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Oxygen-dependent secretion of a bioactive hepcidin-GFP chimera

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ABSTRACT

Hepcidin, a hepatic hormone, regulates serum iron levels by controlling both intestinal iron absorption and iron release from macrophages. Although transcription of hepcidin is controlled by diverse stimuli, it remains elusive if post-transcriptional steps of its production are also regulated. To address this issue, GFP was fused to the C-terminus of hepcidin and the chimeric hepcidin-GFP protein was expressed in hepatoma Huh7 cells. Expression and secretion of hepcidin-GFP were analyzed by fluorescence microscopy or western blotting and its activity was assessed by in vitro biological assays. Transient over-expression of hepcidin-GFP resulted in production and secretion of premature forms. On the other hand, stable low-level expression led to synthesis and secretion of a properly matured hepcidin-GFP. This form was biologically active since it affected appropriately the levels of IRP2 and ferritin in human THP1 monocytes and targeted ferroportin in mouse J774 macrophages. Treatment of hepcidin-GFP expressing cells with hypoxia (0.1% O_2) altered the subcellular distribution of pro-hepcidin-GFP and significantly reduced the secretion of mature hepcidin-GFP. Our hepcidin-GFP expression system allows the investigation of post-transcriptional processing of hepcidin and implicates hypoxia in its secretion control.

1. Introduction

Hepcidin is a liver-derived circulating peptide with antimicrobial properties, initially identified in human urine [1]. It was later discovered that hepcidin predominantly acts as an iron regulatory hormone [2]. Specifically, hepcidin targets ferroportin, the transmembrane iron transporter in duodenal enterocytes and macrophages. Binding of hepcidin to ferroportin provokes its internalization and subsequent degradation [3]. This results to decreased macrophage iron release and intestinal iron absorption and leads to diminished serum iron levels [4].

Hepcidin is regulated at the transcriptional level by distinct iron-and cytokine-dependent pathways [3,4]. Inflammatory signals act through IL-6 and STAT-3 to transcriptionally upregulate hepcidin, thus causing anemia of inflammation [5]. Hepcidin gene expression is down-regulated in response to low body iron stores, anemia, increased erythropoietic activity or hypoxia [6]. The negative effect of hypoxia is also observed in cultured hepatoma cells suggesting that low oxygen affects hepcidin synthesis [7]. A possible link between Hypoxia Inducible Factor-1 (HIF-1), which is the main transcription factor activated by hypoxia, and hepcidin down-regulation was suggested by a study using hepatocyte-specific knockout mice [8]. However, we [9] as well as others [10,11] have shown that hypoxic regulation of hepcidin gene transcription does not directly involve HIF-1. Interestingly, two proteases, furin and matriptase (TMPRSS6), that are regulated by HIF-1α [12,13] can also negatively control hepcidin indirectly via hemojuvelin cleavage [14,15].

Despite the extensive data on the transcriptional control of hepcidin, the post-transcriptional steps of hepcidin production, processing and secretion are poorly characterized. Hepcidin is produced as an 84-amino acid pre-pro-peptide, which is subsequently processed into a 60- to 64-amino acid pro-hepcidin peptide. This is mainly localized in the Golgi and vesicular structures of the secretory pathway [16]. Finally, pro-hepcidin can be cleaved by the prohormone convertase furin [17–19] to produce the biologically active 25-amino acid mature hepcidin form (known as hepcidin 25), which is secreted into the serum.

In the present study we established a cell line expressing stably a biologically active hepcidin-GFP chimera. This system can be

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used to study post-transcriptional production of hepcidin. Our data suggest a novel role of hypoxia in the regulation of hepcidin secretion.

2. Materials and methods

2.1. Plasmid constructs

The cDNA of pre-pro-hepcidin (249 bp) was amplified by PCR from plasmid pcDNA3-hepcidin [20] using as primers: forward: 5′-TTTTT*GTCGAC*ATG GCACTGAGCTCCCACATCTGGGCCGC-3′; reverse: 5′-TTTTT*GGATC* CCGTCTTGCAGCACATCCCACAC-3′ (SalI and BamHI sites in italics, start codon of hepcidin ORF underlined) and cloned into the pEGFP-N3 vector (Clontech).

