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HNF1α and CDX2 Transcriptional Factors Bind to Cadherin-17 (CDH17) Gene Promoter and Modulate Its Expression in Hepatocellular Carcinoma

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ABSTRACT

Cadherin-17 (CDH17) belongs to the cell adhesion cadherin family with a prominent role in tumorigenesis. It is highly expressed in human hepatocellular carcinoma (HCC) and is proposed to be a biomarker and therapeutic molecule for liver malignancy. The present study aims to identify the transcription factors which interact and regulate CDH17 promoter activity that might contribute to the up-regulation of CDH17 gene in human HCC. A 1-kb upstream sequence of CDH17 gene was cloned and the promoter activity was studied by luciferase reporter assay. By bioinformatics analysis, deletion and mutation assays, and chromatin immunoprecipitation studies, we identified hepatic nuclear factor 1α (HNF1α) and caudal-related homeobox 2 (CDX2) binding sites at the proximal promoter region which modulate the CDH17 promoter activities in two HCC cell lines (Hep3B and MHCC97L). A consistent down-regulation of CDH17 and the two transcriptional activators (HNF1α and CDX2) expression was found in the liver of mouse during development, as well as in human liver cancer cells with less metastatic potential. Suppression of HNF1α and CDX2 expression by small interfering RNA (siRNA) significantly down-regulated expressions of CDH17 and its downstream target cyclin D1 and the viability of HCC cells in vitro. In summary, we identified the minimal promoter region of CDH17 that is regulated by HNF1α and CDX2 transcriptional factors. The present findings enhance our understanding on the regulatory mechanisms of CDH17 oncogene in HCC, and may shed new insights into targeting CDH17 expression as potential therapeutic intervention for cancer treatment. J. Cell. Biochem. 111: 618–626, 2010.

KEY WORDS: CDH17; HEPATOCELLULAR CARCINOMAS; HEPATIC NUCLEAR FACTOR 1α; CAUDAL-RELATED HOMEOBOX 2; PROMOTER

Hepatocellular carcinoma (HCC) ranks fifth as the most common malignancy worldwide [Parkin et al., 2005; Hao et al., 2009]. HCC patients usually have poor prognosis largely because of lacking accurate biomarkers, ineffective treatments, and aggressive nature of the tumor, making HCC as one of the most common causes of cancer-related death. In view of this clinical situation, identifying biomarkers for early cancer detection and developing new treatment approaches are two long-term goals in improving patient management of HCC.

Cadherin-17 (CDH17) or liver intestine-cadherin (LI-cadherin) is a member of non-classical cadherin family [Jung et al., 2004], implicating its functional role in cell adhesion. Yet, CDH17 was initially identified as a proton-dependent peptide transporter-1 in human colon adenocarcinoma Caco-2 cells [Dantzig et al., 1994]. CDH17 is over-expressed in gastric metaplasia and gastric carcinoma associated with lymph node metastasis [Grotzinger et al., 2001; Ko et al., 2004, 2005; Kaposi-Novak et al., 2006], but down-regulated in pancreatic carcinoma [Takamura et al., 2003]. It is also a useful immunohistochemical marker for diagnosis of adenocarcinomas of the digestive system [Su et al., 2008]. Using oncoproteomics approach [Sun et al., 2007], our group has recently identified cadherin-17 (CDH17) as a biomarker and therapeutic

Rui Zhu and Kwong-Fai Wong contributed equally.

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molecule for HCC [Wong et al., 2003; Wang et al., 2005; Liu et al., 2009; Hutchinson, 2010]. Over-expression of CDH17 is frequently detected in ~80% HCC cases, but the corresponding regulatory mechanisms remain largely unclear [Lee et al., 2010].

Recently, caudal-related homeobox 2 (CDX2) was found to play important roles in up-regulation of CDH17 expression in colorectal cancer cell lines and gastric adenocarcinoma [Hinoi et al., 2002; Ko et al., 2005; Ge et al., 2008], which was in turn regulated by bone morphogenetic protein 2 (BMP2) and BMP4 in gastric cancer cells [Barros et al., 2008]. Hepatic nuclear factor 1 (HNF1) is one of the key transcriptional factors (TF) that regulate serum α-fetoprotein levels in hepatocellular carcinoma [Nakabayashi et al., 2004]. It is predominantly expressed in liver and kidney, and binds to the promoters of a variety of genes that are expressed exclusively in the liver including fibrinogen-α and -β, albumin, α-1-antitrypsin, liver-type pyruvate kinase, transthyretin, aldolase B, and hepatitis B virus large surface protein [Courtois et al., 1987; Xu et al., 2001]. In addition, over-expression of HNF1α was found in well-differentiated HCC than in the surrounding non-HCC tissues [Wang et al., 1998].

In this study, we aimed to dissect the regulation of CDH17 expression in HCC and in mouse liver during development, and characterize two transcription factors (HNF1α and CDX2) on CDH17 expression. The specific regulation of these TFs was further confirmed by siRNA approach in HCC cells.

MATERIALS AND METHODS

CELL CULTURE AND MOUSE LIVER

Human HCC cells (MHCC97L and Hep3B) and immortalized normal hepatocytes (MIHA) were cultured in Dulbecco's minimal essential medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 50 U/ml penicillin, 50 U/ml streptomycin, and 2 mM L-glutamine at 37°C and 5% CO_2 as previously reported [Wong et al., 2003; Yi et al., 2008; Liu et al., 2009]. The protocol for using animals in this study was approved by the Committee on the Use of Live Animals for Teaching and Research (CULATR), The University of Hong Kong. Mouse livers from different developmental stages, embryonic day 13.5 (E13.5), E16.5, new-born (NB), 3-week, and adult, were isolated [Lee et al., 2008].

