulted in a dose-dependent up-regulation of hepcidin expression shown by qPCR (Figure 1A, 1B). After 24 hours of treatment with 50 ng/ml *FGF23*, the increase in hepcidin expression reached a level of 1.2-fold above baseline (Figure 1B). These results were confirmed by immunoblot analyses. At the protein level, hepcidin expression in HepG2 cells was upregulated after stimulation with 50 ng/mL *FGF23* for 24 hours (Figure 1E). Contrariwise, incubation of HepG2 cells with different concentrations of *FGF23* (10 and 50 ng/mL) resulted in a dose-dependent down-regulation of ferroportin expression shown by qPCR (Figure 1C, 1D). A down-regulation of ferroportin was detected with small amounts of *FGF23* (10 ng/mL). Ferroportin expression was decreased to approximately 50% of control levels at 24 hours (Figure 1D). This down-regulation could also be detected at the protein level. After *FGF23* stimulation of the HepG2 cells, ferroportin expression was significantly lower than in the untreated control cells detected by the decrease of immunoreactivity of the 60 kDa band (Figure 1E). Furthermore, because an increase in hepcidin expression with a high dose of *FGF23* was marginal compared to that with uremic serum, we investigated the effects of extending the time to 48 hours and 72 hours (Figure S1). After 48 and 72 hours of treatment with 50 ng/ml *FGF23*, a down-regulation of hepcidin was detected.

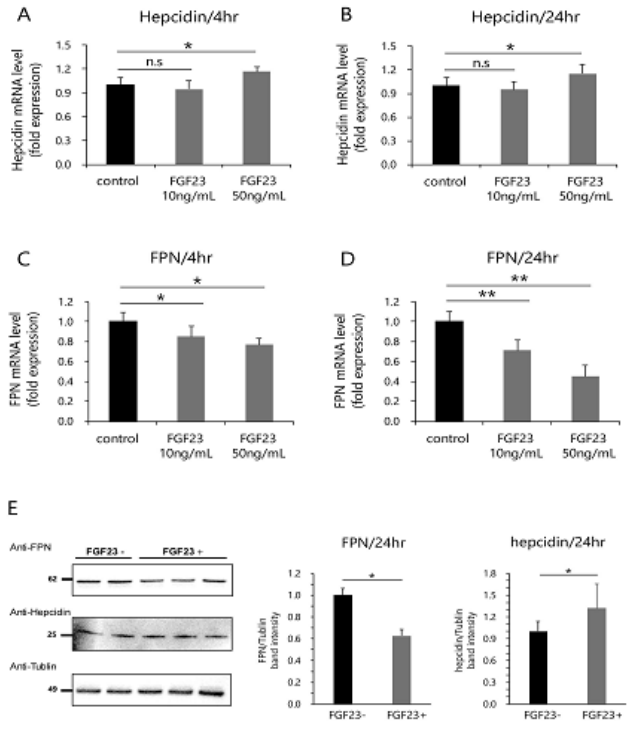

Figure 1: Effects of *FGF23* on hepcidin and *FPN* gene (A-D) and protein expression (E) in HepG2 cells. HepG2 cells were incubated with or without 10 ng/mL *FGF23*, or 50ng/mL *FGF23* for 4 hours (A, C) or 24 h hours (B, D), respectively. Total RNAs were transcribed and amplified by qPCR. Data were normalized by the intensity of GAPDH mRNA-derived signals. Statistical significance was analyzed for the indicated columns (*p < 0.05, **p < 0.01, n.s. = no significant; Student's t-test). N = 3 for each group. HepG2 cells were treated with or without 50 ng/ml *FGF23* for 24 hours (E). Proteins were extracted from HepG2 cells, and conditioned medium was collected and concentrated 20-fold using an Amicon ultrafiltration system. Then 20 μg proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Representative results of western blots of hepcidin, FPN, and β-actin are shown in the left panel. The expression level of hepcidin and FPN proteins in each sample were normalized by that of tublin and related to the value of *FGF23*-non-treated cells. The right panel shows the quantitative data. Statistical significance was analyzed for the indicated columns (*p < 0.05; Student's t-test). N = 3 for each group.

