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Chronic Administration of 2-Acetylaminofluorene Alters the Cellular Iron Metabolism in Rat Liver

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Dysregulated intracellular iron homeostasis has been found not only in rodent and human hepatocellular carcinomas but also in several preneoplastic pathological states associated with hepatocarcinogenesis; however, the precise underlying mechanisms of metabolic iron disturbances in preneoplastic liver and the role of these disturbances remain unexplored. In the present study, using an *in vivo* model of rat hepatocarcinogenesis induced by 2-acetylaminofluorene, we found extensive alterations in cellular iron metabolism at preneoplastic stages of liver carcinogenesis. These were characterized by a substantial decrease in the levels of cytoplasmic non-heme iron in foci of initiated hepatocytes and altered expression of the major genes responsible for the proper maintenance of intracellular iron homeostasis. Gene expression analysis revealed that the decreased intracellular levels of iron in preneoplastic foci might be attributed to increased iron export from the cells, driven by upregulation of ferroportin (*Fpn1*), the only known non-heme iron exporter. Likewise, increased *Fpn1* gene expression was found *in vitro* in TRL1215 rat liver cells with an acquired malignant phenotype, suggesting that upregulation of *Fpn1* might be a specific feature of neoplastically transformed cells. Other changes observed *in vivo* included the downregulation of hepcidin (*Hamp*) gene, a key regulator of Fpn1, and this was accompanied by decreased levels of CCAAT/enhancer binding proteins alpha and beta, especially at the *Hamp* promoter. In conclusion, our results demonstrate the significance of altered intracellular iron metabolism in the progression of liver carcinogenesis and suggest that correction of these alterations could possibly affect liver cancer development.

Key Words: **2-acetylaminofluorene; carcinogenesis; iron metabolism; liver; rat.**

Intracellular iron homeostasis in normal cells is tightly regulated by the coordinated functioning of several proteins responsible for the uptake, intracellular storage, and removal of iron from cells (Hentze *et al*., 2010; Richardson and Ponka, 1997). On the other hand, cancer cells, including liver cancer cells, are characterized by profound aberrations in intracellular iron metabolism (Chen and Chloupková, 2009; Deugnier, 2003; Toyokuni, 2009). Dysregulated iron homeostasis has been found not only in rodent and human hepatocellular carcinomas but also in several preneoplastic pathological states associated with liver cancer development, including hemochromatosis (Kowdley, 2004), chronic hepatitis C virus infection (Isom *et al*., 2009), and nonalcoholic fatty liver disease (Starley *et al*., 2010).

The role of deregulated iron homeostasis in the initiation of liver carcinogenesis has been studied extensively (Eaton and Qian, 2002; Kew, 2009). Particularly, results of several comprehensive studies demonstrating an association between hepatic iron overload development of hepatocellular carcinoma have suggested a possible carcinogenic and/or cocarcinogenic role for iron in chronic liver diseases (Kew, 2009; Ko *et al*., 2007; Kowdley, 2004). Mechanistically, altered iron metabolism may be linked to several fundamental carcinogenic events, including the induction of oxidative stress (Kew, 2009) and damage to lysosomes and mitochondria (Eaton and Qian, 2002). In contrast, there is a lack of conclusive information to clarify the role of iron disturbances in the pathogenesis of liver carcinogenesis that is not associated with iron overload and especially in premalignant liver lesions. Williams and coworkers (Furuya *et al*., 1984; Hirota and Williams, 1982; Williams *et al*., 1976) reported the reduction of non-heme iron content in preneoplastic hepatic foci and suggested that this might be an event that promotes liver carcinogenesis; however, the precise mechanistic role of these iron metabolic disturbances in selectively accelerating the progression of preneoplastic foci has remained unexplored.

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Based on these considerations, the current study was conducted to characterize the underlying molecular mechanisms of intracellular iron homeostasis changes during preneoplastic stages of liver carcinogenesis. Using an *in vivo* model of rat hepatocarcinogenesis induced by 2-acetylaminofluorene (2-AAF), we found a substantial reduction of intracellular non-heme iron, which was accompanied by decreased levels of cytoplasmic ferritin heavy chain protein (Fth) in preneoplastic foci. Additionally, analysis of the gene expression patterns revealed prominent upregulation of the ferroportin (*Fpn1*) gene and downregulation of the hepcidin (*Hamp*) gene. This indicates that the decreased intracellular levels of non-heme iron in preneoplastic foci may be attributed to its increased export from the cells, driven by the loss of an inhibitory effect of hepcidin on ferroportin, the only known non-heme iron exporter.

