NF-κB increased expression of 17β-hydroxysteroid dehydrogenase 4 promotes HepG2 proliferation via inactivating estradiol

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Hepatocellular carcinoma (HCC) arises in a setting of chronic inflammation induced by inflammatory cytokines, such as nuclear factor-kappaB (NF-κB). HCC is a male-predominant cancer that can be attenuated by estradiol (E2) in vitro and in vivo. Although 17β-hydroxysteroid dehydrogenase 4 (HSD17B4) has been implicated as an estradiol-inactivating enzyme, and its promoter sequence contains two putative NF-κB elements: it is currently unknown whether HSD17B4 is the link between inflammation, estradiol and proliferation in hepatoma cells. In this study, HepG2 cells were used to investigate the role of HSD17B4 in the proliferation of liver cancer cells treated with the NF-κB activator, tumor necrosis factor-alpha (TNF-α), with the inhibitor of NF-κB activation, pyrrolidinedithiocarbamate (PDTC), or with a related specific siRNA. We demonstrated that the human HSD17B4 gene is a target for NF-κB activation in the distal NF-κB-responsive element via TNF-α stimulation, which then promotes cell proliferation by decreasing the levels of E2 and enhancing the expression of interleukin 6 (IL-6), cyclin D1 and proliferating cell nuclear antigen (PCAN). These results from HepG2 cells are consistent with the observation that HSD17B4 is highly expressed and activated NF-κB is highly co-localized with the NF-κB-responsive element of HSD17B4 in liver tumor tissues from HCC patients. Our findings indicate for the first time that HSD17B4 plays an important role in aggravated HCC progression and provides a novel therapeutic target for HCC.

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1. Introduction

Human hepatocellular carcinoma (HCC), which is one of the most malignant carcinomas in the worldwide, is closely associated with a history of chronic hepatitis caused by viral infection, such as hepatitis B or C virus, metabolic injury, toxic insults or autoimmune reactions (Mandrekar and Szabo, 2009; Reddy and Rao, 2006). In the majority of cases, HCC arises in a setting of chronic inflammation and subsequent liver fibrosis and cirrhosis (Fattovich et al., 2004). Additionally, the enhanced production of inflammatory cytokines causes hepatic inflammation and the activation of oncogenes, which then results in liver damage, promoting HCC progression (Park et al., 2006). Nuclear factor-kappaB (NF-κB) is one such inflammatory cytokine. NF-κB links inflammation to cancer development and progression. In un-stimulated cells, NF-κB exists in the cytoplasm in an inactive form associated with an inhibitor of κB, IκB. There are several inducers of NF-κB activity, including tumor necrosis factor-alpha (TNF-α), interleukin 1-beta (IL-1β), and bacterial lipopolysaccharides (LPS). These inducers may trigger the phosphorylation, ubiquitination, and degradation of I-κB proteins through the receptor on the surface of cell (Luedde et al., 2007, 2008). Upon degradation of I-κB, NF-κB is activated and free to enter the nucleus, where NF-κB promotes the expression of its downstream genes that contain binding sites for NF-κB. Because many downstream genes are involved in inflammation and proliferation, NF-κB activation is a frequent and early event in human liver cancers of viral or nonviral etiologies and has been associated with the acquisition of a transformed phenotype during hepatocarcinogenesis (Liu et al., 2002).

