

HNF-4 α Negatively Regulates Heparin Expression Through BMPR1A in HepG2 Cells

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Abstract Heparin synthesis is reported to be inadequate according to the body iron store in patients with non-alcoholic fatty liver disease (NAFLD) undergoing hepatic iron overload (HIO). However, the underlying mechanisms remain unclear. We hypothesize that hepatocyte nuclear factor-4 α (HNF-4 α) may negatively regulate heparin expression and contribute to heparin deficiency in NAFLD patients. The effect of HNF-4 α on heparin expression was observed by transfecting specific HNF-4 α small interfering RNA (siRNA) or plasmids into HepG2 cells. Both direct and indirect mechanisms involved in the regulation of HNF-4 α on heparin were detected by real-time PCR, Western blotting, chromatin immunoprecipitation (chIP), and reporter genes. It was found that HNF-4 α suppressed heparin messenger RNA (mRNA) and protein expressions in HepG2 cells, and this suppressive effect was independent of the potential HNF-4 α response elements. Phosphorylation of SMAD1 but not STAT3 was inactivated by HNF-4 α , and the SMAD4 response element was found essential to HNF-4 α -induced heparin reduction. Neither inhibitory SMADs, SMAD6, and SMAD7 nor BMPR ligands, BMP2, BMP4, BMP6, and BMP7 were regulated by HNF-4 α

in HepG2 cells. BMPR1A, but not BMPR1B, BMPR2, ActR2A, ActR2B, or HJV, was decreased by HNF-4 α , and HNF4 α -knockdown-induced stimulation of heparin could be entirely blocked when BMPR1A was interfered with at the same time. In conclusion, the present study suggests that HNF-4 α has a suppressive effect on heparin expression by inactivating the BMP pathway, specifically via BMPR1A, in HepG2 cells.

Keywords HNF-4 α · heparin · pSMAD1/5/8 · BMPR1A

Introduction

Heparin is a liver-derived peptide hormone and plays a key role in iron homeostasis by negatively regulating intestinal iron absorption and iron release from macrophages [1]. Hepatic iron overload (HIO) is frequently observed in patients with non-alcoholic fatty liver disease (NAFLD) [2, 3] and has been confirmed to promote disease progression by increasing hepatic fibrosis and liver cholesterol synthesis [4, 5]. Dysregulation of heparin was frequently observed in patients with NAFLD with HIO [6–8]. Although serum or liver heparin was found to be increased in NAFLD patients, the production of heparin that was normalized to serum ferritin or hepatic iron score (HIS) was still insufficient compared to the elevation of body iron stores [9, 10]. Impaired heparin response was considered to be closely related with the severe form of HIO in NAFLD [11, 12] and has also been proved to play an important role in HIO in some other metabolic diseases, such as type 2 diabetes [13, 14]. However, the mechanisms underlying heparin deficiency in NAFLD patients remain elusive.

Hepatocyte nuclear factor-4 α (HNF-4 α) is a liver-enriched transcription factor that is critical in lipid and glucose

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metabolism. Saturated fatty acid, the most important independent risk factor of NAFLD [15–17], can significantly stimulate HNF-4 α expression [18–20]. The protein level of HNF-4 α was also found significantly increased in mice with leptin-deficient (ob/ob) or diet-induced obesity mimicking the onset of NAFLD [21, 22]. What is more, Courselaud et al. [23] found that HEPC transcripts were increased in liver-specific HNF4 α -null mice. All these findings suggest that HNF-4 α may play a putative role in relative hepcidin deficiency in NAFLD patients. However, the molecular mechanism underlying how HNF-4 α regulates hepcidin expression remains unclear.

The aim of the present study was to dissect the effect of HNF-4 α on hepcidin expression in HepG2 cells in vitro and further clarify its molecular mechanisms, in an attempt to gain deeper insights into the etiology of hepcidin deficiency in NAFLD patients with HIO so as to provide a potential new therapeutic target.

Materials and Methods

Cell Culture, Treatment, and Transfection of siRNA and Plasmids

The human hepatoma cell line HepG2 (Chinese Academy of Sciences, Shanghai, China) was grown at 37 °C in a mixture of high glucose DMEM medium (Hyclone, USA) supplemented with 10 % fetal bovine serum (FBS) (Hyclone, USA) (v:v), 100 IU/ml penicillin, and 50 μ g/ml streptomycin sulfate (Hyclone, USA). For HNF-4 α , SMAD4, or/and BMPR1A silencing, cells were transferred to 6-well plates and transfected with HNF-4 α , SMAD4, or/and BMPR1A siRNA products (sc-35573, sc-92315, and sc-40216, Santa Cruz Biotechnology, USA) that generally consist of pools of three to five target-specific 19–25 nt siRNAs designed to knockdown gene expression or a negative invalid control siRNA in the presence of lipofectamine RNAiMAX in normal growth medium with antibiotics, according to the manufacturer's instructions (Invitrogen, USA). After a 12 h transfection, the medium was replaced and cells were incubated for additional 36 h. For HNF-4 α over-expression, HepG2 cells were seeded in 6-well plates and transfected with 2 μ g HNF-4 α or empty plasmids (Genepharma, Shanghai, China) in the presence of FuGENE HD transfection reagent (E2311, Promega, Madison, WI, USA) and then incubated for 12 h, then the medium was replaced and cells were incubated for additional 36 h. For BMP inhibitor experiments, HepG2 cells were transfected with HNF-4 α siRNA in the presence or absence of the BMP inhibitor LDN193189 (100 nM; Selleck Chemicals, Houston, USA). After a 12 h transfection, the medium was replaced and cells were incubated for additional 36 h with or without additional LDN193189.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (chIP) assay was performed using a commercially available kit (Upstate Biotechnology, Lake Placid, NY, USA) as described by Diedra M [24]. The antibody against HNF-4 α was purchased from Santa Cruz (CA, USA) and SMAD4 and STAT3 from CST (Cell Signaling Technology, Danvers, MA, USA). The primer sequences used for PCR were as follows: Hpc forward 5'-CGCT CTGT TCCC GCTT AT-3' and Hpc reverse 5'-CGAG TGAC AGTC GCTT TTAT G-3' that amplified 129 bp of human hepcidin promoter containing the response elements of SMAD4, STAT3, and HNF-4 α (the proximal one); Hpc forward 5'-ATGGG GTCTC CCTAT GTTGC-3' and Hpc reverse 5'-CCTTG AGATG TGGCT CTGGT AA-3' that amplified 184 bp of human hepcidin promoter containing the response element of HNF-4 α (the distal one).

