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Secretion of bioactive hepcidin-25 by liver cells correlates with its gene transcription and points towards synergism between iron and inflammation signaling pathways

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ABSTRACT

Hepcidin is a small liver-derived peptide central in the regulation of systemic iron homeostasis. Although the gene regulation has been extensively studied at transcriptional level, the corresponding effects on the production of bioactive peptide are largely unknown. We therefore applied a proteomics-based approach by combining immunocapture with time-of-flight mass spectrometry to characterize hepcidin-25 produced by hepatocyte-derived cell lines. Similar to its transcriptional regulation, mature hepcidin-25 was strongly secreted upon stimulation with BMPs and IL-6. The immunocaptured peptide down-modulated iron-exporter ferroportin on the monocyte/macrophage surface. Further mass spectrometry-based analyses indicated that hepcidin-25 in its bioactive conformation was very stable in serum and urine and not converted into its smaller isoforms. Hepcidin-25 was processed in the Golgi apparatus from its precursor, while the unprocessed prohepcidin was secreted only when furin-like protease activity was intracellularly inhibited. Furthermore, the amounts of hepatocytic secretion of hepcidin-25 are highly correlated with the gene transcript levels. An unexpected observation was the synergistic effect of BMPs and IL-6 on hepcidin-25 secretion, which points towards cross-talk between iron and inflammatory stimuli. The study underscores hepcidin-25 quantification as a valuable tool to unravel regulatory pathways in iron metabolism.

1. Introduction

Hepcidin is the key regulatory protein in iron homeostasis [1,2]. Its synthesis is attenuated in hereditary hemochromatosis and elevated in anemia of inflammation [1–3]. The peptide promotes internalization and degradation of ferroportin [4], the sole mammalian iron exporter [5]. With this functionality, hepcidin negatively regulates iron absorption from the intestine, and iron release from recycling macrophages and hepatic stores [6].

Hepcidin is encoded by the *HAMP* gene. Bone morphogenic protein (BMP)-2, -4, -9 and interleukin-6 (IL-6) are among the stimulatory cytokines that promote *HAMP* transcription [7–9]. BMP-2 and -4 but not BMP-9 have been proposed to be involved in hemojuvelin-mediated hepcidin synthesis [10–12]. The BMPs are members of the TGF-β superfamily, and all act via the same set of SMA-mothers against decapentalegic (*Drosophila*) homologs (SMADs) [13]. During inflammation and systemic infection, IL-6 promotes hepcidin synthesis [7,8,14] utilizing signal transducers and activators of transcription-3 (STAT-3) as the transcription initiating factor [15–17]. IL-6-induced hepcidin expression is thought to play a role in anemia of chronic disease [1–3]. A recent *HAMP* promoter analysis suggests that IL-6 promotes hepcidin expression independently of BMP pathways [18,19]. However, mice with a liver specific conditional knock-out of *Smad-4* showed attenuated hepcidin synthesis in response to IL-6 stimulation [20].

In urine, using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), three isoforms of hepcidin, namely hepcidin-20, hepcidin-22 and hepcidin-25, have been identified, which are composed of 20, 22 and 25 residues respectively [21]. However, only the 25-residue isoform promotes ferroportin internalization and degradation [22], the well-characterized central role of hepcidin in iron metabolism [6]. Importantly, proteomics-based mass spectrometry technology has been recently developed to semi-quantify hepcidin-25, in biological liquids such as urine and serum [23–27].

Abbreviations: BFA, brefeldin-A; BMP, bone morphogenetic protein; D-CMK, decanoyl-Arg-Val-Lys-Arg-CMK; HPRT, hypoxanthine phosphoribosyltransferase; IL, interleukin; SMAD, SMA-mothers against decapentalegic (*Drosophila*) homolog; STAT, signal transducers and activators of transcription-3; FACS, fluorescence-activated cell sorting; SELDI-TOF-MS, surface-enhanced laser desorption/ionization time-of-flight; Q-PCR, real-time quantitative polymerase chain reaction; PBS-Tx, PBS supplemented with 0.1% Triton X-100, pH 7.4

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Although RNA hybridization and quantification techniques show the presence of *HAMP* transcripts in several organs [28–32], potential release and stability of bioactive hepcidin from these organs are still yet to be determined. Furthermore, many fascinating *in vitro*, *ex vivo* and animal studies of hepcidin synthesis rely on *HAMP* transcript detection [8,10,12,15,16,18]. Difficulties in detecting this low abundant and small peptide have prevented it from being quantified. In some studies, antibodies have been developed to detect hepcidin. However, often used antibodies in histochemistry only recognize the proform of hepcidin, whereas technical restrictions limit discrimination between hepcidin-25, hepcidin-22, and hepcidin-20 isoforms when antibodies are used for immunoblotting approaches [30–34]. In both cases the results may not fully reflect the levels of bioactive hepcidin-25. In humans, liver *HAMP* transcript levels have been previously correlated with the levels of immunoblot-based quantified urinary hepcidin [35]. However, in one study, kidney cells have been shown to produce hepcidin independently of the liver [31]. Thus at this moment, there is still no direct proof that measurement of liver *HAMP* transcript levels is a valid approach to evaluate secretion of bioactive hepcidin-25 by the liver.

This indicates that a rapid method with highly reliable specificity to detect potential release of bioactive hepcidin-25 and its smaller-22 and -20 isoforms from liver cells and other types of cells is desirable. This method would be useful to unravel the complex molecular signaling of hepcidin and to further explore its downstream effects on iron homeostasis. In this study, we aimed to exploit a mass spectrometry-based method to provide a high through-put detection quality with reliable specificity for measurement of bioactive hepcidin-25 secreted by liver-derived cells, especially upon BMPs and IL-6 activation and to evaluate the significance of this measurement by following the processing events in hepcidin maturation.

2. Experimental procedures

2.1. Cell cultures

The human hepatocyte-derived cell lines, Huh7 and Hep3B, were cultured in a humidified 37 °C incubator with 5% CO_2 using PC-1 medium (Cambrex, MD, USA). Cells at the density of 1×10^6 cells/mL were grown in 6-well plates for 24 h to allow full confluency. One mL total volume of medium was used in each well during treatment for 18 h with interleukin-6 (IL-6) (R&D Systems, MN, USA) or bone morphogenetic protein (BMP)-2 and -9 (R&D Systems) at specified concentrations, and if required additionally with 10 nM brefeldin-A (BFA) (Calbiochem, Darmstadt, Germany) or 50 μM decanoyl-Arg-Val-Lys-Arg-CMK (D-CMK, Calbiochem).

