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ZHX2 is a repressor of α-fetoprotein expression in human hepatoma cell lines

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Abstract

The zinc-fingers and homeoboxes 2 (ZHX2) protein was shown previously to be involved in postnatal repression of α-fetoprotein (AFP) in mice. More recently, loss of ZHX2 expression was often found in human hepatcellular carcinoma (HCC), where AFP is frequently reactivated. Using HepG2 and HepG2.2.15, which express high AFP levels, we show that ZHX2 overexpression significantly decreases of AFP secretion in a dose dependent manner. Furthermore, using LO2 and SMMC7721 cells, which express low AFP levels, we use siRNA inhibition to show that AFP is de-repressed when ZHX2 levels are reduced. This represents the first direct evidence that ZHX2 represses AFP. Co-transfections of ZHX2 and AFP-luciferase reporter genes demonstrate ZHX2 repression is governed by the AFP promoter and requires intact HNF1 binding sites. These data support the idea that ZHX2 contributes to AFP repression in the liver after birth and may also be involved in AFP reactivation in liver cancer.

Keyword: Zinc-fingers and homeoboxes 2 • α-Fetoprotein • hepatoma cell lines • gene regulation

Introduction

The zinc-fingers and homeoboxes 2 (ZHX2) gene belongs to a small family of transcription factors that also includes ZHX1 and ZHX3 [1–3]. All ZHX proteins contain two C2-H2 zinc-finger motifs and four homeodomains, are ubiquitously expressed and localized to the nuclei of cells, and appear to function as transcriptional repressors [2–5]. Human ZHX2 has a predicted molecular mass of 92 kD and shares 87% amino acid identity with mouse ZHX2. Northern analysis indicates that ZHX2 is encoded in a 4.4 kb mRNA and is expressed ubiquitously, but that levels vary in different tissues examined [3].

ZHX2 was first cloned from a size-fractionated brain cDNA library [6]. Using yeast 2-hybrid analysis and *in vitro* pull-down assays, Kawata *et al.* demonstrated a direct interaction between ZHX3 and ZHX2 [7]. ZHX2 can also form homodimers *in vivo* and *in vitro*. Immunoprecipitation analysis detected an interaction between ZHX2 and NY-FA in human embryonic kidney cells [3]. Furthermore, ZHX2 was able to repress reporter activity driven by the cdc25C promoter, which contains three NF-Y binding sequences. Taken together, these data indicate that ZHX2 can form homodimers or heterodimers with other ZHX members and NF-YA and may function as a transcriptional repressor [3, 7].

Insight into the biological function of ZHX2 has come from the analysis of α-fetoprotein (AFP) expression in BALB/cJ mice. AFP is expressed abundantly in the foetal liver and dramatically repressed at birth; AFP levels remain very low in the normal adult liver but can be reactivated in liver cancer and liver regeneration [8]. However, BALB/cJ mice exhibit roughly 20-fold higher AFP mRNA levels in the adult liver than do other mouse strains [9]. Perincheri *et al.* used a positionally cloning approach to show that continued AFP expression in BALB/cJ liver was due to a mutation in the mouse ZHX2 gene [10]. Overexpression of a ZHX2 transgene in BALB/cJ mice led to complete silencing of AFP in the adult liver, providing further evidence that ZHX2 is required for postnatal AFP silencing in the adult liver. H19 and glypican 3 are two additional targets of ZHX2 in the liver [11]. More recent studies suggest that ZHX2 expression is silenced in hepatocellular carcinomas (HCCs) in a manner that might involve epigenetic silencing by hypermethylation of the ZHX2 promoter [12].

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Taken together, these data indicate that ZHX2 may be an important regulator of hepatic gene regulation in liver development and cancer.

The studies described above indicate that ZHX2 is an important regulator of AFP expression in the developing liver and in liver cancer. Here, we have investigated the role of ZHX2 in human AFP expression. We found an inverse correlation between ZHX2 and AFP levels in cultured liver cell lines, consistent with the possibility of ZHX2 acting to repress AFP expression. Using transient transfections, we also show that overexpression of human ZHX2 in hepatoma cell lines inhibited endogenous and transfected AFP genes. Furthermore, siRNA-mediate inhibition of ZHX2 led to increased AFP expression. Further studies indicate that this repression acts through the AFP promoter and may require the hepatocyte nuclear factor (HNF)1 binding sites found in this region. These data provide direct evidence that ZHX2 controls human AFP promoter activity.

Materials and methods

Construction of plasmids

A human ZHX2 expression vector was generated by RT-PCR amplification of human liver mRNA using with forward (5′CCGGTACCCCCTCCAAAAATAAGCC 3′) and reverse (5′CGGAATTCCCTAAGCGTAGTCTGGTACGTCGTAAGGGTAGGCCTGGCCAGCCTCTGCAG3′) primers; the reverse primer encoded the hemagglutinin (HA) peptide tag fused in frame with ZHX2. The amplified 2605 bp amplified fragment was cloned into the *KpnI*/*Eco*RI sites of pcDNA3 (Invitrogen, Beijing,China) to generate the expression vector pcDNA-ZHX2-HA. The human AFP promoter (−251 to + 18) was amplified from human genomic DNA by PCR using forward (AFP-F; 5′ GGGGTACCATTCTGTAGTTTGAGGAG 3′) and reverse (AFP-R; 5′ CCGCTCGAGGTGGTGGAAGCACAATATGG 3′) primers and then inserted into the *KpnI*/*Xho*I sites of pGL3-Basic (Promega, Jinan, China) to construct the reporter plasmid phAF269.