CONSTRUCTION OF THE WILD-TYPE AND MUTANT PROMOTER REGION OF CDH17 GENE

The NCBI Map Viewer (http://www.ncbi.nlm.nih.gov/mapview) was used to predict the promoter region of human *CDH17* (NCBI GeneID: 1015, NC_000008.10) and region flanking −909 and +55 bp was chosen. Two bioinformatics programs, ALGGEN-PROMO (http://alggen.lsi.upc.es) and MatInspector (http://www.genomatix.de) were used to identify potential transcription factors at the −909/+55 promoter region and the results were compared to the transcription factor database TRANSFAC (version 8.3). Polymerase chain reaction (PCR) was employed to amplify this region using forward (5′-TAG AGA GTG GGC TGG GCT C-3′) and reverse (5′-TGG TCG AGA CTC TTG CTA CG-3′) primers. The CDH17 gene promoter was cloned by directional restriction cloning method [Luk et al., 2003, 2004]. Five deletion constructs (−686/+55, −332/+55, −100/+55, −81/+55, and −40/+55) were generated by PCR cloning with the following forward primers (5′-GAA GCC TTG ACT TGA GAA AT-3′, 5′-TGA TCA AGT CTC CTG TGC T-3′, 5′-GAC ACT TTT TAT GAT ACC CA-3′, 5′-AGT GGC TCT CGA AGA GCA AT-3′, and 5′-TTT GAC TGA AGC TGA AGG G-3′), respectively. The wild-type (wt) and mutant promoter constructs were cloned into pGL3-Basic vector by *Xho*I/*Kpn*I restriction digestions. For site-directed mutagenesis, mutated promoter constructs were generated using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). All deletion and point-mutation constructs were confirmed by DNA sequencing.

LUCIFERASE REPORTER ASSAY

Assayed cells were grown to about 90% confluence in a 24-well tissue culture plate. Cells were then transfected with 1 μg purified plasmid, 0.1 μg pRL-TK vector as internal control reporter in serum-free medium using Lipofectamine 2000 reagent (Invitrogen) as previously described [Liu et al., 2009]. After 48 h, the promoter activity was assessed using Dual-Luciferase reporter assay system (Promega, Madison, WI) by measuring the intensity of chemiluminescence in a luminometer (Thermo, Waltham, MA). All experiments were performed in duplicate and repeated at least three times with positive (pGL3-Control, Promega) and negative (pGL3-Basic, Promega) controls.

WESTERN BLOTTING

Western blotting was performed using antibodies against CDH17 (Santa Cruz Biotechnology, Santa Cruz, CA), HNF1α (Santa Cruz Biotechnology), HNF1β (Santa Cruz Biotechnology), CDX2 (Millipore, Billerica, MA), and β-actin (Sigma, St Louis, MO) as previously described [Yi et al., 2008; Lee et al., 2009]. In brief, cells were washed twice with ice-cold phosphate buffered saline (PBS) and then incubated in the lysis buffer (50 mM Tris–HCl pH 8, 150 mM NaCl, 1% NP-40) on ice for 30 min. After ultracentrifugation at 4°C for 15 min at 14,000*g*, the lysate supernatant (30 μg per lane) was resolved on a 10% SDS–PAGE by electrophoresis. Proteins on the gel were then transferred onto a polyvinylidene fluoride membrane (0.4-μm pore size; Millipore) which was blocked with Tris-buffered saline containing 0.1% Tween 20 (TBST) and 5% non-fat milk at 4°C overnight [Lee et al., 2004]. After washing three times with TBST, the membrane was incubated with the above-mentioned primary antibodies (1:500 in TBST containing 5% non-fat milk) at RT for 1 h. Bound antibodies were detected by horseradish peroxidase (HRP)-conjugated secondary antibodies (1:20,000 dilution) and ECL reagent (Amersham Biosciences, Pittsburgh, PA). The immunoreactive signals were developed on Kodak® Biomax™ MS film.

QUANTITATIVE POLYMERASE CHAIN REACTION (QPCR)

Gene expression of CDH17, CDX2, HNF1α, and cyclin D1 was measured using qPCR that was performed as previously described [Yu et al., 2005]. A 5 μl aliquot of 1:10 diluted cDNA was mixed with 10 μl SYBR Green qPCR superMix (Invitrogen) and 0.5 μM of the designated primers. The thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. All reactions were done at least twice and the products were analyzed using the ABI Prism 7700 detection system (Applied Biosystems Inc.). Ribosomal 18S RNA was included in all

TABLE I. Primers Used for Real-Time PCR for Detection of Transcripts in Human Liver Cells and Mouse Liver Tissues

Primer name		Sequence	Product size (bp)
Human			
CDH17	Forward	5′-GGGGGAGATACTCCAGTCGT-3′	167
	Reverse	5′-TCCAGTTGCCAAATAAAGCA-3′	
CDX2	Forward	5′-GAACCTGTGCGAGTGGATG-3′	158
	Reverse	5′-TCCTCCGGATGGTGATGTAG-3′	
HNF1α	Forward	5′-CCATCCTCAAAGAGCTGGAG-3′	173
	Reverse	5′-CTGGTTGAGGCCAGTGGTAT-3′	
Cyclin D1	Forward	5′-CGTGCCCGTGTGCATGTCCT-3′	120
	Reverse	5′-GTGGCCTTTCCCGACCCTGC-3′	
Mouse			
CDH17	Forward	5′-GGCCAAGAACCGAGTCAAGTC-3′	190
	Reverse	5′-CTCCATGAGAATCCAAGGCTG-3′	
CDX2	Forward	5′-CAAGGACGTGAGCATGTATCC-3′	106
	Reverse	5′-GTAACCACCGTAGTCCGGGTA-3′	
HNF1α	Forward	5′-GACCTGACCGAGTTGCCTAAT-3′	103
	Reverse	5′-CCGGCTCTTTCAGAATGGGT-3′	