The human monocyte/macrophage-like cell line, THP1, was cultured in RPMI1640 (Gibco, Breda, The Netherlands) supplemented with 2 mM L-glutamine and 10% fetal calf serum (Gibco).

2.2. Real-time quantitative polymerase chain reaction (Q-PCR)

Isolation of total RNA and subsequent synthesis of cDNA were performed as described previously [36] and followed by Q-PCR of *HAMP*. For normalization of expression, the housekeeping gene hypoxanthine phosphoribosyltransferase (*HPRT*) was quantified [36]. Each Q-PCR was performed in a 25 μL total reaction mixture using TaqMan Universal Master Mix (PE Applied Biosystems, CA, USA). After enzyme activation for 10 min at 95 °C, 40 two-step cycles were performed (30 s, 95 °C; 1 min, 60 °C) in an ABI Prism 7700 (PE Applied Biosystems). All primers and probes were purchased from PE Applied Biosystems (*HAMP*, assay no. Hs00221783_m1; *HPRT*, assay no. 4310890E). Relative expression of target gene in comparison to reference housekeeping gene was calculated based on a mathematical model previously described [37].

2.3. Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS)

Both cell lysates and culture medium of Huh7 and Hep3B were analyzed by SELDI-TOF MS. Each cell lysate was prepared at 4 °C using M-PER protein extraction reagent (Pierce), supplemented with 1× complete proteinase inhibitors (Roche, Mannheim, Germany). After being equilibrated using 100 mM ammonium acetate, pH = 3.0 (Merck, Darmstadt, Germany), 10 μL sample was applied to each spot surface of cation exchange ProteinChip arrays, CM10 (Bio-Rad, CA, USA). The arrays were incubated in a humidity chamber for 30 min, and washed according to manufacturer's instructions. 1 μL energy-absorbing matrix, containing 12.5 g/L sinapinic acid (Bio-Rad) in 500 mL/L acetonitrile (Lab-Scan, Dublin, Ireland) and 5 mL/L trifluoroacetic acid (J.T.Baker, NJ, USA) was applied twice onto each spot using polymer-free polypropylene pipette tips and allowed to air-dry for 5 min. Mass to charge spectra (*m/z*) were generated using a Ciphergen Protein Biology System IIc TOF mass spectrometer (Ciphergen Biosystems, CA, USA) at 165 laser intensity, 10 detector sensitivity, and 50 kDa mass to acquire with optimization from 5 to 15 kDa. External mass calibration was performed with a mixture of synthetic human hepcidin-25 (2789 Da, Peptide International, KY, USA), hepcidin-22- and hepcidin-20 (2436 Da and 2192 Da respectively, kindly provided by E. Nemeth and T. Ganz, University of California, LA, USA) as previously described. Hepcidin-24 (2674 Da) obtained from Peptide International was used as an internal standard. This internal standard was chosen since it can be effectively distinguished from, and at various concentrations, it does not affect the peak height and position of the other naturally occurring hepcidin isoforms [38]. Furthermore, hepcidin-24 (lacking the amino-terminal aspartic acid residue) is highly cationic with p*I* similar to that of hepcidin-25, hepcidin-22 and hepcidin-20 (Table 1). Thus, at binding and washing conditions at neutral pH as applied, these highly cationic peptides have equal binding affinities for the CM10 ProteinChip surface. Stock synthetic peptide solutions were at concentration of 100 μM in deionized water. Five nM internal standard was used for each measurement. Peak annotation was performed with Ciphergen ProteinChip Software version 3.2.0. Hepcidin-25 levels were determined by assessing its peak intensity relative to the peak intensity of the internal standard with known quantity. The lowest detection limit of hepcidin-25 was approximately 0.01 nM, while its peak intensity increased linearly with increased concentrations up to 20 nM. The intra-assay and inter-assay variations (ratios of the standard deviations to the means multiplied by 100%) for the assay were assessed using four measurements repeated within one assay and repeated in different days, respectively.

2.4. Immunocapture and immunodepletion

When appropriate, immunocapture was performed using Magna-Bind™ Protein-G beads (Pierce, IL, USA), coupled to polyclonal rabbit antihepcidin-25 antibodies (generous gift from E. Nemeth and T. Ganz, University of California, LA, USA). Binding of the polyclonal to the beads (1:50) was allowed at room temperature for 1 h, using phosphate-buffered saline (PBS) (Sigma, MD, USA) supplemented

Table 1
p*I* and MW of the precursor and isoforms of hepcidin based on their amino acid sequence, and assuming no post-translational modifications

Form	Predicted p*I*	Predicted MW
Prepropeptide	9.24	9408
Propeptide	9.37	6937
Hepcidin-25	8.22	2797
Hepcidin-22	8.53	2444
Hepcidin-20	8.53	2200
Hepcidin-24 (synthetic standard)	8.51	2682

p*I* = isoelectric point; MW = molecular weight in Da, for the oxidized states.

with 0.1% Triton X-100 (Sigma), pH 7.4 (PBS-Tx). To remove unbound serum proteins, the beads were washed 3 times with PBS-Tx. An external magnetic field was used to collect the beads from the suspension. 1× volume of medium or cell lysate was then added to the beads. After 2 h at room temperature, immunodepleted fractions were collected and the beads were washed 6 times to remove unbound and non-specifically bound proteins. Bound/immunocaptured proteins were then eluted using 0.1 M glycine (Sigma), pH = 3.0. Each eluate was immediately neutralized using 10% 1 M Tris (ICN Biochemicals, Ohio, USA), pH = 9.0. Neutralized eluates and their corresponding immunodepleted fractions were applied to SELDI-TOF MS.

2.5. Fluorescence-activated cell sorting (FACS)

THP1 cells at a density of 5×10^5 cells/mL, were incubated for 18 h with or without 20 nM native hepcidin, isolated from the stimulated Huh7 cells, in the presence of 10 μM Fe(III)ammoniumcitrate (Sigma). After treatment, THP1 cells were fixated using PBS containing 4% formaldehyde (Merck) for 5 min. Blocking of Fc receptors was carried out using PBS containing 20% normal human serum for 30 min at 4 °C. Incubation with 1:25 ferroportin antibody (a generous gift from G.O. Latunde-dada and A.T. Mckie, King's College, London, UK), or 5 μg/mL rabbit IgG isotype control (Rockland, Gilbertsville, USA) was for 3 h in PBS containing 10% NHS at 4 °C. After washing, alexa-fluor-488-labelled secondary antibody (Molecular Probes, Eugene, USA) was applied. 1×10^4 cells were analysed for each flow cytometric measurement in a Becton Dickinson FACScan (Oxnard, CA, USA).

