

# Pancreatic $\beta$ -cells express hepcidin, an iron-uptake regulatory peptide

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## Abstract

Body iron is involved in various vital functions. Its uptake in the intestine is regulated by hepcidin, a bioactive peptide originally identified in plasma and urine and subsequently in the liver. In the present study, we provide evidence at the transcriptional and translational levels that hepcidin is also expressed in the pancreas of rat and man. Immunohistochemical studies localized the peptide exclusively to  $\beta$ -cells of the islets of Langerhans. Immunoelectron microscopical analyses revealed that hepcidin is confined to the insulin-storing  $\beta$ -cell secretory granules. As demonstrated in insulinoma-derived RINm5F cells, the

expression of hepcidin in  $\beta$ -cells is regulated by iron. Based on the present findings we conclude that pancreatic islets are an additional source of the peptide hepcidin. The localization of this peptide to  $\beta$ -cells suggests that pancreatic  $\beta$ -cells may be involved in iron metabolism in addition to their genuine function in blood glucose regulation. In view of the various linked iron/glucose disorders in the pancreas, the present findings may provide an insight into the phenomenology of intriguing mutual relationships between iron and glucose metabolisms.

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## Introduction

Iron is an essential component of hemoglobin and myoglobin and of many enzymes involved in redox reactions and energy metabolism. Low levels of iron in the circulation may cause severe dysfunctions (e.g., anemia, hypoxia) while iron overload may be toxic because of its ability to generate reactive oxygen species. Thus, blood iron concentration is finely regulated. Since humans and other mammals lack effective mechanisms to excrete excess iron, the regulation of absorption of dietary iron, which happens predominantly in the proximal intestine, is the only way of maintaining iron balance. Excessive dietary iron uptake may cause iron deposition in many vital organs, including the liver, heart, skin, and especially pancreas leading to diabetes mellitus that is found in hereditary hemochromatosis, a group of genetic disorders characterized by the dysregulation of iron absorption (Pietrangelo 2004, Beutler 2006).

Hence, iron homeostasis by adequate regulation of iron uptake is of particular importance. In this respect, the small peptide hepcidin proved to function as the key regulator of iron metabolism. Hepcidin was isolated as a 25-amino acid peptide in human plasma and urine, exhibiting antimicrobial activity (Krause *et al.* 2000, Pigeon *et al.* 2001). Ongoing studies hitherto discovered the peptide mainly in the liver (Krause *et al.* 2000, Pigeon *et al.* 2001, Kulaksiz *et al.* 2004, Pietrangelo 2004, Beutler 2006). Animal and cell culture studies could demonstrate that hepcidin acts as a negative regulator of iron absorption in the small intestine and of iron release from macrophages

(Hentze *et al.* 2004, Ganz 2005). Secondary iron overload induced a higher expression of hepcidin in the liver (Pigeon *et al.* 2001, Montosi *et al.* 2005). Recent studies demonstrated an abnormal low hepcidin expression and disrupted hepcidin regulation in most types of hemochromatosis (Bridle *et al.* 2003, Muckenthaler *et al.* 2003, Kulaksiz *et al.* 2004, Pietrangelo 2004, Beutler 2006) and the association of hepcidin mutations with severe juvenile hemochromatosis (Roetto *et al.* 2003).

It has been repeatedly reported that iron influences glucose metabolism, which in turn affects iron metabolic pathways (Fernandez-Real *et al.* 2002a). Likewise, body iron stores are positively correlated with serum insulin and blood glucose concentrations (Tuomainen *et al.* 1997).

Inspired by these observations we analyzed in the present study the existence of the iron-regulatory peptide hepcidin in the human pancreas at the transcriptional and translational levels as well as its cellular and subcellular localization and regulation by iron. The findings will show that the endocrine pancreas is an additional source of hepcidin; its co-localization with insulin in  $\beta$ -cells is discussed perspectively.

## Materials and Methods

### *Tissues and tissue preparation*

Adult male Wistar rats ( $n=6$ , body weight of 250–300 g) were used for the light and electron microscopic investigations. The principles of laboratory animal care and specific national

laws were followed. Rats were killed by decapitation under carbon dioxide anesthesia. The dissected tissue specimens were then quenched in isopentane precooled with liquid nitrogen, freeze-dried for 48 h and fixed by vapor-phase paraformaldehyde (Kulaksiz *et al.* 2002). Surgical specimens of non-diseased pancreatic regions from patients (three females, five males, age range 34–79) suffering from insulinoma ( $n=3$ ) or glucagonoma ( $n=3$ ) or from ductal adenocarcinoma ( $n=2$ ) were treated in the same way. In addition, normal pancreatic tissue samples ( $n=3$ ) were obtained through an organ donor program when no suitable recipient for pancreas transplantation was found. Healthy human liver samples ( $n=7$ ) were obtained after hemi-hepatectomy in adult patients with liver metastases. Pancreatic tissue from a female, 54-year-old patient with hemochromatosis homozygous for the C282Y mutation was resected during autopsy. After liver transplantation, she died due to multiorgan failure.

The investigations were approved by the local ethical committee. All animal experiments described here were conducted in accordance with the accepted standards of human animal care. Investigations with human tissues described here were conducted in accordance with the guidelines in the Declaration of Helsinki.

#### *Peptide synthesis, immunization procedure, and antibodies*

From the published pro-hepcidin sequence (Krause *et al.* 2000, Pigeon *et al.* 2001) the peptides hepcidin (28–47) and hepcidin (70–84) were synthesized as C-terminal amides using a standard Fmoc protocol (Kulaksiz *et al.* 2002). The peptides were coupled to a keyhole limpet hemocyanin using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester, and two specific pathogen free (SPF) rabbits (Charles River, Sulzfeld, Germany Iffa Credo) were immunized with each peptide conjugate (Eurogentec, Seraing, Belgium). After testing the titer by ELISA, the antisera EG(1)-HepC, EG(2)-HepC, each directed against hepcidin (70–84) and EG(1)-HepN, and EG(2)-HepN, each directed against hepcidin (28–47) (Kulaksiz *et al.* 2004, 2005), were used in the present study. The peptide epitopes used for the generation of the antibodies displayed no homology with any hitherto reported protein except hepcidin as confirmed by the BLAST P2 search. Human and rat hepcidin show a sequence homology of 88% why the antisera against human hepcidin could be successfully employed in the rat pancreas.