A relevant aspect of HCC epidemiology is the gender difference in the incidence of this neoplasm. The incidence of HCC is three to five times higher in men than women (Huang et al., 2011). Male mice treated with estradiol have a lesser chance of developing liver tumors than control males; however, ovariectomized female mice have a higher risk of developing liver tumors than normal female mice during chemically induced carcinogenesis (Nagler et al., 2007; Shimizu et al., 1998b; Tsutsumi et al., 1992). Clearly, these results suggest that the pathogenesis and development of HCC may be affected by sex hormones, particularly estradiol levels. Estradiol is a siren qua non steroid hormone that mediates important physiological functions, such as acting as an antioxidant to reduce atherosclerosis in the vasculature (Wing et al., 2009), preventing
apoptosis and necrosis of cardiac and endothelial cells (Masuda et al., 2007; Strehlow et al., 2003), and inhibiting macrophase alternative activation (Yang et al., 2012). Estrogens include estradiol (E2), estrone (E1) and estriol (E3). Estradiol is the most abundant of these and has the most important physiological activity. The physiological and pathological effects of estrogens are dependent on the local levels of estrogen and its metabolite, and the microenvironment in the target tissues (Straub, 2007). The level of E2 is decreased in the livers of HCC patients (Shimizu et al., 2007; Tanaka et al., 2000), and an identical result was confirmed in chemically induced carcinogenesis in male rats (Shimizu et al., 1998a). E2 can suppress the pathological progression of HCC (Naugler et al., 2007; Rogers et al., 2007; Xu et al., 2012; Yang et al., 2012).

17β-Hydroxysteroid dehydrogenase 4 (HSD17B4) is found ubiquitously, with the highest levels in the liver, and participates in estradiol metabolism. HSD17B4 catalyzes the conversion of E2, which is the most potent and active form of estrogen, to E1, which is the less active form of estrogen (Breitling et al., 2001; de Launoit and Adamski, 1999). HSD17B4 over-expression was observed in prostate cancer tissues (Rasiah et al., 2009). In addition, the analysis of the human HSD17B4 upstream flanking DNA identified multiple putative NF-xB responsive DNA elements in the promoter region (Grilli et al., 1993). Furthermore, Rogers et al. suggested that the mechanism of inflammation-associated carcinogenesis is consistent with the male-predominant HCC risk (Rogers et al., 2007). Therefore, HSD17B4 is most likely the link between inflammation, estradiol and proliferation in hepatoma cells.

In this study, we used HepG2 cells to investigate the role of HSD17B4 in hepatoma cell proliferation induced by TNF-α and a possible link among NF-xB, HSD17B4 and E2. We demonstrated that HSD17B4, as an NF-xB target gene, was upregulated in HepG2 cells by TNF-α stimulation to promote HepG2 cell proliferation by decreasing the levels of E2 to enhance interleukin 6 (IL-6), cyclin D1 and proliferating cell nuclear antigen (PCAN) expression. These results from HepG2 cells were consistent with the observation that HSD17B4 is highly expressed and activated NF-xB is highly co-localized with the NF-xB-responsive element of HSD17B4 in liver tumor tissues from HCC patients. Taken together, our findings indicate for the first time that HSD17B4 plays an important role in aggravated HCC progression and identifies a novel therapeutic target for HCC.

2. Materials and methods

2.1. Preparation of liver tissue from patients with HCC

Human liver tissues were obtained from the surgical resection of 16 male patients with HCC in the Second and the Third Affiliated Hospital of Hebei Medical University (Shijiazhuang, China) between August 2012 and December 2013. Informed consent for this study was obtained from each patient before surgery. The tumor and adjacent tissue excised at 1 cm from the tumor margin were identified according to the pathology results. The liver tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin routinely for immunohistochemistry assay, immunofluorescence staining and fluorescence in situ hybridization.

2.2. Cell culture and treatment

The human hepatocellular carcinoma cell line, HepG2, was obtained from the American Type Culture Collection (Rockville, MD, USA). HepG2 cells were maintained in RPMI-1640 medium (Gibco Invitrogen Corp.) containing 10% fetal bovine serum (FBS), penicillin and streptomycin (100 and 100 units/ml, respectively) in a humidified environment at 37 °C and 5% CO2. For TNF-α (Peprotech) administration, the cells were seeded at a density of 3 × 104 cells per cm², cultivated for 24 h, and then grown in FBS-free RPMI-1640 media for an additional 24 h. The NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC) (Beyotime), was added 2 h before TNF-α administration.