2.6. Biostatistical analysis

Results are expressed as means ± standard deviations. Differences in quantitative measures were tested for significance using Student's *t*-test. Significance was established when $P < 0.050$. To assess linear relationship between two continuous variables, two-tailed Pearson regression coefficient (R) was computed. All statistics were performed using the GraphPad Prism 4 statistical program (San Diego, USA).

3. Results

3.1. Identification of hepcidin-25 secreted from hepatocyte-derived cells

By employing SELDI-TOF MS calibrated with hepcidin isoforms (Fig. 1Aa), we intended to identify hepcidin-25 secreted from cultured cells. Both Huh7 and Hep3B cells were used in this study. However since both showed similar pattern of results, we presented the data mainly from Huh7 cells throughout this manuscript.

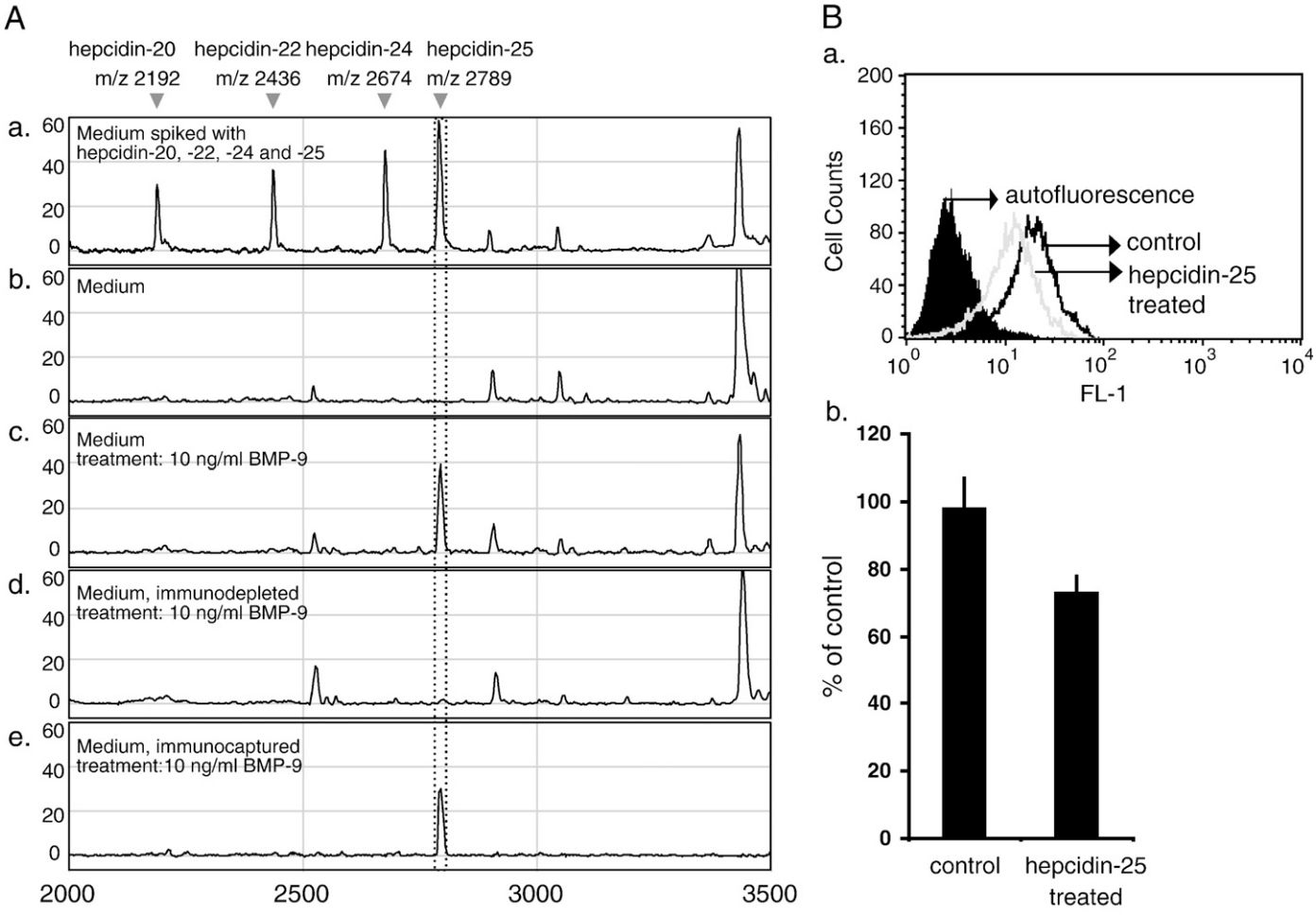

Fig. 1. Characterization of hepcidin-25 secreted from human hepatocyte-derived cells. (A) Representative mass spectra (x = *m/z*; y = signal intensity) of (a) PC-1 medium spiked with synthetic 20-, 22-, 24- and 25-residue hepcidin, (b) the growth medium, (c) medium of Huh7 cells treated with 10 ng/mL BMP-9, (d) medium of Huh7 cells treated with 10 ng/mL BMP-9, and immunodepleted, or (e) immunocaptured using antibody against human hepcidin-25. $n = 3$–4. (B) Functional analysis of native hepcidin-25 using FACS and antibody against ferroportin. (a) Representative FACS analysis (x = fluorescence intensity; y = cell counts) of THP1 autofluorescence from the IgG isotype control, THP1 treated for 18 h with 10 μM Fe(III)ammonium citrate as control, and cotreated with immunocaptured native hepcidin-25. An increased FL-1 fluorescence intensity indicated a higher level of surface ferroportin expression. (b) A plot of relative surface ferroportin expression based on the FACS analysis. Each measurement was the mean value ± standard deviation, $n = 3$.

The MS profile of culture medium did not show marked peaks corresponding to any hepcidin isoforms (Fig. 1Ab). To induce the expression of hepcidin, Huh7 cells were treated with 10 ng/mL BMP-9 for 24 h. A marked peak at *m/z* 2789 was detected on the profile (Fig. 1Ac), which corresponds to hepcidin-25 with intact four disulphide bonds. Notably, hepcidin-22 and -20 were not detectable in this condition. Medium was then subjected to immunodepletion procedure using antibodies raised against hepcidin-25. This successfully depleted the *m/z* 2789, suggesting that this peptide is hepcidin-25 (Fig. 1Ad). Furthermore, the immunocapture procedure recovered exclusively this peptide (Fig. 1Ae), confirming its identity as hepcidin-25.