MATERIALS AND METHODS

Animals and treatment. Weaning male and female Sprague-Dawley rats were obtained from the National Center for Toxicological Research (NCTR) breeding facility, housed in a temperature-controlled (24°C) room with a 12-h light-dark cycle, and given *ad libitum* access to water and NIH-31 laboratory diet. At 6 weeks of age, the rats (mean body weight 150 g) were allocated randomly to receive either NIH-31 diet containing 0.02% of 2-AAF or control NIH-31 diet. Diets were stored at 4°C and given *ad libitum*, with twice a week replacement. After 24 weeks of 2-AAF administration, the rats ($n = 5$ per each control and experimental group) were euthanized by exsanguination following deep isoflurane anesthesia. The livers were excised and a slice of the medial lobe was fixed in 10% neutral buffered formalin for 48 h prior to histopathological examination. The remaining liver was frozen immediately in liquid nitrogen and stored at −80°C for subsequent analyses. All animal experimental procedures were performed in accordance with an animal study protocol approved by the NCTR Animal Care and Use Committee.

Cell culture and treatments. TRL1215 rat liver epithelial cells were cultured in control Wiiliams' E medium and in medium containing 1μM sodium arsenite (Sigma-Aldrich, St Louis, MO) as described previously in detail (Kojima *et al*., 2009). After 18 weeks of culturing, cells were harvested, washed in PBS, and RNAs were extracted using TRI Reagent (Ambion, Austin, TX) according to the manufacturer's instructions.

Non-heme iron histochemistry. The level of non-heme iron in the liver sections was determined by histochemistry using a modified Perls method (Meguro *et al*., 2007). Briefly, formalin-fixed paraffin-embedded liver sections were deparaffinized and rehydrated. The sections were then incubated with Perls reagent (2% HCl mixed 1:1 with 2% potassium ferrocyanide Fe(II) (Sigma-Aldrich) for 60 min in a humid chamber at 60°C. After incubation, endogenous peroxidase was inhibited by incubating with freshly prepared 3% hydrogen peroxide with 0.1% sodium azide for 10 min at room temperature. Staining was developed in 3,3′-diaminobenzidine (Sigma-Aldrich), and tissue sections were counterstained with hematoxylin and mounted with Permount (Fisher Scientific, Pittsburgh, PA).

Immunohistochemistry. To detect expression of the placental form of glutathione-*S*-transferase (Gstp1), formalin-fixed paraffin-embedded liver sections were deparaffinized, rehydrated, and endogenous peroxidase was inhibited as described above. Nonspecific staining was blocked with normal goat 10% serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The sections were then incubated with primary rabbit polyclonal anti-human GSTP primary antibody (DAKO, Carpinteria, CA) at a concentration of 10 μg/ml for 1 h at room temperature. After incubation with the primary antibody, tissue sections were incubated with biotinylated goat anti-rabbit IgG (ExtrAvidin Kit) (Sigma-Aldrich) at a dilution of 1:30 for 30 min at room temperature and then with streptavidin-conjugated horseradish peroxidase at a dilution of 1:30 for 30 min at room temperature. Staining was developed with 3,3′-diaminobenzidine for 5 min at room temperature, and tissue sections were counterstained with hematoxylin and mounted with Permount. For a negative control, 10 μg/ml rabbit IgG (Jackson ImmunoResearch Laboratories) replaced the primary antibody.

To detect ferritin heavy chain (Fth) or light chain (Ftl) protein expression, formalin-fixed paraffin-embedded liver sections were deparaffinized, rehydrated, and placed in an antigen retrieval solution (10mM sodium citrate buffer, pH 6.0) for 15 min in a microwave oven at 100°C. Endogenous peroxidase was inhibited as described above. Nonspecific staining was blocked with 0.5% casein for 20 min at room temperature. Separate sets of serial sections were then incubated with primary goat polyclonal anti-human FTH or FTL primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at concentrations of 2.0 and 1.0 μg/ml, respectively. After incubation with the primary antibodies, tissue sections were incubated with horse anti-goat horseradish peroxidase–conjugated secondary antibody (1:50) for 30 min at room temperature. Staining was developed with 3,3′-diaminobenzidine, and tissue sections were counterstained with hematoxylin and mounted with Permount. To minimize variability in staining caused by handling of the slides, control and 2-AAF–treated rat liver sections were stained simultaneously (in the same run). For a negative control, 2.0 μg/ml goat IgG (Jackson ImmunoResearch Laboratories) replaced the primary antibody. All sections were examined by light microscopy (BX40 Olympus, Tokyo, Japan).

Image analysis. Stained liver sections were scanned, and digital images were obtained with an Aperio Scanscope System (Aperio Technologies, Inc., Vista, CA). The intensity of non-heme iron, Fth, and Ftl staining was evaluated with the Positive Pixel Count Algorithm (Aperio Technologies). This algorithm quantifies the amount of specific stain present in a digital slide by evaluating an average intensity of all pixels for subsequent calculation of optical density.

Quantitative real-time PCR. Total RNA was extracted from rat liver tissues using TRI Reagent. Total RNA (2 μg) was reverse transcribed using random primers and a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The levels of transferrin receptor (*Tfrc*); solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2 (*Slc11a2; Dmt1*); solute carrier family 39 (iron-regulated transporter), member 1 (*Slc40a1*; *Fpn1*); *Hamp*; and *Gstp1* gene transcripts were determined by quantitative real-time PCR (qRT-PCR) using TaqMan Gene Expression Assays (Applied Biosystems) according to the manufacturer's protocol. Relative quantification of gene expression was performed by using β-actin as an internal control. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative amount of the target RNA (Schmittgen and Livak, 2008). The qRT-PCR was performed at least three times and always included a no-template sample as a negative control.