reactions for internal house-keeping control and normalization. All of the primers used for qPCR are showed in Table I.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Nuclear protein extracts from MHCC97L and Hep3B cells were prepared as described previously [Luk et al., 2004; Wong et al., 2007] and the protein concentration was estimated using the Bradford protein assay kit (Bio-Rad, Hercules, CA). Double-strand oligonucleotide probes were synthesized with annealing buffer (200 mM Tris–HCl; pH 9.0, 40 mM $MgCl_2$, 1 M NaCl, 20 mM EDTA) and labeled with [γ-^{32}P] ATP (PerkinElmer, Waltham, MA) using the Ready-To-Go T4 Polynucleotide Kinase kit (Amersham Biosciences) with T4 polynucleotide kinase. Unincorporated nucleotides were removed by spin column in a Tris–EDTA buffer, pH 8.0. An aliquot (10 μg) of nuclear extract was incubated with or without 1 μl unlabeled oligonucleotide probes or the previously described antibodies in 1× binding buffer [4% glycerol, 1 μg poly(dI–dC).(dI–dC), 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, and 0.5 mM DTT] in a 10 μl reaction volume for 20 min at room temperature. Radiolabeled probe was added to each reaction tube and the tubes were incubated for a further 20 min at room temperature. The reaction products were then resolved on a 5% non-denaturing polyacrylamide gel. The gels were dried under vacuum and autoradiographed on Kodak® Biomax™ MS films overnight at −70°C.

CHROMATIN IMMUNOPRECIPITATION (CHIP) ASSAY

Standard ChIP assays were performed with Millipore ChIP Assay kit according to the manufacturer's instructions. Briefly, approximately 1×10^6 cells were cross-linked using 1% formaldehyde at 37°C for 10 min and resuspended in 200 μl SDS lysis buffer containing 1× protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) and 10 mM of DTT. The cells were then sonicated (Sonic Dismembrator Model 100, Fisher Scientific, Waltham, MA) and the lysate was centrifuged at 4°C for 15 min at 14,000*g*. The resultant supernatant was diluted 1:10 with a dilution buffer containing a protease inhibitor. Twenty microliters of the diluted supernatant was collected as input control prior to adding the antibodies. After pre-cleared with 75 μl of salmon sperm DNA/protein A agarose, the immune complexes were incubated at 4°C overnight with either 2 μg of primary antibodies or IgG control, and then recovered with 60 μl of salmon sperm DNA/protein A agarose. Precipitates were washed and incubated at room temperature for 15 min with 250 μl of elution buffer containing 1% SDS and 100 mM sodium bicarbonate. The eluate was reverse cross-linked by heating at 65°C for 4 h with 200 mM NaCl and 200 mg/ml proteinase K. The DNA was recovered and amplified by PCR with forward primer 5′-GTG CTA AGT GTT GGG GGT ACA A-3′ and reverse primer 5′-TTG ACT GAA GCT GAA GGG AGA G-3′. PCR was carried out at 94°C for 5 min and then 30 cycles at 94°C for 40 s, 60°C for 1 min, and 72°C for 1 min, and a final extension step at 72°C for 10 min in a final volume of 25 μl.

SUPPRESSION OF HNF1α AND CDX2 USING SIRNA

Small interfering RNA (siRNA) was used to suppress HNF1α and CDX2 in HCC cells (MHCC97L). RNA oligonucleotides were designed according to the published sequences [Hinoi et al., 2005; Song et al., 2006]. HCC cells were transfected with the designated siRNA duplex or scramble control using Lipofectamine 2000 (Invitrogen) as described above. After 72-h incubation, the efficacy of gene silencing was evaluated by real-time PCR and Western blotting analysis as described above.

MTT ASSAY

To perform MTT assay, 1.5×10^3 MHCC97L cells was seeded into 96-well plates with DMEM medium (Invitrogen). Cells were then transfected with the designated siRNA duplex or scramble control as above. At 24 and 48 h after transfection, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added, and the resulting signals were measured by a 96-well plate reader (BioRad).

STATISTICAL ANALYSIS

All results are represented as mean ± SD. Statistical comparisons were performed by one-way ANOVA or paired Student's *t*-test using the SPSS statistical software package version 13.0 for Window (SPSS Inc., Chicago, IL) and $P < 0.05$ was considered to be statistically significant.

RESULTS

CLONING AND CHARACTERIZATION OF CDH17 GENE PROMOTER

CDH17 promoter region was amplified by PCR and cloned into pGL3-Basic reporter vector. Figure 1A show the positions of the primer sequences used to generate the parental fragment and five deletion constructs. The human CDH17 gene promoter has a conserved CAAT box at −65/−62 position, but lacks the TATA box, an initiator element in the predominant control region of typical eukaryotic genes [Suzuki et al., 2001]. Two putative transcription factors, HNF1 and CDX2 were identified using ALGGEN-PROMO and MatInspector. At this stage, the binding of HNF1α from HNF1β cannot be differentiated as they shared similar binding sequences. Based on the computational analysis, two putative binding sites for HNF1 (one half-site and one palidromic sequence) and three for CDX2 were identified in the CDH17 promoter, respectively.