2.3. Cell proliferation assay

HepG2 cell proliferation assays were performed using a bromodeoxyuridine (BrdU) Cell Proliferation Assay Kit (Millipore) and a Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturers’ recommendations. The cells were labeled for 6 h with BrdU before the termination of HSD17B4 plasmid incubation. The OD readings were performed at 450 nm to measure the incorporation of BrdU. The cells were treated for 3 h with CCK-8 before the termination of HSD17B4 plasmid incubation. The OD readings were performed at 450 nm to determine the numbers of viable cells. All groups were evaluated in an average of three separate wells per experiment. The HSD17B4 expression plasmid was created by the placement of human HSD17B4 cDNA into the pLL3.7 vector (Addgene).

2.4. Western blotting

The cells were lysed in lysis buffer A (50 mM PBS, pH 7.5, 200 mM NaCl, 0.5 mM EDTA, pH 8.0, 2 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 0.5% Tween-20) for cytoplasmic protein extraction and in lysis buffer B (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, pH 8.0, 0.1 mM EGTA, pH 8.0, 1 mM phenylmethylsulfonyl fluoride, and 0.6% NP-40) for nuclear protein extraction. After incubation on ice for 30 min, the cell lysates were centrifuged at 8000 g for 10 min for nuclear proteins or 12,000 g for 20 min for cytoplasmic proteins, and the supernatants were stored until use. Equal amounts of protein (100–200 μg) were separated by 10% SDS-PAGE and electrotransferred onto a PVDF membrane. The membranes were blocked with 5% skim milk powder for 1 h at room temperature, and incubated overnight at 4 °C with the following primary antibodies: 1:2000 rabbit anti-HSD17B4 (Homemade) (Jiang et al., 1996), 1:500 rabbit anti-NF-xB p65 (Epitomics, Inc., catalog no. 1546–1), 1:1000 rabbit anti-cyclin D1 (Cell Signaling, Inc., catalog no. 2922S), 1:1000 rabbit anti-PCNA (Cell Signaling, Inc., catalog no. 13110), 1:500 rabbit anti-β-tubulin (Santa Cruz Biotechnology, Inc., catalog no. sc-5274) and 1:1000 mouse anti-β-actin (Santa Cruz Biotechnology, Inc., catalog no. sc-47778). Following incubation with the appropriate secondary fluorescence antibodies for 1 h at room temperature, the antibody–antigen complexes were imaged using a Chemiluminescence Plus Western Immunoblot Analysis Kit (Santa Cruz Biotechnology). The related grayscale indicated the expression of various genes, with β-actin as the protein loading control.

2.5. RT and real-time quantitative PCR

Total RNA was isolated from the cells using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The cDNA was synthesized using a SuperScript Reverse Transcription Kit (Invitrogen) and was used as a template for real-time quantitative PCR with a SYBR Green PCR Master Mix kit (TaKaRa). Quantitative real-time PCR was performed with a Rotor-Gene 3000 Detection System (Gene Biosystems). The PCR cycles consisted of an initial denaturation step at 95 °C for 30 s, followed by 40 cycles of denaturation for 5 s at 95 °C, annealing for 30 s at 55 °C, and elongation for 30 s at 72 °C. As an internal control, 18s rRNA was used for RNA template normalization. All PCRs were performed in triplicate. The mRNA relative expression level was expressed as 2−ΔΔCt (Ct sample – Ct control). The primer pairs are shown in Table 1.
Note: Bold font is mutated bases.

2.6. Small interfering RNA (siRNA) transfection

The specific siRNA against NF-κB p65 (siNF-κB p65), containing 5′-GAUGAGAUCUUCCUAGGAdTdT-3′ and 5′-ACAGUAGGGTTGGGGTCAATT-3′, was purchased by Sigma. The specific siRNA against HSD17B4 (siHSD17B4), containing 5′-GAUGAGAUCUUCCUAGGAdTdT-3′; reverse, biotin-5′-TACTACGGTGAGGGTAC-3′, which corresponds to 1618/−1597 bp of the human HSD17B4 upstream region with biotin added to their 5′-end of the strand were used. The sequences of these oligonucleotides are as follows: the NF-κB-binding site A: forward, biotin-5′-CTTCTAGCCCTCTTCAAAACTTCTC-3′; reverse, biotin-5′-ACAGTCCCCTGATGTTTGC-3′.