Western blot analysis of protein expression. Liver tissue lysates were prepared by homogenization of 50 mg of tissue in 500 μl of lysis buffer (50mM Tris-HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150mM NaCl; 1mM EDTA; 1mM PMSF; 1 μg/ml each of aprotinin, leupeptin, and pepstatin; 1mM Na_3VO_4; and 1mM NaF), sonication, and incubation at 4°C for 30 min, followed by centrifugation at 10,000 × g at 4°C for 20 min. Extracts containing equal quantities of proteins were separated by SDS-polyacrylamide gel electrophoresis on 8–15% polyacrylamide gels and transferred to PVDF membranes. Membranes were probed with primary antibodies against Fth (1:200) and CCAAT/enhancer binding proteins alpha (Cebpa; 1:200) and beta (Cebpb; 1:200). All primary antibodies were obtained from Santa Cruz Biotechnology. Horseradish peroxidase–coupled donkey anti-goat secondary antibody (Santa Cruz Biotechnology) was used for visualization. Chemiluminescence detection was performed with the Immobilon Western Chemiluminescent HRP Substrate (Millipore Corporation, Billerica, MA) and measured directly by a UVP BioSpectrum Imaging System (Upland, CA). Equal protein loading was confirmed by immunostaining against β-actin (1:5000) (Sigma-Aldrich).