2.2. Cell culture and treatment

Human Huh7 hepatoma cells and mouse J774 macrophages were grown in DMEM (high glucose; Gibco) supplemented with 10% heat-inactivated low endotoxin FBS (Biochrom AG), 100 U/ml penicillin–streptomycin (Gibco). Genetisin (Gibco) was used in a concentration of 400 μg/ml for the stable cell lines. Human THP1 monocytic cells were cultured in supplemented RPMI. For hypoxic treatment Huh7 cells were exposed for 4–24 h to 0.1% O_2, 94.9% N_2 and 5% CO_2 in a tightly sealed hypoxia chamber INVIVO$_2$ 200 (RUSKINN Life Sciences). Cells were treated when required with 20 ng/ml IL-6 (Sigma) and 100 μM hemin for 24 h or 100 μM desferrioxamine (DFO) for 16 h.

2.3. Co-culture

THP1 cells grown in suspension were treated with DFO and placed on top of monolayers of mock or hepcidin-GFP expressing Huh7 cells for 4 h. Following co-culture, THP-1 cells were obtained from the medium by centrifugation and the cell pellet was lysed in RIPA buffer. J774 cells, previously treated with DFO or hemin, were treated with conditional medium obtained from 24 h cultures of mock or hepcidin-GFP expressing Huh7 cells. Following treatment, J774 cells were lysed either in RIPA buffer (for detection of IRP-2 and ferritin), or in Laemmli buffer (for detection of ferroportin).

2.4. Cell transfection

Transient or stable transfections were carried out in 12-well plates using Lipofectamine 2000 (InVitrogen) in Huh7 cells. When required, 24 h post-transfection cells were treated for 18 h with 1 μg/ml brefeldin A (BFA) (Sigma). Individual colonies of stably transfected cells were isolated and picked from a plate using the cloning cylinders (Sigma) according to the manufacturer's instructions. The colonies were further checked for hepcidin-GFP expression by western blot and fluorescence microscopy analysis.

2.5. TCA precipitation, SDS–PAGE and western analysis

For TCA precipitation, culture medium from transiently or stably transfected cells was incubated for 1 h in 10% TCA. After centrifugation, the pellet was washed twice with acetone and was resuspended in 2× SDS sample buffer. Cell lysates and TCA precipitates were analyzed by 12% SDS–PAGE and Western blotting using the following antibodies: anti-GFP serum, anti-human albumin (Cell Signaling), anti-ferritin (DakoCytomation Inc.), anti-IRP2 [21] and anti-ferroportin [22]. Detection of immunoreactive protein bands and calculation of their apparent MW was done using the UVITEC Alliance 4.7 imaging system and UVIBAND software (Cambridge, UK).

2.6. Fluorescence microscopy

Huh7 cells expressing hepcidin-GFP were fixed with 3% formaldehyde in PBS for 5 min at room temperature, counterstained with DAPI, mounted on slides and viewed by a Leica DFC480 camera (LAS software ver. V2.3.1.R1) on an Axioscope 40 Zeiss microscope.

3. Results

3.1. Expression and secretion of hepcidin-GFP from transiently transfected Huh7 cells

To study the post-transcriptional synthesis of hepcidin we constructed a plasmid (pHepc-GFP) coding for the 84 amino acid pre-pro-hepcidin tagged with GFP at its C-terminus under the control of a CMV promoter. Transient transfection of hepatoma Huh7 cells