Generation of Hepcidin Promoter Plasmid Constructs, Cell Transfection, and Luciferase Reporter Assays

pGL3-basic luciferase reporter (Promega, Southampton, UK) with full-length human hepcidin promoter was generated by obtaining genomic DNA from HepG2 cells and cloning the proximal 942 bp of human HAMP promoter into the pGL3-basic luciferase reporter vector, as described by Courselaud et al. [23]. Site-directed mutagenesis (Quickchange II, Stratagene, Stockport, UK) on putative BMPs response elements was performed as described by Ling et al. [25] and the putative HNF-4 α response element was depleted according to Courselaud et al. [23]. HepG2 cells were transferred to 48-well plates and transfected with empty pGL3-basic vectors, certain HAMP reporter constructs, and HNF-4 α plasmids (Genepharma, Shanghai, China) using FuGENE HD Transfection Reagent (Promega, Madison, WI, UK) according to the manufacturer's instructions. To normalize the transfection efficiency, an internal control pRL-SV40 Renilla luciferase plasmids (Promega, Southampton, UK) was co-transfected alongside the HAMP constructs in a 1:200 ratio in serum-free medium. After a 48 h equilibration, luciferase activity was determined in triplicate in at least three independent experiments using the Dual Luciferase Reporter Assay, according to the manufacturer's instructions (Promega, Southampton, UK).

RNA Isolation and Real-Time Quantitative PCR

Real-time qPCR was used to analyze HNF-4 α and hepcidin mRNA levels in HepG2 cells. Total RNA was extracted from HepG2 cells using a single step extraction method and TRIzol reagent (Invitrogen, USA), as previously described [26]. Total RNA was reverse-transcribed using RT reagent Kit (PrimerscriptTM, TAKARA, Japan). Real-time qPCR was performed in 1 \times Universal Master Mix (Applied

Biosystems, USA) with gene-specific primers by the ABI Prism 7300 Sequence Detection System (Applied Biosystems, USA), and mRNA levels of specific genes were normalized to the β -actin levels of the same sample.

Western Blotting

HepG2 cells were washed twice in Hank's balanced salt solution and homogenized in lysis buffer for the preparation of whole protein extracts. NE-PER Reagents (Pierce Biotechnology, Rockford, IL, USA) was used to extract nuclear and cytosolic proteins according to the manufacturer's instructions. Protein concentrations were determined using the BCA Protein Assay kit (Pierce Biotechnology). Cell lysates (30 μ g) were loaded and then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10 % ~ 15 %). After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) using the wet Trans-Blot Cell (Bio-Rad Laboratories, Hercules, CA, USA). Then, the membranes were blocked with 5 % (*w/v*) nonfat milk powder in TBS/T (0.5 % Tween 20 in 1 \times Tris-buffered saline) for 2 h at room temperature. The blots were then incubated overnight at 4 $^{\circ}$ C with primary antibodies, followed by incubation with an IRDye 800CW-conjugated secondary antibody and detection with LI-COR imaging system (LI-COR Biosciences). The following primary antibodies were used: HNF-4 α (Santa Cruz, USA; 1:500 dilution), hepcidin (ab30760, Abcam, UK; 1:100 dilution), SMAD1 (Cell Signaling Technology, USA; 1:1000 dilution), pSMAD1/5/8 (Cell Signaling Technology, USA; 1:1000 dilution), SMAD4 (BBI, China; 1:1000 dilution), STAT3 (Cell Signaling Technology, USA; 1:1000 dilution), pSTAT3 (Cell Signaling Technology, USA; 1:1000 dilution), BMPR1A (Epitomics, USA; 1:500 dilution), BMPR1B (Abcam, UK; 1:500 dilution), BMPR2 (Epitomics, USA; 1:500 dilution), ActR2A (BBI, China; 1:1000 dilution), ActR2B (BBI, China; 1:1000 dilution), HJV (Abcam, UK; 1:100 dilution), Id1 (Santa Cruz, USA; 1:200 dilution), and β -actin (Bioworld, USA; 1:5000 dilution). The immunoreactive bands were quantitated by densitometric scanning. After a wash with TBS/T containing 0.5 % Tween 20, the blots with monoclonal antibodies were incubated with peroxidase-conjugated affinity-pure rabbit anti-goat or goat anti-rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories, USA; 1:4000 dilution). Peroxidase-conjugated antibodies were detected by the enhanced chemiluminescence (ECL) method (Amersham). Signals quantitated by densitometry were normalized to β -actin levels or, in the case of phosphoproteins, to the total levels of the same protein.

Statistics

Values are represented as mean \pm standard error mean (S.E.M.). Statistical analysis was performed using the Statview software (SAS Institute Inc., SAS campus drive, USA). Statistical difference between two groups was assessed by the Independent *t* test. One-way ANOVA, followed by LSD *t* post hoc test, was performed to analyze the difference between the three or more groups. $P < 0.05$ is considered statistically significant.

Results

HNF-4 α Inhibits Hepcidin Expression in HepG2 Cells

To directly evaluate the effect of HNF-4 α on hepcidin expression, HNF-4 α siRNA or plasmid was transfected into HepG2 cells, and hepcidin mRNA and protein levels were detected by real-time qPCR and Western Blot. Compared with the blank control group, hepcidin mRNA and protein levels were both markedly increased when HNF-4 α was knocked down (for hepcidin, si-HNF4 α vs. control, $p < 0.01$) (Fig. 1a, c) and significantly reduced when HNF-4 α was over-expressed (for hepcidin, oe-HNF4 α vs. control, $p < 0.001$ or $p < 0.01$) (Fig. 1b, c).

HNF4 α -Induced Reduction of Hepcidin is not Dependent on its Potential Response Elements on Hepcidin Promoter

Knowing that two potential response elements were reported on the flanking region of human HEPC gene [23], we first verified whether HNF-4 α could bind to human HEPC promoter through chromatin immunoprecipitation (chIP) experiments. Our data showed that HNF-4 α bound to human hepcidin promoter through the proximal binding site but not the distal one (for distal binding site, potential HRE1; for proximal binding site, potential HRE2) (Fig. 2a). Next, we observed the role of the chIP-verified response element in the HNF4 α -induced reduction of hepcidin through experiments with reporter genes. HepG2 cells were co-transfected with human hepcidin promoter with or without the HNF-4 α binding sites and plasmids encoding HNF-4 α (for wild-type HEPC promoter, HAMP; for HEPC promoter with depletion of HNF-4 α binding site, Δ HNF-4 α) (Fig. 2b). HNF-4 α plasmids showed an obvious inhibitory effect on the luciferase activities of the wild full-length hepcidin promoter (HAMP + HNF-4 α vs. HAMP, $p < 0.001$) (Fig. 2b). Furthermore, depletion of the HNF-4 α binding sites significantly suppressed the basal level of the hepcidin promoter activity (Δ HNF-4 α vs. HAMP, $p < 0.001$) (Fig. 2b), but showed no blocking effect on the HNF4 α -induced reduction (Δ HNF-4 α + HNF-4 α vs. Δ HNF-4 α , $p < 0.001$) (Fig. 2b).