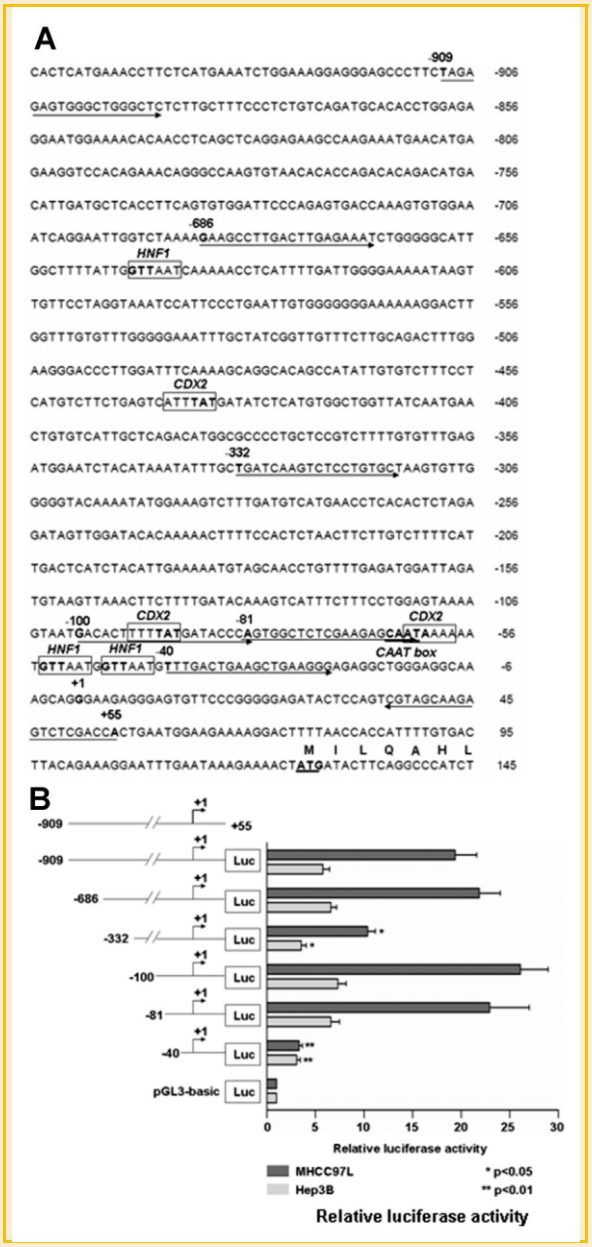

Fig. 1. Cloning and characterization of CDH17 gene promoter. A: The nucleotide sequences of the 5′-flanking region of human CDH17 gene and the putative transcriptional regulatory elements from −955 to +145 were depicted. The G at position +1 indicates the transcriptional initiation site based on the GenBank database. The translation start site (ATG) is underlined and the amino acid sequences are indicated by bold and capital letters above nucleotide sequences. The primer sequences used for generating the parental fragment and the five deletion constructs were marked with arrows indicating the forward and reverse primers. B: The promoter activity of CDH17 deletion constructs in Hep3B and MHCC97L cells was demonstrated. The pGL3-332/+55Luc and pGL3-40/+55Luc constructs exhibited drastic reduction in luciferase activity ($^{*}P<0.05$ and $^{**}P<0.01$, respectively) when compared with the control fragment (pGL3-909/+55Luc), whereas pGL3-686/+55Luc, pGL3-100/+55Luc and pGL3-81/+55Luc showed comparable luciferase activities with the control. The luciferase activity of the empty expression vector pGL3-Basic was assigned a value of 1 and the relative activity of each construct was normalized with the pRL-TK expression level. Values are represented as mean ± SD from at least three independent experiments.

Figure 1B shows the luciferase reporter activities of different promoter deletion constructs when compared to the parental (−909/+55) control in two different HCC cell lines: MHCC97L (CDH17^{high}) and Hep3B (CDH17^{low}). HNF1α and CDX2 were demonstrated to be two critical factors for CDH17 transcription as there were significantly reduced luciferase activities from the deletion constructs pGL3-332/+55Luc and pGL3-40/+55Luc. Both cell lines exhibited similar activity patterns although the signals were generally stronger in the MHCC97L cell line.

To confirm the functional roles of these TF, point mutations were introduced at the TF core-binding sites in the CDH17 promoter sequences. Reporter activities were markedly reduced for HNF1 mutant (−47/−45, $P<0.01$), followed by CDX2 mutant (−91/−89, $P<0.05$; Fig. 2). Mutations on other HNF1 and CDX2 binding sites did not drastically reduce promoter activities. Double mutations of the HNF1 (−47/−45) and CDX2 (−91/−89) binding sites reduced the CDH17 reporter activity near to basal level.

BINDING OF CDX2 AND HNF1α TO CDH17 PROMOTER IN VITRO AND IN VIVO

The physical bindings of HNF1 and CDX2 to the CDH17 gene promoter in MHCC97L and Hep3B cells were studied by EMSA and ChIP assays. The wt probes for CDX2 spanned from −103 to −79 since it covered CDX2 critical binding sites. Similarly, we chose −56 to −30 for synthesizing HNF1α probes. For the mutant (mut) counterparts, we used probes with mutation of −91 to −89 for CDX2 and −47 to −45 for HNF1α, respectively. The formation of DNA–protein complex can be clearly detected in EMSA (Fig. 3A). Furthermore, addition of 10- and 50-fold excess of ''cold'' wt probes, but not the mutant probes could displace or compete with the binding. Moreover, CDX2, HNF1α, but not HNF1β antibodies were able to retard the migration of the DNA–protein complex, suggesting the binding of the nuclear extract is specific. In addition, ChIP assay was performed to study DNA–protein interactions in vivo. The primers we used were spanning from −318 to −18 of CDH17 promoter, which did not contain HNF1α and CDX2 sequences in terms of primers themselves. After immunoprecipitation by designated antibodies, the recovered DNA was amplified by PCR. The addition of HNF1α and CDX2, but not HNF1β and IgG control antibodies, were able to pull down DNA–protein complexes and resulted in positive signals (Fig. 3B).