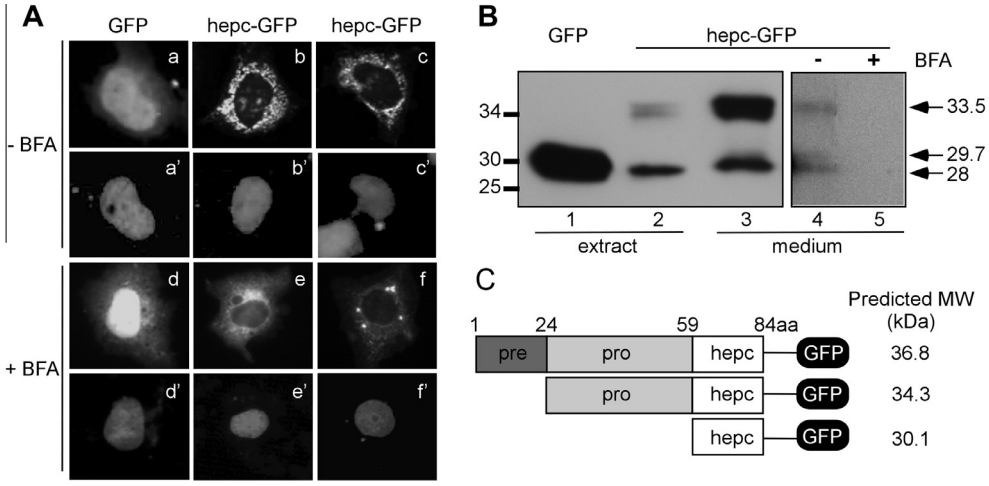

Fig. 1. Expression and secretion of pro-hepcidin-GFP from transiently transfected Huh7 cells. (A) Fluorescence microscopy imaging of Huh7 cells 24 h post-transfection with pEGFP-N3 alone (a, d) or pHepc-GFP (b, c, e, f) and 18 h of treatment without (b, c) or with 1 μg/ml brefeldin A (e, f). The position of the cell nucleus is visualized by DAPI staining (a′–f′). (B) Western blot analysis of extracts (lanes 1 and 2) or culture media TCA precipitates (lanes 3 to 5) from Huh7 cells transiently expressing GFP (lane 1) or hepcidin-GFP (lanes 2 to 5) and incubated for 18 h in the absence (lanes 1 to 4) or presence of 1 μg/ml brefeldin A (lane 5) using an anti-GFP antibody. Numbers on the left show the positions of MW markers (in kDa) and arrows on the right indicate the position and apparent MW of detected bands. (C) Schematic representation of hepcidin-GFP, its precursor forms and their predicted MW according to their amino acid sequence.

with pHepc-GFP led to the expression of a hepcidin-GFP fusion protein that was detected by fluorescent microscopy in cytoplasmic organelles of the secretory pathway such as endoplasmic reticulum and Golgi apparatus (Fig. 1A, panels b and c). This localization was specific for hepcidin-GFP since GFP alone, when expressed in the same cells, showed no specific pattern and was evenly distributed between cytoplasm and nucleus (Fig. 1A, panel a). Analysis of cell lysates and culture medium from hepcidin-GFP transiently expressing cells revealed the presence of GFP and slower migrating GFP-fusion proteins (Fig. 1B, lanes 2 and 3, marked with arrows) in both cell extracts and medium. The presence of free GFP in the extracts and medium indicates excessive proteolysis of the fusion protein that removes the hepcidin part. The slower migrating GFP-fusion protein (a doublet with apparent MW of approx. 33.5 kDa) most likely corresponds to the pro-hepcidin-GFP form (predicted MW 34.3 kDa, Fig. 1C). The lack of pre-pro-hepcidin-GFP suggests very fast and efficient removal of the pre-region (signal sequence, Fig. 1C). A faint band with approx. MW 29.7 kDa, migrating very close to GFP and only present in the medium, may represent mature hepcidin-GFP (predicted MW 30.1 kDa, Fig. 1C) and its low abundance suggests that the final processing step is inhibited. This inhibition can be explained by saturation of the cleavage system caused by high overexpression of the fusion protein, a fact that may also explain secretion of pro-hepcidin-GFP to the culture medium. This is in agreement with a previous study which has shown rapid cleavage of the pre-region of native pre-pro-hepcidin and efficient secretion of pro-hepcidin when the last cleavage step is impaired [17]. To confirm that the GFP-fusion proteins detected in the medium are products of active secretion, we treated the hepcidin-GFP expressing cells with BFA, which blocks protein secretion by disrupting the Golgi complex. This treatment affected the reticular distribution of pro-hepcidin-GFP (Fig. 1A, e and f) and led to the disappearence of GFP-fusion proteins from TCA-precipitates of the culture medium (Fig. 1B, lane 5). These data suggest that the GFP moiety does not apparently affect expression and initial procesing of pre-pro-hepcidin-GFP, although the final maturation step is inhibited probably due to overexpression. To avoid this, we procceeded in establishing stably transfected cell lines with more physiological expression levels of the fusion protein.