Using PCR-based site direct mutagenesis, luciferase reporter constructs with the proximal and/or distal HNF-1 binding sites of the AFP promoter were constructed. Briefly, primers were used to change the distal HNF1 site (centred at −125) from GCTATGCTGTTAATTATTGGCA to GCTATGCTGTCGATTATTGGCA and the proximal HNF1 site (centred at −55) from CAACAAAAGGTTACTAGTTAAC to CAACAAAAGGCGACTAGTTAAC (altered sequences underlined). Mutated promoter constructs with the distal mutation, proximal mutation, or both mutations are designated pAF269-A1, pAF269-A2 and pAF269-A3, respectively. (Details for primers and mutagenesis protocol are available upon request). In order to increase the AFP promoter activity in reporter plasmids, the cytomegalovirus (CMV) enhancer was cloned upstream of pAF269, pAF269-A1, pAF269-A2 and pAF269-A3 to generate pEAF269, pEAF269-A1, pEAF269-A2 and pEAF269-A3, respectively.

Cell culture and transient transfections

Human hepatoma cell lines HepG2 (ATCC HB-8065) HepG2.215, and SMMC7721 and the immortalized human liver cell line LO2 were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences and chosen based on differential levels of AFP expression. LO2 cells were cultured in RPMI 1640 medium; whereas the other three cell lines were cultured in Minimum Essential Medium (MEM) with 1 mM sodium pymvate; all media were supplemented with 10% foetal calf serum (FCS), 50 U/ml penicillin, and 50 μg/ml streptomycin (Gibco). Cells were incubated at 37°C in 5% CO_2.

Cells were transiently transfected with plasmid DNA using Lipofectamine™ 2000(Invitrogen, Beijing, China). Twenty-four hours prior to transfection, HepG2(3×10^5), COS-7(4×10^4), HepG2.215(3×10^5), SMMC7721(2.5×10^5) or LO2 (1.5×10^5) cells were seeded in 24-well plates. At the time of transfection, the cells were incubated in 0.5 ml MEM medium without FCS containing 1.0 μg of different plasmid DNA. Six hours after transfection, the culture medium was replaced by fresh medium with 10% FCS.

Measurement of AFP and albumin in culture medium

ELISA was used to measure the level of AFP secreted into culture medium using the ACCESS® immuno-assay system (Beckman Coulter, USA), Albumin (Alb) secretion was measured using the Alb Assay kit by the BCG Method (MAKER, Sichuan, China). Cells were cultured in 24-well plates for 48 hrs with or without treatment (ZHX2 overexpression or siRNA transfection). Media were collected and cell number/well was determined. The amount of protein secreted was expressed as ng/mL. Three to five wells were analysed for each experiment and each experiment repeated at least three times.

RT-PCR analysis for assessment of ZHX2 mRNA expression

ZHX2 mRNA levels was determined using RT-PCR. Briefly, 5×10^5 cells cultured for 24 hrs were washed twice with phosphate-buffered saline (PBS) and total RNA was extracted using TRIZOL (Sangon, Shanghai, China) according to the manufacturer's protocol. Primers, designed using Premier Primer v5.0 software_Premier Biosoft, Palo Alto, CA, USA), were as follows: ZHX2, 5′CCCCCAATGGTGCTCTGT (sense) and 5′TTGCTTTCCTTGCTACGG(antisense); β-actin, 5′GGCATCGTGATGGACTCCG (sense) and 5′GCTGGAAGGTGGACAGCGA3 (antisense). Reverse transcription was performed with 3μg of total RNA, 1 μl of oligo(dT), 2 μl of 10nmol/L dNTP and M-MLV reverse transcriptase (200U; Promega, USA). Reverse transcription products were subjected to PCR (Applied Biosystems Germany with 0.5 nM specific primers using 2×Taq PCR MasterMix (TIANGEN, Beijing, China). Reactions were carried out for 25 (β-actin) or 35 (ZHX2) cycles of 94°C denaturation for 45 sec., 55°C annealing for 45 sec., and 72 extension for 45 sec., followed by a final 10 min. extension at 72°C. PCR products were separated on 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Western blot analysis

Cells were lysed in gel-loading buffer [50mM Tris-HCl (pH 6.8), 100 mM dithiothreitol (DTT), 2% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue and 10% glycerol]. After boiling for 10 min., 50 μg of protein were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotted onto a nitrocellulose membrane (Millipore Co., Jinan, China). Membranes were blocked with PBS containing 5% non-fat milk and incubated with monoclonal antibody directed against the hemagglutanin (HA) epitope (Covance Co., Berkeley, CA, USA). After washing in PBS containing 0.5% Tween 20, the bound primary antibody was detected with antimouse