DIFFERENTIAL EXPRESSION OF CDH17, HNF1, AND CDX2 IN HCC CELLS AND FETAL MOUSE LIVER

Luciferase reporter assays revealed differential promoter activities in Hep3B and MHCC97L cell lines (Figs. 1B and 2). To study if the endogenous CDH17 transcript level correlates with HNF1α and CDX2 expression levels, we determined the expression of these transcripts in HCC cells and mouse livers at different developmental stages. MHCC97L cells which has a high-metastatic potential also expressed a high level of CDH17 transcript as well as CDX2 and HNF1α transcripts; whereas low-metastatic potential HCC cells (Hep3B), and MIHA cells expressed low or undetectable levels of the gene (Fig. 4A). Since CDH17 plays important roles during embryonic liver development, the expression of CDH17 and TFs levels in mouse

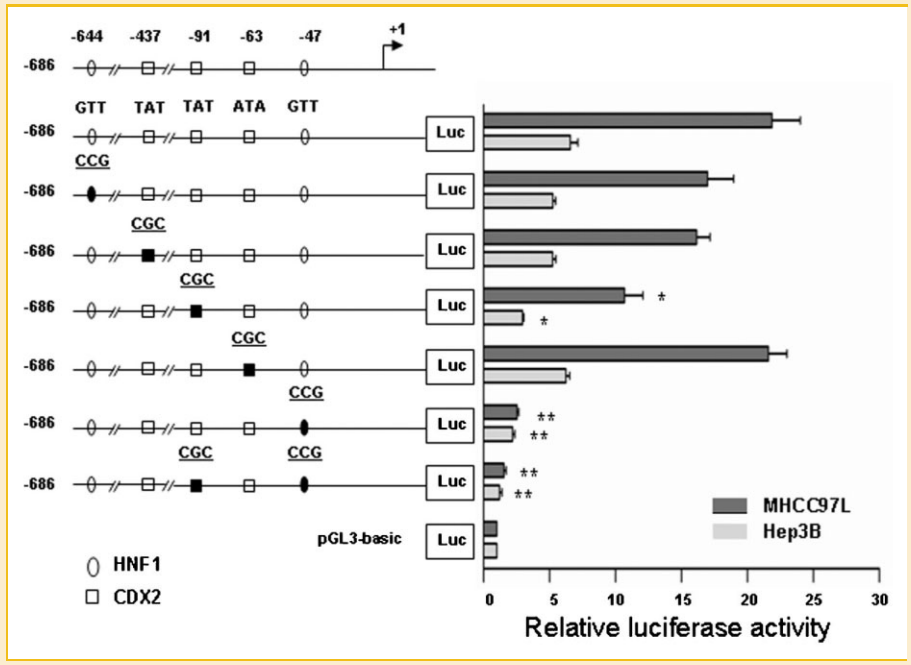

Fig. 2. Mutation of transcriptional factor binding sites on CDH17 promoter. The CDH17 promoter activities after mutating HNF1 and CDX2 binding sites were studied in Hep3B and MHCC97L cells. The HNF1 (GTTAAT) and CDX2 (ATTTAT and TTTTAT, and ATAAAA in forward and reverse orientation, respectively) binding sites on the CDH17 promoter were displayed on top, followed by the mutant HNF1 (filled oval, CCGAAT) and/or CDX2 (filled rectangle, ATTCGC and TTTCGC, and CGCAAA, respectively) binding sites. Mutation at CDX2 (−91/−89 position) and/or HNF1 (−47/−45 position) binding site(s) significantly reduced the luciferase activity ($^{*}P < 0.05$ and $^{**}P < 0.01$, respectively) when compared with the wild-type control. Combined mutation of HNF1 and CDX2 sites resulted in significant reduction ($^{**}P < 0.01$) in luciferase activity, but comparable to single HNF1 mutation at −47/−45 position. Values are expressed as mean ± SD of at least three independent experiments.

livers at different development stages (E13.5, E16.5, NB, 3-week, and adult) were measured. We found that CDH17 expressed abundantly in the early embryonic mouse livers, but its expression becomes undetectable in late-fetal stages and adult livers (Fig. 4A). Similar to the human HCC findings, the differential expression of CDH17 transcript in mouse livers largely correlated with the patterns of HNF1α and CDX2 expressions. This result was supported by Western blotting analysis of the CDH17, HNF1α, and CDX2 protein levels (Fig. 4B). Interestingly, the decreases of CDH17 and HNF1α were more drastic in both human HCC cells and mouse livers than the CDX2. In addition, by using ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html), we found these TF-binding sites on CDH17 promoter among mouse, rat, and human are highly conserved (data not shown), suggesting a conserved regulatory mechanism of CDH17 expression among different animal species.

KNOCKING DOWN CDX2 AND HNF1α SUPPRESSED CDH17 EXPRESSION AND REDUCED CELL PROLIFERATION AND THE WNT PATHWAY ACTIVITY OF MHCC97L CELLS

To further demonstrate HNF1α and CDX2 can regulate CDH17 expression, we used siRNA approach to knockdown HNF1α and CDX2 expression and observed the expression of CDH17 in MHCC97L cells (Fig. 5). RNAi-mediated targeting HNF1α or CDX2 lowered the mRNA level of the TFs (HNF1α or CDX2), when compared with the vehicle or scramble control. These down-regulations were accompanied with approximate 60% reduction of CDH17 transcript level in MHCC97L cells ($P < 0.05$). An additive effect of combined HNF1α and CDX2 RNAi treatment on CDH17 suppression was also observed, indicating a coordinate regulation of HNF1α and CDX2 in the cells (Fig. 5A). Knock-down expression of CDX2, HNF1α, and CDH17 by siRNA could significantly suppress the mRNA and protein levels of the respective molecules (Fig. 5B). To address the question of whether these changes could alter the cancer behaviors, we measured the cell proliferation activity by MTT assay after siRNA treatments and the subsequent effect on cyclin D1 transcript level, which is a down-stream target of the CDH17-mediated Wnt signaling pathway in MHCC97L. As shown in Figure 5C, knocking down the expression of CDX2 and HNF1α transcriptional activators could reduce the expression of cyclin D1 (Fig. 5C) and diminished cell viability by 40 to 60% ($P < 0.001$) at 24 and 48 h following the siRNA treatments (Fig. 5D).