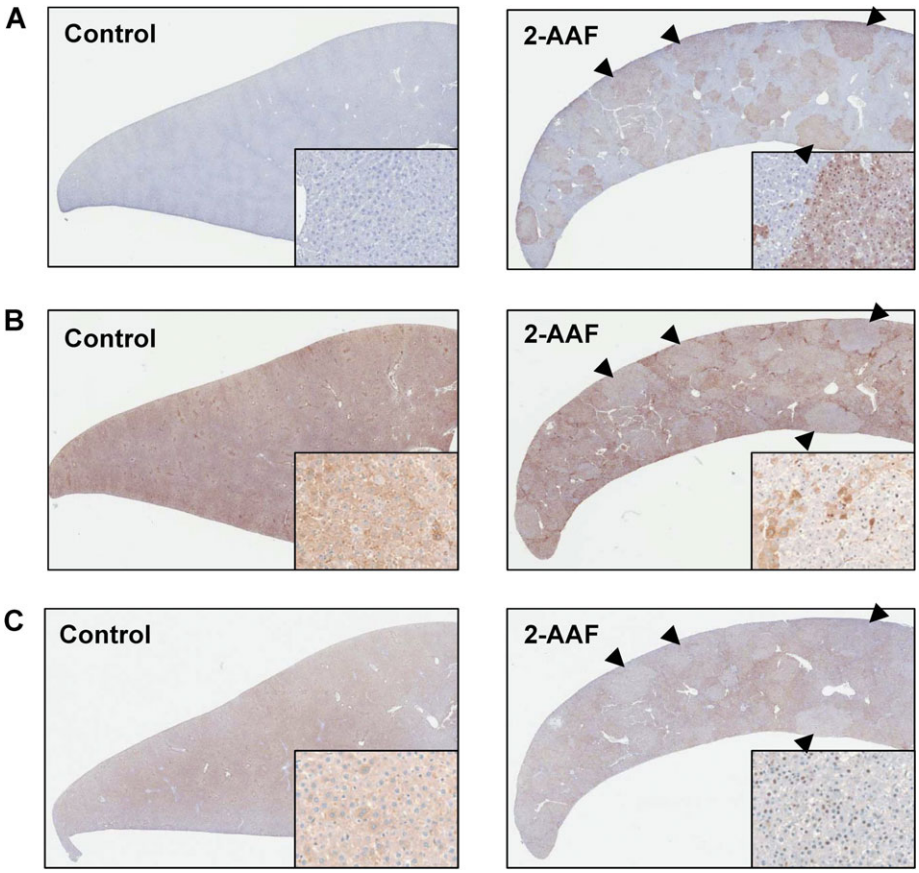

FIG. 1. Non-heme iron and Fth levels in the livers of control rats and 2-AAF–treated rats for 24 weeks. (A) Immunohistochemical demonstration of Gstp1 staining in liver sections of a control male rat and a male rat fed the 2-AAF–containing diet for 24 weeks (×40). Liver sections of 2-AAF–fed male rats consisted of large foci of Gstp1-positive hepatocytes (arrowheads) evenly distributed throughout the entire section of the liver. (B) Histochemical non-heme iron staining in liver sections of a control male rat and a male rat exposed to 2-AAF for 24 weeks (×40). Preneoplastic Gstp1-positive foci in liver sections of 2-AAF–treated male rats have a markedly reduced accumulation of the cytoplasmic non-heme iron (arrowheads and insert; ×200). (C) Immunohistochemical Fth staining in liver sections of a control male rat and a male rat fed the 2-AAF–containing diet for 24 weeks (×40). Preneoplastic Gstp1-positive foci exhibit lower Fth staining intensity (arrowheads and insert; ×200) as compared with Gstp-negative tissue or control livers.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assays, with primary antibodies against Cebpa and Cebpb proteins, were performed as described previously (Tryndyak *et al*., 2010). Immunoprecipitated DNA was analyzed by quantitative real-time PCR (qPCR) with ChampionChIP qPCR primers for rat *Hamp* (NM_053469.1; SABiosciences, Frederick, MD).

Statistical analyses. Results are presented as mean ± SD. Comparisons between control rats and rats fed the 2-AAF–containing diet were made by Student's *t*-test. When necessary, the data were natural log transformed before conducting the analyses. p values < 0.05 were considered significant.

RESULTS

Dysregulation of Intracellular Iron Metabolism in Preneoplastic Gstp1-Positive Foci

Immunohistochemical staining of liver sections of 2-AAF–fed male Sprague-Dawley rats revealed large foci consisting of Gstp1-positive hepatocytes evenly distributed throughout the entire section of the liver, whereas no Gstp1-positive foci or even single Gstp-positive hepatocytes were detected in the livers of rats fed the control diet (Fig. 1A). Only single Gstp1-positive hepatocytes or isolated minifoci were detected in the livers of 2-AAF–exposed female rats. This difference in Gstp1-immunohistochemical staining in the livers of male and female rats exposed to 2-AAF was confirmed further by qRT-PCR that demonstrated a 1.8-fold change in *Gstp1* gene expression in female rats compared with 43 times greater expression of *Gstp1* in the livers of male rats exposed to 2-AAF compared with the control rats (Table 1).

Previously, Williams and coworkers (Furuya *et al*., 1984; Hirota and Williams, 1982; Williams *et al*., 1976) reported the depletion of non-heme iron in preneoplastic foci during 2-AAF–induced hepatocarcinogenesis. Similarly, we demonstrate that preneoplastic Gstp1-positive foci in liver sections of 2-AAF–treated rats have a markedly reduced accumulation of the cytoplasmic non-heme iron (Figs. 1B and 2), whereas in control liver sections, non-heme iron was evenly distributed in hepatocytes, predominantly in the cytoplasm.

TABLE 1
Gene Expression in the Livers of Control Rats and Rats Treated with 2-AAF for 24 Weeks

		Gene expression				
Groups	Sex	*Gstp1*	*Tfrc*	*Dmt1*	*Fpn1*	*Hamp*
Control	Male	1.1 ± 0.2	1.0 ± 0.1	1.0 ± 0.5	1.0 ± 0.1	1.0 ± 0.2
2-AAF		43.3 ± 0.2*	1.7 ± 0.1*	1.0 ± 0.2	3.0 ± 0.4*	0.5 ± 0.04*
Control	Female	1.2 ± 0.3	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
2-AAF		2.2 ± 0.6*	1.1 ± 0.3	1.2 ± 0.3	1.1 ± 0.2	1.3 ± 0.3

Note. The data presented as the mean ± SD, $n = 5$.
*Significantly different from a control rats of the same sex ($p < 0.05$).

Effect of 2-AAF Exposure on Expression of Proteins Involved in Cellular Iron Storage

Intracellular iron homeostasis is tightly regulated by the coordination of several proteins that are responsible for the uptake, intracellular storage, and removal of iron from cells. Therefore, the observed reduction in the levels of non-heme iron prompted us to investigate the status of these proteins in the preneoplastic livers of 2-AAF–exposed rats.

The decrease in intracellular non-heme iron accumulation was accompanied by the reduction in the levels of cytoplasmic Fth protein in the preneoplastic liver tissue, which was evidenced by a substantially lower Fth staining intensity specifically in preneoplastic Gstp1-positive foci as compared with Gstp1-negative liver tissue (Figs. 1C and 2). Interestingly, progression of liver carcinogenesis was accompanied by the advancement of these molecular intracellular iron homeostasis abnormalities. This was evidenced by an even more prominent depletion of non-heme iron and reduction of the Fth protein levels in hepatocellular carcinomas (Supplementary Fig. 1).

Effect of 2-AAF Exposure on Regulation of Cellular Iron Uptake and Export

Table 1 shows that feeding male rats a 2-AAF–containing diet resulted in a 1.7-fold upregulation of the *Tfrc* gene, whose product is responsible for Fe(III) uptake into cells. No changes in the expression of the *Dmt1* gene, whose product is responsible for Fe(II) uptake, were found in the livers of rats exposed to 2-AAF. Interestingly, 2-AAF caused opposite trends in expression changes of *Fpn1* and *Hamp* genes (Table 1). Specifically, administration of 2-AAF to male rats for 24 weeks resulted in an upregulation of the *Fpn1* gene, which encodes the iron exporter ferroportin and downregulation of the *Hamp* gene. At this time, the expression of the *Fpn1* gene in the livers of 2-AAF–treated male rats was three times greater than in the control animals, whereas the expression of the *Hamp* gene was two times lower (Table 1).