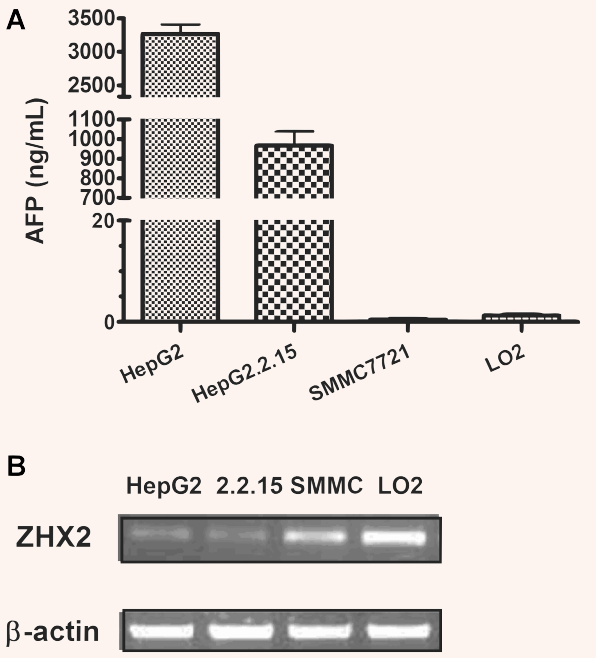

Fig. 1 Alpha-fetoprotein (AFP) secretion and zinc-fingers and homeoboxes 2 (ZHX2) mRNA levels in various human liver cell lines. Analysis was performed in the human hepatoma cell lines HepG2, HepG2.2.15, and SMMC7721 and the immortalized human liver line LO2. (**A**) ELISA analysis of AFP secretion. (**B**) RT-PCR analysis of ZHX2 expression, using oligonucleotides for β-actin to insure that equal amounts of RNA were used from each cell line.

IgG (Zhongshan Co. Beijing, China). Following washes, the antibody-bound protein was visualized by enhanced chemiluminescence.

Luciferase report assays

HepG2 cells were seeded into 24-well plates and transfected with the indicated plasmids using lipofectamine^{TM}2000 (Invitrogen, USA). To monitor ZHX2 inhibition of the AFP promoter, 0.6 μg of phAF269 and increasing doses of pCDNA-ZHX2-HA (0, 0.1, 0.2, 0.3, 0.4μg) were co-transfected into HepG2 cells. For analysis of the role of the HNF1 binding sites, 0.6 μg of pAF269 or mutant AFP promoter reporter plasmids (with or without the CMV enhancer) were co-transfected with 0.3 μg of pCDNA-ZHX2-HA. When necessary, pcDNA3 DNA was included to bring the total amount of DNA in each transfection to 1 μg; 20 ng of pRL-TK plasmid DNA (Promega, USA) was also included in each group to normalize transfection efficiency. Cells were harvested 48 hrs after transfection.

For luciferase assays, cell pellets were re-suspended in 100 μl of lysis buffer (Promega, USA) and lysed for 15 min. at room temperature and stored at −80°C. Dual-luciferase reporter assays were performed in triplicate following the Promega protocol using 20 μl of cell extracts and 100 μl of Luciferase Assay Reagent II Reagent and 100 μl Stop&Glo^{TM} Reagent with the Luminoskan TL plus (Labsystems, Frankfurt, Germany). Firefly luciferase activity was normalized against the renilla reniformis luciferase activity from the co-transfected pRL-TK to control for variations in transfection efficiency. All of the data shown in this study were obtained from at least three independent experiments.

Knockdown of ZHX2 by siRNAs

ZHX2-specific siRNA plasmids were constructed to reduce ZHX2 in cultured cells. Oligonucleotides targeting regions centered on 1674nt and 2360nt of the transcribed region of the human ZHX2 gene, respectively, were designed and synthesized by Allele Biotechnology & Pharmaceuticals, Inc. Guangzhou, China (Details for oligos are available upon request). The oligos were annealed and cloned into BamH I /Hind III sites of pSilencer 3.0 to construct siRNA plasmids ps1674 and ps2360. A third oligo, which has no homology to ZHX2, was synthesized to construct a negative control siRNA plasmid. The efficiency of siRNAs efficiency was verified by cotransfection with the pcDNA-ZHX2-HA expression plasmid in HepG2 cells.

For siRNA knockdown assays, LO2 and SMMC7721 cells were seeded into 24-well plates and transfected with 1μg of ps1674, ps2360 or the control siRNA vector as described above. Fifty-six hours after transfection, supernatants and cells were collected to assay AFP protein and ZHX2 mRNA expression as described above. All transfections were performed three to five times.