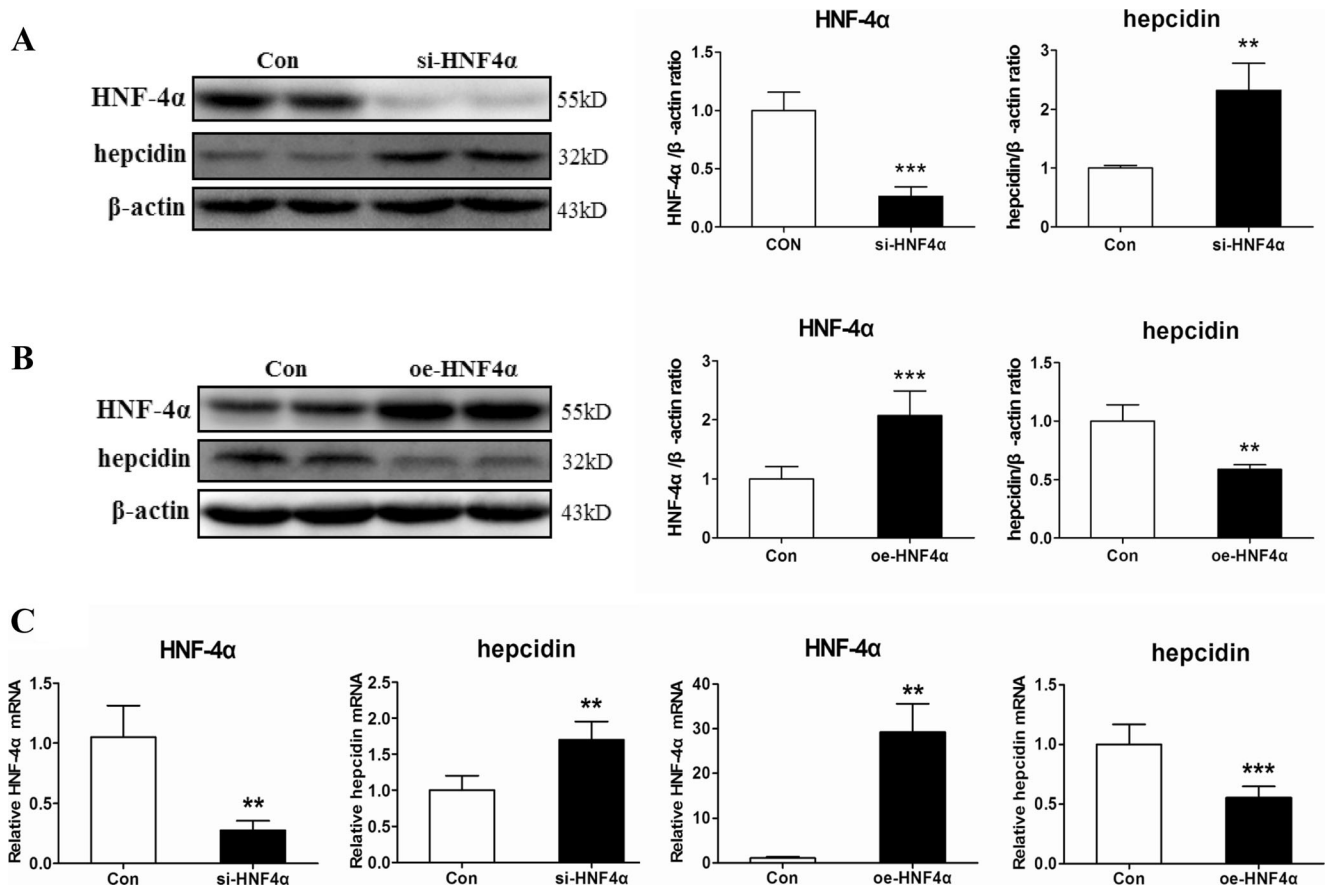


Fig. 1 HNF-4 α reduces hepcidin expression in HepG2 cells. **a,b** HepG2 cells were transferred to 6-well plates and incubated with growth medium containing negative invalid control siRNA (Con) or specific HNF-4 α siRNA (si-HNF4 α) for 12 h and normal growth medium without siRNA for additional 36 h. **b,c** HepG2 cells were seeded in 6-well plates and incubated with growth medium containing empty control plasmids (Con) or plasmids encoding HNF-4 α (oe-HNF4 α) for 12 h

and normal growth medium without plasmids for additional 36 h. Hepcidin protein (**a, b**) and mRNA (**c**) level were measured by Western blotting and Real-time qPCR. **, significantly different from control, $p < 0.01$ (***, $p < 0.001$). Values are expressed as mean \pm S.E.M. and determined in three independent experiments. Statistical difference between two groups was assessed by the Independent t test

HNF-4 α negatively Regulates Hepcidin by Inactivating BMP but not IL-6 Pathway

STAT3 and SMADs are known to mediate hepcidin response to IL-6 and BMPs, respectively. To determine whether these two novel pathways were involved in HNF4 α -induced hepcidin repression, we observed the effect of HNF-4 α on the total and/or phosphorylated protein levels of STAT3, SMAD1, and SMAD4. It was found that the phosphorylation of SMADs was markedly inhibited by HNF-4 α plasmids and markedly enhanced when HNF-4 α was knocked down by specific siRNA (oe-HNF4 α vs. control, $p < 0.05$; si-HNF4 α vs. control, $p < 0.05$) (Fig. 3), without altering the total level of SMAD1 and SMAD4 ($p > 0.05$) (Fig. 3). Besides, no significant alteration was found in the total or phosphorylated protein levels of STAT3 when HNF-4 α was interfered with or over-expressed ($p > 0.05$) (Fig. 3).

