Serpinb3 is overexpressed in the liver in presence of iron overload

Cristian Turato,[1] Patricia Kent,[2] Giada Sebastiani,[2] Stefania Cannito,[3] Elisabetta Morello,[3] Liliana Terrin,[4] Alessandra Biasiolo,[4] Davide Simonato,[4] Maurizio Parola,[3] Kostas Pantopoulos,[2] Patrizia Pontisso[1]

[1]Veneto Institute of Oncology, IOV - IRCCS, Padua, Italy
[2]McGill University, Montreal, Quebec, Canada
[3]Department of Clinical and Biological Sciences, University of Torino, Torino, Italy
[4]Department of Medicine, University of Padova, Padova, Italy

Correspondence to
Professor Patrizia Pontisso, Department of Medicine, University of Padua, Via Giustiniani 2, Padua 35128, Italy; patrizia@unipd.it

Accepted 2 July 2017
Published Online First 1 November 2017

ABSTRACT

Iron overload results in cellular toxicity, tissue injury, organ fibrosis and increased risk of neoplastic transformation. SerpinB3 is a serine protease inhibitor overexpressed in the liver in oxidative stress conditions, able to induce fibrosis and increased risk of malignant transformation. Aim of the present study was to assess the effect of iron overload on SerpinB3 expression in the liver using in vivo and in vitro models.

The expression of Serpinb3 was assessed in the liver of hemojuvelin knockout mice (Hjv−/−), an established model of hereditary hemochromatosis, and of wild type control mice, following dietary or pharmacological iron manipulation. To assess the direct effect of iron in vitro, cell lines were treated with different concentration of hemin or with an iron chelator.

Hepatic Serpinb3 mRNA and protein were highly expressed in Hjv−/− mice, but not in wild type controls fed with a standard diet. Serpinb3 became detectable in wild type mice fed with a high iron diet or injected with iron dextran; these treatments further induced Serpinb3 expression in Hjv−/− mice. Livers expressing Serpinb3 showed a positive staining also for HIF-2α in the same areas. Hemin promoted induction of SerpinB3 mRNA in HeLa and HA22T/VGH cells, but a mild stimulation of SerpinB3 promoter activity in HeLa and Huh7 cells. In conclusion, Serpinb3 is strongly induced by iron in the mouse liver. The molecular link between iron, ROS and SerpinB3 seems to be HIF-2α, which is induced by iron overload and was previously found capable of up-regulating SerpinB3 at the transcriptional level.

Significance of this study

What is already known about this subject?

- Iron overload may cause cellular toxicity, organ fibrosis and increased risk of neoplastic transformation.
- SerpinB3 is overexpressed in the liver under oxidative stress conditions and has the capacity to induce fibrosis and promote malignant transformation.
- HIF-2α is induced by reactive oxygen species (ROS) and can directly activate the SerpinB3 promoter.

What are the new findings?

- SerpinB3 is upregulated in the liver of mice with severe iron overload (hemojuvelin knockout mice, a model of hereditary iron overload).
- Dietary or parenteral iron loading induced Serpinb3 overexpression also in wild-type control mice.
- SerpinB3 expression is induced by iron treatment in vitro.
- HIF-2α was also induced in the liver of iron-overloaded mice.

How might these results change the focus of research or clinical practice?

- The molecular link between iron, ROS and SerpinB3 seems to be HIF-2α, which is induced by iron overload and was previously found capable of upregulating SerpinB3 at the transcriptional level.

INTRODUCTION

Iron overload may cause severe disease and death in patients with hereditary hemochromatosis. The key molecular event that brings to excessive iron accumulation is inappropriate decrease of hepcidin, a liver peptide that acts as the central regulator of iron homeostasis.[1 2] Iron-dependent hepcidin expression is mediated by the bone morphogenetic proteins/SMAD (BMP/SMAD) signaling cascade, which involves bone morphogenetic proteins (BMP2 and BMP6), the BMP coreceptor hemojuvelin (HJV) and other cofactors. From the functional point of view, the intact BMP signalling pathway is essential for hepcidin expression,[2] and hepatic HJV is indispensable for BMP activation.[3]

Deregulation of this pathway in hereditary hemochromatosis leads to hepatic iron overload and iron-mediated liver injury. This is primarily caused by overproduction of reactive oxygen species (ROS), catalyzed by excess redox-active iron,[4] and can lead to liver fibrosis, cirrhosis and hepatocellular cancer.[5]

SerpinB3 is a member of the ov-serpin family of serine protease inhibitors. It is expressed in normal epithelial cells and overexpressed in cancerous cells and damaged hepatocytes.[6–8] SerpinB3 was recently shown to prevent

To cite: Turato C, Kent P, Sebastiani G, *et al*. *J Investig Med* 2018;**66**:32–38.