Results

Negative correlation between AFP expression and ZHX2 mRNA in human cell lines

AFP is one of the most useful tumuor markers for detecting HCC [8], with serum AFP level of 200 ng/ml or higher showing a specificity of 100% for HCC [9]. Recent studies demonstrated reduced ZHX2 expression in HCC [12, 13]. Furthermore, the ZHX2 gene regulates AFP silencing in the mouse liver after birth [10]. Based on these studies, we investigated whether ZHX2 regulates the human AFP gene. We first analysed AFP protein and ZHX2 mRNA expression by ELISA and RT-PCR, respectively, in a panel of human liver cell lines (Fig. 1). Two hepatoma cell lines with high AFP expression, HepG2 and HepG2.2.15, express low ZHX2 mRNA levels. In contrast, in the other two human liver cell lines that secrete low levels of AFP, hepatoma cell line SMMC7721 and immortalized liver cell line LO2, have comparatively lower ZHX2 mRNA levels. This inverse correlation of AFP secretion and ZHX2 mRNA indicates that ZHX2 could be responsible for the suppression of AFP in human liver cell lines.

ZHX2 overexpression reduces AFP secretion in a dose-dependent manner

To investigate further the role of ZHX2 in AFP regulation, we generated a HA hemagglutinin-tagged human ZHX2 expression vector,

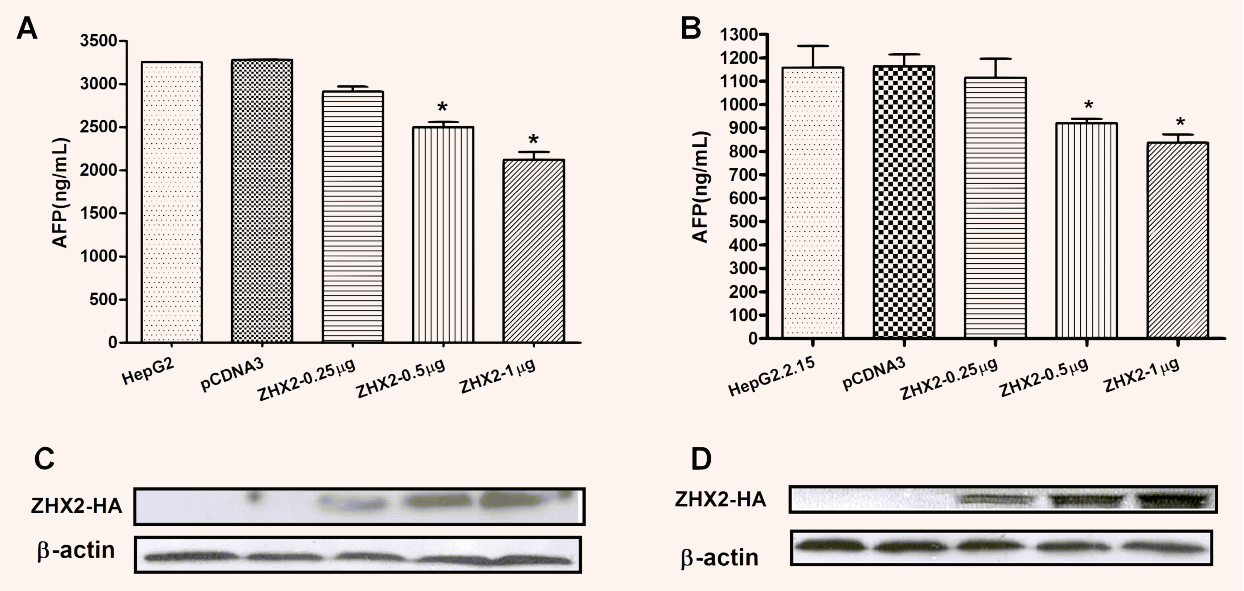

Fig. 2 ZHX2 overexpression reduced AFP secretion in human hepatoma cells. HepG2 (panel **A**) and HepG2.2.15 (panel **B**) were untransfected or transfected with control pcDNA3 vector or increasing doses (0.25, 0.5 or 1.0 μg) of the pcDNA-ZHX2-HA vector. Media was collected 48 hours after transfection and AFP levels were measured by ELISA. A significant decrease (*P<0.01) was seen in both cell lines transfected with 0.5 μg or 1.0 μg of the pcDNA-ZHX2-HA compared to non-transfected controls. Western analysis with anti-HA antibodies confirmed that increasing levels of ZHX2-HA were present in transfected HepG2 and HepG2.2.15 cells (panels **C** and **D**, respectively). Analysis of β-actin confirmed that equal amounts of protein were present in each lane.

pcDNA-ZHX2-HA. Western analysis confirmed the expression of *in vitro* synthesized ZHX2-HA from this vector (data not shown). Increasing doses of pcDNA-ZHX2-HA plasmids were transfected into AFP-expressing HepG2 cells (Fig. 2). AFP levels were essentially the same in untransfected cells and those expressing the control pcDNA3.0 vector. However, increasing pcDNA-ZHX2-HA levels reduced AFP secretion in a dose-dependent manner (Fig. 2A). One microgram of pcDNA-ZHX2-HA plasmid significantly downregulated AFP expression from 3280±13.4 to 2122.333±269.021 ng/mL when compared to empty vector transfected group (P<0.01). Western analysis of transfected cells confirmed the increased ZHX2-HA levels in transfected cells (Fig. 2C). Similar results were obtained AFP-expressing HepG2.2.15 (Fig. 2B and D). These data indicate that forced ZHX2 expression can reduce endogenous AFP expression in two different cell lines.