In order to further verify the role of the BMP signaling pathway in HNF4 α -mediated hepcidin suppression, chIP, reporter gene assay, and co-transfection with HNF-4 α and

SMAD4 siRNA was performed (for wild-type HEPC promoter, HAMP; for HEPC promoter with mutation on SMAD4 binding site, Δ SMAD4; for HNF-4 α silence, Δ H; for SMAD4 silence, Δ S; for co-silence, Δ H + S). The results of chIP showed that the amount of SMAD4 binding to hepcidin promoter was increased markedly in HepG2 cells transfected with HNF-4 α siRNA (SI) and decreased obviously when HNF-4 α was over-expressed (OE) by encoding plasmids (SMAD4: SI vs. control, $p < 0.01$; OE vs. Control, $p < 0.001$) (Fig. 4a), but the amount of STAT3 remained unchanged under both of the above conditions (Fig. 4a). The luciferase activity of hepcidin promoter with mutation SMAD4 binding site was no longer decreased and significantly increased when HNF-4 α plasmids were co-transfected (HAMP + HNF-4 α vs. HAMP, $p < 0.001$; Δ SMAD4 + HNF-4 α vs. Δ SMAD4, $p < 0.001$) (Fig. 4b). The expression of hepcidin in HepG2 cells with low levels of HNF-4 α was elevated markedly, which could be entirely blocked by co-transfection of SMAD4 siRNA (Δ H vs. control, $p < 0.05$; Δ H + S vs. Δ S, $p > 0.05$) (Fig. 4c).

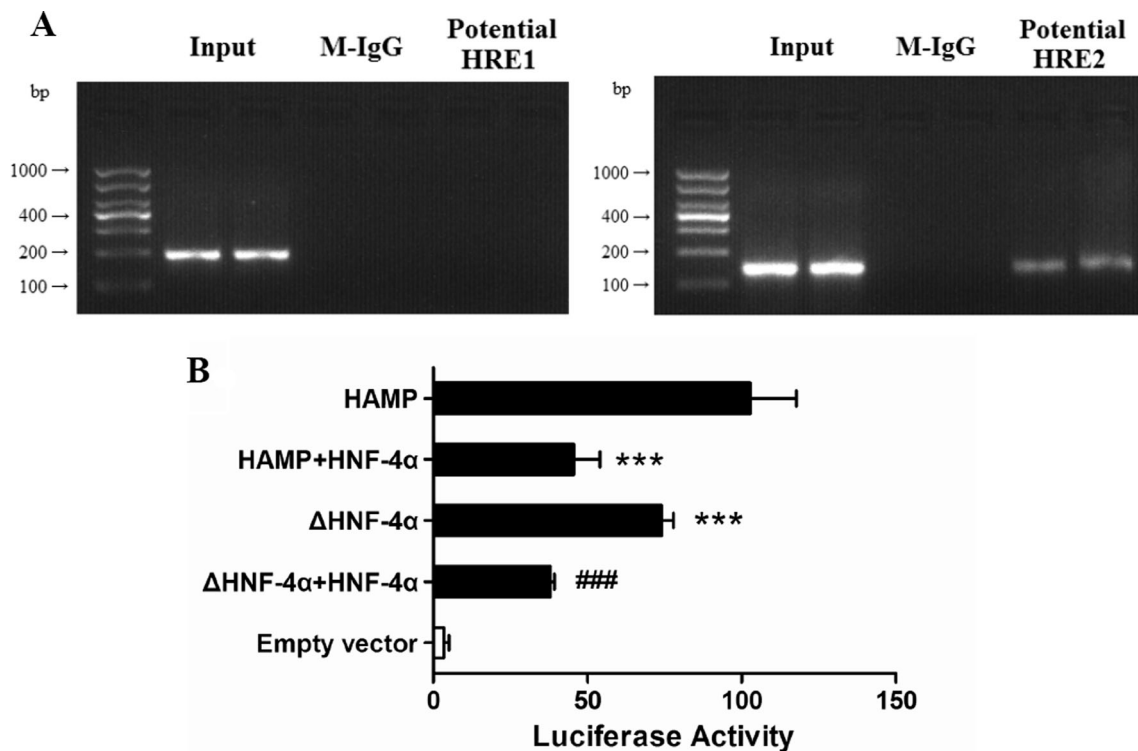


Fig. 2 The suppressive effect of HNF-4 α on hepcidin is independent of potential HNF-4 α response elements on hepcidin promoter. **a** DNA binding activity of HNF-4 α with human hepcidin promoter region was determined by ChIP in HepG2 cells. **b** HepG2 cells were co-transfected with luciferase reporter constructs of empty vector, hepcidin promoter of full-length (HAMP), or hepcidin promoter with depletion of potential

HNF-4 α binding sites (Δ HNF-4 α) and HNF-4 α encoding plasmids. The luciferase activity was measured after 48 h. ***, significantly different from HAMP, $p < 0.001$; ###, compared with Δ HNF-4 α , $p < 0.001$. Values are expressed as mean \pm S.E.M. and determined in three independent experiments. One way ANOVA, followed by LSD t post hoc test, was performed to analyze difference between the groups

In addition, we also found Id1, another well-characterized transcriptional target of BMP-activated SMADs, was significantly inhibited by HNF-4 α over-expression and obviously upregulated when HNF-4 α was knocked down (si-HNF4 α vs. control, $p < 0.001$; oe-HNF4 α vs. control, $p < 0.001$) (Supplemental Data, Fig. 1). Besides, we also showed that BMP2-stimulated hepcidin expression disappeared when HNF-4 α was over-expressed in HepG2 cells (OE + BMP2 vs. control, $p < 0.001$) (Supplemental Data, Fig. 2).

The Suppressive Effect of HNF-4 α on BMP Pathway and Hepcidin is Specifically Mediated by BMPR1A

Protein levels of BMP receptors (BMPR1A, BMPR1B, BMPR2, ActR2A, and ActR2B) [27] and the co-receptor HJV [1] were analyzed by Western blot to better clarify how HNF-4 α repressed the activation of the BMP pathway, or more precisely, the phosphorylation of SMAD1. The results showed that the silencing of HNF-4 α increased the level of BMPR1A, while over-expression decreased it (si-HNF4 α vs. control, $p < 0.01$; oe-HNF4 α vs. control, $p < 0.01$) (Fig. 5), without significantly altering BMPR1B, BMPR2, ActR2A, ActR2B, and HJV ($p > 0.05$) (Fig. 5).

To better investigate the role of BMPR type I receptor and/or BMPR1A in the suppressive effect of HNF-4 α on hepcidin, we observed the effect of BMP type I receptor inhibitor, LDN193189, on HNF-4 α regulation of hepcidin, and also the hepcidin expression in HepG2 cells co-transfected with HNF-4 α and BMPR1A siRNA (for BMP inhibitor LDN193189, L; for HNF-4 α silence in the presence of LDN193189, L + Δ H; for HNF-4 α silence, Δ H; for BMPR1A silence, Δ B; and for co-silence, Δ H + B) (Fig. 6a, b). It was found that the upregulation of hepcidin by HNF-4 α silence could be entirely blocked by either administration of LDN193189 or knocking down BMPR1A (Δ H vs. Control, $p < 0.05$; L + Δ H vs. L, $p > 0.05$; Δ H vs. Control, $p < 0.05$; Δ H + B vs. Δ B, $p > 0.05$) (Fig. 6a, b). In addition, we also found that neither BMPR1A ligands nor intracellular inhibitory SMADs could be regulated by HNF-4 α ($p > 0.05$) (Supplemental Data, Figs. 3 and 4).