2.7. Reporter gene plasmid construction and luciferase assay

According to an analysis of the human HSD17B4 upstream flanking DNA, multiple putative NF-κB responsive DNA elements are located upstream of the transcription start −1657/−8 (contain site A and site B) and −708/−8 (contains site B). The wild and mutant regions of site A and site B were obtained by PCR with the normal and mutagenic primer pairs (Table 1), respectively. The PCR products were cloned into the pGL3-Basic Vector (Promega) using T4 DNA ligase (Fermentas) to generate the various wild types (site AB-wt) and mutant (site A-mut, site B-mut and site AB-mut) HSD17B4 promoter-reporter plasmids. All constructs were verified by DNA sequence analysis.

The cells were seeded into each well of a 24-well plate and grown for 24 h before co-transfection with the reporter gene plasmids and the control pTK-RL (Renilla Luciferase) plasmid. Transfection was performed using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer’s instructions. Luciferase assays were performed 48 h after transfection using a dual luciferase assay kit (Promega). The transcription activity of region A or B was measured as the relative firefly luciferase activity normalized with respect to the activity of the control Renilla luciferase. Each assay was performed in triplicate.

2.8. Chromatin immunoprecipitation (ChIP) assay

The cells were fixed with 1% formaldehyde for 15 min, which cross-links and preserves protein/DNA interactions. The cross-linking was stopped with the adding of 125 mM glycine for 5 min at room temperature. The cells were washed with ice-cold PBS and then snaped into PBS plus protease inhibitors and collected by centrifugation at 4000 rpm for 5 min. The cells were lysed in cell lysis buffer (5 mM KOH, pH 8.0, 85 mM KCl, 0.5% NP-40) for 10 min, and the nuclei were then pelleted by centrifugation at 5000 rpm for 5 min. The DNA was incubated for 10 min in nuclear lysis buffer (50 mM Tris, pH 8.1, 10 mM EDTA, 1% SDS), and then sonicated into an average size of 200–600 bp small uniform fragments. The DNA/protein complexes were immunoprecipitated using either rabbit IgG (Bioworld, Inc., catalog no. BD0051) (as a negative control) or antibody NF-κB p65 (Epitomics, Inc., catalog no. 1546-1) overnight. The immune complexes were collected with 10 μl of protein A agarose slurry for 1 h at 4 °C with rotation. Following immunoprecipitation, DNA cross-linking was reversed at 45 °C for 2 h, and the proteins removed with proteinase K treatment. After DNA purification, real-time PCR analysis was performed to compare the enriched amounts of immunoprecipitated DNA. The primer pairs used for ChIP assays are described in Table 1.

2.9. Oligonucleotide pull down assay

The oligonucleotides containing the putative NF-κB binding site of the human HSD17B4 upstream region with biotin added to their 5′-end of the strand were used. The sequences of these oligonucleotides are as follows: the NF-κB-binding site A: forward, biotin-5′-AAGCTTAAGGCCTGCAACGACA-3′; reverse, biotin-5′-TGAGAGAUCUUCCUAGGAdTdT-3′. The oligonucleotides were annealed following standard protocols (Invitrogen) to form the biotinylated double-stranded oligonucleotides. Cell nuclear protein extract was pre-cleared using ImmunoPure streptavidin–agarose beads (20 μl/sample, Pierce, Rockford, IL, USA) for 1 h at 4 °C. After centrifugation for 1 min at 5000 g, the supernatant was incubated with 100 pmol of biotinylated double-stranded oligonucleotides and 10 μg of poly (dl-dc)·poly (dl-dc) overnight at 4 °C with gentle rocking. Then, 30 μl of streptavidin–agarose beads was added, followed by further incubation for 1 h at 4 °C. The protein–DNA–streptavidin–agarose complex was washed with lysis buffer B four times, then the protein was separated on a 10% SDS–PAGE and subjected to western blot analysis with an antibody against NF-κB p65 (Input as a positive control).