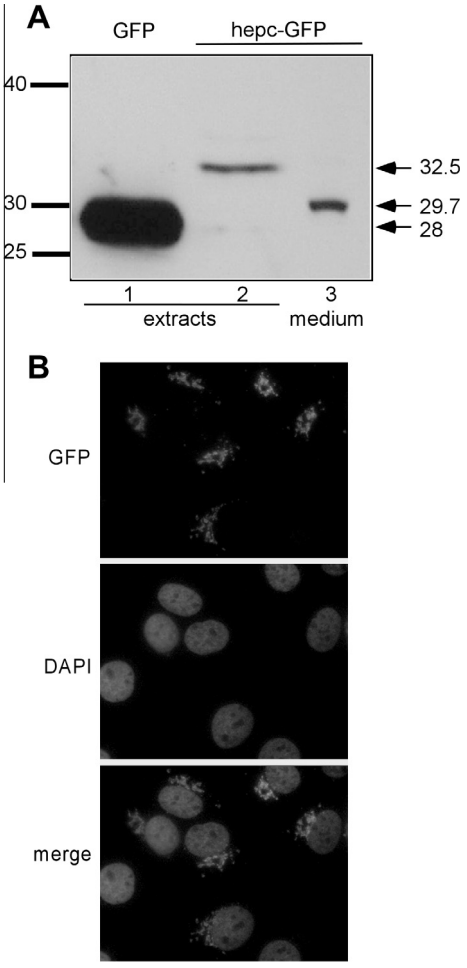

Fig. 2. Stable expression and secretion of hepcidin-GFP from Huh7 cells. (A) Western blot analysis of cell extracts (lanes 1 and 2) and culture medium TCA precipitate (lane 3) obtained from cells stably expressing GFP (lane 1) or hepcidin-GFP (lanes 2 and 3). Numbers on the left show the positions of MW markers (in kDa) and arrows on the right indicate the position and apparent MW of detected bands. (B) Fluorescence microscopy imaging of Huh7 cells stably expressing hepcidin-GFP. The position of the cell nucleus is visualized by DAPI.

3.2. Secretion of mature hepcidin-GFP from stably transfected Huh7 cells

After stable transfection of pHepc-GFP into Huh7 cells we obtained a clone that constitutively expressed hepcidin-GFP. Western blot analysis showed the absence of free GFP from both cell extracts and media, suggesting that the fusion protein avoids detectable degradation when produced at low levels (Fig. 2A, lanes 2 and 3). Furthermore, a single fusion protein was detected in cell extracts, which, according to its apparent MW, corresponds to pro-hepcidin-GFP (Fig. 2A, lane 2). Interestingly, a single protein was detected in the culture medium that, according to its apparent MW, corresponds to the mature and fully processed hepcidin-GFP (Fig. 2A, lane 3). Therefore, Huh7 cells express, process and secrete hepcidin-GFP in a manner analogous to native hepcidin. This is confirmed by fluorescent microscopy analysis of the stable clones that revealed localization of the intracellular GFP-fusion (which according to the western blot analysis corresponds to pro-hepcidin-GFP) at confined perinuclear sites representing the Golgi apparatus (Fig. 2B).

3.3. Hepcidin-GFP promotes iron accumulation in monocytic cells and targets ferroportin

To find out if hepcidin-GFP produced from the stable clone was biologically active, THP1 monocytic cells, treated with DFO in order to deplete iron, were co-cultured with untransfected (mock) or hepcidin-GFP expressing Huh7 cells and analyzed for the expression of IRP2, a cytoplasmic post-transcriptional regulator which undergoes proteasomal degradation in response to iron loading [23]. Co-culturing of THP1 with [IL-6]-stimulated mock Huh7 cells decreased the steady-state levels of IRP2 (Fig. 3A top panel, lane 2) as expected by the fact that IL-6 stimulation increases production of endogenous hepcidin by Huh7 cells [20], which then inhibits iron efflux from THP1 cells. Interestingly, IRP2 was further decreased when THP1 cells were co-cultured with the Huh7 clone expressing hepcidin-GFP (Fig. 3A, top panel, lane 3). Furthermore, ferritin expression, which is de-repressed when intracellular iron retention is high [23], was up-regulated after co-culture with the hepcidin-GFP expressing clone (Fig. 3B, middle panel, lane 3). Moreover, when mouse J774 macrophages, that were previously treated with DFO or hemin for elevating the endogenous levels of IRP2 or ferroportin, respectively, were cultured with conditioned medium derived from hepcidin-GFP producing Huh7 cells, significant down-regulation of both IRP2 and ferroportin levels was observed (Fig. 3B, lanes 2 and 4). Overall, these data suggest that hepcidin-GFP is biologically active and can promote iron accumulation in monocytes by targeting ferroportin.