#### *Cell culture*

RINm5F, rat insulinoma cells, were grown in RPMI 1640 medium (PAA, Pasching, Austria) containing L-glutamine, supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, penicillin (100 units/ml), and streptomycin (100 µg/ml). The cells were cultivated at 37 °C in 5% CO<sub>2</sub>. They were incubated with increasing concentrations of Fe-NTA from 1 µM up to 65 µM for 72 h ( $n=10$ ) according to a previously published protocol (Gehrke *et al.* 2003). After stimulation experiments, total RNA or proteins were

extracted from RINm5F cells and analyzed by quantitative PCR and immunoblot assays.

The human hepatoma HepG2 cells were obtained from the German Collection of Microorganisms and Cell Culture (Braunschweig, Germany) and grown at 37 °C in 5% CO<sub>2</sub> in RPMI 1640 media (Gibco) supplemented with 10% (vol/vol) heat-inactivated FBS, penicillin (100 units/ml), and streptomycin (100 µg/ml). These cells were used as control.

#### *Expression analyses in pancreas and RINm5F cells*

RNA isolation was performed using the Qiagen RNeasy kit including DNase digestion ( $n=15$ ). Reverse transcription (RT)-PCR analysis was performed as described previously (Kulaksiz *et al.* 2004, 2005) using the following primers and specifications given in 5′-3′ orientation: human hepcidin (GenBank database accession no. NM021175) – 5′-CCT GAC CAG TGG CTC TGT TT-3′ and 5′-GGT TCT ACG TCT TGC AGC AC-3′, corresponding to positions 130–149 and 330–311 and rat hepcidin (GenBank database accession no. NM053469) – 5′-GGC AGA AAG CAA GAC TGATGAC-3′ and 5′-ACA GGA ATA AAT AAT GGG GCG-3′, corresponding to positions 146–168 and 346–332. Rat ferroportin (GenBank database accession no. AF394785) – 5′-GCT AGA ATC GGT CTT TGG TCC-3′ and 5′-ACC ATG ATG AAA TGC AGA AGG-3′; corresponding to positions 1646–1666 and 1765–1745.

After an initial denaturation of 94 °C for 4 min, the reactions were subjected to 30 cycles of the following thermal program: 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s; this program was followed by a final 5 min elongation step at 72 °C. The amplification products were run on an ethidium bromide-stained 1.8% of 89 mM Tris/89 mM boric acid/2 mM EDTA (pH 8.3) agarose gel. To exclude possible genomic DNA contamination, control samples were employed in which the reverse transcriptase enzyme was omitted from the cDNA synthesis reaction. As specificity controls, the amplified PCR products were sequenced on a 377 DNA sequencer (Perkin-Elmer). The sequence analyses revealed that the PCR-generated products have a complete homology with the cDNAs of the corresponding proteins.

#### *Quantitative RT-PCR*

Real-time quantification of mRNA transcripts was performed according to the protocol published (Gehrke *et al.* 2003;  $n=10$ ). In brief, a two-step RT-PCR was performed using the LightCycler system and Relative Quantification Software Version 1.0 (Roche). In the first step, cDNA synthesis was performed using the First Strand cDNA Synthesis Kit for RT-PCR (Roche) according to the manufacturer's instructions. In a second step, transcripts of hepcidin were amplified in duplicate with specific sense and antisense primers. All transcripts were detected using SYBR green I according to the manufacturer's instructions and were normalized to β-actin as the internal control.

### Immunohistochemistry

After embedding the specimens in epoxy resin (Araldite), serial semithin sections were cut at 0.5 µm and processed for immunohistochemical staining. Details on the antibodies used for the detection of pancreatic hormones (insulin, glucagon, somatostatin, and pancreatic polypeptide) have been published earlier (Cetin *et al.* 1993).

Immunostainings according to the avidin–biotin–peroxidase complex (ABC) technique and method controls were performed as outlined previously (Cetin *et al.* 1993, Kulaksiz *et al.* 2002, 2005). Briefly, the ABC method included incubation of semithin sections with primary antibodies (1:2000) in PBS containing 0.1% Triton X-100 (v/v) for 24 h at 4 °C, followed by incubation for 30 min at room temperature with the second antibody, biotin-labeled goat anti-rabbit IgG (Jackson Immuno Research, West Grove, USA). The sections were then incubated for 30 min with a preformed complex of biotin–peroxidase/streptavidin (Jackson Immuno Research), and the peroxidase activity was revealed using 0.02% diaminobenzidine hydrochloride (w/v) with 0.3% nickel sulfate (w/v) as the chromogen. The specificity of the antibodies was tested by preincubation of all antibodies with homologous and heterologous antigens according to previously published protocols (Kulaksiz *et al.* 2002, 2004, 2005). The preadsorption of hepcidin antibodies with the homologous antigens (hepcidin (28–47) or hepcidin (70–84)) at concentrations as low as 6.25 µg/ml completely blocked immunostaining in the pancreas as well as in the reference organ liver. Preadsorption of the antibodies with heterologous antigens and with insulin, limpet hemocyanin (both from Sigma) at concentrations up to 100 µg/ml had no effect on immunostaining.

### Electron microscopy

For immunogold electron microscopy, anesthetized rats were transcardially perfused with prewash and fixative solutions. Briefly, the fixative consisted of a mixture of 4% (v/v) depolymerized paraformaldehyde, 0.1% glutaraldehyde, and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.4) containing 0.5 mM calcium chloride. Following fixation of the dissected tissue in the same fixative, the specimens were embedded either in Epon or in Unicryl (British BioCell, Cardiff, UK), as described previously (Cetin *et al.* 1993). Surgical specimens of normal pancreatic tissue obtained from patients suffering from glucagonoma or nesidioblastosis were fixed in a mixture of cacodylate-buffered 2% paraformaldehyde and 2.5% glutaraldehyde or in cacodylate-buffered 1.4% glutaraldehyde, and subsequently embedded in Epon. On-grid labeling of thin sections was performed as outlined elsewhere (Cetin *et al.* 1993) using secondary antibodies conjugated with 10 or 12 nm colloidal gold (British BioCell; Jackson Immuno Research). Images of micrographs were digitalized and their contrast and brightness were adjusted using Adobe Photoshop software.

### Immunofluorescence microscopy

RINm5F cells were grown on cover slides, fixed in cold methanol, and incubated with hepcidin antibodies at a dilution of 1:1000 overnight at 4 °C (Kulaksiz *et al.* 2004). After washing in PBS (pH 7.0), the cells were incubated with an anti-rabbit antibody (diluted 1:200) labeled with Cy3 (Dianova, Hamburg, Germany). The nucleus of the cells was stained using PBS containing 10 µg/ml of 4',6-diamidino-2-phenylindole (Sigma).

Immunostaining was investigated using an Olympus AX-70 microscope. Micrographs were taken using a digital camera (colorview 12, soft imaging system SIS, Münster, Germany) and analySIS software (SIS, Münster, Germany).