siRNA-mediated ZHX2 knockdown de-represses AFP synthesis in hepatoma cell lines

To confirm the specificity of ZHX2 repression of AFP in HCC cell lines, we carried out siRNA knockdown experiments. Two siRNA plasmids, targeting the regions centered at 1674nt and 2360nt of human ZHX2 gene (both in exon 3), were constructed and co-transfected with pcDNA-ZHX2-HA into HepG2 cells (Fig. 3). Western analysis confirmed that both siRNAs efficiently suppressed levels of ZHX2-HA whereas the control siRNA had no affect on ZHX2-HA levels (Fig. 3A). These two siRNAs were then transfected into LO2 and SMMC7721 cells, which both exhibit high ZHX2 and low AFP expression (see Fig. 1). Fifty-six hours after transfection, AFP levels increased roughly 1.5-fold in SMMC7721 and 2.8-fold in LO2 cells with either ZXH2 siRNA (Fig. 3B and D). In both cells, the negative control siRNA had no affect on AFP levels. In contrast to AFP, the ZHX2 siRNAs did not change serum Alb levels (Fig. 3B and D); this is expected since Alb is not a target of ZHX2. ps1674 and ps2360 transfection led to 1.5- to 2.8- times more AFP expression than that of untreated cells. On the other hand, negative control siRNA, which has no homology to ZHX2, had no effect on AFP expression. In order to confirm the specific suppression of AFP by the siRNAs, Alb secretion was analysed in the cell supernatant. RT-PCR confirmed the specific reduction of ZHX2 mRNA levels by the transfected siRNAs (Fig. 3C and E). These results provide further evidence that ZHX2 specifically inhibits AFP expression in human hepatoma cell lines.

Human AFP promoter activity can be down-regulated by ZHX2

Previous studies in transgenic mice indicated that ZHX2 regulates AFP through the 250 bp mouse AFP promoter [14].