DISCUSSION

Liver–intestine cadherin or CDH17 is expressed in the rat liver and intestinal epithelial cells [Angres et al., 2001], as well as in the embryonic mouse liver, but no CDH17 expression was detected in normal liver tissue of mature humans and mice [Takamura et al., 2003]. Physiological functions of CDH17 include the maintenance of tissue integrity, tissue growth and differentiation [Berndorff et al., 1994]. Nevertheless, aberrant expression of CDH17 mRNA and protein was found in upper gastrointestinal malignancies

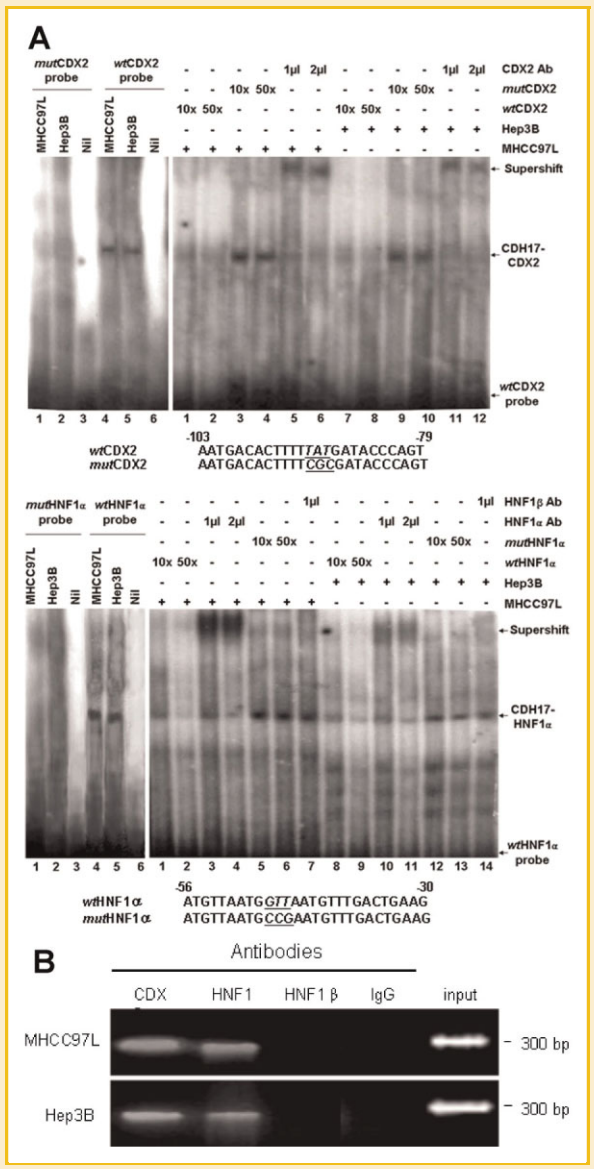

Fig. 3. Binding of HNF1 and CDX2 on CDH17 promoter sequences in vitro and in vivo. A: Radiolabeled wild-type CDX2 and HNF1 probes were incubated with Hep3B and MHCC97L nuclear extracts and then resolved on a 5% polyacrylamide gel. The presence of DNA–protein (either CDX2 or HNF1α) complexes in the middle of the gel suggested binding of transcriptional factor with DNA in vitro. Unbound probes migrated to the bottom of gels. Addition of excessive (10× and 50×) unlabeled wild-type (wt), but not mutant (mut) probes reduced the signal intensity. While the presence of specific antibody binds to the protein–DNA complex further retarded the migration of the complex in the gel (supershift). The sequences of the probes were shown at the bottom of the gels. The wild-type and mutant sequences were underlined for comparison. B: ChIP assay was used to detect the presence of DNA–protein (CDX2 and HNF1α) complexes in MHCC97L and Hep3B cells in vivo. Antibodies against CDX2, HNF1α, HNF1β, and IgG control, were used to immunoprecipitate the DNA–protein complexes. After recovery, the purified DNA was amplified by CDH17 promoter-specific primers. Input control genomic DNA was extracted from cross-linked MHCC97L and Hep3B cells prior addition of antibodies for immunoprecipitation.