Discussion

Hepcidin is a liver-derived peptide hormone that plays a key role in iron homeostasis by negatively regulating body iron stores [1]. It was reported that, although elevated, the

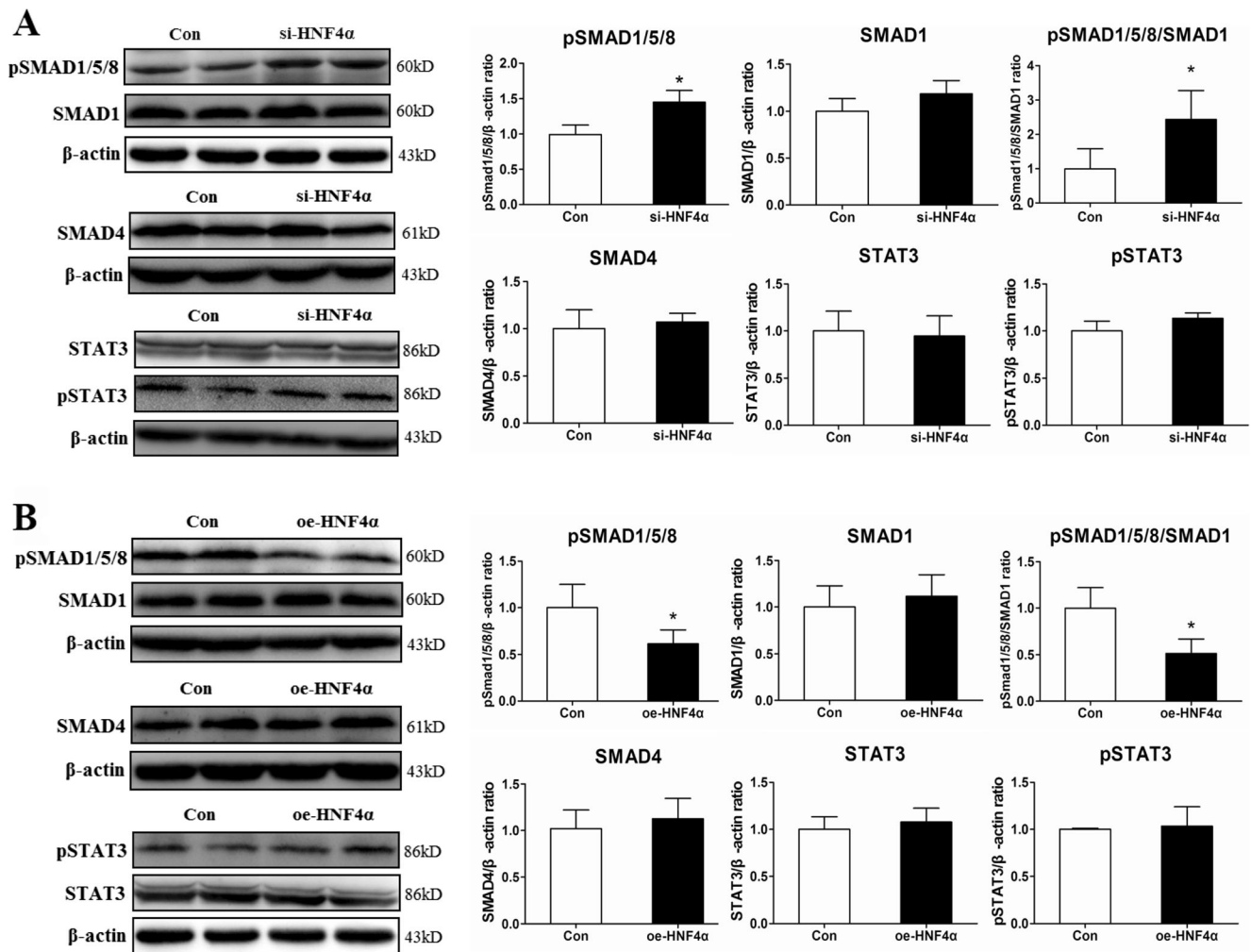


Fig. 3 HNF-4 α inhibits the phosphorylation of SMAD1 but has no significant effect on STAT3. **a** HepG2 cells were transferred to 6-well plates and incubated with growth medium containing negative invalid control siRNA (Con) or specific HNF-4 α siRNA (si-HNF4 α) for 12 h and normal growth medium without siRNA for additional 36 h. **b** HepG2 cells were seeded in 6-well plates and incubated with growth medium containing empty control plasmids (Con) or plasmids encoding HNF-4 α (oe-HNF4 α) for 12 h and normal growth medium without plasmids for

additional 36 h. Protein levels of STAT3, pSTAT3, SMAD4, SMAD1, and pSMAD1/5/8 were detected by Western Blot and the phosphorylation ratio of SMAD1 was calculated by pSMAD1/5/8/SMAD1. *, significantly different from control, $p < 0.05$. Values are expressed as mean \pm S.E.M. and determined in three independent experiments. Statistical difference between two groups was assessed by the Independent t test

production of hepcidin mRNA in NAFLD patients with HIO was still insufficient [9, 10], which is believed to play an important role in the occurrence of HIO [9, 15]. However, the mechanism underlying the impairment to hepcidin expression remains unclear. The orphan nuclear receptor HNF-4 α is an essential transcription factor and master regulatory protein of lipid and glucose metabolism [28, 29]. In the present study, we demonstrated that HNF-4 α inhibited the activity of the BMP pathway specifically through BMPR1A and negatively regulated hepcidin expression in HepG2 cells, providing a probable new link between HNF-4 α , or furthermore, lipid metabolic disorder and iron metabolism.