To further characterize this peptide, we tested its functionality. In this experiment, we used THP1 cells, known to express ferroportin [39]. Incubation of THP1 cells with the immunocaptured peptide significantly decreased the expression of ferroportin on the cell surface, as analyzed by flow cytometry (Fig. 1Ba and b). This demonstrates the peptide biofunctionality. We therefore conclude that the *m/z* 2789 secreted from cultured hepatocyte-derived cells is indeed bioactive hepcidin-25.

3.2. Post-translational processing of hepcidin precursor

MS is useful to characterize processed isoforms and post-translational modifications of a protein without prior knowledge of the types of modifications. We therefore aimed to use this MS approach to characterize hepcidin processing events. As predicted by SignalP 3.0 [40], hepcidin precursor contains an endoplasmic reticulum signal peptide with a putative cleavage site between Gly^{24} and Ser^{25}. The 60-residues propeptide, predicted by TargetP1.1 [40], is localized in the secretory pathway, and predicted by ProP1.0 [41] cleaved by a furin-like subtilisin/kexin protease to hepcidin-25. Based on the calculated p*I* values, hepcidin precursor as well as its mature isoforms are all strongly cationic (Table 1), therefore CM10 array capable of binding cationic proteins was chosen for this MS analysis.

First, we confirmed the involvement of furin-like proteases in hepcidin processing using the specific inhibitor of furin-like proteases, D-CMK [42]. MS was calibrated using hepcidin isoforms (Fig. 2a) and the non-natural internal standard, hepcidin-24, was included to illustrate the secretion level of hepcidin (Fig. 2). After stimulation with BMP-9, Huh-7 produced and highly secreted hepcidin-25 to the medium (Fig. 2b and f). When Huh7 cells were pretreated for 6 h with D-CMK and further cotreated for 18 h with D-CMK during stimulation with BMP-9, hepcidin-25 was no longer observed in both the medium and lysate (Fig. 2c and g). However, without pretreatment, D-CMK failed to completely block hepcidin-25 secretion (not shown). Since D-CMK has low efficiency of penetration into cells [43], the result suggests that blocking of this cleavage process requires intracellular inhibition. Since most of furin-like processing enzymes reside in the Golgi apparatus, we investigated whether hepcidin-25 processing occurs in this organelle. The medium and lysate profile of Huh7 cells stimulated with BMP-9 and cotreated with BFA, a specific inhibitor of the Golgi secretory pathway [44], showed no hepcidin-25 peak (Fig. 2d and h). This missing hepcidin-25 peak was not due to alteration of *HAMP* transcription by the inhibitors, D-CMK and BFA, as monitored by Q-PCR (not shown). Taken together, our results suggest that hepcidin precursor is processed by furin-like enzymes in the Golgi apparatus.

We next aimed to characterize the formation of the smaller isoforms, namely hepcidin-20 and hepcidin-22. Both isoforms have been purified from urine [21] while only hepcidin-20 was characterized in serum [24,27]. First, we investigated whether these isoforms are extracellular degradation products of hepcidin-25. Incubation of medium containing hepcidin-25 for 72 h at 37 °C did not result in the formation of smaller isoforms (not shown). Similarly, immunocaptured hepcidin-25, was not processed to smaller isoforms (Fig. 3a–e), when incubated for 72 h at 37 °C in urine or serum of a patient with juvenile hemochromatosis [45] which was shown to have endogenous

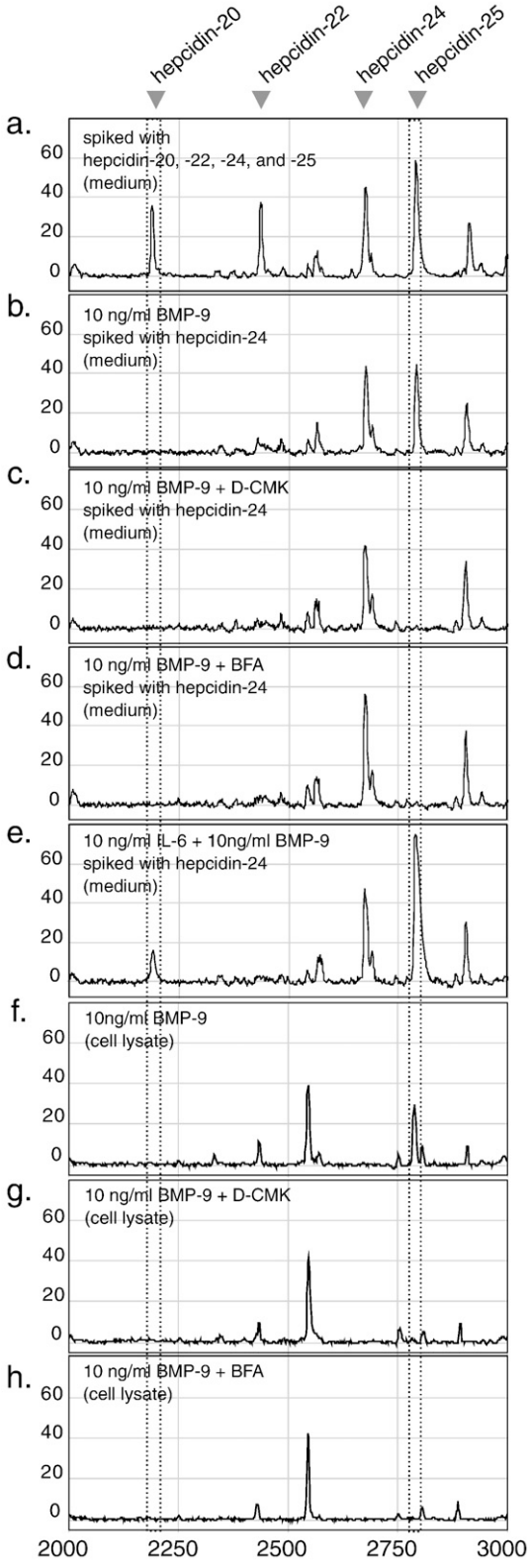

Fig. 2. Identification of post-translational processing of hepcidin precursor in Huh7 cells. Representative mass spectra of (a) PC-1 medium spiked with synthetic 20-, 22-, 24- and 25-residue hepcidin, (b) medium of Huh7 cells treated with 10 ng/mL BMP-9 for 18 h, (c) and pre-(6 h) and cotreated with D-CMK, (d) or cotreated with BFA. Secretion of hepcidin-25 was blocked in both (c and d). (e) Medium of Huh7 cells treated with 10 ng/mL BMP-9 in combination with 10 ng/mL IL-6. The 20-residue hepcidin was detected in this profile. (f–h) represent spectra of Huh7 cell lysates treated as in b, c and d respectively, $n=2–3$.