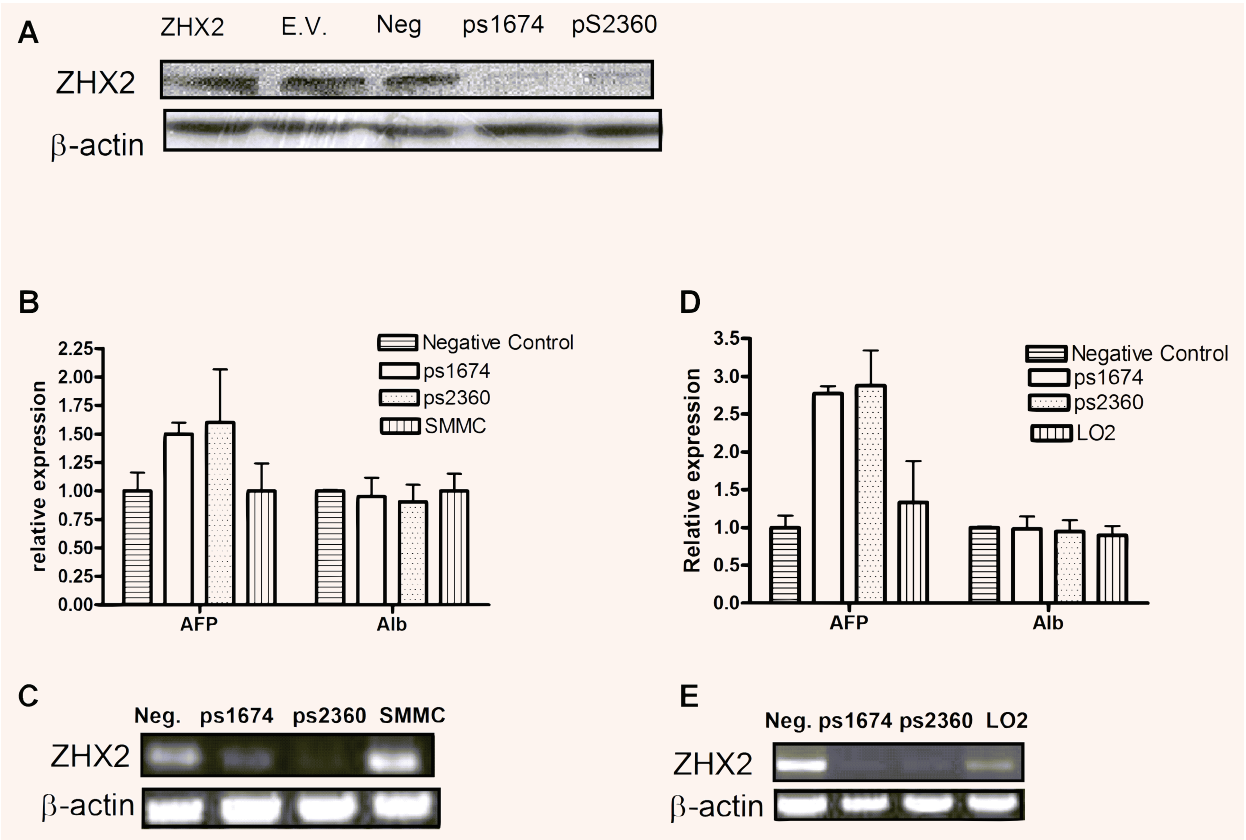

Fig. 3 Knockdown of ZHX2 by siRNA leads to increase AFP secretion. (**A**) HepG2 cells were transfected with pcDNA3-ZHX2-HA alone (ZHX2) or with siRNA empty vector (E.V.), non-specific siRNA (Neg), or siRNAs that target ZHX2 (ps1674 and ps2640). Extracts from transfected cells were prepared after 48 hrs and western analysis was performed with anti-HA antibody; β-actin was used as a loading control. (**B**) SMMC721 cells were untransfected (SMMC) or transfected with non-specific siRNA (negative control), ps1674, or ps2360. ELISA analysis after 56 hrs demonstrates increased AFP secretion in cells transfected with ps1674 and ps2360. Albumin levels were not affected by transfected siRNAs. (**C**) RT-PCR confirmed that ZHX2 mRNA levels were reduced by ps1674 (lane 2) and ps2360 (lane 3) but not by the control siRNA (lane 1) when compared to non-transfected cells (lane 4). The β-actin levels confirmed the equal levels of RNA in the four samples. Data in (**D**) and (**E**) are similar to (**B**) and (**C**) except that experiments were performed in LO2 cells. A significant decrease was seen of ZHX2 mRNA levels. Meanwhile a significant increase was seen of AFP secretion.

Previous studies also showed that AFP gene expression is controlled mainly at the transcriptional level [15]. The regulatory regions that control human AFP gene expression are located roughly within 6 kb of DNA upstream of the AFP transcription start site; these regions, especially the AFP promoter, are conserved between mouse and human [16]. We therefore performed co-transfections to test whether ZHX2 can regulate the human AFP promoter. The phAF269 reporter plasmid, in which the Luciferase gene was linked to the 269-bp human AFP promoter, was co-transfected with increasing doses of the pcDNA-ZHX2-HA plasmids into HepG2 cells. Normalized Luciferase assay results clearly showed that ZHX2 down-regulated human AFP promoter activity in a dose dependant manner in HepG2 cells (Fig 4).

ZHX2 repress AFP expression dependant on HNF-1 binding sites in AFP promoter

Several liver-enriched factors involved in AFP promoter regulation, including Foxa, C/EBP, HNF1, FTF and Nkx2.8, have been identified using biochemical, molecular genetic and tissue culture system [17, 18]. Among them HNF1 is particularly interesting. Genetic analysis showed that hereditary persistence of α-fetoprotein (HPAFP) in several families was due to mutations in either the proximal or distal HNF1 site of the AFP promoter [19–21]. Since this demonstrated a possible role for HNF1 binding in postnatal AFP levels, we analysed whether the HNF1 binding sites in AFP core promoter are involved in ZHX2-mediated repression.