In contrast, the expression of *Tfrc*, *Dmt1*, *Fpn1,* and *Hamp* genes in the livers of female rats exposed to 2-AAF did not differ from the control female rats (Table 1).

The fact that the livers of 2-AAF–exposed male rats consist of predominantly preneoplastic lesions suggested that these changes in gene expression might be specific feature of neoplastically transformed cells. In order to test this hypothesis, we measured the expression of *Tfrc*, *Dmt1*, *Fpn1*, and *Hamp* genes in TRL1215 rat liver cells that have been continuously exposed to sodium arsenite for 18 weeks *in vitro*. As a result of

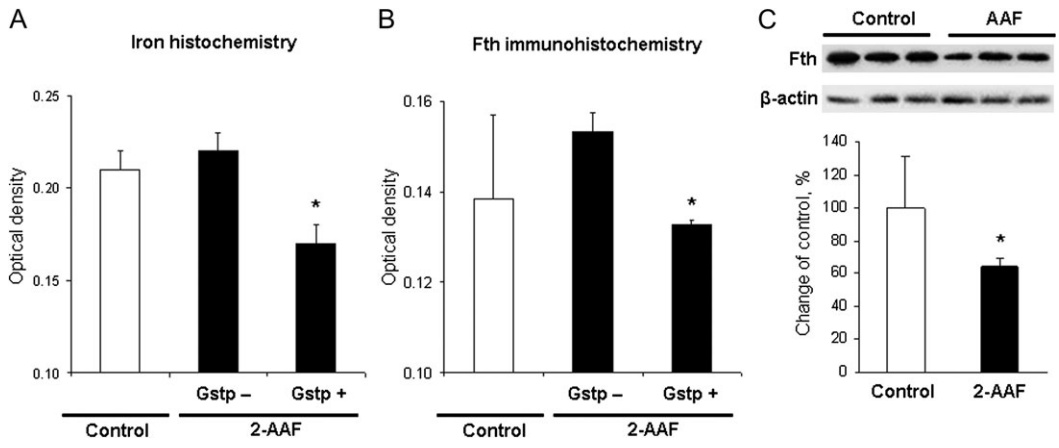

FIG. 2. Quantitative analysis of non-heme iron and Fth in the liver sections of control and 2-AAF–treated male rats. (A) Quantitative analysis of non-heme iron liver sections of a control male rat and a male rat fed the 2-AAF–containing diet for 24 weeks. (B) Quantitative analysis of Fth immunostaining liver sections of a control male rat and a male rat fed the 2-AAF–containing diet for 24 weeks. Positive Pixel Count Algorithm (Aperio Technologies) was used for evaluation of non-heme iron and Fth staining intensity. Data are presented as change in optical density ($n = 5$, mean ± SD). *Significantly different from Gstp1-negative areas. (C) Western blot analysis of Fth protein in the livers of control and 2-AAF–exposed male rats. Liver tissue lysates were separated by SDS-polyacrylamide gel electrophoresis and subjected to Western immunoblotting using specific antibodies against Fth. Equal sample loading was confirmed by immunostaining against β-actin. These results were reproduced in two independent experiments. Representative Western immunoblot images are shown. Chemiluminescence detection was performed with the Immobilon Western Chemiluminescent HRP Substrate (Millipore Corporation) and measured directly by a BioSpectrum Imaging System (UVP). Data are presented as percent change from control male rats ($n = 5$, means ± SD). *Significantly different from control male rats ($p < 0.05$).

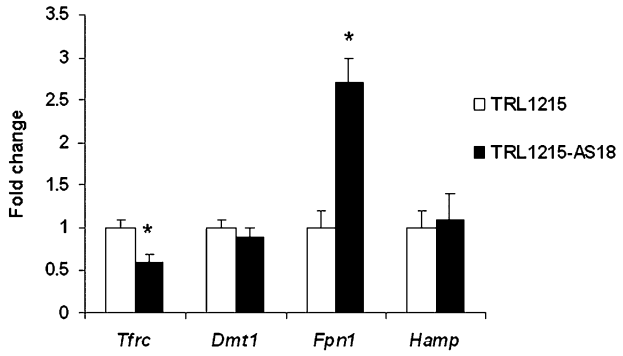

FIG. 3. Expression of *Trfc*, *Dmt1*, *Hamp*, and *Fpn1* genes in TRL1215 rat epithelial liver cells. TRL1215 cells maintained in control medium (TRL1215; white bars) or in medium containing 1μM sodium arsenite (TRL1215-AS18; black bars) for 18 weeks. Data are presented as fold change relative to control cells (mean ± SD, $n = 3$). These results were reproduced in two independent experiments. *Significantly different from control TRL1215 cells.