oxidative stress-induced cell death.[9 10] This antioxidant effect was interpreted as the result of SerpinB3 binding to intramitochondrial respiratory complex I, leading to a decrease of complex I activity and mitochondrial ROS generation, resulting in an antiapoptotic outcome. Another study provided a link between SerpinB3, hypoxia and ROS by showing that hypoxia inducible factor 2α (HIF-2α) acts as a direct transcriptional inducer of the SerpinB3 gene in liver cancer cells.[10] HIF-2α exerts a central role in coordinating oxygen sensing and is stabilized by hypoxia and ROS.[11] Interestingly, HIF-2α regulates transcription of several iron metabolism genes[12 13] and is subjected to regulation by iron regulatory protein 1 (IRP1). Thus, IRP1 binds with high affinity to the 5'UTR site of HIF-2α mRNA, inhibiting its translation. The inhibition is relieved in hypoxic and iron-repleted cells, allowing HIF-2α synthesis.[14 15]

In light of these findings, the aim of the present study was to assess whether iron overload can modulate the expression of SerpinB3 in vitro and in vivo.

MATERIALS AND METHODS

Animals model

Hemojuvelin knockout mice (Hjv−/−), a model of hereditary iron overload (hemochromatosis) were originally provided by Dr N. Andrews[16] and backcrossed onto the C57BL/6 background.[17] C57BL/6 mice were purchased from Jackson Laboratories. Animals were housed in macrolone cages (up to five mice/cage, 12:12 hours light–dark cycle: 07:00–19:00; 22°C±1°C, 60%±5% humidity) with free access to food and water, according to standard institutional guidelines. At the age of 6 months, male Hjv−/− and wild-type mice (n=10 for each genotype) were sacrificed; the livers were snap frozen in liquid nitrogen and stored at −80°C for molecular studies. To assess the possible modulatory effect of iron on SerpinbB3 expression, 10-week-old male C57BL/6 Hjv−/− mice (n=22) and isogenic wild-type controls (n=24) were placed on diets with variable iron content (Harlan Laboratories, Indianapolis, Indiana, USA), or intraperitoneally injected with iron dextran (1 g/kg body weight) once per week for 4 weeks. The standard diet contained 225 ppm iron (2018 Teklad) and the low-iron diet 75–100 ppm iron (TD.05616). The high-iron diet was the standard, enriched with 2% carbonyl iron (TD.09521).[17] At the endpoint, the animals were sacrificed by cervical dislocation. All experimental procedures were approved by the Animal Care Committee of McGill University (protocol 4966).

Cell culture

Huh7 (human hepatoma cell line) and HA22T/VGH (liver endothelial cell line) were maintained in Dulbecco's modified Eagle's medium 1× with 4.5 g/L glucose, L-glutamine, and sodium pyruvate supplemented with 10% fetal bovine serum (FBS) 1× penicillin/streptomycin, and 1× non-essential amino acids in a humidified incubator at 37°C with 5% CO_2. HeLa cells (epitheloid cervix carcinoma cell line) were cultured in the same manner, excluding 1× non-essential amino acids. To determine iron-dependent regulation, cells were treated overnight with 50 µM hemin (Sigma), or with 100 µM of the iron chelator deferoxamine (DFO, Pharma Science).

Quantitative real-time (qRT)-PCR

Total RNA was extracted using RNase Trizol (Invitrogen, Carlsbad, California, USA), according to the manufacturer's instructions. After determination of the purity and the integrity total RNA, complementary DNA synthesis, qRT-PCR reactions were carried out as previously described.[18] To characterize the mouse-homologous serpin isoforms, the following set of conserved primers was used: sense: 5′-TTTTACACAAGTCCTTTGTGGAGG-3′, antisense: 5′-CTGGACACATGGAAGAGACACCAC-3′, which allows the amplification of the mouse *Serpinb3a*, *-b3b* and *-b3c*, homologous to human *SerpinB3* and *SerpinB4*.[19] To characterize the expression of human *SerpinB3* in human cell lines the following set of specific primer was used: sense: 5'-aactcctgggtggaaagtcaa-3'; antisense: 5'- accaatgtggtattgctgccaa-3'. *β-Actin* (for mouse models) and *GAPDH* (for human cell lines) were used as internal reference and coamplified with target samples using identical qRT-PCR conditions.

Immunohistochemistry (IHC)

IHC was performed on paraffin liver sections specimens. Briefly, paraffin sections (2 µm thick), mounted on poly-L-lysine coated slides, were incubated with polyclonal antibody specific anti-SerpinB3 protein (Hepa-Ab, kind gift of Dr Fassina, Xeptagen, Venice, Italy) or against HIF-2α (Novus Biologicals, Cambridge, UK), as previously