2.10. HSD17B4 activity assay

Because HSD17B4 catalyzes the conversion of E2 to E1, the catalytic activity of HSD17B4 was indicated by the remaining amount of E2 under the present conditions. Briefly, cells were treated with E2 (10 nM) for 2 h and the concentration of E2 in the culture media was then determined using a RIA kit (Jiuding, Tianjin, China) according to the manufacturer’s recommendations. The amount of E2 was expressed as ng/ml culture media.

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Table 1

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Note: Bold font is mutated bases.
2.11. Immunohistochemistry (IHC) assay

Immunohistochemistry was performed on paraffin sections (4 μm) as described previously (Fishbein et al., 2003). Briefly, liver sections were blocked with goat serum and incubated with a rabbit anti-HSD17B4 antibody (Jiang et al., 1996). After extensive washes, the sections were incubated with biotinylated anti-rabbit IgG, horse-radish peroxidase-avidin D, and then developed with DAB kit (Vector Labs SK-4100). The sections were also counterstained with hematoxylin. The staining intensities were determined by measuring the integrated optical density (IOD) with light microscopy using a computer-based Image-Pro Morphometric System (Medial Cybernetics, Bethesda, MD, USA).

2.12. Immunofluorescence staining (IF) assay

HSD17B4 expression and location in the liver tissues were determined by immunofluorescence staining as described previously (Cook et al., 2006). Briefly, sections were permeabilized with 0.1% Triton X-100 in PBS. After blocking with goat serum, the sections were incubated a rabbit anti-HSD17B4 antibody (1:500) followed by the appropriate FITC-conjugated secondary antibodies (KLP). Unbound antibody was removed by washing, and then the tissues were stained with DAPI and visualized and photographed under a confocal microscope with the Confocal Laser Scanning Microscope Systems (Leica).

2.13. Fluorescence in situ hybridization (FISH) combined with IF assay

Co-location of NF-κB-binding putative site A sequence and NF-κB in liver tissues was detected by FISH combined with IF as described previously (Cook et al., 2006). Briefly, liver tissue sections rehydrated in a graded series of ethanol washes were digested with proteinase K (1 μg/ml). Hybridization was performed on the sections with a FAM-labeled probe complementary to the NF-κB-binding site A sequence (forward, FAM-5′-CGGGTTTTTTCCCCACCGTGAGTA-3′; reverse, FAM-5′-TACTACGGTGCGAAAACCCCG-3′). After hybridization, the free probes were washed off with PBS, and the sections were incubated with an anti-NF-κB p65 primary antibody followed by the appropriate TRITC-conjugated secondary antibodies (KLP). After removing the unbound antibody, the tissues were stained with DAPI and visualized and photographed under a confocal microscope with the Confocal Laser Scanning Microscope Systems (Leica).

2.14. Statistical analysis

The results are presented as the means ± standard deviation (SD) of the mean. The statistical analyses of differences between the compared groups were performed using a one-way ANOVA. The differences were considered statistically significant at the p < 0.05 level. All statistical analyses were performed using the software SPSS 13.0.

3. Results

3.1. Increased HSD17B4 is involved in the HepG2 proliferation

As shown in Fig. 1, HSD17B4 over-expression increased the cell number (Fig. 1A), BrdU incorporation (Fig. 1B) and the protein levels of cyclin D1 and PCNA (Fig. 1E). By contrast, the basic-induced cell numbers (Fig. 1C), BrdU incorporation (Fig. 1D) and the protein contents of cyclin D1 and PCNA (Fig. 1F) were significantly decreased by the knockdown of endogenous HSD17B4 using siRNAs. These results indicated that HSD17B4 affects HepG2 proliferation.