### Immunoblot analysis

For western blot analysis ( $n=10$ ), the protein extracts were incubated for 7 min at 94 °C in sample buffer with 4% (w/v) SDS (Merck), 50 mM Tris–HCl (pH 8.45), 1 mM EDTA, 3.24 mM dithiothreitol (Roth, Karlsruhe, Germany), 12.5% (w/v) glycerol (Merck), and 0.002% bromophenol blue (Merck). To detect hepcidin, a 16.5% tricine–SDS–polyacrylamide gel was used according to the published protocols (Kulaksiz *et al.* 2002, 2004, 2005). The immunoreaction on the western blot was specifically blocked after preincubation of the antibodies with the corresponding peptide immunogens. The cross-reactivity with the second goat anti-rabbit antibody was excluded by appropriate controls (Kulaksiz *et al.* 2002, 2004, 2005).

### Hepcidin measurement by ELISA

Hepcidin was measured in the supernatants of RINm5F cells by ELISA (DRG Instruments GmbH, Marburg, Germany) according to the published protocol (Kulaksiz *et al.* 2004, 2005).

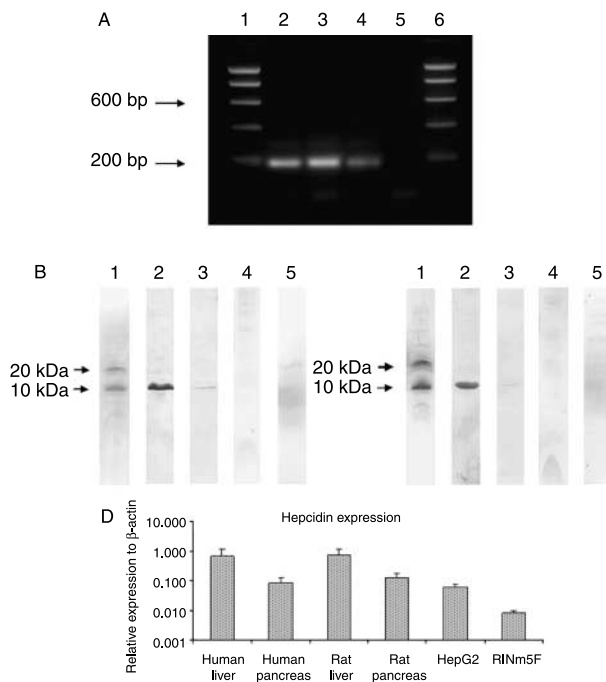
### Statistical analysis

Statistical analysis of quantitative variables was performed using the non-parametric Mann–Whitney test.  $P<0.05$  was considered significant. All statistical analyses were performed using SAS, version 9.1 (SAS Institute, Cary, NJ, USA).

## Results

### Expression of hepcidin in the human and rat pancreas

RT-PCR analyses using specific primers revealed that hepcidin is highly expressed in human and rat pancreas (Fig. 1). The amplification yielded an expected transcript of 201 bp. Sequence analyses of the amplification products confirmed a complete homology with the hepcidin cDNAs of rat and man (Krause *et al.* 2000, Pigeon *et al.* 2001). As determined by quantitative RT-PCR, the expression of hepcidin in the pancreas was considerable but not as high as



**Figure 1** Expression of hepcidin in the pancreas. (A) RT-PCR analysis of human pancreas (lane 2), rat pancreas (lane 3), RIN m5F cells (lane 4), and control of pancreas lacking reverse transcription (lane 5). A bp DNA ladder has been indicated (lanes 1 and 6). (B and C) Immunoblot analysis of hepcidin in the extracts of human liver (lane 1), human (lane 2), and rat (lane 3) pancreas. Note the immunoreactive peptide bands at 10 kDa obtained with both N- and C-terminal antibodies EG(2)-HepN (B) and EG(2)-HepC (C). Note that no immunoreactive peptides are observed in the human pancreatic extracts with preimmunesera (lane 4) or after preincubation of the antibodies with the corresponding peptide immunogens (lane 5). (D) Quantitative RT-PCR ( $n=6$ ). The expression levels of hepcidin (means) are given as the amount relative to the expression of the housekeeping gene actin in each sample.

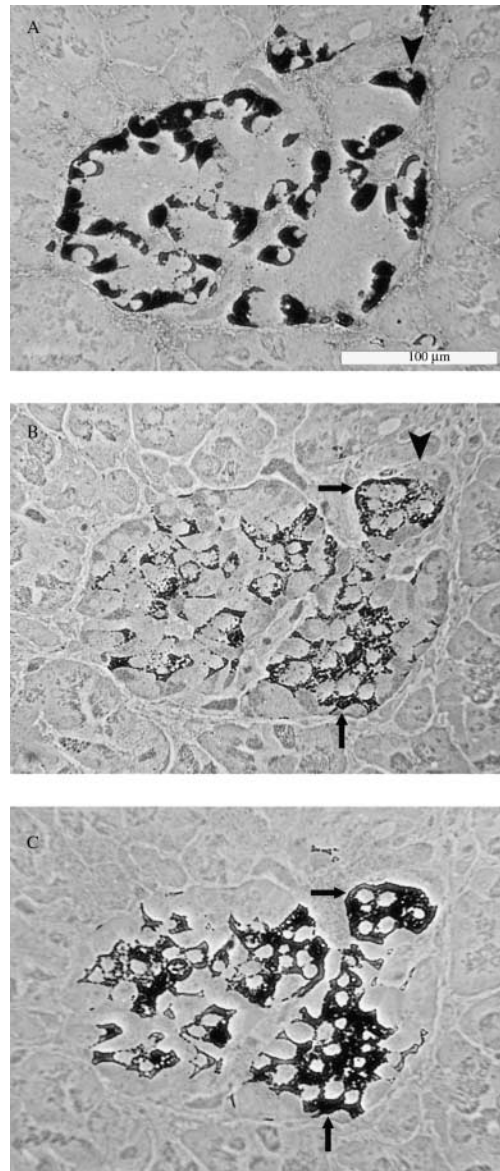
that in the liver (Fig. 1). In this respect, it has to be noted that in contrast to the liver the islets of Langerhans of the pancreas, where hepcidin is expressed (see below), constitute merely a small portion of the total pancreatic parenchyma. The existence of hepcidin at the translational level was verified by immunoblotting analyses in human and rat pancreatic extracts. Both N- and C-terminal region-specific hepcidin antisera concurrently identified the immunoreactive peptide of  $\sim 10$  kDa (Fig. 1). The size of this pancreatic peptide co-migrating with the immunoreactive band observed in homogenates of the liver (positive control) corresponds to the molecular mass of hepcidin deduced from the respective cDNA sequence (Pigeon *et al.* 2001, Kulaksiz *et al.* 2004).