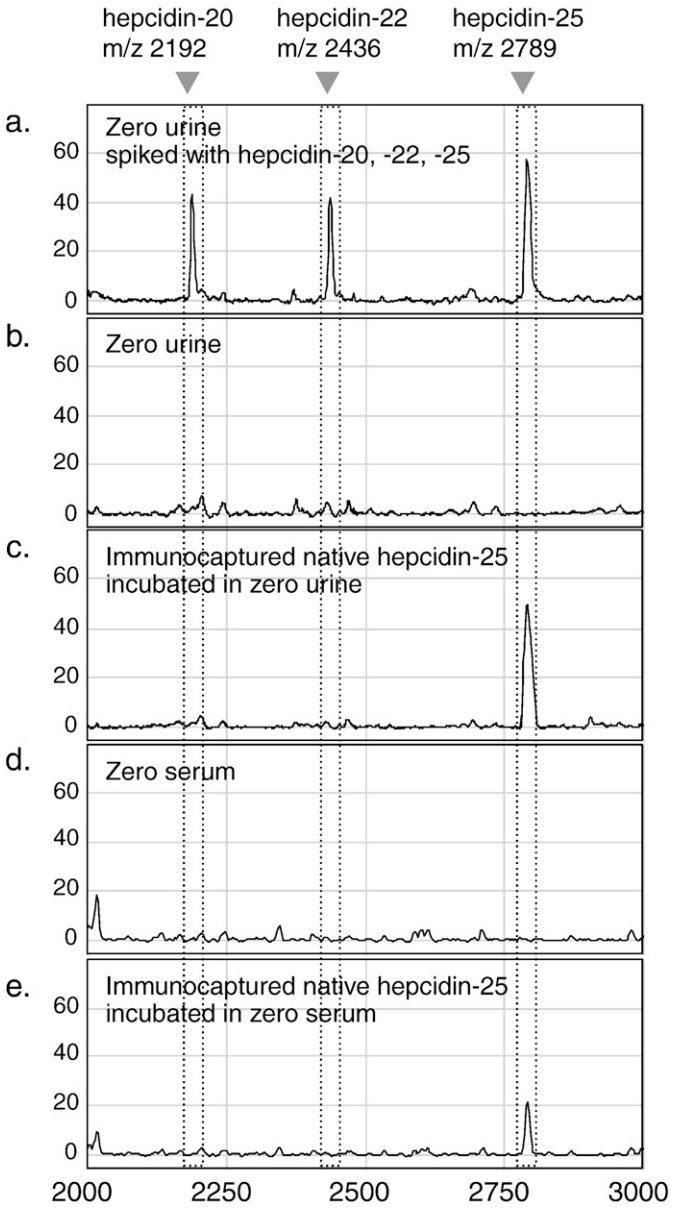

Fig. 3. Processing analysis of native hepcidin-25 in human serum and urine. No smaller isoforms of hepcidin were detected after 78 h incubation at 37 °C. Representative mass spectra of (a) urine containing negligible amount of endogenous hepcidin (zero-urine) spiked with synthetic 20-, 22-, and 25-residue hepcidin, (b) zero-urine, (c) immunocaptured native hepcidin-25 incubated in zero-urine at 37 °C, (d) serum containing negligible amount of endogenous hepcidin (25% zero-serum), (e) immunocaptured native hepcidin-25 incubated in 25% zero-serum at 37 °C, n=2–3.

hepcidin levels below the detection limit of the method (Fig. 3b and d). In this experiment, 25% serum was used to reduce detection saturation and increase CM10 sensitivity towards hepcidin isoforms. Together these suggest that the formation of smaller hepcidin isoforms may occur intracellularly or may only occur in specific organs. Indeed, we observed the presence of hepcidin-20 in the medium profile of the overly stimulated Huh7 cells (Fig. 2e). Similar to hepcidin-25, we also observed blocked hepcidin-20 secretion, when D-CMK was added (not shown).

Previous publications have repeatedly measured serum prohepcidin as an alternative measure of hepcidin in the circulation [27,46,47]. However, the results show no significant different prohepcidin levels in the various groups of patients, including congenital anemia and hereditary hemochromatosis, that have unusual urinary and serum hepcidin-25 levels [23–27,48,49]. Using MS coupled with an immunocapture method, we aimed to identify hepcidin precursor from hepatocyte-derived cells, and determined whether this protein could be secreted.

No peak at m/z 9408 corresponding to preprohepcidin (Table 1) was detected in the lysate profiles of cells stimulated with BMP-9 or both stimulated with BMP-9 and treated with D-CMK (Fig. 4a and b). This suggest that the precursor protein may be rapidly processed to prohepcidin after its translation. In agreement with this finding, we observed a peak of m/z 9408 corresponding to preprohepcidin, when BFA was applied (Fig. 4c). As many other preproprotein, this protein may have been accumulated in the endoplasmic reticulum, when the Golgi apparatus is disassembled due to BFA action. This conforms that hepcidin maturation follows the Golgi secretory pathway.

A peak at m/z 6937, corresponding to prohepcidin (Table 1) was also not detected, however a peak at m/z 6929 was detected in the