3.2. Increased HSD17B4 expression in HepG2 is associated with NF-κB activation

To confirm that the up-regulation of HSD17B4 expression was related to NF-κB activation, HepG2 cells were treated with TNF-α to activate NF-κB, PDTC to inhibit TNF-α activation, or siRNA to knockdown NF-κB expression. TNF-α caused an increase (Fig. 2A and B) and PDTC caused a decrease (Fig. 2C and D) in the expression of HSD17B4, and the NF-κB targets, IL-6 and IL-8, were accompanied by NF-κB activation and inhibition, respectively, in a concentration-dependent manner. Furthermore, decreases in activated NF-κB HSD17B4 and the NF-κB targets IL-6 and IL-8 were observed following NF-κB knockdown by siRNA (Fig. 2E and F). These results suggest that the activation of NF-κB promoted HSD17B4 expression.

3.3. The distal NF-κB element in the human HSD17B4 promoter responds to activated NF-κB

To identify the link between HSD17B4 and NF-κB, sequence analysis of the HSD17B4 promoter region was performed and revealed two putative NF-κB-responsive elements (Fig. 3A). To determine whether the sequences respond to NF-κB activation, two fragments of the HSD17B4 promoter were ligated to the luciferase reporter gene and transfected into HepG2 cells. These cells were treated with either the NF-κB activator, TNF-α alone, or the inhibitor, PDTC, alone. As shown in Fig. 3B, the luciferase activity of the region –1657 to –8 (site AB-wt) of the HSD17B4 promoter was increased by TNF-α treatment and decreased by PDTC pretreatment before TNF-α treatment. However, no significant changes in the luciferase activity of the proximal region –708 to –8 (site B-wt) of the HSD17B4 promoter were observed by identical treatments, suggesting that the region –1657 to –708 containing putative site A mediates the NF-κB-α-induced transactivation of HSD17B4. Compared with empty vector, little luciferase activity of mutations of the HSD17B4 promoter regions was still observed. When site A or site B were mutated, however, the luciferase activation of HSD17B4 promoter could not be induced by TNF-α, or inhibited by PDTC (Fig. 3C). This suggests that site A in the region of the HSD17B4 promoter was required for NF-κB binding to activate HSD17B4 expression.

To further confirm that putative site A mediates the responses to TNF-α via NF-κB binding, a ChIP assay and an oligonucleotide pull-down assay were performed. The DNA fragments that bind to NF-κB were precipitated using an antibody against NF-κB p65, and the DNA fragments were identified as encompassing putative site A or site B by real-time PCR. As shown in Fig. 3D, an increase in size A DNA fragments was associated with TNF-α treatment and the increase was prevented by PDTC pretreatment before TNF-α treatment, whereas site B DNA fragments were not significantly affected by identical treatments. The proteins that bind specifically to the biotinylated double-stranded oligonucleotides containing putative site A or site B were precipitated with streptavidin-agarose beads identified by western blot using an anti-NF-κB p65 antibody. As shown in Fig. 3E, NF-κB p65 binding to site A was increased by TNF-α treatment or prevented by PDTC pretreatment before TNF-α treatment, whereas NF-κB p65 binding to site B was not significantly affected by identical treatments. These results indicate that the putative site A in the HSD17B4 promoter is an enhancer element that binds to NF-κB to mediate the TNF-α-induced transcription of HSD17B4 in HepG2 cells.

3.4. NF-κB-upregulated expression of HSD17B4 decreases E2 levels

The relevance of HSD17B4 both to E2 and NF-κB led to the hypothesis that the NF-κB-upregulated expression of HSD17B4 decreases the levels and the role of E2 in HepG2 proliferation. To
confirm this hypothesis, HepG2 cells were treated with TNF-α or transfected with a HSD17B4 expression plasmid. Then, these cells were treated with E2. As shown in Fig. 4A and B, TNF-α treatment and HSD17B4 over-expression reduced the levels of E2 in the culture medium compared with their respective controls. However, TNF-α treatment does not affect the expression of HSD17B2, 8 and 10, which also catalyze E2 to E1 (data not shown).