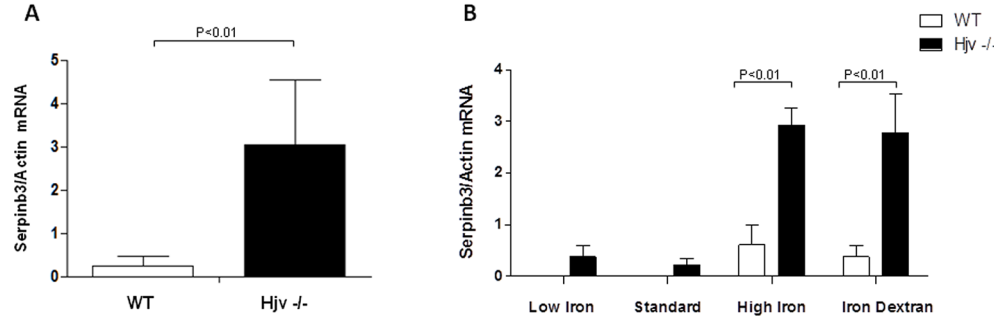

Figure 1 Expression of SerpinB3 mRNA is induced by hepatic iron overload.(A) *SerpinB3* mRNA expression in 6-month-old Hjv−/− mice (black bar) and wild-type (wt) mice (white bar) on a standard diet. (B) *SerpinB3* mRNA expression in 10-week-old Hjv−/− and wt mice subjected to dietary iron manipulations or challenged with iron dextran injection. Results are expressed as means±SE. Hjv, hemojuvelin.

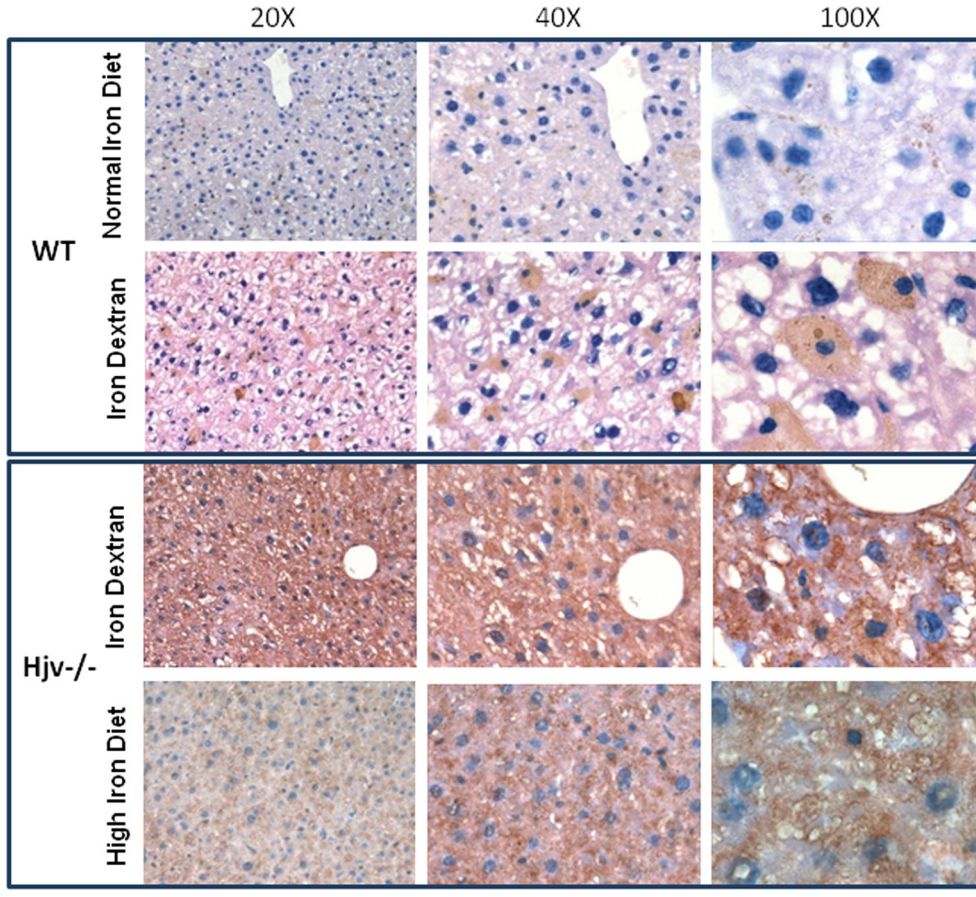

Figure 2 Immunohistochemal detection of iron-dependent SerpinB3 induction in mouse liver. Representative image of immunohistochemistry for SerpinB3 in the liver of wt and Hjv–/– mice challenged with iron dextran showing a marked increase of SerpinB3 protein in the liver of the Hjv–/– mice.

described.[10] After blocking endogenous peroxidase activity with 3% hydrogen peroxide and performing microwave antigen retrieval, primary antibodies were labelled by using EnVision, HRP-labeled System (DAKO) and visualized by 3′-diaminobenzidine substrate. For negative controls, the primary antibodies were replaced by isotype-matched and concentrations-matched irrelevant antibody.