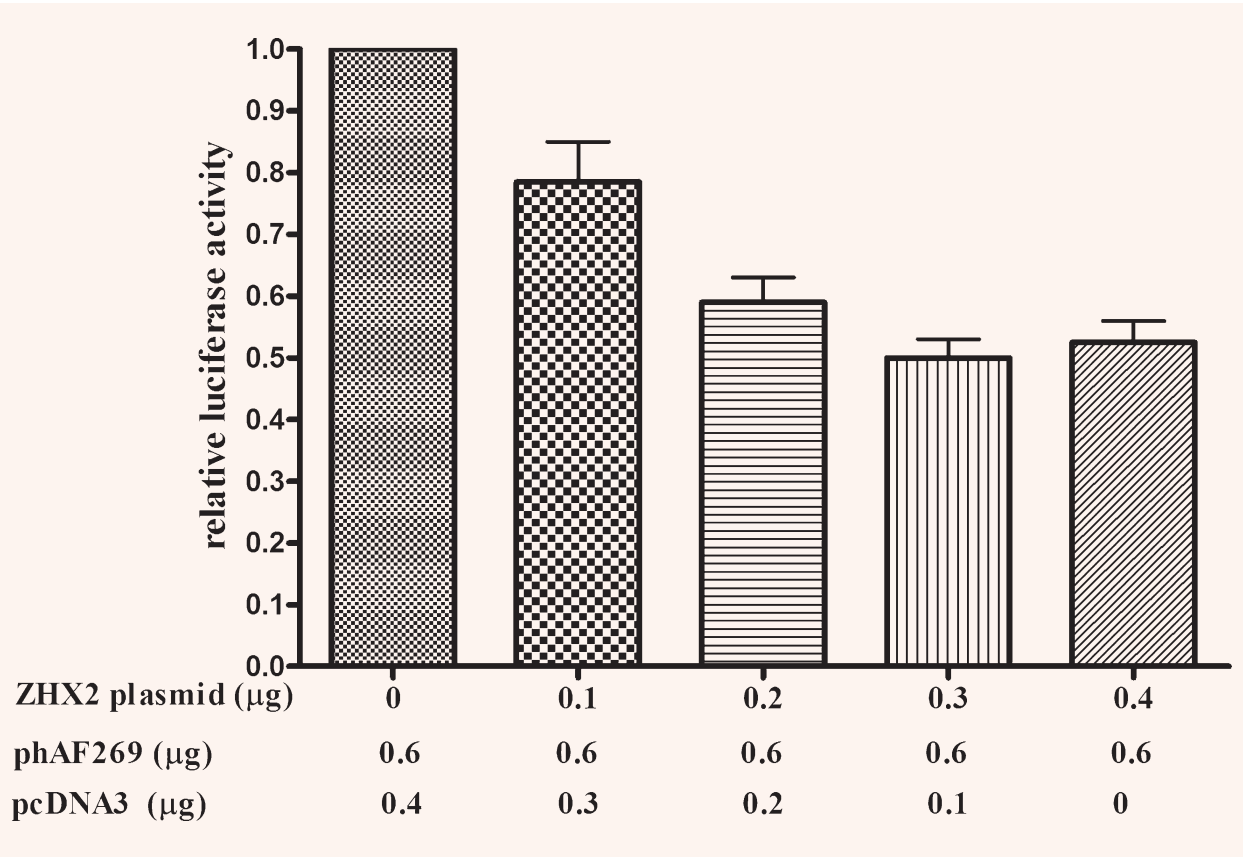

Fig. 4 ZHX2 inhibits the activity of the co-transfected AFP promoter in HepG2 cells. The phAF269 luciferase reporter plasmid was co-transfected with increasing amounts of the pcDNA3-ZHX2-HA (0 μg to 0.4 μg). pcDNA3 was used to bring the total DNA in each transfection to 1.0 μg. After 48 hrs, cells were harvested and assayed for luciferase levels. Luciferase activity was normalized to SV40-Renilla luciferase relative light units (means ± SDs of three separate experiments). Luciferase activity of pAF269 was set to 1.0 when pcDNA3-ZHX2-HA was 0 μg.

To accomplish this, co-transfections were performed with AFP promoter constructs in which one or both the HNF1 sites were mutations (Fig. 5). These promoters were analysed alone or in the presence of the CMV enhancer. In all cases, the lose of either HNF1 site reduced promoter activity, whereas the absence of both sites resulted in a promoter with little, if any, transcriptional activity. Furthermore, the mutated promoter constructs were no longer responsive to ZHX2-mediated repression. These data indicate that HNF1 binding plays an important role in ZHX2-mediated repression of the AFP promoter in HCC cell lines.

Discussion

AFP has served as an important model of liver-specific gene regulation during development and in disease [22]. Since first identified as an oncofoetal protein in 1963, AFP continues to be an important diagnostic marker for liver cancer [8, 23]. In addition to its role as a transporter for various serum ligands including fatty acids, retinoids, steroids, drugs, dyes and heavy metals, AFP has been reported to also exhibit growth regulatory properties [24–26]. For example, recent studies suggest that AFP induced apoptosis in tumour cells and promote the escape of liver cancer cells from immune surveillance, suggesting that AFP reactivation plays a key role during tumourigenesis in the liver [27, 28]. These immunoregulatory and growth regulatory activities make AFP an attractive therapeutic target and emphasize the importance of studying AFP regulation for developing new anticancer therapies.

Mouse studies demonstrated that ZHX2 is involved in postnatal repression [10, 15]. Transgenic studies indicated that ZHX2 acted through the AFP promoter region [14]. The data provided in these papers support the idea that ZHX2 acts to repress AFP at birth. This possibility is supported by data from Kawata *et al.*, who showed that ZHX2 can repress NF-Y-mediated activation of the cdc25C promoter when assayed in *Drosophila*