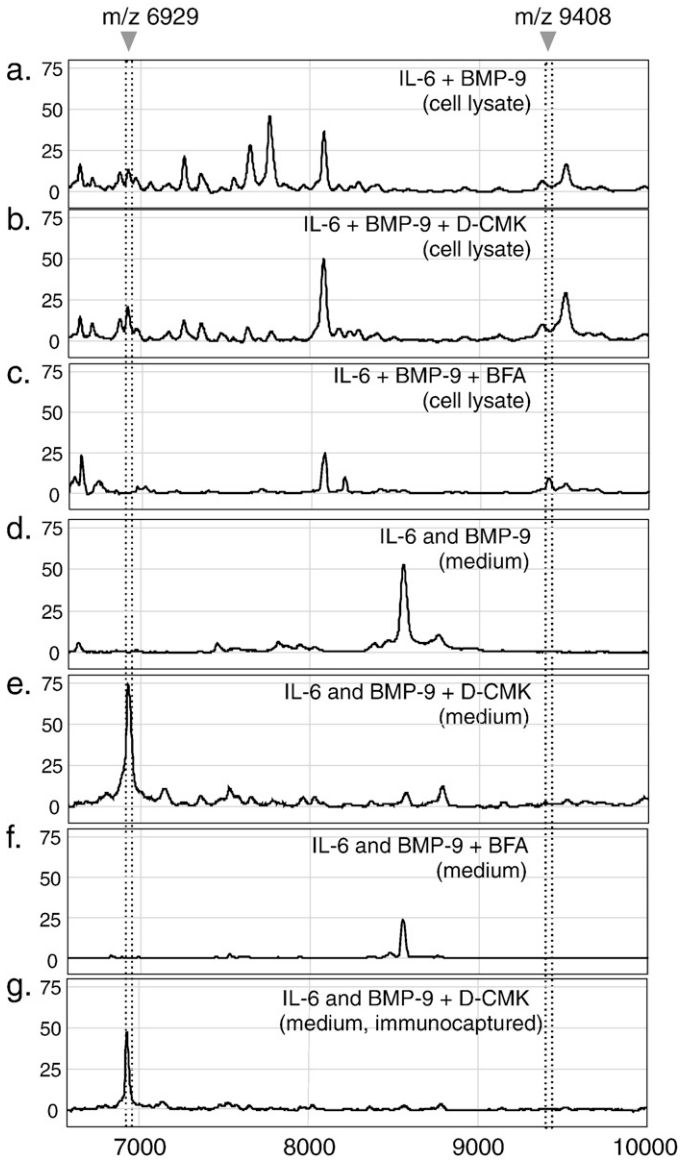

Fig. 4. Identification of hepcidin precursor from Huh7 cells using anti-human hepcidin immunocapture method coupled with SELDI-TOF MS. Representative mass spectra of (a) Huh7 lysate after treatment with 10 ng/mL IL-6+10 ng/mL BMP-9 for 18 h or (b) in the presence of D-CMK, or (c) in the presence of BFA, (d) medium of Huh7 after treatment with 10 ng/mL IL-6+10 ng/mL BMP-9 for 18 h, (e) in the presence of D-CMK or (f) in the presence of BFA, (g) immunocaptured medium of Huh7 cells treated with 10 ng/mL BMP-9 for 18 h in the presence of D-CMK. The peak at m/z 6929 corresponds to prohepcidin, and the peak at m/z 9408 corresponds to preprohepcidin, n=2–3.

lysate spectra (Fig. 4a and b). The same peak was highly present in medium of the stimulated cells that were pretreated with D-CMK (Fig. 4e). However, this peak was absent, when furin-like proteases were not inhibited (Fig. 4d) or when the cells were treated with BFA (Fig. 4f). To further confirm the identity of this peak, we performed immunocapture experiment using the antihepcidin antibodies. The *m/z* 6929 peak was exclusively reidentified (Fig. 4g), confirming its identity as prohepcidin. The identified prohepcidin was 8 Da smaller than the predicted molecular weight (Table 1), suggesting the presence of four disulphide bonds in this molecule. Thus, similar to the processing of many percursor proteins [50], the disulphide bonds may have been formed in the endoplasmic reticulum, before prohepcidin enters the Golgi network. Taken together these results suggest that prohepcidin is a transient intermediate formed during hepcidin maturation, and can only be secreted when furin-like proteases are inactive.

Additionally, in this study, we could not observe the residual propeptide of *m/z* 4157, p*I* 12.12, both in the medium and in the cell lysate spectra, which is theoretically formed when prohepcidin is cleaved to hepcidin-25. We therefore speculate, that this residual propeptide may be proteolitically degraded immediately after cleavage, or followed modifications that would change its predicted molecular weight.

3.3. Quantification of secreted hepcidin-25 and comparison with its transcript levels

Several cytokines including IL-6 and BMPs have been shown to promote *HAMP* expression in hepatocytes at transcriptional level [8,10,12,15,16,18], however the levels of secreted bioactive protein are not known. In this investigation we aimed to provide a quantitative method to measure hepcidin-25 secreted from liver cells.

HAMP transcripts were measured in both Huh7 and Hep3B cells after treatment with increasing concentrations of IL-6 and BMP-9 (Fig. 5A). Although the basal *HAMP* mRNA levels of Huh7 cells are 60 folds higher than Hep3B cells, both cell lines showed similar expression patterns upon different stimulations (Fig. 5A and B). Up to 10 ng/mL, both IL-6 and BMP-9 induced *HAMP* expression in a concentration-dependent manner, while above 10 ng/mL, *HAMP* expression plateaued (Fig. 5A). Combination treatment with 10 ng/mL IL-6 and 10 ng/mL BMP-9 unexpectedly resulted in synergistically higher levels of *HAMP* transcripts (Fig. 5B) compared to transcript levels obtained after single treatments with IL-6 or BMP-9 (Fig. 5A), or calculated by summing up the levels from individual treatments with 10 ng/mL IL-6 and 10 ng/mL BMP-9 (Fig. 5B). This synergistic effect was also observed using combination treatments with 10 ng/mL IL-6 and 10 ng/mL BMP-2 (Fig. 5C).

In MS, the amount of peptide in a sample does not necessarily correlate with the ion-current intensity of its mass spectrometric signal. Therefore quantification of a specific peptide using MS requires incorporation of an internal standard. In this study, using SELDI-TOF MS together with the non-natural internal standard, hepcidin-24, we quantified hepcidin-25 secreted to culture medium from the differently treated cells (Fig. 6A). Hepcidin-25 of 0.03 nM was measured in the overnight culture medium of Huh7 cells (Fig. 6B), but not in the Hep3B culture medium, which may be due to its very low basal *HAMP* expression levels as shown by Q-PCR analysis. After 10 ng/mL IL-6 treatment, concentrations of hepcidin-25 measured in culture medium of Hep3B and Huh7 cells were 0.05 nM and 1.2 nM hepcidin-25 respectively, while after 10 ng/mL BMP-9 treatment, 2.1 nM and 3.8 nM were measured respectively. Combination of IL-6 and BMP-9 resulted in synergistic secretion of hepcidin-25 to 4.7 nM and 6.5 nM from Hep3B and Huh7 cells respectively (Fig. 6B). Similar pattern was also observed when BMP-2 was used (not shown). The intra-assay and inter-assay variations were 4.7 and 7.2% respectively. Similar to its transcript, this shows synergistic signaling by IL-6 and BMPs pathways in the synthesis of hepcidin. Moreover, from all data collected after IL-6, BMP, or IL6-BMP-combined treatments, hepcidin-25 levels highly correlated with *HAMP* transcript levels in both Hep3B (R=0.91) and Huh7 (R=0.92) cells.