#### *Hepcidin is localized to pancreatic $\beta$ -cells*

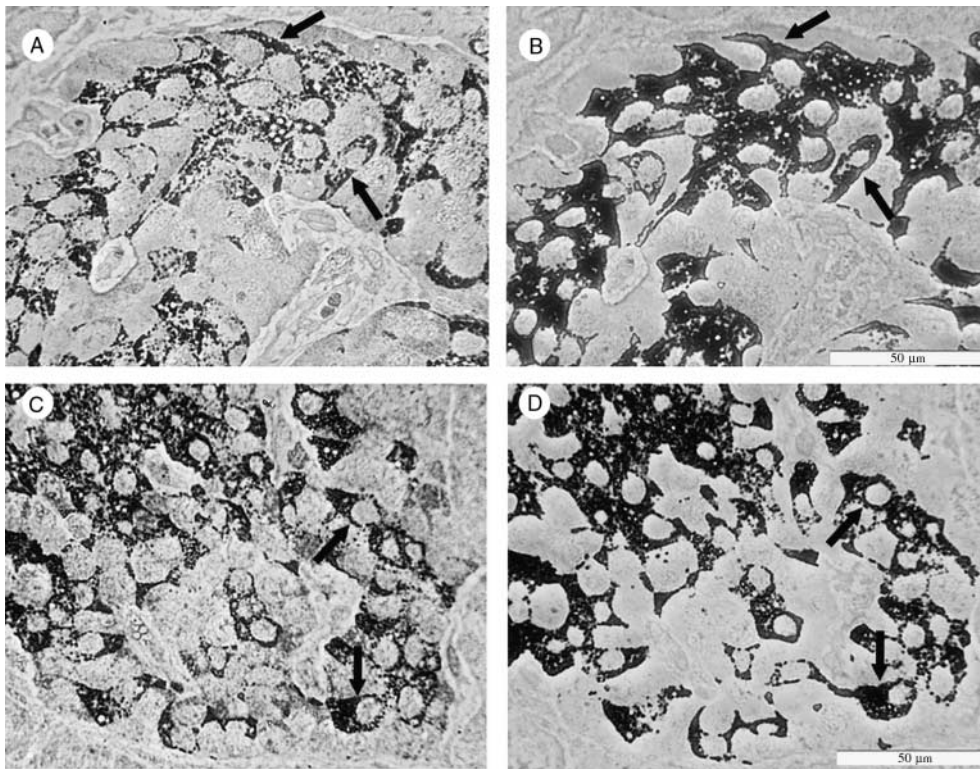
All hepcidin antisera under study coincidentally immunostained distinct cells within the human Langerhans islets, which, by their typical location within the islets and by co-localization with

insulin, were clearly identified as  $\beta$ -cells (Figs 2 and 3). Other endocrine cell types (i.e., glucagon  $\alpha$ , somatostatin  $\delta$ , and pancreatic polypeptide PP-cells) or the exocrine gland cells were completely unreactive for hepcidin. The same cellular distributive pattern was found in the rat pancreas where a strong hepcidin immunoreactivity was detected in the  $\beta$ -cells (Fig. 4).

At the subcellular level, hepcidin was confined to the typical secretory granules of the  $\beta$ -cells, where insulin is



**Figure 2** Cellular and subcellular localization of hepcidin in the pancreas. Three serial semithin ( $0.5 \mu\text{m}$ ) sections through an islet of Langerhans in the human pancreas immunostained for (A) glucagon, (B) hepcidin, and (C) insulin. In the pancreatic islet, hepcidin immunoreactivity is exclusively localized to insulin ( $\beta$ -) cells (arrows). No immunoreactivity is found in glucagon ( $\alpha$ ) cells (arrowheads). Scale bar,  $100 \mu\text{m}$ .



**Figure 3** Granular immunoreactivity pattern for hepcidin is shown with N- and C-terminal hepcidin antisera ((A) EG(2)-HepN and (C) EG(2)-HepC) in serial semithin sections in pancreatic insulin  $\beta$ -cells (B and D) (arrows). Within the pancreatic islet, hepcidin and insulin are co-localized in the same cells. Scale bars, 50  $\mu$ m.

localized (Fig. 5). Hepcidin immunoreactivity exhibited a distinct intragranular topology; the immunoreactivity of this peptide was absent in the electron-lucent halo but was confined to the dense core of the  $\beta$ -cell secretory granules, i.e. the compartment where insulin is also resident.

#### *Hepcidin expression in $\beta$ -cells is regulated by iron*

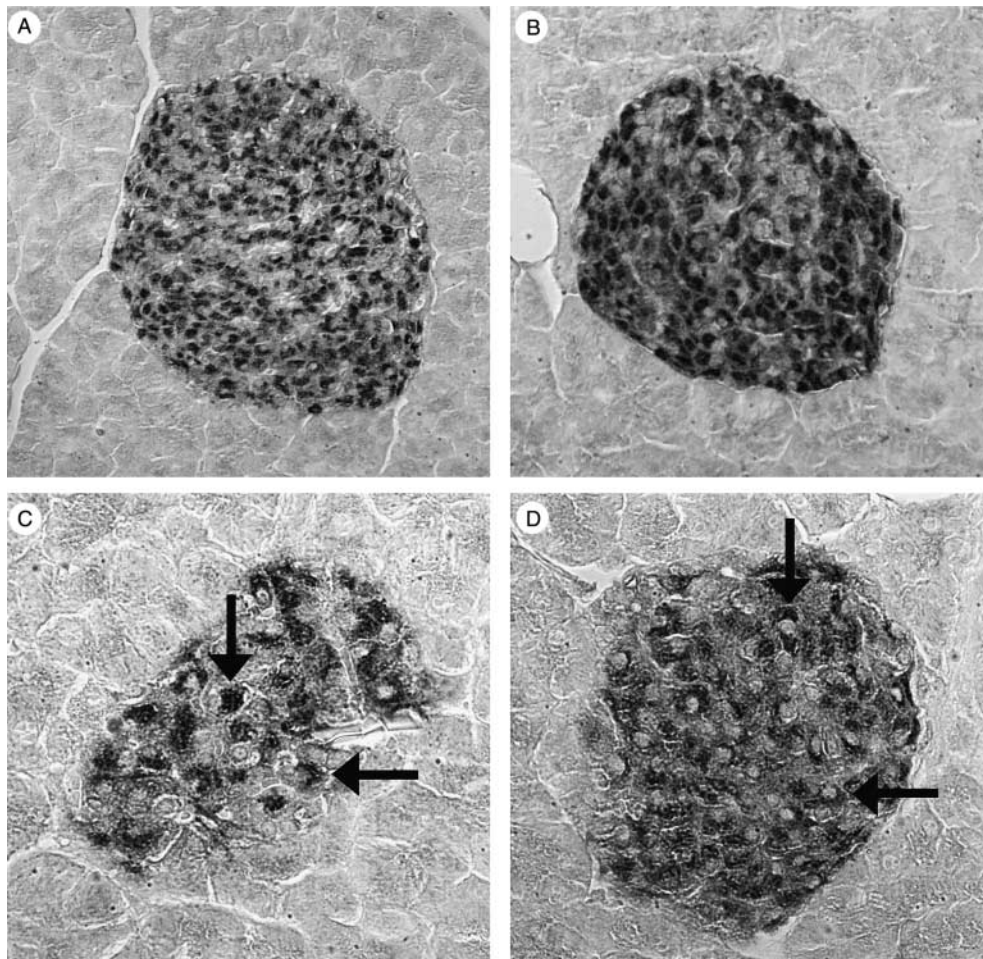
RINm5F cells, rat insulinoma cells of the pancreas, exhibiting preserved  $\beta$ -cell features represent a suitable model for  $\beta$ -cell experiments (Gazdar *et al.* 1980). RT-PCR analyses using the appropriate primer specifications and combinations successfully employed in the rat liver and pancreas identified the expression of hepcidin in RINm5F cells (Fig. 1). In analogy to the human and rat pancreas, the amplification product in RINm5F cells was also of 201 bp, which after sequencing revealed a complete homology to the rat hepcidin cDNA (Pigeon *et al.* 2001). The existence of the respective translated proteins in these cells was verified by immunocytochemistry and immunoblotting experiments using the region-specific hepcidin antisera (Figs 6 and 7).