We found that the expression of hepcidin protein was significantly suppressed by HNF-4 α plasmids and markedly elevated when HNF-4 α expression was interfered with by

specific siRNA, suggesting that HNF-4 α had an inhibitory effect on hepcidin synthesis. The same result was also found in the mRNA level of hepcidin, furthermore, implying that the regulatory effect of HNF-4 α on hepcidin probably occurred at the transcriptional level. Knowing that HNF-4 α has two potential binding sites in the HEPIC 5'-flanking promoter region [23], we then evaluated whether HNF-4 α could bind to the hepcidin and play a direct inhibitory effect on hepcidin transcription. It was found that HNF-4 α could bind to the proximal potential binding site in the human hepcidin promoter. However, the data of reporter gene experiments further demonstrated that HNF-4 α induced reduction on fluorescence activity of hepcidin promoter could not be blocked by deletion of the ChIP-verified HNF4-binding sites, suggesting that HNF-4 α may indirectly, but not directly, suppress the

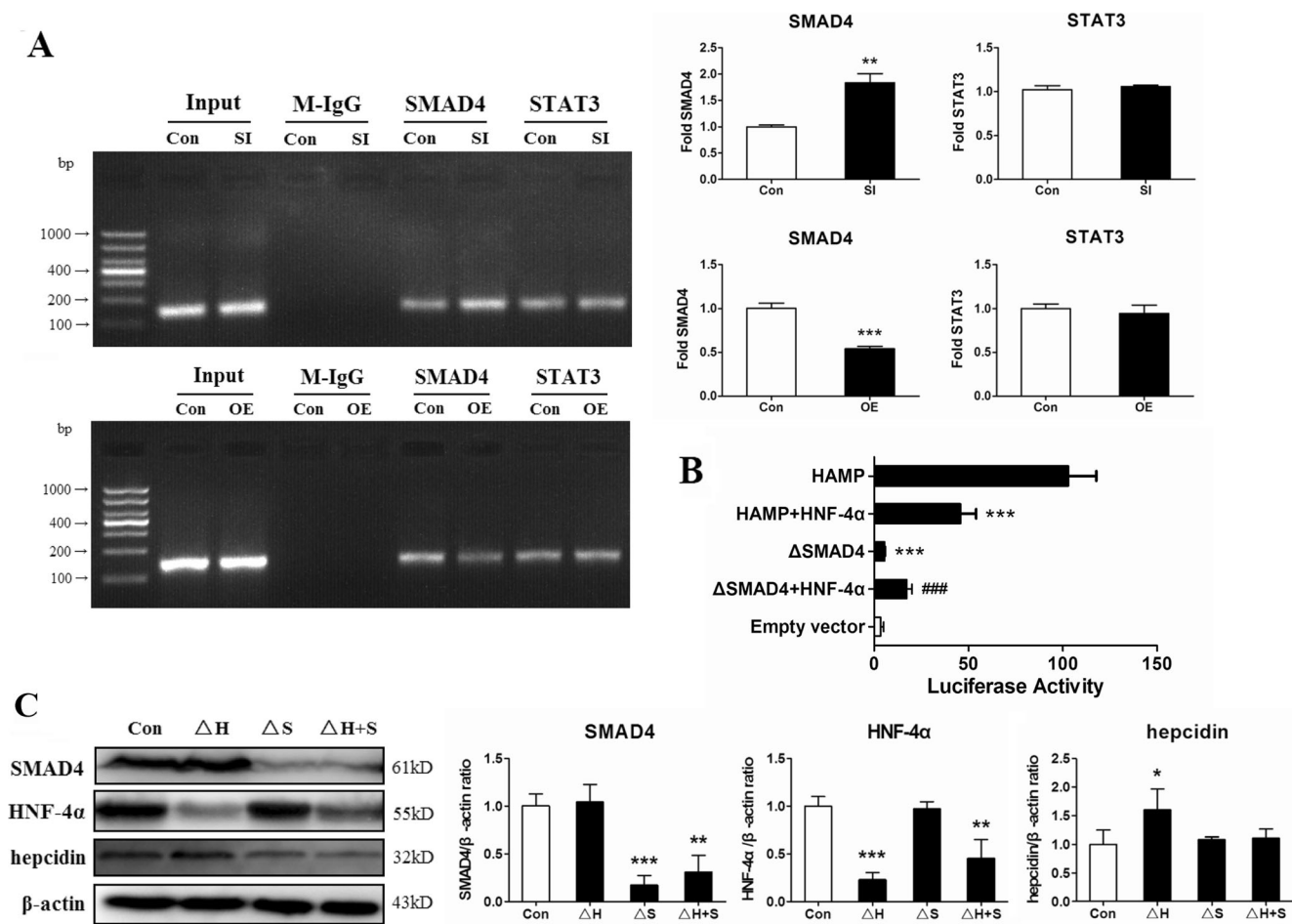


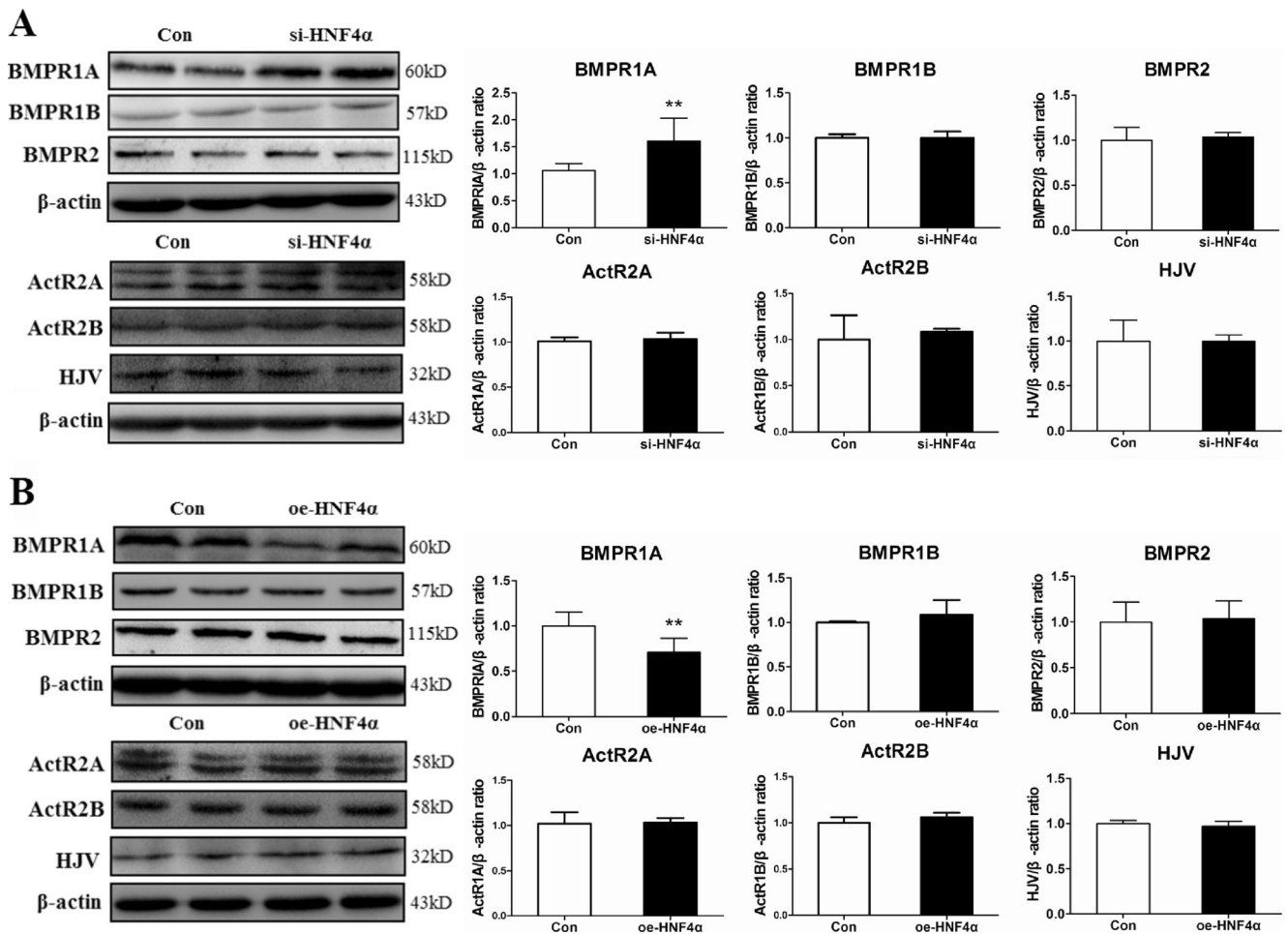
Fig. 4 HNF-4 α reduces hepcidin expression specifically via blocking BMP pathway. **a** HepG2 cells were incubated with growth medium containing negative invalid control siRNA (Con) or specific HNF-4 α siRNA (si-HNF4 α) for 12 h and normal growth medium without siRNA for additional 36 h or incubated with empty control plasmids (Con) or plasmids encoding HNF-4 α (oe-HNF4 α) for 12 h and normal growth medium without plasmids for additional 36 h. DNA binding activity of SMAD4 and STAT3 with hepcidin promoter was determined by ChIP. **, significantly different from control, $p < 0.01$ (***, $p < 0.001$). **b** Mutation of SMAD4-binding site on hepcidin promoter, refer to Matak et al. [45]. HepG2 cells were co-transfected with luciferase reporter constructs of hepcidin promoter of SMAD4-mutation, and HNF-4 α