4. Discussion

Hepcidin is the key molecule in iron homeostasis and plays a central role in anemia of inflammation [1,2]. A reliable measurement of this peptide is crucial in order to understand its regulation and role in iron homeostasis. Using SELDI-TOF MS, we have developed a rapid quantification method with high specificity to measure bioactive hepcidin-25 secreted from human hepatocyte-derived cell lines. The identified hepcidin-25 was able to down-regulate surface ferroportin, confirming its functionality. Although further development is still needed to sufficiently detect very low levels of hepcidin-25, the method could potentially be extended to detect potential release of hepcidin from different cell types and organs, in which the presence of *HAMP* transcripts have been previously reported [28–32].

Utilizing MS, we observed hepcidin maturation, which required active furin-like proteases, in line with recent studies employing pulse-chase labeling in liver cells [34,51]. However, due to low

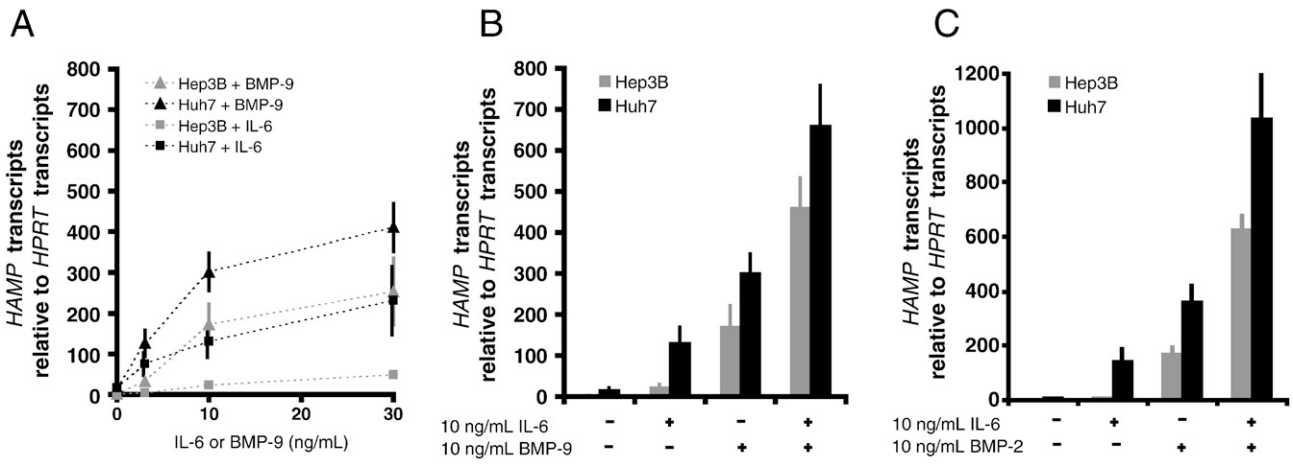

Fig. 5. Quantification of *HAMP* transcript levels in Huh7 and Hep3B cells using Q-PCR. (A–C) *HAMP* transcripts in Huh7 and Hep3B cells, normalized with human *HPRT* transcripts, after indicated treatment for 18 h. Each measurement was the mean value±standard deviation, n=6.