Promoter activity

To investigate the mechanism of induction, an in vitro reporter assay to test the iron responsiveness of the SerpinB3 promoter was generated by luciferase assay. Huh7 or HeLa cells were seeded at a density of 7.5×10^4 cells/mL or 1.5×10^5 cells/mL, respectively, in a 12-well dish in media lacking antibiotics and incubated overnight. Transient transfections were performed the next day using Lipofectamine2000 (Invitrogen) as per the manufacturer's protocol for a 24-well dish. To generate the promoter constructs of SerpinB3, fragments immediately upstream of the transcription start site of SerpinB3 (online supplementary figure 1) were cloned into the promoterless pGL3 basic luciferase (luc) reporter vector. Luminescence from luciferase was measured and normalized to *renilla* luminescence using the Dual Luciferase Reporter Assay (Promega). Values were reported as percentages of *luc* activity. The 2.7 Kb Hepc-pGL3 plasmid, containing 2.7 Kb of the hepcidin promoter cloned into the pGL3 basic vector, used as positive control, was kindly provided by Dr M. Muckenthaler.[20]

Western blot

Cells proteins were extracted on ice using RIPA (50 mM Tris-HCL pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 1% Triton X-100) and boiled at 95°C for 5 min in sample buffer (62.5 mM Tris-HCL pH 6.8, 50% glycerol, 2% SDS, and 50 mM DTT) as previously described.[21] Sodium dodecyl sulphate - polyAcrylamide gel electrophoresis (SDS-PAGE) gels of 10%–15% were used to separate proteins, which were transferred onto nitrocellulose membranes (BioRad) and blocked in 5% milk TBS-T (Tris Buffered Saline – tween 20) for 1 hour at room temperature. Membranes were probed overnight at 4°C with primary antibodies: anti-β actin (1:5000, Sigma-Aldrich, Milan, Italy), heme oxygenase 1 (HO-1, 1:1000, Thermo Fisher Scientific, Milan, Italy), ferritin (1:1000, Novus Biologicals, Cambridge, UK), SerpinB3 (Hepa-Ab, 8 µg/mL, Xeptagen, Venice, Italy), HiF2 (1:500, Novus Biologicals, Cambridge, UK) in 5% milk TBS-T. Membranes were washed for 3×10 mins with fresh tris-buffered saline-Tween 20 (TBS-T) and incubated with appropriate HRP-conjugated secondary antibodies, washed and the detection of peroxidase-coupled antibodies was performed with the enhanced chemiluminescence method (Perkin-Elmer). Sample loading was evaluated by reblotting the same membrane with β-actin antibody.

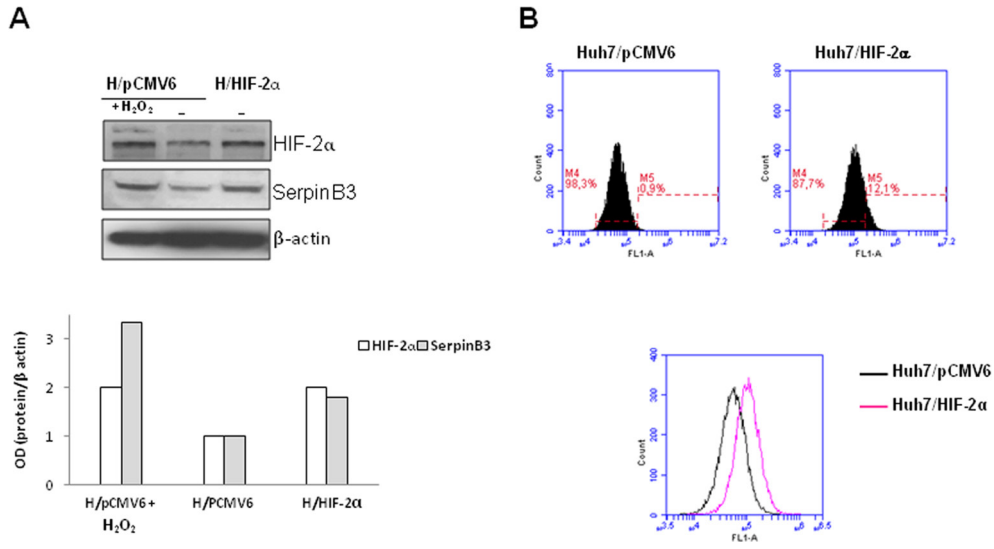

Figure 3 SerpinB3 and ROS in human liver cells overexpressing HIF-2α.(A) Western blot analysis of HIF-2α and SerpinB3 in Huh7 cells transfected with empty vector (H/pCMV6) or overexpressing HIF-2α (H/HIF-2α) after 24 hours from plating. Huh7 with empty vector were treated with H_2O_2 50 µM, used as oxidative stress positive control. Equal loading was evaluated by reprobing the same membranes with monoclonal anti-β-actin antibody. (B) Intracellular generation of ROS in Huh7 cells transfected with empty vector (Huh7/pCMV6) or overexpressing HIF-2α (Huh7/HIF-2α) after 24 hours from plating. ROS were evaluated as the conversion of 2′,7′-dichlorodihydrofluorescein diacetate by flow cytometric analysis. HIF-2α, hypoxia inducible factor 2α; ROS, reactive oxygen species.