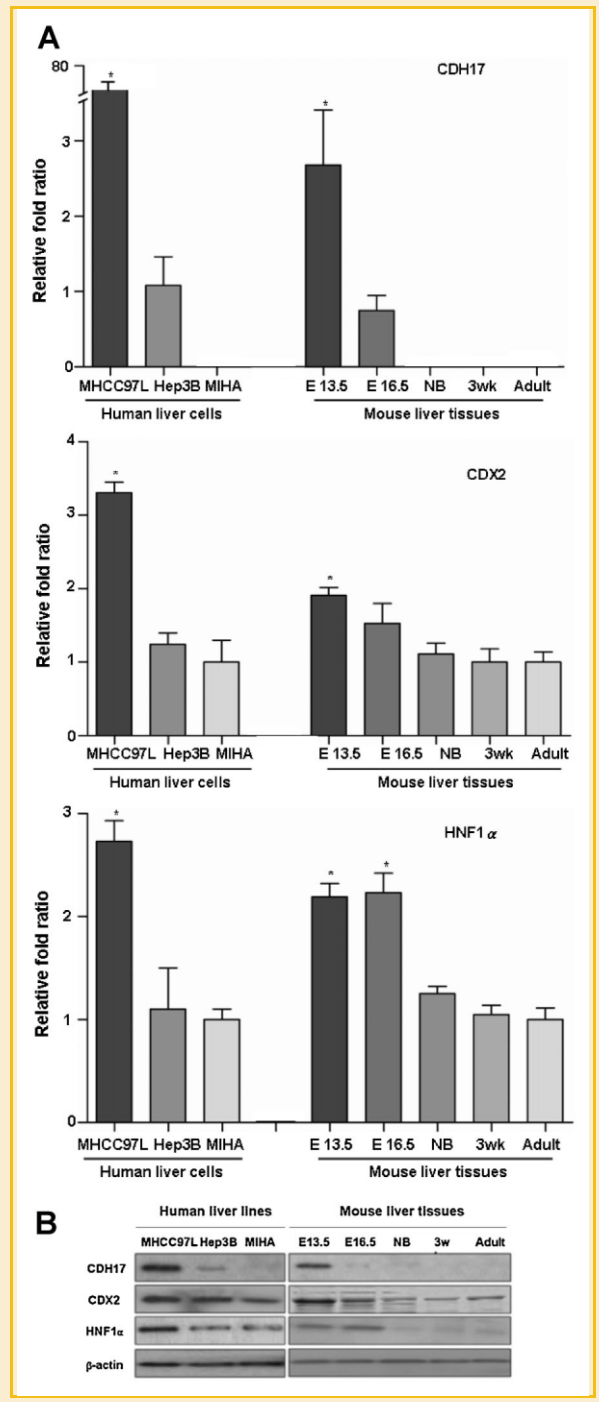

Fig. 4. Expression of CDH17, HNF1 and CDX2 transcripts in human HCC cells and mouse liver. A: Quantitative RT-PCR was used to quantify the expression of CDH17, HNF1α, and CDX2 transcripts. The expression of CDH17, HNF1α, and CDX2 transcripts were higher in human MHCC97L and lower in Hep3B and MIHA cells; while gradually decreases from E13.5, E16.5, new born (NB), 3-week old, and adult mouse livers. B: Western blotting confirmed a higher expression level of CDH17, HNF1α, and CDX2 proteins in MHCC97L cells. A lower expression level was found in Hep3B and MIHA cells. The protein expression levels of CDH17 and HNF1α decreased drastically in mouse fetal liver from E13.5 until born. The expression of CDX2 protein showed less prominent reduction through development. The expression of β-actin protein was used as loading control.