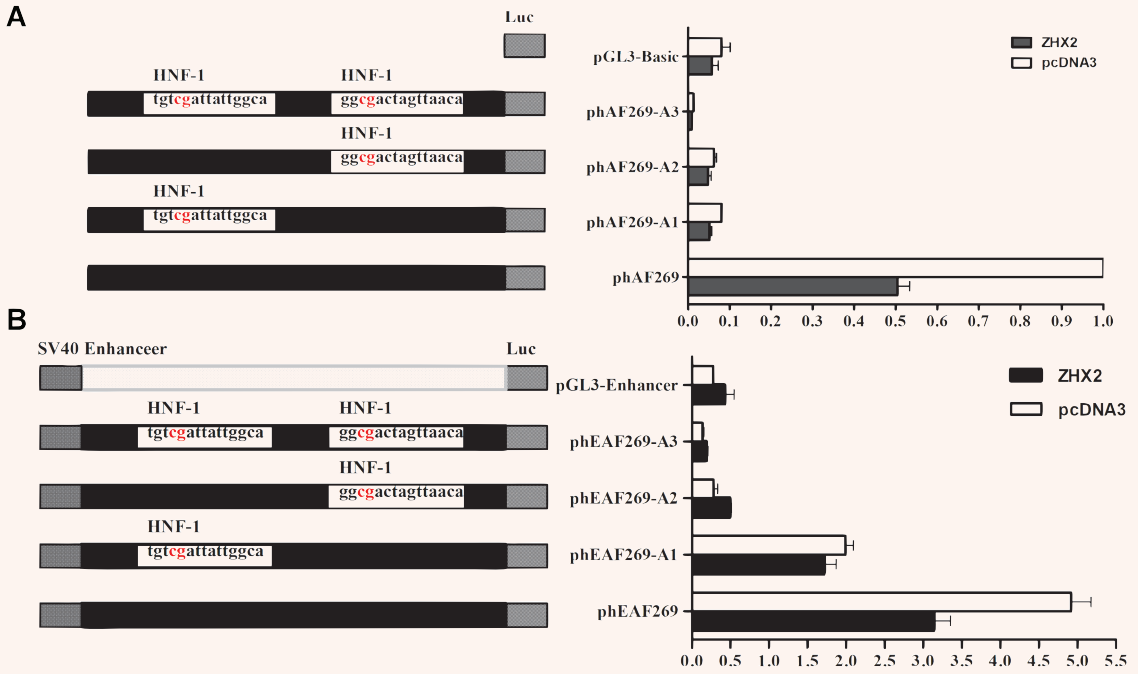

Fig. 5 Mutation of HNF1 sites in AFP promoter relieves ZHX2-mediated repression. AFP promoter luciferase vectors were generated with mutations in the distal, proximal, or both HNF1 sites (as shown on left). These were co-transfected with pcDNA-ZHX2-HA into HepG2 cells and luciferase levels were monitored 48 hours after transfection. AFP promoter activity was monitored in the absence (**A**) or presence (**B**) of the cytomegalovirus (CMV) enhancer region. pGL3-basic and pGL3-Enhancer were used as controls in (**A**) and (**B**), respectively. Luciferase activity was normalized to SV40-Renilla luciferase relative light units (means ± SDs of three separate experiments). And luciferase activity of pAF269 was set to 1.0 when pcDNA3-ZHX2-HA was 0 μg. Transfection of pcDNA3-ZHX2-HA could significantly decrease AFP promoter activity either in the absence (**A**) or presence (**B**) of the CMV enhancer region. However, mutation of HNF-1 binding site demolished the suppression.

cells [3]. However, it is not known if the cdc25C promoter is a natural target of ZHX2. Our co-transfections and siRNA knockdown studies indicate that ZHX2 represses the activity of the AFP promoter, which is known to be a ZHX2 target. Furthermore, our studies were done in human HCC cells, where AFP reactivation frequently occurs. These data we present here provide the first direct evidence that ZHX2 acts on the AFP promoter and indicates that ZHX2 represses AFP in humans, similarly to the repression seen in mice.

By using methylation-sensitive restriction fingerprinting (MSRF), a sensitive assay for differences in methylation between two cell populations, Lu *et al.* demonstrated that a 199 bp CpG island of the ZHX2 gene is often (46.9% of 32 samples) hypermethyated in human HCC samples in which AFP is re-activated [12]. This hypermethylation was associated with a decrease in ZHX2 expression. These data, along with our siRNA knockdown results, indicates that a loss or reduction of ZHX2 results in increased AFP expression.

The AFP promoter contains binding sites for ubiquitous and tissue-specific transcription factors, such as binding sites for HNF-1/NF-1, *nkx*2.8, fetoprotein transcription factor (FTF) and FoxA [29–31]. These sites are conserved among the mouse, rat and human AFP promoters. While the function of these sites have been determined primarily from *in vitro* studies, human studies have indicated a role for HNF1 in AFP expression [32]. These data indicate that several cases of hereditary persistence of AFP, in which AFP is not completely repressed after birth, are due to mutations in the distal or proximal HNF1 sites. These mutations increase binding for HNF1 to these sites and suggest that HNF1 may be involved in postnatal AFP regulation. Here, using reporter plasmids with mutations in the HNF1 sites, we have shown that both the proximal and distal HNF-1 binding sites in AFP core promoter are important to ZHX2 repression on AFP (Fig. 5). Whether this is due to direct interactions between HNF1 and ZHX2 will require further investigation. Previous studies showed ZHX2 can form heterodimer with ZHX1, ZHX3 and NF-YA [3, 7], raising the possibility that ZHX2 might also physically interact with HNF1. Clearly, the detailed mechanism of ZHX2 regulation of AFP promoter activity needs further analysis.

In summary, we have shown that ZHX2 represses AFP expression in human HCC cell lines, and that this involves the

AFP core promoter and requires HNF1 binding sites. A better understanding of how ZHX2 acts to repress transcription and interacts with other factors is needed. Since AFP is frequently reactivated in liver tumours and ZHX2 appears to be silenced in some HCC samples, our studies may provide new insight into the development of HCC and may also provide new targets for drug development.

Acknowledgements

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