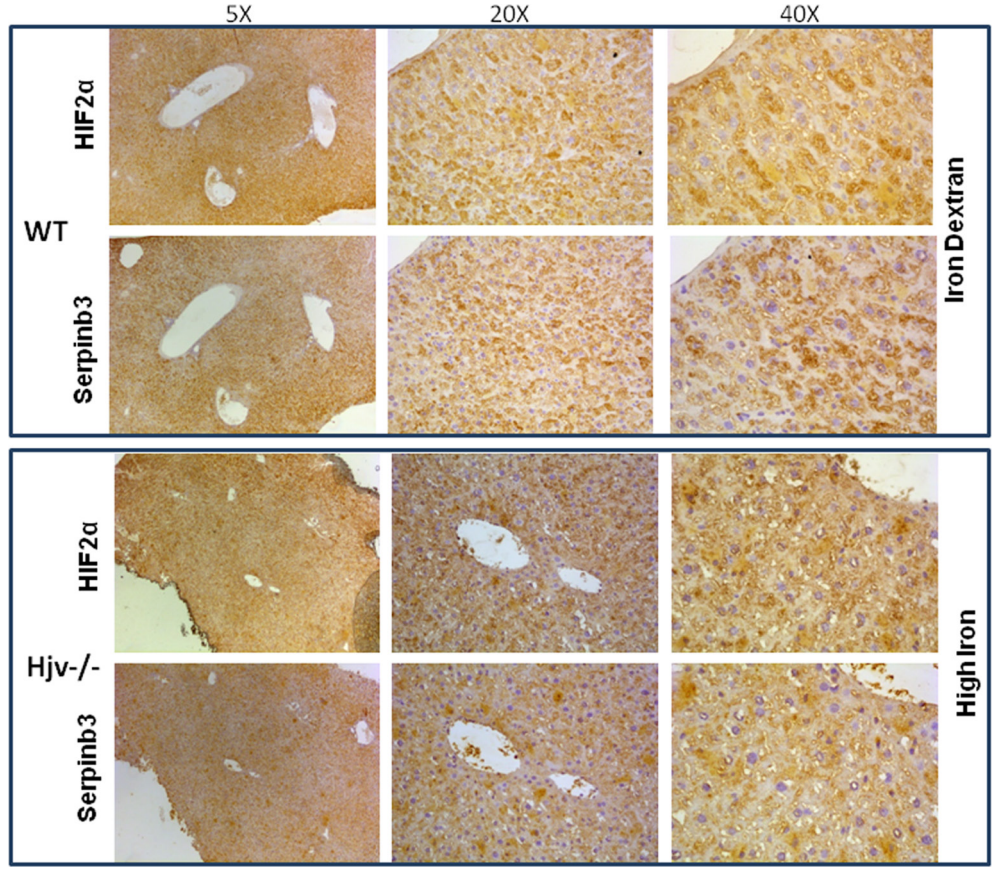

Figure 4 HIF-2α and SerpinB3 are both upregulated in mouse liver following high iron intake. Representative immunohistochemistry of SerpinB3 and HIF-2α expression in the liver of wt mice and of Hjv−/− mice challenged with iron. The induced iron overload promoted HIF-2α expression in the same areas that were positive for SerpinB3.

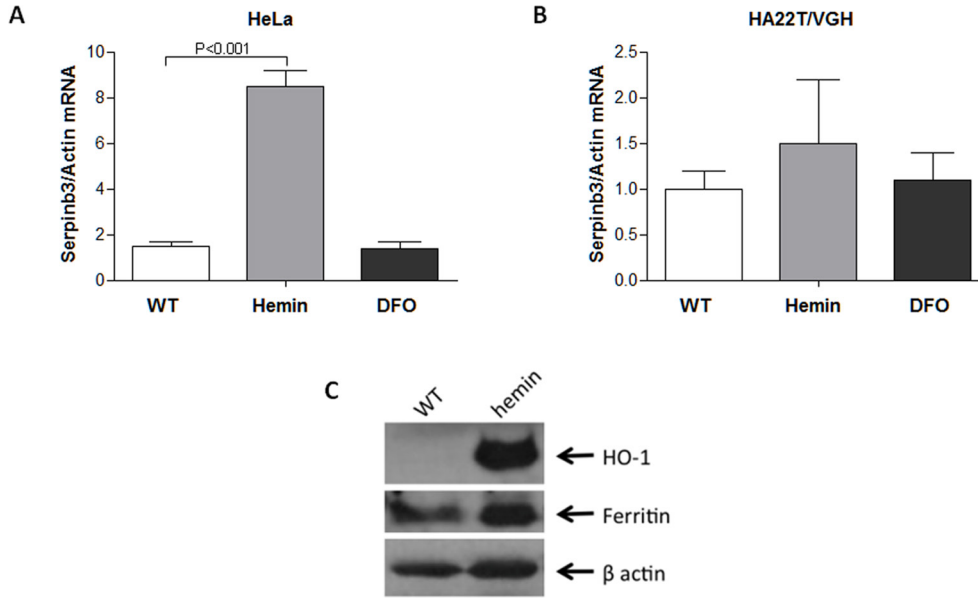

Figure 5 Iron increases SerpinB3 mRNA expression in cultured cells. (A and B) *SerpinB3* mRNA expression was performed on HeLa hepatoma and HA22T/VGH cells treated overnight with hemin (50 µM) or DFO (100 µM) and reported as fold changes from untreated (wild-type) cells. (C) To prove iron delivery into HeLa cells after hemin treatment, Western blot was performed to measure heme oxygenase 1 (HO-1) and ferritin, biomarkers of heme metabolism and iron overload. DFO, deferoxamine.