this exposure, these cells acquired a malignant phenotype, which was evidenced by increased invasiveness, anchorage-independent growth, and the ability to produce malignant tumors in nude mice (Kojima *et al.*, 2009). Figure 3 shows a decrease in *Tfrc* expression and extensive upregulation of *Fpn1* in neoplastically transformed TRL1215 cells compared with TRL1215 cells that were not exposed to sodium arsenite. In contrast to *in vivo* data demonstrating a downregulation of the *Hamp* gene in preneoplastic livers, we did not detect similar changes in *Hamp* expression in neoplastically transformed TRL1215 cells. This discrepancy may be explained by either very low expression of the *Hamp* gene in control TRL1215 cells or indicate the existence of additional mechanisms responsible for the *Fpn1* upregulation.

Recently, hepcidin, a product of the *Hamp* gene, has emerged as a central regulator of iron homeostasis, in general, and as a key negative regulator of ferroportin, in particular (Fleming, 2008; Ganz and Nemeth, 2011; Hentze *et al.*, 2010; Pietrangelo, 2011). The observed decrease in *Hamp* expression in the livers of male rats with 2-AAF prompted us to investigate further the possible mechanisms of *Hamp* gene downregulation. The results of several studies have demonstrated fundamental roles for Cebpa and Cebpb proteins in the regulation of *Hamp* transcription in the liver (Courselaud *et al.*, 2002; Sow *et al.*, 2009). Specifically, hepatic deletion of the *Cebpa* or *Cebpb* genes by siRNA inhibited expression of the *Hamp* gene (Courselaud *et al.*, 2002; Sow *et al.*, 2009). Therefore, we investigated the expression of Cebpb and Cebpa in the livers of control male rats and male rats fed the 2-AAF–containing diet. Figure 4A shows that the levels of Cebpb and Cebpa proteins in the livers of 2-AAF–treated male rats were significantly decreased by 33 and 29%, respectively, as compared with the control male rats. Furthermore, Figure 4B shows lower Cebpa staining intensity in the preneoplastic livers and in hepatocellular carcinomas of 2-AAF–treated rats as compared with normal liver tissue.

In addition to the reduction in global levels of Cebpb and Cebpa proteins, we detected a substantial decrease of Cebpb at the promoter of the *Hamp* gene in preneoplastic livers as measured by ChIP analysis (Fig. 5). These findings indicate that decreased levels of Cebpb and Cebpa proteins may contribute to downregulation of *Hamp* expression in the livers of 2-AAF–exposed rats.

DISCUSSION

Accumulated evidence indicates clearly that liver carcinogenesis induced by exposure to genotoxic carcinogens, including 2-AAF, involves both genotoxic and non-genotoxic events (Bagnyukova *et al.*, 2008; Heflich and Neft, 1994; Neumann, 2007; Neumann *et al.*, 1994). It is widely believed that these carcinogen-induced non-genotoxic tumor-promoting alterations in vital cellular pathways force cells toward neoplastic transformation and tumor development (Bagnyukova *et al.*, 2008; Neumann, 2007). Specifically, it has been demonstrated that selective clonal expansion of initiated hepatocytes driven by various cellular aberrations plays a fundamental role in liver carcinogenesis (Pitot, 2007).

The results of several studies conducted by Williams and coworkers (Furuya *et al.*, 1984; Hirota and Williams, 1982; Holmström *et al.*, 2006; Williams *et al.*, 1976) have demonstrated decreased iron content in preneoplastic and neoplastic lesions as compared with surrounding nontumor liver tissues and age-matched control liver tissues. Similarly, the formation of an iron-deficient phenotype in hepatic adenomas in mice induced by diethylnitrosamine exposure has been reported recently (Youn *et al.*, 2009). These findings suggest that uniform changes in intracellular iron metabolism induced by various carcinogens may also play a major role in liver carcinogenesis. The results of the present study demonstrating reduced accumulation of non-heme iron along with decreased levels of Fth in the cytoplasm in preneoplastic and neoplastic lesions in the livers of 2-AAF–exposed male rats confirmed previous findings and provided additional support to that suggestion. Interestingly, no changes were found in the livers of female rats exposed to 2-AAF (data not shown). This may be explained by a different degree of preneoplastic morphological changes in the livers of male and female rats. Particularly, the livers of male rats are characterized by large Gstp1-positive foci evenly distributed throughout the entire section of the liver, whereas only single Gstp1-positive hepatocytes or isolated minifoci were detected in the livers of 2-AAF–exposed female rats.

To elucidate the mechanisms responsible for this alteration, we investigated the expression of major genes responsible for the proper maintenance of accurate intracellular homeostasis. Analysis of the gene expression patterns revealed the predominant upregulation of the *Fpn1* gene and downregulation of the *Hamp* gene in the livers of male 2-AAF–treated male rats (Table 1).