FGF23 Decrease Hepcidin Expression Induced by *BMP-6* or *IL-6*

It is known that *BMP-6* and *IL-6* increased hepcidin expression [5]; we examined the mechanism of *FGF23* here. *BMP-6* treatment resulted in an up-regulation of hepcidin expression, and this effect was decreased in the presence of 50 ng/mL *FGF23* (Figure 2A, 2B). *FGF23* also decreased the *IL-6*-induced up-regulation of hepcidin

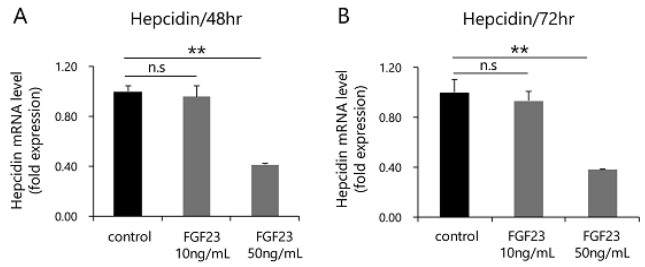

Figure S1: Effects of *FGF23* on hepcidin gene expression in HepG2 cells. HepG2 cells were incubated with or without 10ng/mL *FGF23*, or 50 ng/mL *FGF23* for 48 hours (A) or 72 hours (B), respectively. Total RNAs were transcribed and amplified by real-time PCR. Data were normalized by the intensity of GAPDH mRNA-derived signals. Statistical significance was analyzed for the indicated columns (**p < 0.01, n.s. = no significant; Student's t-test). N = 3 for each group.

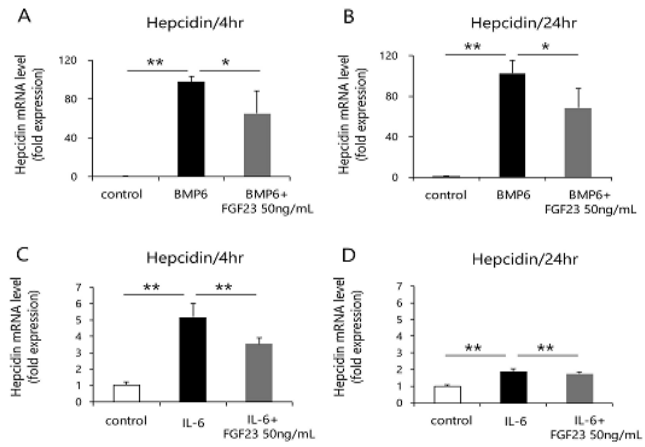

Figure 2: Effects of *FGF23* on hepcidin gene expression in HepG2 cells. HepG2 cells were incubated with or without 200 ng/mL *BMP-6* in the presence or absence of 50 ng/mL *FGF23* for 4 hours (A) or 24 hours (B). HepG2 cells were incubated with or without 50 ng/mL *IL-6* in the presence or absence of 50 ng/mL *FGF23* for 4 hours (C) or 24 hours (D). Total RNAs were transcribed and amplified by qPCR. Data were normalized by the intensity of 18S rRNA-derived signals. Statistical significance was analyzed for the indicated columns (*p < 0.05, **p < 0.01; Student's t-test). N = 3 for each group.

mRNA levels in HepG2 cells (Figure 2C and 2D). Even after additional experiments, we could not recognize an increase at 10 ng/ml of *FGF23* (data not shown), but a definite decrease of more than 50% could be found at 50 ng/ml, conversely.

FGF23 Decrease FPN Expression after Stimulation by *BMP-6* or *IL-6*

In general, an increase in hepcidin accompanies a decrease in FPN [3]. As such, we next studied the effect of *FGF23* on the *BMP-6*- or *IL-6*-mediated ferroportin reduction. *BMP-6* treatment caused a slight down-regulation of ferroportin mRNA levels in HepG2 cells and this effect was promoted in the presence of 50 ng/mL *FGF23* (Figure 3A, 3B). *IL-6* treatment also resulted in a down-regulation of ferroportin expression to as high as 50% of the expression of control cells, and this effect was accelerated in the presence of 50 ng/mL *FGF23* (Figure 3C and 3D).

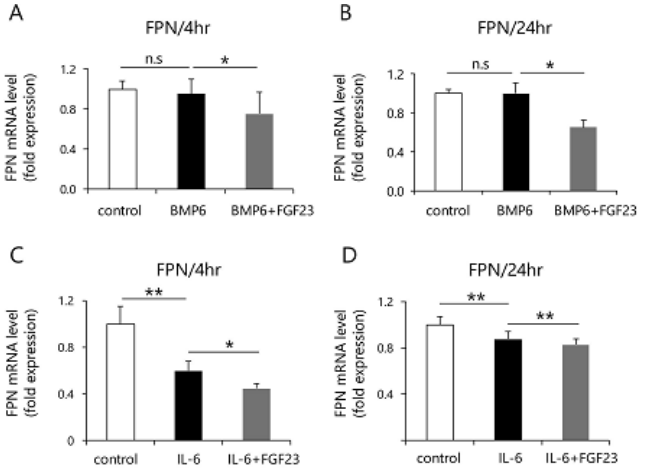

Figure 3: Effects of *FGF23* on *FPN* gene expression in HepG2 cells. HepG2 cells were incubated with or without 50 ng/mL *FGF23* in the presence of 200 ng/mL *BMP-6* for 4 hours (A) or 24 hours (B). HepG2 cells were incubated with or without 50ng/mL *FGF23* in the presence of 50 ng/mL *IL-6* for 4 hours (C) or 24 hours (D). Total RNAs were transcribed and amplified by qPCR. Data were normalized by the intensity of 18S rRNA-derived signals. Statistical significance was analyzed for the indicated columns (*p < 0.05, **p < 0.01, n.s. = no significant; Student's t-test). N = 3 for each group.