Detection of intracellular generation of ROS

Huh7 cells transfected with empty vector (Huh7/pCMV6, control) or Huh7 cells overexpressing HIF-2α were seeded in 35×10 mm culture dishes (5×10^5 cells per well), for 24 hours. For comparative purposes, control cells were treated with 50 µM H_2O_2 under normoxic conditions for 3 hours (as positive control).

Intracellular generation of ROS was detected evaluating the conversion of 2′,7′-dichlorodihydrofluorescein diacetate (used at 5 µM concentration), once taken up by cells and deacetylated by esterase, into the corresponding fluorescent derivative[21] in flow cytometric analysis. The samples were analyzed by flow cytometer (BD Accurri C6); 10 000 events were collected per sample. Results were provided as mean±SD of two experiments conducted in triplicate.

Statistical analysis

Data are expressed as mean±SE mean (SEM). Analysis of multiple groups was performed with one-way analysis of variance. A probability value of $p<0.05$ was considered as statistically significant. The calculations were carried out with GraphPad InStat Software (San Diego, California, USA).

RESULTS

SerpinB3 is upregulated in livers of Hjv−/− mice in an iron-dependent manner

To explore the effects of genetic iron overload in the in vivo expression of Serpinb3, we evaluated *Serpinb3* mRNA levels in livers of Hjv−/− mice and isogenic wild-type control mice (wt) on a standard diet. The data in figure 1A show a

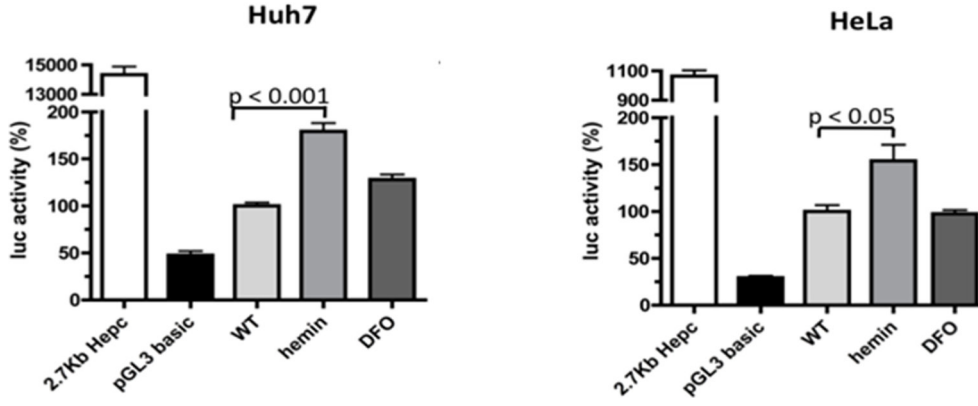

Figure 6 Iron stimulates SerpinB3 promoter activity. The SerpinB3 5 Kb promoter was transiently transfected into either Huh7 or HeLa cells and cells were then treated overnight with hemin (50 µM) or with the iron chelator deferoxamine (DFO) (100 µM). A mild but significant induction of SerpinB3 promoter activity was seen with hemin treatments in both Huh7 and HeLa cells, while DFO did not modify its basal levels.

15-fold increase in expression of *Serpinb3* in Hjv−/− livers compared with controls ($p<0.01$) (figure 1A).

To assess possible effects of dietary or pharmacological iron on Serpinb3 regulation, wt and Hjv−/− mice were fed with standard, low or high iron diets or injected intraperitoneally with iron dextran (to bypass the hepcidin-controlled enterocyte gate). In wt mice, liver *Serpinb3* mRNA became detectable only in animals fed with high iron diet or intraperitoneally injected with iron dextran. In Hjv−/− mice, *Serpinb3* mRNA expression was already detectable in the liver of mice fed with the low diet and progressively increased proportionally to dietary iron content. Iron dextran injection induced *Serpinb3* mRNA similar to high iron diet (figure 1B).

Immunohistochemical analysis in serial paraffin sections was consistent with Serpinb3 mRNA expression, showing that hepatic SerpinB3 immunopositivity was virtually absent in sections from control WT mice, increased in WT mice following treatment with iron dextran and was strongly exacerbated in the liver of Hjv−/− mice fed with iron supplemented diet or treated with iron dextran (figure 2).

HIF-2α and Serpinb3 in human liver cells and in livers of iron-overloaded mice

In order to confirm the recent study describing SerpinB3 as a novel hypoxia-responsive and ROS-responsive protein in the liver,[10] the tumor liver cell line Huh7, stably transfected to overexpress HIF-2α, was used. At protein level, we observed an increase of SerpinB3 (figure 3A), associated with intracellular generation of ROS (figure 3B) in cells overexpressing HIF-2α, compared with parental control cells.