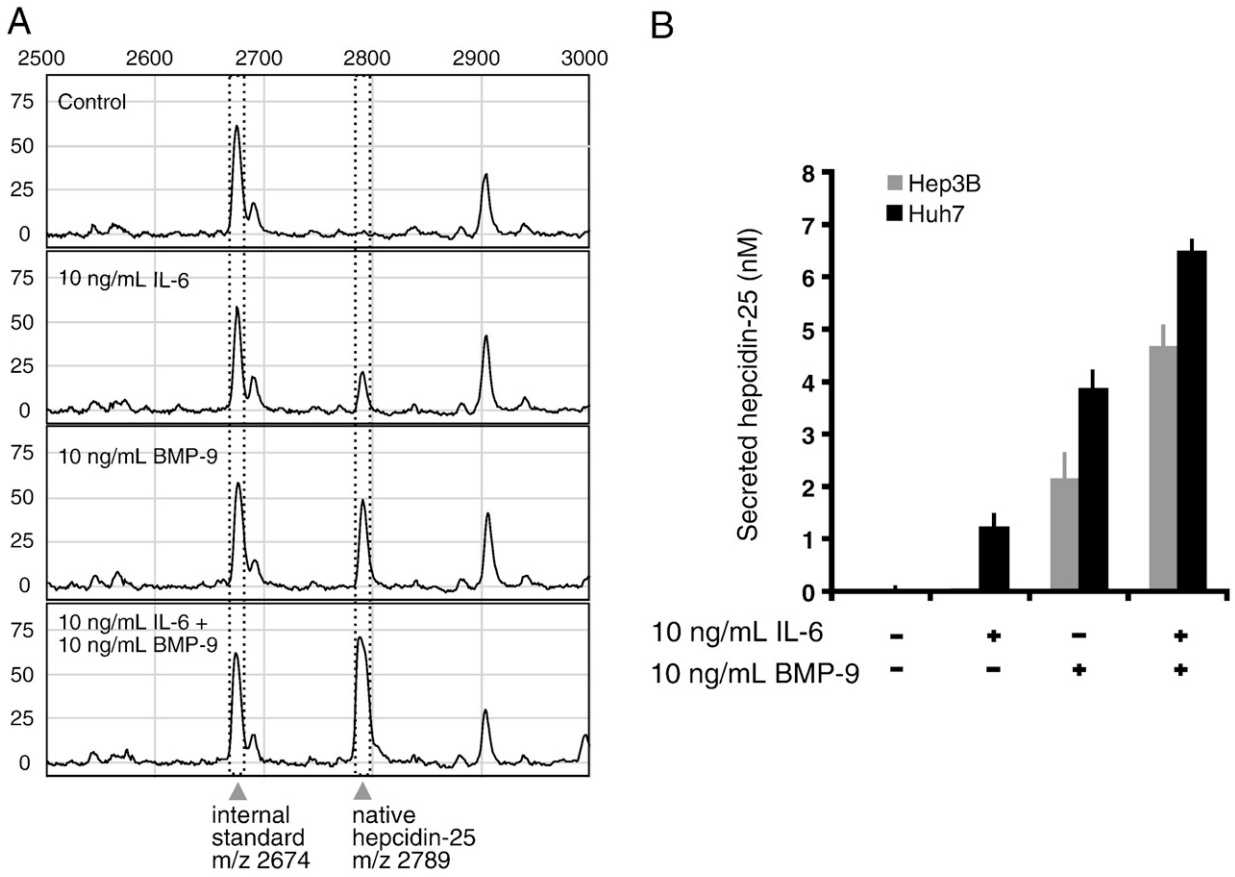

Fig. 6. Quantification of hepcidin-25 secreted from Huh7 and Hep3B cells using SELDI-TOF MS and an internal standard. (A) Representative protein mass profiles of PC-1 medium of Huh7 cells with indicated treatment for 18 h, spiked with 5 nM internal standard, (B) secreted hepcidin-25 from Huh7 and Hep3B cells with indicated treatment measured using SELDI-TOF MS with the internal standard. Each measurement was the mean value ± standard deviation, n = 4–5.

resolution of the polyacrylamide gel electrophoresis approach, recognition of different hepcidin isoforms was not possible and thus data interpretation from this study was not optimal. Therefore we exploited our MS approach to accurately characterize both prohepcidin and the mature isoforms of hepcidin. Using this method, we show that prohepcidin processing occurs in the Golgi apparatus. This is in line with the previous study showing the presence of prohepcidin in the Golgi complex [52]. We also observed that the highly stimulated liver-derived cells also secreted hepcidin-20, suggesting that both hepcidin-20 and -25 are directly produced and secreted from the liver. This observation was corroborated by the fact that prolonged incubation of native hepcidin-25 in cell culture medium, human serum and urine, did not result in the conversion of hepcidin-25 into smaller isoforms. Together, this provides first evidence that hepcidin-20 is formed intracellularly. Furin-like proteases may alternatively cleave prohepcidin to yield mature hepcidin-20. However, processing by an unknown intracellular protease that can only use hepcidin-25 as a substrate or intracellular hydrolysis of the proline–isoleucine peptide bond within hepcidin-25 can currently not be excluded. In addition, we did not observe hepcidin-22 secreted from these cells. This is indeed in line with the observation that this isoform is absent in the circulation [24,27], whereas present in urine [21]. Since hepcidin-22 was also not formed upon prolonged incubation of hepcidin-25 in serum and urine, we speculate that this form may be the result of differential processing and secretion by the kidney *in vivo*. Further investigations are certainly warranted to characterize the origins and functions of the two smaller isoforms.

By applying proteomics-based approach, we have detected prohepcidin in the hepatocyte growth medium, after furin-like protease activity was intracellularly inhibited. This therefore makes it unlikely that *in vivo* conditions arise to promote regulated secretion of prohepcidin from the liver. Moreover, cytokines like IL-12 [53] and TGF-β/SMADs [54,55] are capable of promoting furin expression, suggesting that the growing demand in hepcidin maturation in such inflammatory conditions could be fulfilled by increased furin expression. Thus, this questions the physiological relevance of prohepcidin measurements in the circulation. In this respect, it should be realized that antibodies used in prohepcidin assays [32] also recognize the cleaved residual propeptide that can be co-secreted with hepcidin-25 by hepatocytes. However, the fate of this residual propeptide in the circulation is currently fully unknown, which makes proper interpretation of prohepcidin measurements very difficult.

In this study, we further quantified both *HAMP* transcripts and hepcidin-25 secreted from both hepatocyte-derived cells upon IL-6 and BMPs stimulation. The levels of mRNA and secreted hepcidin-25 were highly correlated in these cells, suggesting that measurement of liver *HAMP* transcript levels may serve as a valid approach to evaluate secretion of bioactive hepcidin-25 by the liver. Such significant correlations however may not always be the case when different cell types are used. This therefore underscores the necessity to measure secreted bioactive hepcidin in addition to its transcript levels.

Recent hepcidin promoter studies show that distinct regulatory elements are used by BMPs/iron and IL-6/LPS pathways to promote hepcidin synthesis [18,19], suggesting that the two pathways independently signal for hepcidin. On the contrary, in this study we noted the unexpected synergistic effect of BMPs and IL-6 on hepcidin synthesis, which points towards an intertwinement between the two

regulatory pathways. For instance, when the transcription factors, SMAD and STAT, responsible for BMPs and IL-6 signaling respectively, are both present, although bind at separated regions of the *HAMP* promoter, they may be able to interact. It can be envisaged that this interaction may provide a synergistic induction of hepcidin expression, as the interaction between SMAD-1 and STAT-3 was previously shown to promote synergistic signaling of the glial fibrillary acidic protein (GFAP) promoter when bridged by the transcriptional activator p300 [56]. Although BMP-9 and BMP-2 signal via two distinct receptors, both use SMAD-1 to promote intracellular signaling. Thus the synergism from stimulation using BMPs and IL-6 could arise at the level of SMAD-1-STAT-3 interaction. Indeed, a BMP responsive element on hepcidin promoter which lies in close proximity to STAT-3 binding site has been shown to facilitate IL-6 stimulation [57]. Moreover, blocking of the BMP-SMAD signaling pathway inhibits IL-6 stimulation [11]. IL-6 effect was also blunted in *Smad-4* liver specific knock-out mice [20]. These studies and our results therefore suggest that an adequate response towards IL-6 stimulation is dependent on the SMAD pathway. The intertwinement between the two regulatory pathways may be relevant during inflammation, especially in inflammatory conditions, such as vascular endothelial injury, joint diseases and liver injury, in which BMPs are up-regulated by proinflammatory cytokines, to promote tissue repair. The synergistic interaction between iron and inflammation shown in our study also implies that iron supplementation will likely aggravate hepcidin production especially when being applied in anemic conditions due to inflammatory diseases or infections. Further studies are certainly warranted to unravel how the iron and inflammation pathways converge to promote hepcidin expression.

In summary, using MS-based approaches, we have developed a rapid method for detection and quantification of the low abundant hepcidin-25 secreted by liver cells. Our study indicates the necessity of such assays for characterization of and discrimination between hepcidin isoforms. The observation whereby BMP/iron and IL-6 pathways act synergistically in promoting secretion of bioactive hepcidin-25 further underscore that detection and measurement of hepcidin-25 will greatly assist studies on iron metabolism. The uncovered synergistic interaction between iron and inflammation pathways in hepcidin regulation holds potential implications especially in the management of iron disorder conditions.

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