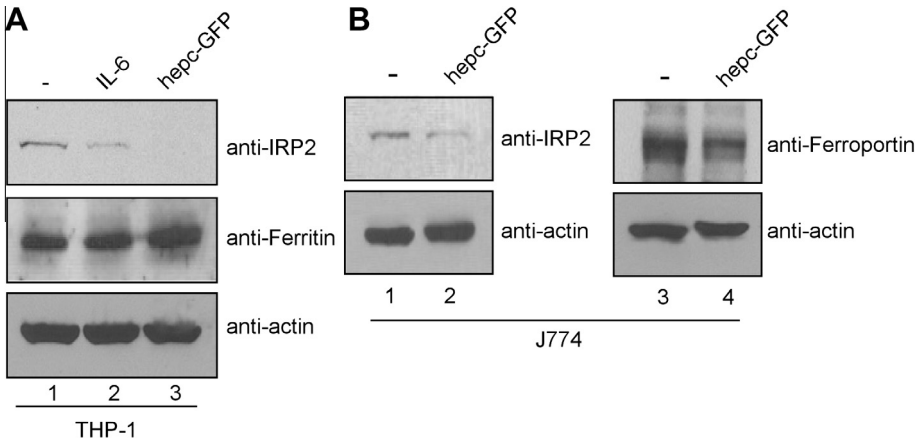

Fig. 3. Hepcidin-GFP promotes iron accumulation in monocytic cells by targeting ferroportin. (A) Western blot analysis of THP-1 cell lysates after pre-treatment of DFO and 4 h co-culture with mock Huh7 cells (lane 1), IL-6 treated Huh7 (lane 2) or Huh7 cells stably expressing hepcidin-GFP (lane 3) followed by detection for IRP2 (upper panel), ferritin (middle panel), and actin (lower panel). (B) Western blot analysis of J774 cells pre-treated with DFO (left panels), or hemin (right panels) followed by treatment with conditional medium from mock Huh7 cells (lanes 1 and 3) or cells stably expressing hepcidin-GFP (lanes 2 and 4).

3.4. Hypoxia impairs secretion of hepcidin-GFP

We previously showed that inhibition of 2-oxoglutarate dependent oxygenases, which mimics hypoxia, down-regulates hepcidin gene transcription [9]. To investigate whether hypoxia has a further post-transcriptional impact on hepcidin, we exposed the hepcidin-GFP-expressing Huh7 clone to 0.1% O_2. Of note, expression of hepcidin-GFP in these cells is under the control of a heterologous (CMV) promoter and transcriptional effects of hypoxia can be excluded. As generation of the GFP chromophore is an oxygen-dependent process [24], we first checked if GFP fluorescence is impaired by hypoxia. We observed no significant difference in the GFP signal between normoxia and hypoxia using cells expressing cytoplasmic GFP-tagged HIF-1α peptides, confirming that 0.1% of oxygen is sufficient for proper chromophore generation and overall folding of GFP (data not shown).

Analysis of the culture medium from cells incubated under hypoxia for 24 h showed a significant 40% reduction of the mature secreted hepcidin-GFP compared to control cells, while the levels of human albumin constitutively secreted by Huh7 cells remained unchanged (Fig. 4A, right panel, compare lanes 3 and 4; Fig. 4B). In contrast, expression levels of intracellular pro-hepcidin-GFP was only slightly reduced (by 10%) by hypoxia (Fig. 4A, left panel, compare lanes 1 and 2; Fig. 4B). Again no change was observed in intracellular human albumin levels. Of note, the antibody against human albumin secreted by Huh7 cells does not cross-react with bovine serum albumin present in the medium TCA precipitates (data not shown).

To further investigate the hypoxia effect, hepcidin-GFP expressing cells were exposed to hypoxia for different time intervals. As shown by fluoresence microscopy (Fig. 4C), pro-hepcidin-GFP gradually (detectably at 8 h and more obviously at 24 h) lost its confined perinuclear Golgi-like localization and gave an increasingly diffuse (and, therefore, weaker) signal, suggesting escape from the secretory pathway. Analysis of the culture medium after 8 h of hypoxic treatment revealed no significant change in the amounts of secreted mature hepcidin-GFP (Fig. 4D, lanes 1 and 2). However, when the culture medium was replaced after 8 h of hypoxic treatment and exposure of the cells to hypoxia continued for another 16 h, we observed a significant reduction in the accumulation of secreted mature hepcidin-GFP while secretion of human albumin was unaffected (Fig. 4D, lanes 3 and 4). Taken together, our data suggest that long-term (24 h) hypoxia does not significantly affect post-transcriptional production of pro-hepcidin but inhibits specifically the secretion process of mature hepcidin.