The regulation of hepcidin expression in RINm5F cells was analyzed upon exposure to iron, since iron regulates hepcidin expression in the liver (Gehrke *et al.* 2003, Pietrangelo 2004, Beutler 2006). The regulatory effect of iron on hepcidin gene expression was measured in these cells

under various Fe-NTA concentrations using quantitative RT-PCR. Under small amounts of iron, i.e., 1  $\mu$ M up to 10  $\mu$ M, a significant up-regulation of hepcidin-mRNA up to threefold was observed in RINm5F cells (Fig. 7), as is also the case in the liver. Remarkably, hepcidin expression was down-regulated when Fe-NTA concentrations of 65  $\mu$ M or higher were used, even if the concentrations of Fe-NTA were not cytotoxic as shown in a neutral red assay (Fig. 7). These results were confirmed by immunoblot analyses (Fig. 7).

#### **Discussion**

The present findings show that hepcidin is expressed in the pancreas of rat and man. RT-PCR analyses with specific primers yielded the expected transcripts, which after sequencing revealed complete homology with the corresponding human and rat cDNAs. In immunoblot experiments, the presence of the peptide in the pancreas was verified by two region-specific hepcidin antisera that identified the peptide of a correct molecular mass of  $\sim$ 10 kDa in the pancreas as well as in the reference organ liver. Hence, these data stress the pancreas as an additional source of the peptide hepcidin that hitherto is known to originate mainly from the liver. However, as revealed by the quantitative RT-PCR analyses, the expression rate of hepcidin



**Figure 4** Cellular localization of hepcidin in rat pancreas. Immunohistochemical sections stained with N- and C-terminal hepcidin antibodies EG(2)-HepN (A and C) and EG(2)-HepC (B and D) showing immunoreactivity in insulin  $\beta$ -cells (arrows). (Magnification: A,  $\times 200$ ; B,  $\times 300$ ; C and D, approximately  $\times 400$ ).

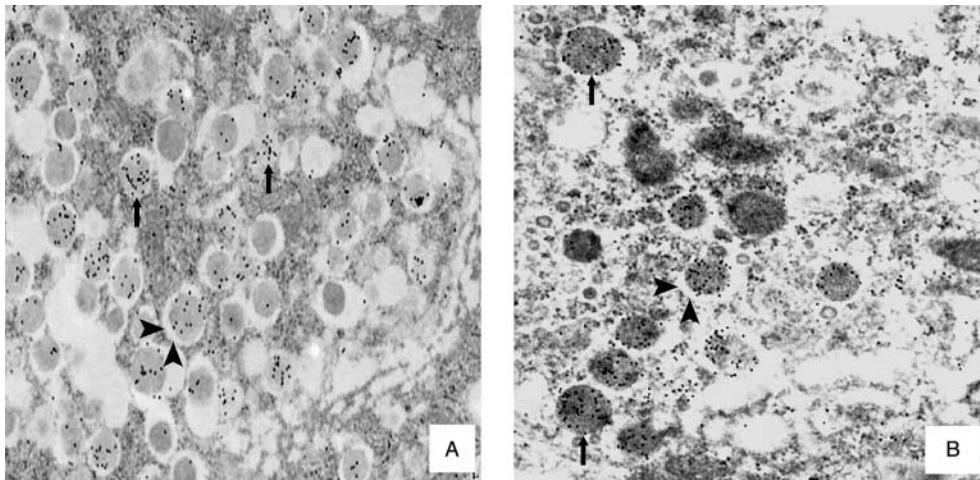
in the pancreas is lower than that in the liver. Interpreting this critically, it has to be noticed that hepcidin in the pancreas is expressed exclusively in the islets of Langerhans, which constitute merely a small compartment of the total pancreatic parenchyma. Thus, the virtually lower expression level of hepcidin in the pancreas may roughly correspond to that in the liver. Consequently, the expression of hepcidin in the pancreas might gain additional importance in degenerative hepatic diseases with suspending production of hepcidin in the liver (e.g., liver cirrhosis).

In previous studies, hepcidin was localized in extra-hepatic key organs like the heart and the kidney (Kulaksiz *et al.* 2005, Merle *et al.* 2007, Sheikh *et al.* 2007), where it responds to an acute phase reaction. In the heart, hepcidin was localized at the intercalated disc area (Merle *et al.* 2007); in the kidney, hepcidin was expressed in the epithelial tubule and the duct cells and secreted into the urine (Kulaksiz *et al.* 2005). These data suggest a local role for hepcidin regulating the intrinsic iron homeostasis in these organs. In contrast, in the pancreas, hepcidin is localized in

the endocrine cells secreting their products into the blood. Thus, the pancreas may contribute to the systemic hepcidin pool.