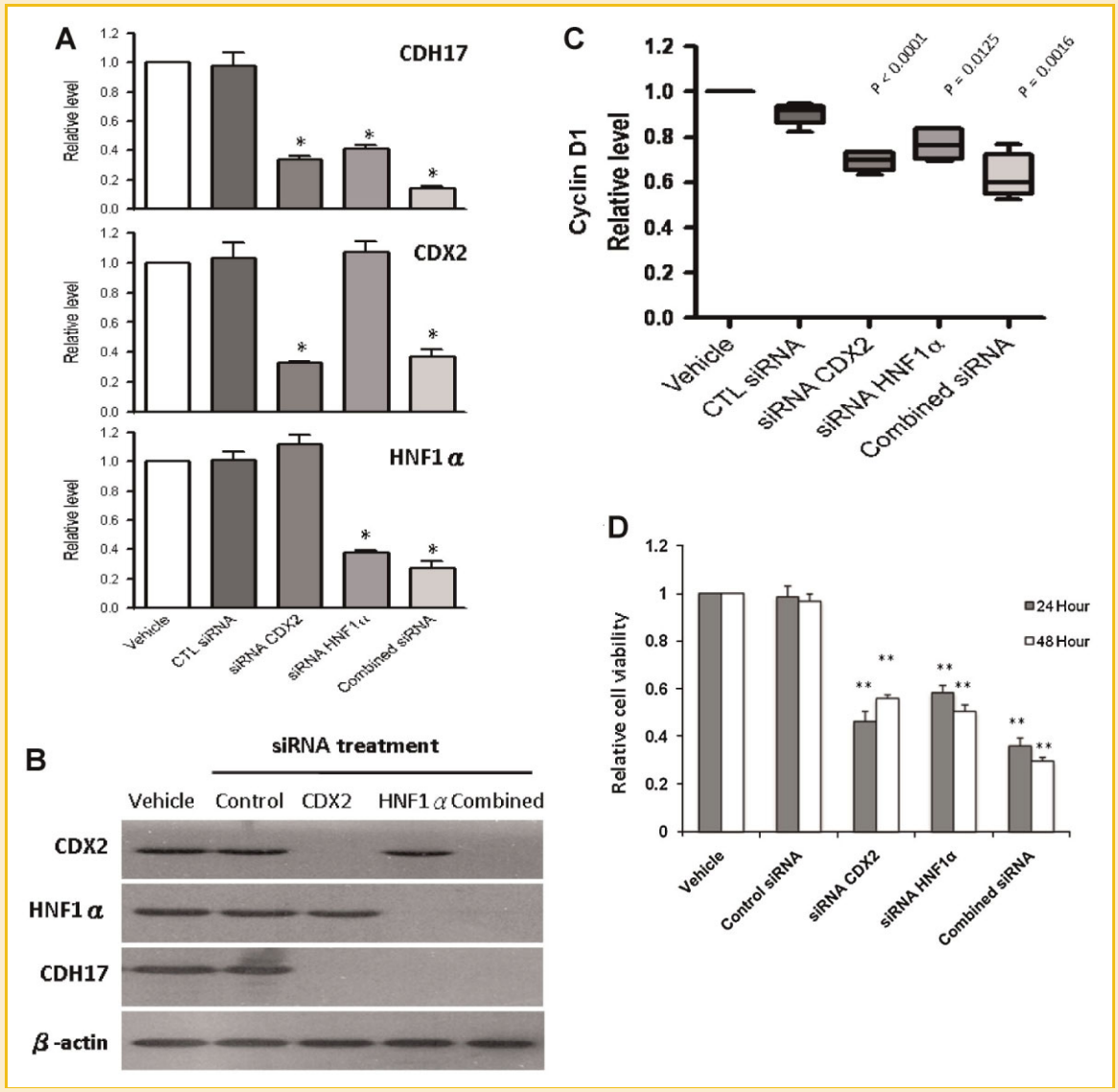

Fig. 5. siRNA-mediated knock-down of HNF1α and CDX2 in MHCC97L cells. A: siRNA oligonucleotides targeted against HNF1α and CDX2 transcripts were transfected into the CDH17 expressing MHCC97L cells. Vehicle (transfection reagent) and control (CTL, scramble siRNA) were included as negative controls in each experiment. The expression of the CDH17, HNF1α, and CDX2 transcripts were measured by real-time RT-PCR. The expression of CDX2 and HNF1α transcripts were significantly reduced by their specific siRNA. Interestingly, either single or combined CDX2 and HNF1α siRNA significantly suppressed CDH17 expression ($^{*}P < 0.05$). Data were shown as mean ± SD and were representative of three independent experiments. B: Western blotting demonstrated that expression of CDX2, HNF1α, and CDH17 protein expression was abrogated after transfection with siRNA against HNF1α, CDX2, or both. C: Expression of cyclin D1 in MHCC97L after siRNA treatment was also determined. Knocking down either CDX2 or HNF1α and both targets could significantly suppress cyclin D1 expression, when compared with that treated with vehicle or control siRNA. Data were shown as mean ± SD and were representative of two independent experiments. D: The relative cell viability of MHCC97L cells transfected with siRNAs was determined by MTT assay. Transfection of CDX2 and/or HNF1α siRNA resulted in a significant reduction in cell viability ($^{**}P < 0.001$) at 24 or 48 h, when compared with that of the controls (vehicle or control siRNA).

including liver cancer. In this study, we demonstrated that the expression of CDH17 is regulated by HNF1α and CDX2 TFs in HCC and mouse liver.

By comparing the promoter activity of pGL3-81/+55Luc with other constructs, the decreased promoter activity of pGL3-40/+55Luc could be due to the deletion of HNF1 and CDX2 binding sites that may regulate CDH17 promoter activity. Yet, mutation of CDX2 at −91, but not −63 position significantly decreases the promoter activity in pGL3-686/+55Luc, confirming CDX2 may play an important role in regulating CDH17 expression. In line with this, mutation of HNF1 site at −47/−45 position drastically suppresses CDH17 expression in MHCC97L and Hep3B cells. Interestingly, the decrease in promoter activity in pGL3-332/+55Luc construct could be explained by the presence of putative repressor elements (e.g., CDP and BCL6 at −166 and −124 position, respectively). Our unpublished data suggested mutation of these repressor sites significantly increase CDH17 promoter activity in vitro. Yet, how these suppressors interact with other TFs in vivo need further investigations.

Furthermore, both EMSA and ChIP assays demonstrated binding of HNF1 and CDX2 to the CDH17 promoter sequences. HNF1α, but

not the HNF1β isoform bound to the CDH17 promoter and the protein–DNA complex was recognized by HNF1α-specific antibody. The temporal expression of HNF1α and CDX2 transcript in relation to CDH17 expression was studied in human liver cell lines and mouse liver tissues. It was found that elevated levels of HNF1α and CDX2 expression were closely associated with a higher expression of CDH17 in metastatic HCC cell line (MHCC97L >> Hep3B > MIHA) as well as in early embryonic mouse liver tissues (E13.5 > E16.5 > NB). The stimulatory effect of CDX2 and HNF1α on CDH17 expression was confirmed by our in vitro findings. Knocking down the TFs by siRNA suppressed CDH17 expression. Of importance, this abrogation on the function of the two TFs reduced cell viability as well as cyclin D1 expression. Cyclin D1 is a key target of CDH17 along the Wnt signaling pathway, and is playing key roles in promoting cancer development [Liu et al., 2009]. All of these findings collectively suggested the modulating effect of CDX2 and HNF1α on CDH17 are contributing to the hepatocellular carcinogenesis.

HNF1 and CDX2 are homeodomain transcription factors that regulate a number of intestine-specific genes including Lewis type 1 antigen synthase (β3Gal-T5) and UDP-glucuronosyltransferases (UGT) [Isshiki et al., 2003; Gregory et al., 2004]. CDX2 regulates CDH17 expression in colorectal cancer cell lines and gastric adenocarcinoma [Hinoi et al., 2002; Ko et al., 2005; Barros et al., 2008; Ge et al., 2008]. In this study, three putative CDX2-binding sites were found in the 1-kb CDH17 promoter region (Fig. 1A) and it is the −91/−89 site that mainly contributed to CDH17 promoter activity and binding in HCC. More importantly, we identified HNF1 binding site that may play a definitive role in regulating CDH17 transcription in human HCC as well as in mouse fetal liver. Although this palindromic sequence contains two HNF1 binding sites in the CDH17 promoter, deletion/mutation studies identified −47/−45 position is crucial in regulating CDH17 expression by site-directed mutagenesis and EMSA (Figs. 1B and 2).

HNF1α and HNF1β are two related TFs that bind to DNA as homo- or heterodimers. They were identified initially as liver-enriched transcription factors that were involved in the expression of several plasma proteins including α1-antitrypsin [Mendel and Crabtree, 1991]. However, no detectable HNF1β transcript and protein was found in the two HCC cell lines studied (data not shown), and HNF1β failed to bind to CDH17 promoter DNA sequence in EMSA or ChIP assays. These findings suggest that HNF1α, but not HNF1β is associated with the regulation of the CDH17 gene in HCC. Interestingly, HNF1β has recently been demonstrated to direct the expression of kidney-specific cadherin (cadherin-16) [Bai et al., 2002].

In conclusion, the present study provides the first evidence that HNF1α plays a critical role, together with CDX2, on the regulation of CDH17 gene expression in HCC as well as in early stages of fetal liver development in mice. Clinically, over-expression of CDH17 in HCC is associated with poor clinical outcomes; HNF1α and CDX2 in accompany with CDH17 may be potential therapeutic targets for disease diagnosis and treatment.

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