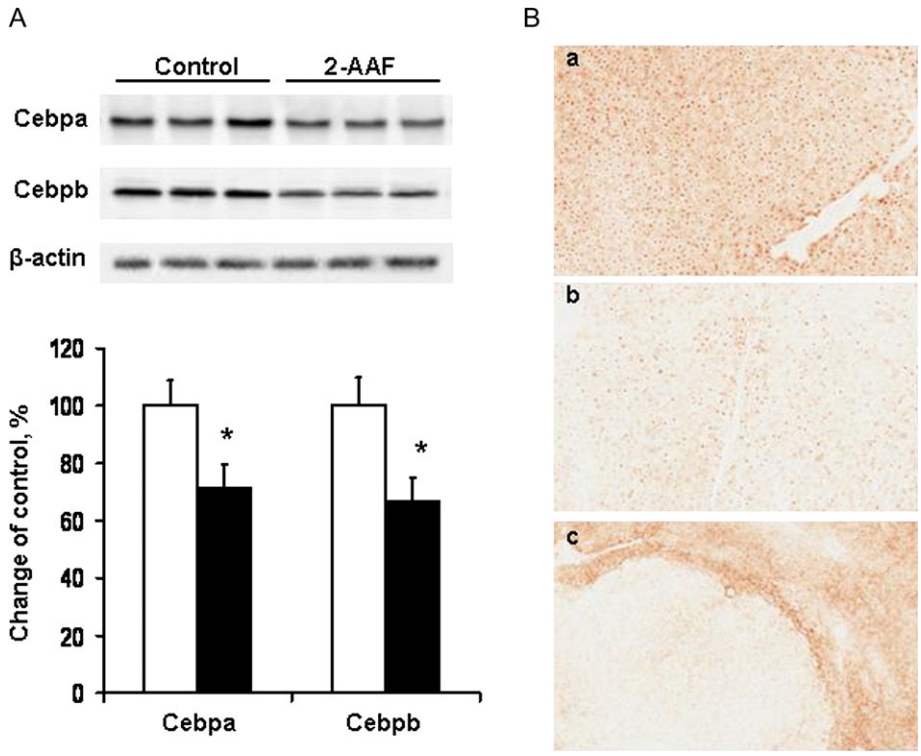

FIG. 4. Levels of Cebpa and Cebpb proteins in the livers of control male and male rats exposed to 2-AAF for 24 weeks. (A) Western blot analysis of Cebpa and Cebpb proteins in the livers of control and 2-AAF–exposed male rats. Liver tissue lysates were separated by SDS-polyacrylamide gel electrophoresis and subjected to Western immunoblotting using specific antibodies against Cebpa and Cebpb. Equal sample loading was confirmed by immunostaining against β-actin. These results were reproduced in two independent experiments. Representative Western immunoblot images are shown. Chemiluminescence detection was performed with the Immobilon Western Chemiluminescent HRP Substrate (Millipore Corporation) and measured directly by a BioSpectrum Imaging System (UVP). Data are presented as percent change from control male rats ($n = 5$, means ± SD). *Significantly different from control male rats ($p < 0.05$). (B) Immunohistochemical Cebpa staining in liver sections of a control rat (a), preneoplastic livers (b), and hepatocellular carcinomas (c) of 2-AAF–treated male rats.

The *Fpn1* gene encodes for ferroportin, the only known exporter of intracellular non-heme–associated iron (Fleming, 2008; Ganz and Nemeth, 2011; Hentze *et al.*, 2010; Pietrangelo, 2011; Pinnix *et al.*, 2010). Therefore, the decreased intracellular level of non-heme iron in preneoplastic foci may be attributed to the increased iron export from the cells. This suggestion is further supported by a 3.0-fold upregulation of the *Fpn1* gene compared with a 1.7-fold increase in expression of the *Tfrc* gene, the product of which is responsible for the intake of iron into the cell, in the livers of 2-AAF–treated male rats (Table 1). A similar increase in the *Fpn1*/*Tfrc* gene expression ratio was found also in TRL1215 rat liver cells with an acquired malignant phenotype induced by continuous exposure to sodium arsenite *in vitro* (Fig. 3). This indicates that overexpression of *Fpn1* may be a specific feature of neoplastically transformed cells that leads to formation and maintenance of the iron-starved phenotype in cancer cells. This suggestion is supported by mounting evidence that demonstrated a prominent and consistent upregulation of *Fpn1* in hepatocellular carcinoma, whereas trends of alterations in Tfrc expression in hepatocellular carcinoma remain inconclusive.