Using immunohistochemistry, hepcidin immunoreactivity was found thoroughly in the islets of Langerhans where the peptide was exclusively localized to pancreatic  $\beta$ -cells. Within these cells, immunoelectron microscopical studies revealed that hepcidin is confined to insulin-storing secretory granules assuming co-secretion of hepcidin and insulin upon the stimulation of pancreatic  $\beta$ -cells, e.g., by glucose. Indeed, we could detect hepcidin not only in the RINm5F cells (see below) but also in the supernatant of these cells measured by ELISA (Kulaksiz *et al.* unpublished data).

The co-localization of both peptides and their assumed co-release is in accordance with the recent findings demonstrating lowered C-peptide secretion (Fernandez-Real *et al.* 2002b) and hepcidin secretion (Nicolas *et al.* 2002, Abraham *et al.* 2006) after phlebotomy-induced iron depletion. Likewise, the reported relationship of body iron stores with serum insulin and blood glucose concentrations (Tuomainen *et al.* 1997,



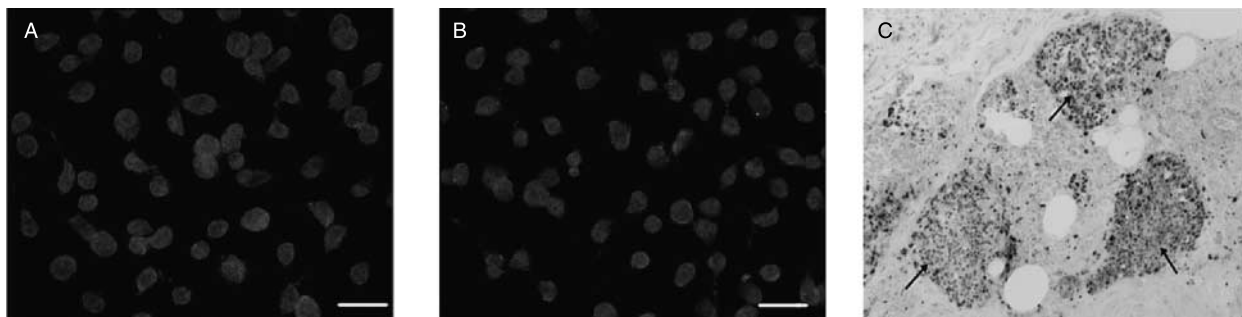
**Figure 5** Subcellular localization of hepcidin in normal human (A) and rat (B) pancreatic  $\beta$ -cells by immunoelectron microscopy. Within the  $\beta$ -cells, hepcidin immunoreactivity is exclusively confined to the secretory granules. Immunoreactivity for this peptide is accumulated in the dense core of the secretory granules (arrows); the electron-lucent halo of these granules exhibits no immunoreactivity for hepcidin (arrowheads). Electron micrograph ( $\times 25\,000$ ).

Abraham *et al.* 2006) are clearly in line with the present data. Hence, the expression of hepcidin in pancreatic  $\beta$ -cells and its localization to insulin-storing secretory granules argues for an iron-regulatory function of the pancreatic  $\beta$ -cells beyond their proper glucose-regulatory function. Very likely, the regulation of iron and glucose metabolisms is distinctly coupled at least at the pancreatic level by the co-release of insulin and hepcidin. Even under pathophysiological conditions, the secretion of insulin and hepcidin seems to be linked since patients with hereditary hemochromatosis revealed not only a decreased insulin secretion (Abraham *et al.* 2006, McClain *et al.* 2006) but also decreased serum hepcidin levels (Bridle *et al.* 2003, Kulaksiz *et al.* 2004).

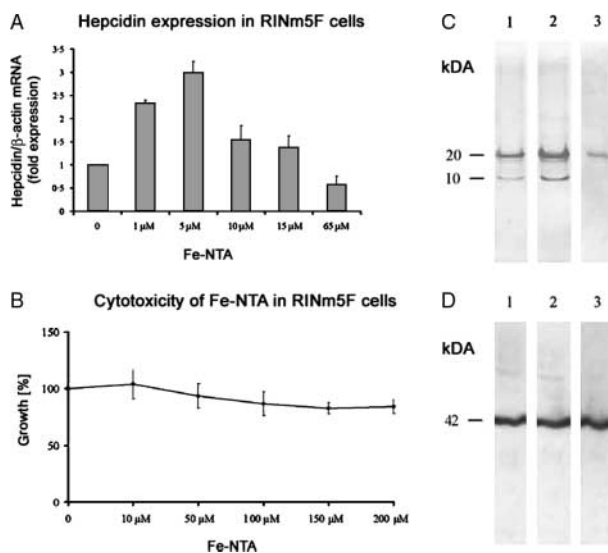
It is of particular importance that diseases of glucose metabolism such as diabetes mellitus are mostly associated with elevated body iron status, and diseases of iron overload are often accompanied by type 2 diabetes (Fernandez-Real *et al.* 2002a, Abraham *et al.* 2006). Phlebotomies, leading to decreased

iron stores, reduce postprandial hyperinsulinemia (Facchini 1998, McClain *et al.* 2006) and improve insulin sensitivity (Hua *et al.* 2001). Moreover, frequent blood donations do not only decrease iron stores but also lead to reduced serum glucose values (Bofill *et al.* 1994). Consequently, a crosstalk between iron and glucose metabolisms has been suggested (Fernandez-Real *et al.* 2002a).

The pancreas is one of those organs mainly affected by hemochromatosis. Hemochromatosis is characterized by systemic iron overload that is due to duodenal hyperabsorption (Andrews 1999) together with symptoms of a high prevalence of abnormal glucose homeostasis secondary to decreased insulin secretion (Abraham *et al.* 2006, McClain *et al.* 2006). It is of particular note that iron deposition in islets is restricted to  $\beta$ -cells (Rahier *et al.* 1987) and that in hemochromatosis only the  $\beta$ -cells of the islets of Langerhans are affected by iron overload (Fig. 6). Furthermore, the (apo)ferritin content proved to be four- to eightfold higher in  $\beta$ -cells of rat islets treated with 20 mmol/l



**Figure 6** Cellular localization of hepcidin and iron in the pancreas. (A and B) Hepcidin expression in RINm5F cells with C- and N-terminal hepcidin antibodies EG(2)-HepC (A) and EG(2)-HepN (B) using immunofluorescence technique with Cy3. Scale bars, 5  $\mu$ m. (C) Iron staining in pancreatic  $\beta$ -cells in a deceased patient with hereditary hemochromatosis ( $\times 250$ ).