Considering that *HIF-2α* mRNA translation is derepressed by iron[15] and that HIF-2α, in turn, can induce the expression of SerpinB3,[10] immunohistochemistry analysis was carried out to investigate possible coexpression of these two molecules in the liver, relative to iron accumulation. These analyses revealed the presence of Serpinb3 and HIF-2α in the same areas of the livers with high iron deposition (figure 4).

SerpinB3 mRNA is upregulated by iron

To explore if *SerpinB3* transcription was iron-dependent in vitro, different cell lines were treated overnight with 50 µM hemin or with 100 µM DFO. Hemin treatment of HeLa cells induced a sixfold induction of SerpinB3 mRNA, while no significant change was observed with DFO treatment (figure 5A). A similar trend was observed in the HA22T/VGH cells, although SerpinB3 increase was not statistically significant (figure 5B). Also in this case DFO did not modify *SerpinB3* mRNA expression and this is likely to reflect low cellular iron levels.

As expected, hemin administration to HeLa cells promoted induction of HO-1, the enzyme-catalyzing heme degradation, and ferritin, the intracellular iron storage protein (figure 5C).

Iron stimulates the Serpinb3 promoter

To determine whether iron directly modulates *Serpinb3* mRNA transcription, we used Huh7 and HeLa cells transfected with a luciferase indicator under the control of a 5 kb Serpinb3 promoter fragment (onlinesupplementary figure 2). Overnight treatment with 50 µM hemin triggered 1.5-fold induction of Serpinb3 promoter activity in both Huh7 and HeLa cells (figure 6). DFO did not elicit any significant effect of Serpinb3 transcription, in agreement with the *Serpinb3* mRNA data shown in figure 5.

It is worth noting that the 2.7 Kb Hepc promoter (containing 2.7 Kb of the hepcidin promoter cloned into the pGL3 basic vector), used as positive control, showed 15-fold increased activity in Huh7 compared with HeLa cells, consistent with the fact that hepcidin is predominantly expressed in hepatocytes.

Taken together, these findings uncover the role of iron as an inducer of *Serpinb3* transcription.

DISCUSSION

Iron is vital for normal cellular physiology, but iron overload results in cellular toxicity, tissue injury, organ fibrosis and increased risk of neoplastic transformation.[22] Since SerpinB3 is profoundly involved in liver fibrogenesis[18 23] and cancer development,[24] we investigated whether iron affects SerpinB3 expression. In the present study, we demonstrate that SerpinB3 is induced by iron accumulation in the liver. Accordingly, we observed high SerpinB3 mRNA and protein expression in the livers of Hjv−/− mice, a model of hereditary hemochromatosis. Furthermore, liver expression of SerpinB3 was positively regulated by dietary or parenteral iron in both Hjv−/− and wild-type mice. In vitro experiments further showed that iron induces SerpinB3 transcription. Nevertheless, the degree of transcriptional induction of SerpinB3 in iron-treated cultured cells was substantially lower to that observed in iron-loaded mouse livers. Thus, our data suggest the involvement of additional mechanisms underlying iron-dependent SerpinB3 induction in vivo.

The iron-induced expression of SerpinB3 in the liver was associated with a parallel increase of HIF-2α expression in the same histological areas. A recent study reported that HIF-2α directly binds to SerpinB3 gene promoter, highlighting SerpinB3 as a novel hypoxia-responsive and ROS-responsive protein in the liver, especially in primary liver cancer.[10] Based on these findings, we speculate that iron could indirectly promote the robust SerpinB3 induction observed in vivo via the generation of ROS and subsequent HIF-2α stabilization, leading to SerpinB3 transcriptional activation. This hypothesis was supported by the increase of ROS and SerpinB3 found in Huh7 cell line genetically modified to overexpress HIF2-α. The increased expression of SerpinB3 in response to iron overload might contribute to hepatocyte survival[9] and fibrosis deposition.[18 23] Along this line, hepatic iron overload was found to increase chemically induced liver fibrogenesis by promoting an oxidative injury and, similarly to SerpinB3, premature induction of profibrogenic cytokines, especially transforming growth factor beta 1 (TGF-β1), endothelin-1 and platelet-derived growth factor (PDGF).[25]

The molecular link between iron, ROS and SerpinB3 seems to be HIF-2α, which is induced by iron overload, and it is capable of upregulating SerpinB3 at the transcriptional level.[10]

This suggested pathway could represent a novel biological mechanism, allowing cell survival under hypoxic and

oxidative stress conditions, like iron overload, and supports the pathophysiological involvement of SerpinB3 in different chronic diseases leading to progressive fibrosis and, eventually, to neoplastic transformation.

Correction notice This article has been corrected since it was published Online First. The affiliation for the first author has been updated to Veneto Institute of Oncology, IOV - IRCCS, Padua, Italy.

Contributors CT, PK, PP, GS, and KP conceived and designed the experiments; CT, PK, SC and EM performed the experiments; CT, LT, AB, and DS analyzed the data; CT wrote the paper; PP, MP, and KP revised the paper. All Authors had final approval of the submitted manuscript.