To further confirm that hepatoma cell proliferation occurs via HSD17B4 over-expression by inactivating E2, HepG2 cells were treated with E1, E2 and transfected with a HSD17B4 expression plasmid or siRNA to knockdown HSD17B4 expression. E2 treatment and HSD17B4 knockdown reduced cell proliferation, and the reduced levels were consistent (Fig. 4D). However, E1 treatment at 100 nM (100-fold more than the physiological concentration) did not affect cell proliferation (Fig. 4C and D). These results indicate that NF-κB, at least partially, up-regulates HSD17B4-aggravated hepatoma cell proliferation by inactivating E2.

3.5. HSD17B4 expression is increased and its promoter site A is co-localized with increased NF-κB in liver tumor tissues from HCC patients

To determine whether HSD17B4 plays a role in aggravated HCC progression, we investigated the expression of HSD17B4 in adjacent and tumor tissues of livers from HCC patients. The immunohistochemistry images and statistical analysis of the integrated optical density (IOD) values of the images and immunofluorescence images all show that HSD17B4 expressed in tumor tissues is increased compared with the tumor adjacent tissue in the livers from HCC patients (Fig. 5A and B).

To confirm that HSD17B4 expression increases during hepatocyte proliferation via NF-κB binding to the element of the HSD17B4 promoter, we assessed the co-localization of NF-κB and the putative NF-κB binding element (Site A) at the HSD17B4 promoter in liver tissues from HCC patients using FISH combined with IF. As shown in Fig. 5C, tumor tissues have more activated NF-κB entering the nuclei and binding to the site A sequence of the HSD17B4 promoter compared with tumor adjacent tissues, in parallel with the increase in HSD17B4 expression (Fig. 5A and B). These results indicate that the enhanced HSD17B4 expression by activated NF-κB is associated with the development of HCC.

4. Discussion

Hepatocellular carcinoma (HCC) is associated with chronic inflammation and the regenerative wound-healing response. Several inflammatory mediators, such as TNF-α, which is involved in the progression of HCC, are activators of NF-κB activation. NF-κB activation is a frequent and early event in liver cancers of viral (HBV
or HCV) or nonviral (alcohol consumption, NAFLD and liver toxins) etiologies and is associated with phenotype transformation during hepatocarcinogenesis (Liu et al., 2002). The activation of NF-κB in hepatoma cells generally promotes hepatocarcinogenesis via proliferative and antiapoptotic effects, as well as the spreading of liver tumors, despite contrary processes reported for some conditions (He et al., 2010; Lu et al., 2010; Maeda et al., 2005; Oakley et al., 2005; Zhang et al., 2010). NF-κB participates in the regulation of genes involved cell proliferation, such as cyclin D1, which is a key regulator of the G1 checkpoint control of cell cycle progression (Hinz et al., 1999), PCNA, which is an S phase marker of the cell cycle, and IL-6, which is an activator of Stat3 pro-carcinogenic signaling pathways (Akaishi et al., 1998; Fan et al., 2013; Gough et al., 2009). Consistent with these observations, TNF-α was observed to promote HepG2
cell proliferation by NF-κB activation, which subsequently up-regulated expression of cyclin D1, PCNA and IL-6.