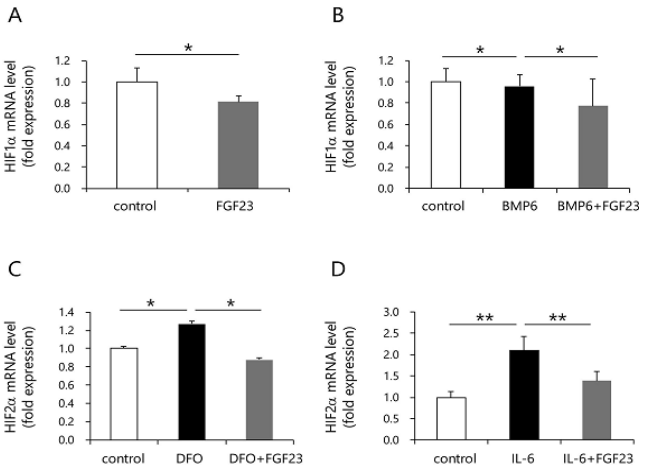

Figure 4: Effects of *FGF23* on *HIF* gene expression in HepG2 cells. HepG2 cells were treated with or without 50 ng/mL *FGF23* for 4 hours (A). HepG2 cells were treated with or without 200 ng/mL *BMP-6* (B), 100 μg/mL deferoxamine (C), or 50 ng/mL *IL-6* (D) in the presence or absence of 50 ng/mL *FGF23* for 4 hours. Total RNAs were transcribed and amplified by qPCR. Expression levels of the *HIF1α* gene were normalized by the intensity of 18S rRNA-derived signals (A, B). Expression levels of the HIF2α gene were normalized by the intensity of 18S rRNA-derived signals (C, D). Statistical significance was analyzed for the indicated columns (*p < 0.05, **p < 0.01; Student's t-test). N = 3 for each group.

FGF23 down-regulates HIF

Previously it has been shown that stabilized HIF promotes decreased hepcidin and increased ferroportin [6]. HIF can also bind directly to the ferroportin promoter and induce its expression. To evaluate HIF expression in response to *FGF23*, HepG2 cells were treated with the iron chelator Deferoxamine (DFO), *BMP-6*, or *IL-6* in the presence of *FGF23* [15]. Stimulation with *FGF23* showed a significant down-regulation of *HIF-1α* expression in HepG2 cells at

mRNA level compared to controls (Figure 4A). When HepG2 cells were treated with *BMP-6*, *HIF-1α* expression was slightly decreased compared to control cells. *FGF23* significantly promoted *BMP-6*-induced down-regulation of *HIF-1α* expression in HepG2 (Figure 4B). DFO and *IL-6* significantly increased *HIF-2α* mRNA levels in HepG2, all of which were inhibited by treatment with 50 ng/mL *FGF23* (Figure 4C and 4D). Immunoblot analysis confirmed these results at the protein level (data not shown). These results indicate that *FGF23* down-regulates HIF expression.

Discussion

More than 10 years ago, Gutierrez et al. [10] first reported a strong connection between *FGF23* and mortality in patients with HD, despite no difference in both serum levels of ferritin and hemoglobin among their patients' subgroups [10]. Whether or not *FGF23* is implicated directly in iron homeostasis in patients with HDs under Erythropoiesis Stimulating Agent (ESA) treatment has become a recently intriguing subject. As well known, hepcidin governs iron homeostasis through degradation of FPN on the membrane [3], and *HIF1α* and/or *HIF2α* (*HIFs*) are very sensitive to iron homeostasis [6]. In the study, we observed *FGF23* treatment on HepG2 cells indicated a decrease not only in FPN (Figure 1,3) and *HIFs* (Figure 4) expression, but also in hepcidin mRNA expression at a high dose of 50 ng/ml, unexpectedly (Figure 2 and S1).