Funding This work obtained financial support by the Associazione Italiana per la Ricerca sul Cancro and the Canadian Institutes for Health Research.

Competing interests None declared.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1 Camaschella C. Treating Iron Overload. *N Engl J Med Overseas Ed* 2013;368:2325–7.
2 Zhao N, Zhang AS, Enns CA. Iron regulation by hepcidin. *J Clin Invest* 2013;123:2337–43.
3 Gkouvatsos K, Wagner J, Papanikolaou G, *et al*. Conditional disruption of mouse HFE2 gene: maintenance of systemic iron homeostasis requires hepatic but not skeletal muscle hemojuvelin. *Hepatology* 2011;54:1800–7.
4 Fraga CG, Oteiza PI. Iron toxicity and antioxidant nutrients. *Toxicology* 2002;180:23–32.
5 Kowdley KV, Iron KKV. Iron, hemochromatosis, and hepatocellular carcinoma. *Gastroenterology* 2004;127:S79–86.
6 Sheshadri N, Catanzaro JM, Bott AJ, *et al*. SCCA1/SERPINB3 promotes oncogenesis and epithelial-mesenchymal transition via the unfolded protein response and IL6 signaling. *Cancer Res* 2014;74:6318–29.
7 Turato C, Vitale A, Fasolato S, *et al*. SERPINB3 is associated with TGF-β1 and cytoplasmic β-catenin expression in hepatocellular carcinomas with poor prognosis. *Br J Cancer* 2014;110:2708–15.
8 Quarta S, Vidalino L, Turato C, *et al*. SERPINB3 induces epithelial-mesenchymal transition. *J Pathol* 2010;221:343–56.
9 Ciscato F, Sciacovelli M, Villano G, *et al*. SERPINB3 protects from oxidative damage by chemotherapeutics through inhibition of mitochondrial respiratory complex I. *Oncotarget* 2014;5:2418–27.
10 Cannito S, Turato C, Paternostro C, *et al*. Hypoxia up-regulates SERPINB3 through HIF-2α in human liver Cancer cells. *Oncotarget* 2015;6:2206–21.
11 Majmundar AJ, Wong WJ, Simon MC. Hypoxia-inducible factors and the response to hypoxic stress. *Mol Cell* 2010;40:294–309.
12 Peyssonnaux C, Nizet V, Johnson RS. Role of the hypoxia inducible factors HIF in iron metabolism. *Cell Cycle* 2008;7:28–32.
13 Shah YM, Xie L. Hypoxia-inducible factors link iron homeostasis and erythropoiesis. *Gastroenterology* 2014;146:630–42.
14 Zimmer M, Lamb J, Ebert BL, *et al*. The connectivity map links iron regulatory protein-1-mediated inhibition of hypoxia-inducible factor-2a translation to the anti-inflammatory 15-deoxy-delta12,14-prostaglandin J2. *Cancer Res* 2010;70:3071–9.
15 Wilkinson N, Pantopoulos K. IRP1 regulates erythropoiesis and systemic iron homeostasis by controlling HIF2α mRNA translation. *Blood* 2013;122:1658–68.
16 Huang FW, Pinkus JL, Pinkus GS, *et al*. A mouse model of juvenile hemochromatosis. *J Clin Invest* 2005;115:2187–91.
17 Gkouvatsos K, Fillebeen C, Daba A, *et al*. Iron-dependent regulation of hepcidin in hjv-/- mice: evidence that hemojuvelin is dispensable for sensing body iron levels. *PLoS One* 2014;9:e85530.
18 Turato C, Calabrese F, Biasiolo A, *et al*. SERPINB3 modulates TGF-beta expression in chronic liver disease. *Lab Invest* 2010;90:1016–23.
19 Villano G, Turato C, Quarta S, *et al*. Hepatic progenitor cells express SerpinB3. *BMC Cell Biol* 2014;15:5.
20 Casanovas G, Mleczko-Sanecka K, Altamura S, *et al*. Bone morphogenetic protein (BMP)-responsive elements located in the proximal and distal hepcidin promoter are critical for its response to HJV/BMP/SMAD. *J Mol Med* 2009;87:471–80.
21 Cannito S, Novo E, Compagnone A, *et al*. Redox mechanisms switch on hypoxia-dependent epithelial-mesenchymal transition in Cancer cells. *Carcinogenesis* 2008;29:2267–78.
22 Ramm GA, Ruddell RG. Hepatotoxicity of iron overload: mechanisms of iron-induced hepatic fibrogenesis. *Semin Liver Dis* 2005;25:433–49.
23 Novo E, Villano G, Turato C, *et al*. SerpinB3 promotes Pro-fibrogenic responses in activated Hepatic Stellate cells. *Sci Rep* 2017;7:3420.
24 Pontisso P. Role of SERPINB3 in hepatocellular carcinoma. *Ann Hepatol* 2014;13:722–7.
25 Sebastiani G, Gkouvatsos K, Maffettone C, *et al*. Accelerated CCl4-induced liver fibrosis in hjv-/- mice, associated with an oxidative burst and precocious profibrogenic gene expression. *PLoS One* 2011;6:e25138.