**Figure 7** Hepcidin expression in  $\beta$ -cells regulated by iron. (A) Hepcidin expression in RINm5F cells after iron (Fe-NTA) stimulation using quantitative RT-PCR ( $n=5$ ) (1  $\mu$ M Fe-NTA,  $P<0.01$ ; 5  $\mu$ M Fe-NTA,  $P<0.001$ ; 10  $\mu$ M Fe-NTA,  $P<0.002$ ; 15  $\mu$ M Fe-NTA,  $P<0.05$ ; 65  $\mu$ M Fe-NTA,  $P<0.05$ ). (B) Cytotoxicity of Fe-NTA in RINm5F cells measured by neutral red assay, 3 days after iron stimulation. Note that no cytotoxicity exists under the iron concentrations used. (C) Immunoblot analysis of iron-stimulated RINm5F cells showing hepcidin immunoreactivity at the range of 10 kDa. Note the up-regulation of hepcidin with 3  $\mu$ M Fe-NTA (2) and down-regulation with 65  $\mu$ M Fe-NTA (3) compared with control (1). The second immunoreactive band at 20 kDa may reflect a dimeric type of hepcidin. (D) No up- or down-regulation was detected in  $\beta$ -actin expression under the same conditions.

glucose than that in  $\beta$ -cells treated with 1 mmol/l glucose (MacDonald *et al.* 1994), which unquestionably underlines the correlation between iron and glucose metabolism in these cells. Interestingly, in a recent study, no correlation between hepcidin and glucose homeostasis could be shown in the hepcidin knockout mouse (Ramey *et al.* 2007). However, in this study, iron overload was detected only in the acini and not in the  $\beta$ -cells. This could be due to interspecies differences. Indeed, in contrast to the human genome, the mouse genome contains two hepcidin genes encoding hepcidin and hepcidin 2; hepcidin is very weakly expressed in the mouse pancreas (Ilyin *et al.* 2003, Krijt *et al.* 2004).

Moreover, recent studies showed that pancreatic  $\beta$ -cells express the divalent metal ion transporter 1 (DMT1) (Koch *et al.* 2003), a protein of the duodenal epithelium crucially involved in cellular iron uptake (Hentze *et al.* 2004). The high levels of (apo)ferritin detected in the  $\beta$ -cells (MacDonald *et al.* 1994) may be explained by high iron fluxes through DMT1. Of note, we also could detect ferroportin, the only known mammalian iron exporter and hepcidin receptor (Nemeth *et al.* 2004), in  $\beta$ -cells (Kulaksiz *et al.* unpublished data). The presence of hepcidin and DMT1/ferroportin in pancreatic  $\beta$ -cells also argues for a local regulatory action in cellular iron transport.

In a set of cell stimulating experiments, we could show that the expression of hepcidin in  $\beta$ -cells is regulated by iron. Using quantitative RT-PCR and immunoblot assays, the regulatory effect of iron on hepcidin gene expression was demonstrated in RINm5F cells that represent a suitable model for  $\beta$ -cell experiments (Gazdar *et al.* 1980). The data obtained at the transcriptional and translational levels clearly show that increasing concentrations of iron result in a significant up-regulation (up to 221%) of hepcidin-mRNA in a dose-dependent manner. On the other hand, repeated experiments with iron concentrations of 65  $\mu$ M or higher clearly revealed a switch-over to down-regulation of hepcidin expression although the concentrations used did not prove to be cytotoxic. Similar effects of iron on hepcidin expression were also observed in HepG2 cells as already shown by us in previous studies (Gehrke *et al.* 2003, Fein *et al.* 2007). Likewise, an up-regulation of hepcidin expression in the liver was obtained after iron stimulation (Pigeon *et al.* 2001, Hentze *et al.* 2004, Montosi *et al.* 2005). Interestingly, in RINm5F cells and as already demonstrated in HepG2 cells (Gehrke *et al.* 2003) the expression of hepcidin decreased to the basal level when stimulating with higher amounts of iron. This could be clearly demonstrated at the mRNA and protein levels respectively. Hence, both cell lines of hepatic and pancreatic origin, i.e., HepG2 and RINm5F cells, equally react on iron; it may be suggested that the regulation of hepcidin expression is similar in the liver and in the endocrine pancreas.

In summary, hepcidin has been recognized as the key regulator of iron homeostasis (Hentze *et al.* 2004, Ganz 2005, Beutler 2006). Hitherto, the liver has been considered as the main site of hepcidin expression (Park *et al.* 2001, Kulaksiz *et al.* 2004). However, the present data clearly show that hepcidin is also expressed in the pancreas where the peptide is specifically attributed to the  $\beta$ -cells of the islets of Langerhans and confined to insulin-storing secretory granules. This assumes an additional iron-regulatory function of the pancreatic  $\beta$ -cells beyond their genuine function in the regulation of blood glucose.

In view of the fact that the pancreas belongs to those organs severely affected by hemochromatosis, the expression of the iron-regulatory peptide hepcidin in the pancreas opens up new vistas in the physiology of iron/glucose regulation and phenomenology of iron-storing diseases at the pancreatic  $\beta$ -cell level.