HSD17B4 has recently been shown to be highly expressed in prostate cancer cells (Romanuik et al., 2010) and prostate tissues from prostate cancer patients (Rasiah et al., 2009; True et al., 2006; Zha et al., 2005). High protein levels of HSD17B4 in paraffin sections from liver cancer patients are also listed in the Human Protein Atlas (http://www.proteinatlas.org/ENSG00000133835/cancer/tissue/liver-cancer). The genome expression studies in hepatocellular carcinoma (http://www.cbioportal.org/public-portal/) have also shown that the increased expression of HSD17B4 at the mRNA level in liver samples from patients with hepatocellular carcinoma was associated with the higher expression of over 1000 other genes. Additionally, there was a linear correlation between HSD17B4 and these genes, such as tumor progress-related gene CDC14B (Pearson correlation coefficient $R^2 = 0.70$), and the inflammation-related genes, ITPR2 (Pearson correlation coefficient $R^2 = 0.66$) and ST3GAL6 (Pearson correlation coefficient $R^2 = 0.65$). These findings suggest that there may be certain relationship between HSD17B4 and cell proliferation under inflammatory conditions. This relationship was confirmed both in HepG2 cells and liver tumor tissues of 16 HCC patients in the present study, which demonstrated that HSD17B4 was not only highly expressed in HepG2 cells and human liver tumor tissues but also promoted HepG2 proliferation via its effects on the expression of cyclin D1, PCNA and IL-6 under TNF-α-stimulated inflammation, although other NF-κB-related genes may also be involved in this proliferation. Additionally, the relationship between HSD17B4 and the cytokine NF-κB was confirmed by our results, showing that endogenous HSD17B4 expression was increased by TNF-α-stimulation and correlated with NF-κB activation. By contrast, the TNF-α-stimulated increase was reversed by an inhibitor of NF-κB activation, PDTC, or by the knockdown of endogenous
NF-κB with siNF-κBp65. These observations led to the identification of the HSD17B4 gene as an NF-κB target. The human HSD17B4 gene upstream flanking region contains two NF-κB binding sites, site A at in −1617/−1608 and site B at in −422/−413. In HepG2 cells, NF-κB constitutively binds these two sites in the HSD17B4 promoter, even in the absence of TNF-α stimulation. However, NF-κB binding to site A was shown to be particularly responsive to the stimulation and inhibition of NF-κB activation in HepG2 cells, suggesting a mechanism linked to NF-κB activation under which HSD17B4 is highly expressed during inflammation. This was supported by the observations that increased activated NF-κB was co-localized with its protective effect may be related to its anti-inflammatory effect (Shi et al., 2014). Yang et al. showed that E2 suppresses tumor growth by regulating the polarization of macrophages (Yang et al., 2012). Consistent with these results, our data show that activated NF-κB promotes cell proliferation by up-regulating HSD17B4 expression, which subsequently converts E2 to E1. As discussed above, E1 can be metabolized into either noncarcinogenic/marginally carcinogenic 2-OH E1 or carcinogenic 4-OH E1 (or to a lesser extent 16α-OHE1) metabolites, which are predicted to initiate cancer through the formation of DNA adducts (Cavaliere and Ragan, 2006; Cavaliere et al., 2000). Rasiah et al. suggested that HSD17B4 over-expression may contribute to prostate cancer progression through altered hormone balance (Rasiah et al., 2009). However, our data demonstrate that E1 had neither a proliferative nor anti-proliferative effect on HepG2 under the present conditions. We propose that, in addition to IL-6, HSD17B4 also contributes to the proliferation of hepatoma cells under inflammation by the following mechanism, which is distinct from that of IL-6: decreasing the levels and functions of E2 to enhance IL-6, cyclic D1 and PCNA expression. However, because anti-estriadiol therapeutic approaches in some clinical trials have proven to be only modestly beneficial (Group, 1998; Liu et al., 2000), it remains to be elucidated whether other mechanisms by which HSD17B4 promotes hepatoma cell proliferation exist.

In summary, our results provide direct evidence that HSD17B4 is an NF-κB target gene, a novel proliferation-promoting protein and...
a possible link between estradiol and inflammation-induced hepatocarcinogenesis. We propose the following mechanism to explain how HSD17B4 promotes the proliferation of hepatoma cells during inflammation: TNF-α activates NF-κB, which stimulates IL-6 and HSD17B4 expression by binding to the NF-κB response element (site A) in gene promoters, which, in turn, promotes hepatoma cell proliferation by up-regulating cyclin D1 and PCNA and inactivating E2 (Fig. 6). The inhibition of HSD17B4 expression may be a novel therapeutic target to ameliorate liver cancer cell dysfunction and prevent HCC.

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**Appendix: Supplementary material**

Supplementary data to this article can be found online at doi:10.1016/j.mce.2014.11.016.
Fig. 6. A proposed mechanism of the relationship between HSD17B4, inflammation and proliferation in HCC.

References