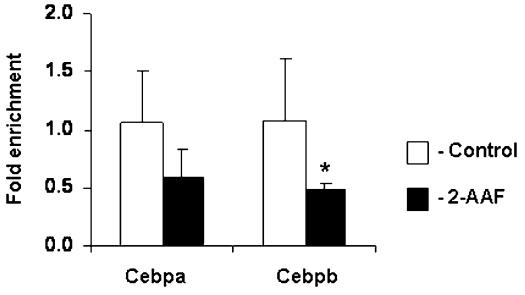

FIG. 5 Level of Cebpa and Cebpb proteins at *Hamp* promoter. A ChIP assay was performed with primary antibodies against Cebpa and Cebpb. Purified DNA from Cebpa- and Cebpb-enriched fragments and from input DNA was amplified by a qPCR with a rat *Hamp*-specific primer set (NM_053469.1; SABiosciences). Data are presented as fold change relative to control male rats after normalization to input DNA ($n = 5$, mean ± SD). *Significantly different from control male rats ($p < 0.05$).

The overexpression of *Fpn1* may be attributed to the decreased expression of the *Hamp* gene, which encodes hepcidin, a 25-amino acid liver-synthesized hormone that interacts with the iron exporter ferroportin and causes its degradation in several tissues, including liver (Ganz and Nemeth, 2011; Pietrangelo, 2011). Several possible explanations exist for the mechanism of hepcidin downregulation. First, it may be due to the inability of preneoplastic liver foci to

express *Hamp* properly. This is supported by evidence that expression of the *Hamp* is suppressed in hepatocellular carcinomas (Chen and Chloupková, 2009; Kijima *et al.*, 2008). Second, downregulation of the *Hamp* gene may be linked to decreased levels of Cebpb and Cebpa proteins (Figs. 3 and 4), which are critical regulators of *Hamp* expression (Courselaud *et al.*, 2002; Sow *et al.*, 2009). Third, downregulation of the *Hamp* gene may be associated with a decreased level of p53 protein. This suggestion is supported by the results of several recent studies. Specifically, a study conducted by Weizer-Stern *et al.* (2008) has shown that the *Hamp* promoter contains a p53 response element and that the silencing of p53 resulted in decreased expression of *Hamp* in hepatoma cells. Additionally, Dongiovanni *et al.* (2010) reported recently that depletion of iron in rat liver *in vivo* increases expression of Mdm2 and decreases expression of p53. In our previous study on 2-AAF–induced hepatocarcinogenesis, using the same liver tissue samples as in the present study, we demonstrated a substantial upregulation of Mdm2 and downregulation of p53 (Pogribny *et al.*, 2009). Any of these alterations may inhibit expression of hepcidin. More importantly, the results of the present study and studies conducted by other investigators (Chen and Chloupková, 2009; Kijima *et al.*, 2008; Tan *et al.*, 2009; Tseng *et al.*, 2009) indicate that suppression of hepcidin may be a critical event linked to the development and progression of hepatocellular carcinomas.

We hypothesize that the reduction of non-heme iron content in Gstp-positive preneoplastic hepatic foci driven by dysregulated functioning of the Fpn1-Hamp interaction may contribute to the progression of liver carcinogenesis by (i) inhibiting apoptosis and (ii) decreasing oxidative stress in initiated cells. Both of these events may favor the selective accelerated and uncontrolled cell proliferation observed in preneoplastic foci. Indeed, this suggestion is supported by the results of our previous study that demonstrated a profound disbalance between cell proliferation and apoptosis in Gstp-positive foci in the livers of 2-AAF–exposed rats (Pogribny *et al.*, 2009).

In addition to regulation of the *Hamp* gene, Cebpb and Cebpa proteins function as negative regulators of the rat *Gstp1* gene (Ikeda *et. al.*, 2006; Osada *et al.*, 1995; Sakai and Muramatsu, 2007). In the normal rat liver, Cebpb and Cebpa bind to Gstp enhancer 1 and suppress expression of *Gstp1* gene. In contrast, a downregulation of Cebpb and Cebpa in the preneoplastic liver foci may reduce their inhibitory effect on the *Gstp1* gene and, consequently, increase *Gstp1* expression (Ikeda *et. al.*, 2006; Osada *et al.*, 1995; Sakai and Muramatsu, 2007). Indeed, the results of our study, as well as previous reports, demonstrate a substantial upregulation of *Gstp1* expression and decreased levels of Cebpb and Cebpa proteins in 2-AAF–treated livers (Table 1; Figs. 4 and 5). This suggests that a common mechanism, driven by loss of an inhibitory regulatory function of Cebpb and Cebpa, may intimately link disturbances in intracellular iron homeostasis and the selective progression of preneoplastic foci during liver carcinogenesis. Similarly, the concomitant downregulation of hepatic Hamp and Cebp has been associated with hepatitis C infection (Miura *et al.*, 2008; Nishina *et al.*, 2008).

In conclusion, the results of the present study demonstrate the importance of altered intracellular iron metabolism in liver carcinogenesis induced by administration of complete carcinogen 2-AAF, particularly the role of dysregulated Fpn1-Hamp interaction in the formation of an iron-deficient phenotype in preneoplastic lesions. Additionally, our study highlights the link between disturbances in iron metabolism and accelerated selective expansion of initiated cells. Therefore, we hypothesize that correction of these alterations may affect liver cancer development; however, the underlying mechanisms leading to these alterations remain to be determined.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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