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## References

- Abraham D, Rogers J, Gault P, Kushner JP & McClain DA 2006 Increased insulin secretory capacity but decreased insulin sensitivity after correction of iron overload by phlebotomy in hereditary haemochromatosis. *Diabetologia* **49** 2546–2551.
- Andrews NC 1999 Disorders of iron metabolism. *New England Journal of Medicine* **341** 1986–1995.
- Beutler E 2006 Hemochromatosis: genetics and pathophysiology. *Annual Review of Medicine* **57** 331–347.
- Bofill C, Joven J, Bages J, Vilella E, Sans T, Cavalle P, Miralles R, Llobet J & Camps J 1994 Response to repeated phlebotomies in patients with non-insulin-dependent diabetes mellitus. *Metabolism* **43** 614–620.
- Bridle KR, Frazer DM, Wilkins SJ, Dixon JL, Purdie DM, Crawford DH, Subramaniam VN, Powell LW, Anderson GJ & Ramm GA 2003 Disrupted hepcidin regulation in HFE-associated haemochromatosis and the liver as a regulator of body iron homeostasis. *Lancet* **361** 669–673.
- Cetin Y, Aunis D, Bader MF, Galindo E, Jorns A, Bargsten G & Grube D 1993 Chromostatin, a chromogranin A-derived bioactive peptide, is present in human pancreatic insulin ( $\beta$ ) cells. *PNAS* **90** 2360–2364.
- Facchini FS 1998 Effect of phlebotomy on plasma glucose and insulin concentrations. *Diabetes Care* **21** 2190.
- Fein E, Merle U, Ehehalt R, Herrmann T & Kulaksiz H 2007 Regulation of hepcidin in HepG2 and RINm5F cells. *Peptides* **28** 951–957.
- Fernandez-Real JM, Lopez-Bermejo A & Ricart W 2002a Cross-talk between iron metabolism and diabetes. *Diabetes* **51** 2348–2354.
- Fernandez-Real JM, Pannaraja G, Castro A, Garcia-Bragado F, Hernandez-Aguado I & Ricart W 2002b Blood letting in high-ferritin type 2 diabetes: effects on insulin sensitivity and  $\beta$ -cell function. *Diabetes* **51** 1000–1004.
- Ganz T 2005 Hepcidin – a regulator of intestinal iron absorption and iron recycling by macrophages. *Best Practice & Research. Clinical Haematology* **18** 171–182.
- Gazdar AF, Chick WL, Oie HK, Sims HL, King DL, Weir GC & Lauris V 1980 Continuous, clonal, insulin- and somatostatin-secreting cell lines established from a transplantable rat islet cell tumor. *PNAS* **77** 3519–3523.
- Gehrke SG, Kulaksiz H, Herrmann T, Riedel HD, Bents K, Veltkamp C & Stremmel W 2003 Expression of hepcidin in hereditary hemochromatosis: evidence for a regulation in response to serum transferrin saturation and non-transferrin-bound iron. *Blood* **102** 371–376.
- Hentze MW, Muckenthaler MU & Andrews NC 2004 Balancing acts: molecular control of mammalian iron metabolism. *Cell* **117** 285–297.
- Hua NW, Stoohs RA & Facchini F 2001 Low iron status and enhanced insulin sensitivity in lacto-ovo vegetarians. *British Journal of Nutrition* **86** 515–519.
- Ilyin G, Courselaud B, Troade MB, Pigeon C, Alizadeh M, Leroyer P, Brissot P & Loréal O 2003 Comparative analysis of mouse hepcidin 1 and 2 genes: evidence for different patterns of expression and co-inducibility during iron overload. *FEBS Letters* **542** 22–26.
- Koch RO, Zoller H, Theuri I, Obrist P, Egg G, Strohmayer W, Vogel W & Weiss G 2003 Distribution of DMT1 within the human glandular system. *Histology and Histopathology* **18** 1095–1101.
- Krause A, Neitz S, Magert HJ, Schulz A, Forssmann WG, Schulz-Knappe P & Adermann K 2000 LEAP-1, a novel highly-disulfide bonded human peptide, exhibits antimicrobial activity. *FEBS Letters* **480** 147–150.
- Krijt J, Cmejla R, Sýkora V, Vokurka M, Vyoral D & Necas E 2004 Different expression pattern of hepcidin genes in the liver and pancreas of C57BL/6N and DBA/2N mice. *Journal of Hepatology* **40** 891–896.
- Kulaksiz H, Schmid A, Honscheid M, Ramaswamy A & Cetin Y 2002 Clara cell impact in air-side activation of CFTR in small pulmonary airways. *PNAS* **99** 6796–6801.
- Kulaksiz H, Gehrke SG, Janetzko A, Rost D, Bruckner T, Kallinowski B & Stremmel W 2004 Pro-hepcidin: expression and cell specific localisation in the liver and its regulation in hereditary haemochromatosis, chronic renal insufficiency, and renal anaemia. *Gut* **53** 735–743.
- Kulaksiz H, Theilig F, Bachmann S, Gehrke SG, Rost D, Janetzko A, Cetin Y & Stremmel W 2005 The iron regulatory peptide hormone hepcidin: expression and cellular localization in the mammalian kidney. *Journal of Endocrinology* **184** 361–370.
- MacDonald MJ, Cook JD, Epstein ML & Flowers CH 1994 Large amount of (apo)ferritin in the pancreatic insulin cell and its stimulation by glucose. *FASEB Journal* **8** 777–781.
- McClain DA, Abraham D, Rogers J, Brady R, Gault P, Ajioka R & Kushner JP 2006 High prevalence of abnormal glucose homeostasis secondary to decreased insulin secretion in individuals with hereditary haemochromatosis. *Diabetologia* **49** 1661–1669.
- Merle U, Fein E, Gehrke SG, Stremmel W & Kulaksiz H 2007 The iron regulatory peptide hepcidin is expressed in the heart and regulated by hypoxia and inflammation. *Endocrinology* **148** 2663–2668.
- Montosi G, Corradini E, Garuti C, Barelli S, Recalcati S, Cairo G, Valli L, Pignatti E, Vecchi C, Ferrara F *et al.* 2005 Kupffer cells and macrophages are not required for hepatic hepcidin activation during iron overload. *Hepatology* **41** 545–552.
- Muckenthaler M, Roy CN, Custodio AO, Minana B, deGraaf J, Montross LK, Andrews NC & Hentze MW 2003 Regulatory defects in liver and intestine implicate abnormal hepcidin and Cyb5I expression in mouse hemochromatosis. *Nature Genetics* **34** 102–107.
- Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, Ganz T & Kaplan J 2004 Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science* **306** 2090–2093.
- Nicolas G, Chauvet C, Viatte L, Danan JL, Bigard X, Devaux I, Beaumont C, Kahn A & Vaulont S 2002 The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. *Journal of Clinical Investigation* **110** 1037–1044.
- Park CH, Valore EV, Waring AJ & Ganz T 2001 Hepcidin, a urinary antimicrobial peptide synthesized in the liver. *Journal of Biological Chemistry* **276** 7806–7810.
- Pietrangelo A 2004 Hereditary hemochromatosis – a new look at an old disease. *New England Journal of Medicine* **350** 2383–2390.
- Pigeon C, Ilyin G, Courselaud B, Leroyer P, Turlin B, Brissot P & Loréal O 2001 A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. *Journal of Biological Chemistry* **276** 7811–7819.
- Rahier JR, Loozen S, Goebbels RM & Abraham M 1987 The hemochromatotic human pancreas: a quantitative immunohistochemical and ultrastructural study. *Diabetologia* **30** 5–12.
- Ramey G, Faye A, Durel B, Viollet B & Vaulont S 2007 Iron overload in Hepc1 (1/1) mice is not impairing glucose homeostasis. *FEBS Letters* **581** 1053–1057.
- Roetto A, Papanikolaou G, Politou M, Alberti F, Girelli D, Christakis J, Loukopoulos D & Camaschella C 2003 Mutant antimicrobial peptide hepcidin is associated with severe juvenile hemochromatosis. *Nature Genetics* **33** 21–22.
- Sheikh N, Dudas J & Ramadori G 2007 Changes of gene expression of iron regulatory proteins during turpentine oil-induced acute-phase response in the rat. *Laboratory Investigation* **87** 713–725.
- Tuomainen TP, Nyyssonen K, Salonen R, Tervahauta A, Korpela H, Lakka T, Kaplan GA & Salonen JT 1997 Body iron stores are associated with serum insulin and blood glucose concentrations. *Diabetes Care* **20** 426–428.

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