4. Discussion

It is currently not known if production and secretion of hepcidin are also controlled at the post-transcriptional level, as is often the case for peptide hormones. Study of hepcidin processing, maturation and secretion has been hampered by its small size and the lack of widely available immunological reagents specifically detecting the mature form. To experimentally address this issue, we constructed a chimeric protein by fusing GFP at the C-terminus of hepcidin hoping that GFP would not interfere with processing and activity of hepcidin but rather offer an easy way to monitor the hepcidin path both intra- and extra-cellularly. Previous reports had indeed shown that recombinant hepcidin forms tagged with His or His-Myc epitopes in their C-termini retained biological activity [25,26].

Our analysis of hepcidin-GFP maturation that was originally performed after transient over-expression in hepatoma cells showed very little and hardly detectable secreted mature hepcidin-GFP. Instead, we observed accumulation of pro-hepcidin-GFP forms both inside the cells and in the culture medium. The presence of these forms in the medium, as shown by treatment with BFA, was the result of active secretion and not leakage of the cells. The pro-form of hepcidin contains a predicted furin cleavage site [1]. Furin is a member of the subtilisin-like pro-protein convertases and localizes to the trans-Golgi network [27]. It is hence possible that even correctly folded but over-expressed pro-hepcidin-GFP overpowers the ability of furin or other proteases to process it [26], explaining its accumulation in the secretory pathway and its release into the culture medium. Unprocessed pro-hepcidin can indeed be found in blood and hepatocyte culture medium [17,18,28] but was shown to be biologically inactive [19].

Problems associated with transient over-expression of hepcidin-GFP were resolved when Huh7 clones were selected that stably expressed the chimera at much lower and presumably closer to physiological levels. Under these conditions, two stable fusion protein forms were detected: pro-hepcidin-GFP, which was only intracellular and localized in Golgi-like structures, and mature hepcidin-GFP, which was only found secreted in the culture medium. We, therefore, concluded that, despite the presence of the bulky GFP-moiety, recombinant hepcidin-GFP was processed and secreted in a manner analogous to endogenous hepcidin. The fusion protein phenocopied the function of hepcidin in terms of cellular iron homeostasis. Co-culture of hepcidin-GFP expressing Huh7 cells with THP1 cells decreased IRP2 and increased ferritin expression in THP1 cells suggesting that hepcidin-GFP is able to