plasmids. Luciferase activity was measured 48 h later. ***, significantly different from HAMP, $p < 0.001$. ###, significantly different from Δ SMAD4, $p < 0.001$. **c** HepG2 cells were transfected with negative invalid control siRNA (Con), specific HNF-4 α siRNA (Δ H), specific SMAD4 siRNA (Δ S), or co-transfected with siRNA for HNF-4 α and SMAD4 (Δ H + S), respectively, and incubated for 48 h with the replacement of growth medium 12 h later. The protein level of hepcidin was detected by Western blotting. *, significantly different from control, $p < 0.05$ (**, $p < 0.01$, ***, $p < 0.001$). Values are expressed as mean \pm S.E.M. and determined in three independent experiments. Statistical difference between groups was assessed by one-way ANOVA followed by LSD t post hoc test

transcriptional activity of hepcidin. In addition, we were surprised to find that the fluorescence activity of hepcidin promoter showed a significant moderate decrease, but not increase, after the knockout of HNF-4 α response element, implying that the direct effect of HNF-4 α on hepcidin might be positive but not negative, which is consistent with the regulatory effect of HNF-4 α on many other target proteins involved in lipid and glucose metabolism [30]. These data strongly suggest that, besides a direct positive effect of HNF-4 α on hepcidin transcription, there exists a dominant indirect mechanism involved in the HNF-4 α -induced hepcidin reduction.

STAT3 and SMADs are novel transcription regulators of hepcidin and mediate its response to IL-6 and BMPs. In the present study, we found that only the level of pSMAD1/5/8

protein was significantly suppressed by HNF-4 α and obviously stimulated when HepG2 was transfected with HNF-4 α siRNA. STAT3, pSTAT3, SMAD1, and SMAD4 all remained unchanged when HNF-4 α was silenced or overexpressed, suggesting that HNF-4 α reduced hepcidin specifically via inactivation of BMP pathway. Our results also demonstrated that the binding activity of SMAD4 to human hepcidin promoter was dramatically enhanced or suppressed under the condition of HNF-4 α knockdown or overexpressed, but no significant change was observed in the binding activity of STAT3. These results further demonstrated that it was BMP but not IL-6 pathway that may be involved in the mechanism underlying the inhibitory effect of HNF-4 α on hepcidin.



control plasmids (Con) or plasmids encoding HNF-4 α (oe-HNF4 α) for 12 h and normal growth medium without plasmids for additional 36 h. Protein levels of BMPR1A, BMPR1B, BMPR2, ActR2A, ActR2B, and HJV were detected by Western blotting. **, significantly different from control, $p < 0.01$. Values are expressed as mean \pm S.E.M. and determined in three independent experiments. Statistical difference between two groups was assessed by the Independent t test

control plasmids (Con) or plasmids encoding HNF-4 α (oe-HNF4 α) for 12 h and normal growth medium without plasmids for additional 36 h. Protein levels of BMPR1A, BMPR1B, BMPR2, ActR2A, ActR2B, and HJV were detected by Western blotting. **, significantly different from control, $p < 0.01$. Values are expressed as mean \pm S.E.M. and determined in three independent experiments. Statistical difference between two groups was assessed by the Independent t test

Besides, we also found that the fluorescence activity of human hepcidin promoter with mutation SMAD4-binding site was no longer decreased in response to HNF-4 α plasmids, once again suggesting that the BMP pathway was essential for the suppressive effect of HNF-4 α on hepcidin. The increase of fluorescence activity of human hepcidin promoter with mutation SMAD4-binding site is consistent with our previous finding that there might be a direct stimulatory effect of HNF-4 α on hepcidin transcription through potential response elements; however, it was much weaker compared with the negative one mediated by BMP pathway and therefore could be observed only when BMP pathway was blocked. On the basis of the above results, we further confirmed the role of BMP pathway by finding that HNF-4 α siRNA-induced elevation of hepcidin totally disappeared in the absence of SMAD4, better supporting the critical role of BMP pathway in HNF-4 α regulation on hepcidin.