FGF23 had been first reported to be one of the negative regulators of both HIF's activity and erythropoiesis in *FGF23*-null mice by Coe et al. [15]. Conversely, Singh et al. recently reported that *FGF23* gave rise to the activation of calcineurin /phospholipase Cγ (PLCγ)/ nuclear factor of activated T cells (NFAT) system through FGF Receptor 4 (FGFR4) in HepG2 cells [14], which stabilizes *HIFs* [16]. In this study, *FGF23* induced the suppression of *HIFs*, in a manner identical to Coe et al.'s study [15]. Although Coe et al.'s study indicated definitely that *HIFs* activity is a prerequisite for functional hematopoiesis and is definitely inhibited by *FGF23*, the molecular mechanism of *FGF23*, by which *HIFs* are inhibited is unclear. Agoro et al. [5] also reported a remarkable suppression of bone marrow *HIF1α* mRNA even in CKD model rats in which serum levels of *FGF23* were not as highly elevated as found in patients with HD [5]. *FGF23* could reportedly induce an inflammatory reaction and enhance the expression of C-reactive protein (CRP) and *IL-6* in the hepatocyte [14]. While inflammation had been also reported to induce *FGF23* mRNA expression *via.* the *HIF1α* in bone tissue from rats with or without CKD [17,18]. By contrast, as above-mentioned, Coe et al. had demonstrated a genetic suppressive effect on *HIFs* by *FGF23* [15]. Additionally, Agoro and colleagues demonstrated a definite recovery of HIF mRNA expression with an *FGF23*-blocking peptide, which suggested that *FGF23* can inhibit HIF expression [5]. Therefore, a negative feedback linkage could be between *HIFs* activity and *FGF23* production *in vivo*. Consistently, in this study, we could recognize that *FGF23* nullified an increase in the expression of *HIFs* mRNA induced by DFO and *IL-6* without effect on the basal expression of HIF mRNA. Although, until now, the effect on FPN expression via HIF has not been studied directly in the hepatocyte, *HIFs* were well known to be directly implicated in the expression of iron-regulating genes including promoters of transferrin receptor [19] and FPN *in vivo* [20]. The decreased expression of FPN found in this study, thus, might be due to a suppressed activity of HIF by *FGF23* in the HepG2cell. However, our results that hepcidin expression was suppressed by *FGF23* at 50 ng/ml was seemingly unreasonable because inflammatory cytokines induce hepcidin production through the *IL-6*/STAT signal pathway [21], and *FGF23*, as mentioned above, directly provoke inflammation in the hepatocyte [14]. Also, Agoro's study indicated that hepatic hepcidin expression was abolished completely by the *FGF23*-blocking peptide, which suggested that *FGF23* induced hepcidin expression in CKD rats [5]. In our study, however, suppression of hepcidin expression by *FGF23* was recognized at 50 ng/ml at 48 hours and 72 hours and after stimulation with *BMP-6* or *IL-6* (Figure 2 and S1). Therefore, we consider that *FGF23* could exert biphasic action in HepG2 cells and conversely inhibit hepcidin expression at a pharmacological dose.

Most factors involved in iron homeostasis are upregulated by *HIFs* [22], but hepcidin is known to be downregulated by *HIFs*. Peyssonnaux et al. [6] had firstly demonstrated that *HIF1α* repressed gene expression encoding hepcidin [6]. In addition, an increased hepcidin expression in Agoro's study was directly abolished by *FGF23* blocking peptide, which was injected intraperitoneally, i.e., a direct route via the portal vein to the liver, suggesting a direct action of *FGF23* to induce hepatic hepcidin expression in CKD rats [5]. Accordingly, direct action and indirect action of *FGF23 via*. suppression of *HIFs* by *FGF23* might be expected to induce synergistic hepcidin expression. However, our study demonstrated the down-regulation of hepcidin expression by *FGF23* at 50 ng/ml, albeit controversially (Figure 2). *BMP-6* was indicated to induce hepcidin expression via the SMAD signal pathway [23]. Similarly, *IL6* was also well known to induce hepcidin expression through the JAK/STAT signal pathway [21]. Therefore, whether or not *FGF23* influences the SMAD or STAT pathway should be further investigated. Furthermore, suppression of hepcidin was generally known to be accompanied by an increase in FPN expression; nevertheless, our study in Figure 3 demonstrated an unreasonable link between hepcidin and FPN in HepG2 cells.

David et al. [17] had revealed the activation of PLCγ/calcineurin via FGFR4 by *FGF23* in the hepatocyte, which induces Ca ion influx in HepG2 cells [17]. Additionally, FGFR4 had been reportedly to be regulated both by calcium and phosphate [14]. We, thus, consider that a high intracellular Ca concentration might contribute to our unreasonable results between FPN and hepcidin [24].

Conclusion

In conclusion, for intravenous iron administration therapy in patients with HD, scientific evidence-based guidelines with iron homeostasis have not yet been established. Although our study suggested a strong direct implication of *FGF23* in iron homeostasis, it remains inconclusive. Moreover, because an implication of *FGF23* on both hepcidin and FPN has not yet investigated directly in vitro, a further systematic study is needed to unveil the underlying mechanisms of *FGF23* on iron homeostasis, especially whether or not *FGF23* acts directly on both hepcidin expression and its function.

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