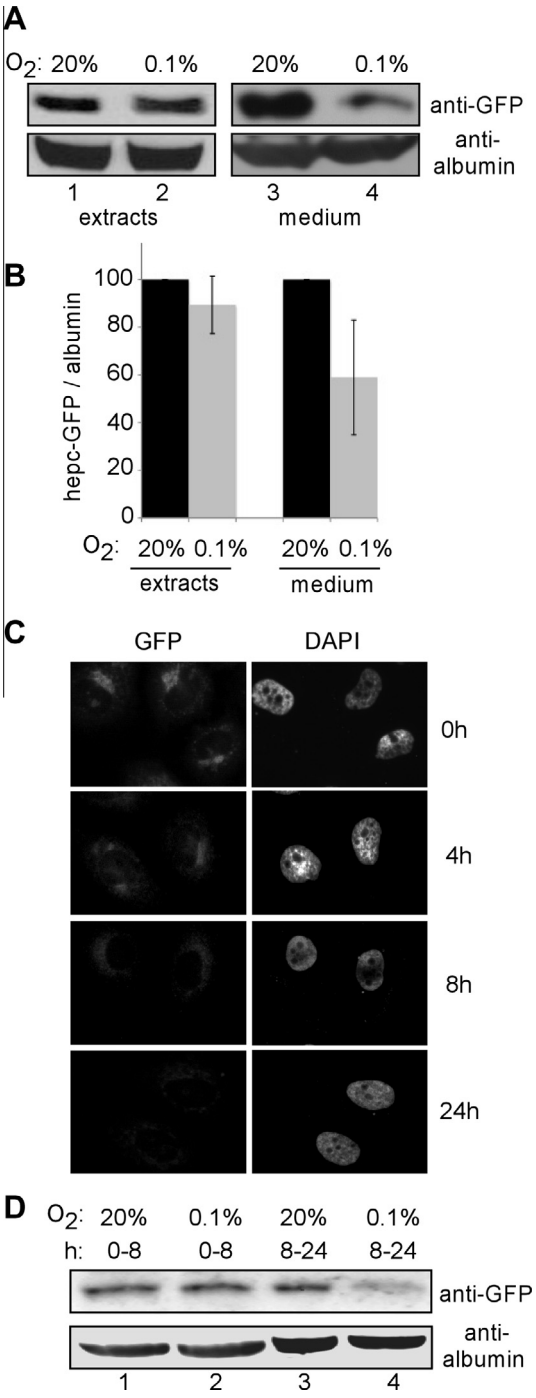

Fig. 4. Hypoxia inhibits secretion of hepcidin-GFP. (A) Western blot analysis of cell extracts (left panel) and culture media TCA precipitates (right panel) collected after 24 h exposure of hepcidin-GFP expressing cells to 20% (normoxia) or 0.1% oxygen (hypoxia). (B) Quantification of the experiment described in (A). Values shown are mean ± SD from three independent experiments. (C) Fluorescence microscopy imaging of Huh7 cells stably expressing hepcidin-GFP incubated under normoxia or 0.1% oxygen for the indicated time periods. The position of the cell nucleus is visualized by DAPI staining. (D) Western blot analysis of culture media TCA precipitates of hepcidin-GFP expressing cells grown under normoxia or hypoxia. The culture medium was collected after 8 h (0–8, lanes 1 and 2), replaced and collected again after 16 h (8–24, lanes 3 and 4) of incubation.

inhibit iron efflux and promote iron accumulation in monocytic cells as previously reported for physiologically generated hepcidin [20]. This was confirmed by showing that treatment with conditioned medium containing hepcidin-GFP down-regulated both IRP2 and ferroportin in mouse J774 macrophages.

Taking advantage of the hepatoma cells secreting active hepcidin-GFP, we were able to examine whether hypoxia, a known negative transcriptional regulator of hepcidin [7], also affects its post-transcriptional production, processing and secretion steps. Our results show that incubation under low oxygen (0.1%) has little effect on the production of pro-hepcidin-GFP but significantly decreases the secretion of mature hepcidin-GFP from Huh7 cells. At the same time, hypoxia affects neither production nor secretion of human albumin, another constitutively Huh7 secreted protein. Although the predominant effects of hypoxia are normally transcriptional, in a few cases low oxygen has been reported to affect post-transcriptional events such as initiation of mRNA translation, via mTOR [29,30] or HIF-2α [31], or protein secretion, by inhibition of cellular trafficking or increased attachment to membranes [32]. Hypoxia may also interrupt protein processing by preventing correct folding or by disrupting disulfide bond formation in the ER [33]. The fact that hypoxia did not cause accumulation of precursor forms of pro-hepcidin-GFP suggests that its co-translational import into the endoplasmic reticulum was not affected. Since the negative effect of hypoxia on the levels of pro-hepcidin-GFP, if any, was very modest, the gradual loss of its Golgi-like perinuclear localization pattern during the course of hypoxia together with the lack of intracellular accumulation of the mature form indicate possible defects in the ER to Golgi transport or intra-Golgi processing steps. Also the fact that pro-hepcidin-GFP does not accumulate inside the cells despite inhibition of mature hepcidin-GFP secretion would be consistent with rapid degradation of the pro-hepcidin-GFP that gets blocked in the ER or Golgi. On the other hand, the continuous and undisturbed secretion of albumin under hypoxia suggests that the overall function of the secretory pathway is not grossly affected by low oxygen.

In summary, we propose that hypoxia inhibits hepcidin secretion at one or more steps after its co-translational import into the ER and probably before its final post-Golgi maturation and exocytosis step. A previous study [17] has shown that processing of hepcidin is not affected by inhibitors of the HIF pathway. Therefore, the negative effects of hypoxia on both hepcidin gene transcription and protein secretion appear to be HIF-independent. A major future task will be the elucidation of the exact molecular mechanism by which hypoxia modulates the secretion of hepcidin. The hepcidin-GFP chimera described herein provides an invaluable experimental tool in this context.

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