To clarify the mechanism underlying the inactivation of BMP pathway by HNF-4 α , we first observed the effect of HNF-4 α on BMP receptors and their co-receptor HJV and found that only BMPR1A could be regulated by HNF-4 α and was markedly decreased, implying that BMPR1A might be involved in the reduction of hepcidin induced by HNF-4 α . LDN193189, a BMP type I receptor inhibitor, was proved to totally blocked hepcidin elevation induced by HNF-4 α knockdown in our present study, and we also found that silencing HNF-4 α could not further stimulate hepcidin expression when BMPR1A was knocked down at the same time. Our results indicated that the suppressive effect of HNF-4 α on BMP pathway and hepcidin expression was dependent on BMP type I receptor, and more precisely, BMPR1A. BMPR1A, and 1B share different bio-functions in vivo. BMPR1A was found to play a specific role in Mullerian duct regression [31], heart morphogenesis [32], and growth control

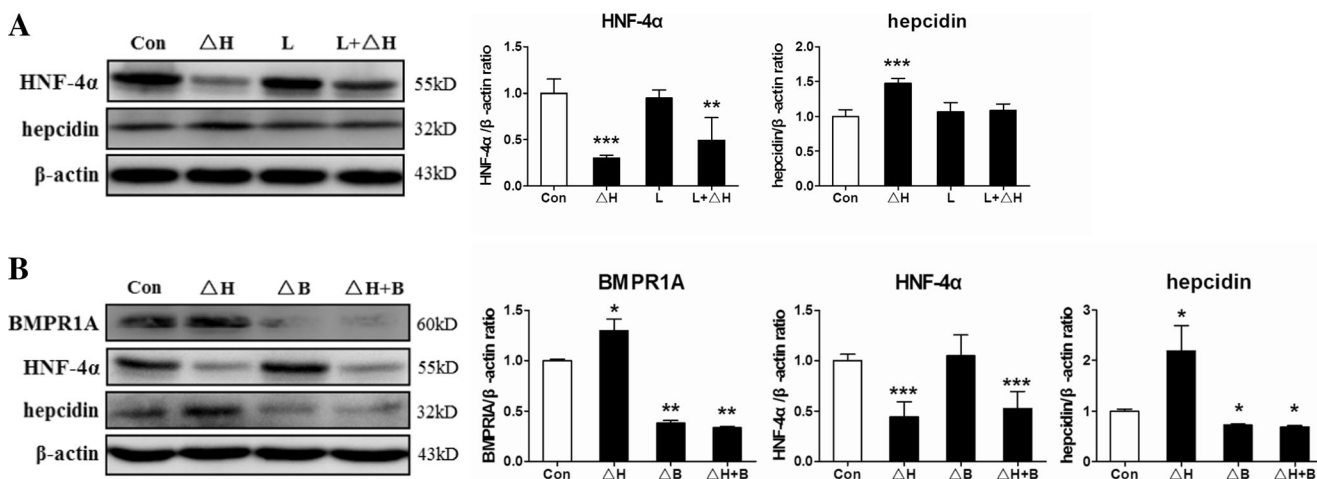


Fig. 6 BMP-SMAD signaling pathway and BMPR1A mediate HNF4 α -induced suppression on hepcidin in HepG2 cells. HepG2 cells were transfected with negative invalid control siRNA (Con), specific HNF-4 α siRNA (Δ H), LDN193189 (L), or transfected with siRNA for HNF-4 α with LDN193189 (L + Δ H) (a) or negative invalid control siRNA (Con), specific HNF-4 α siRNA (Δ H), specific BMPR1A siRNA (Δ B), or co-transfected with siRNA for HNF-4 α and BMPR1A (Δ H + B) (b),

respectively, and incubated for 48 h with the replacement of growth medium 12 h later. The protein level of hepcidin was detected by Western blotting. *, significantly different from control, $p < 0.05$ (**, $p < 0.01$; ***, $p < 0.001$). Values are expressed as mean \pm S.E.M. and determined in three independent experiments. Statistical difference between groups was assessed by one-way ANOVA followed by LSD t post hoc test

of colon epithelial cells [33] while BMPR1B was considered essential in reproductive function [34]. It was even reported that BMPR1A and BMPR1B signaling exerted opposing effects on gliosis after spinal cord injury [35]. BMP1A and 1B also showed different affinity to their upstream ligands. For instance, BMPR1B bound more efficiently to GDF-15 [36] and BMP7 [37]. All these findings illustrate that BMPR1A and 1B have significant distinction on their response to upstream factors and mediation of downstream pathways, which might be part of the reason why HNF-4 α only showed its regulatory effect on BMPR1A.

The molecular mechanism underlying the repression of BMPR1A by HNF-4 α remains undetermined. In silico analysis of BMPR1A, promoter was previously performed with only SP1 response elements were detected [38]. A subsequent study proved that it positively regulated transcription of BMPR1A [39], implying that SP1 may, to some extent, participate in the regulation of HNF-4 α on BMPR1A. Functional synergism of HNF-4 α and Sp1 on the human apolipoprotein CIII (apoCIII) promoter was found in other studies [40, 41], failing to support a possible negative regulatory effect of HNF-4 α on BMPR1A expression. Although the interaction of HNF-4 α with SP1 in their co-regulation of blood coagulation factor X was previously reported to be mutually exclusive, a minor overlap of their binding sites was eventually found [42]. In addition, no binding site for HNF-4 α was identified in the BMPR1A promoter and other type I BMPR. These results imply that HNF-4 α might not repress BMPR1A directly or through interaction with SP1, and therefore, the mechanism underlying the effect of HNF-4 α on BMPR1A expression requires further study.

It was recently reported that miR-122 is positively regulated by HNF-4 α [43] and also plays a direct inhibitory role in hepcidin transcription via its response elements on the promoter [44]. These findings imply that miR-122 may also, to some extent, be involved in the suppressive effect of HNF-4 α on hepcidin, although further evidence for this conclusion is required. BMPR1A mRNA was also reported to have been decreased by miR-122; however, no significant response of pSMAD1/5/8 to miR-122 was determined in the same study [44], suggesting that miR-122 may not be involved in the regulation of HNF-4 α on BMPR1A.

In summary, our study reports HNF-4 α to inhibit hepcidin expression by specifically suppressing BMPR1A in HepG2 cells and thus elucidating a likely novel mechanism of hepcidin regulation. Since HNF-4 α is significantly stimulated by several risk factors of NAFLD, such as saturated fatty acids, our study assists in explaining the relative deficiency of hepcidin in NAFLD.

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Author Contributions Min Li, who designed the concept of the study, made critical revisions of the manuscript and was responsible for obtaining the funding. Wencai Shi and Heyang Wang, who designed the study, provided technical or material support, participated in data acquisition, analysis and interpretation, drafted the manuscript, and performed the statistical analysis. Xuan Zheng, Xin Jiang, Zheng Xu, and Hui Shen participated in data acquisition and technical support. Min Li is the guarantor of this article and, as such, has full access to all the data in the study and is responsible for the integrity of the data and the accuracy of the data analysis.

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