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Characterization of E2F3a Function in HepG2 Liver Cancer Cells

Wei Li,[1,2] Guo-Xin Ni,[1] Ping Zhang,[2] Zheng-Xi Zhang,[1] Wei Li,[3] and Qiang Wu[1,2]*

[1] *Key Laboratory of Systems Biomedicine, Ministry of Education, Shanghai Center for Systems Biomedicine, Shanghai Jiao Tong University, Shanghai 200240, China*

[2] *State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Shanghai Jiao Tong University, Shanghai 200032, China*

[3] *School of Pharmacy, Shanghai Jiao Tong University, Shanghai 200240, China*

ABSTRACT

E2F3a is a transcription factor that has been shown to be overexpressed in liver cancer tissues. To characterize the function of E2F3a in hepatocellular carcinoma (HCC), effects of ectopic overexpression of E2F3a on cell cycle, apoptosis, and gene expression of HepG2 cells were studied. E2F3a significantly enhances the apoptotic rate of HepG2 cells by 33% but only has minor effects on cell proliferation. By using microarray analyses, we identified 162 target genes (160 upregulated and 2 downregulated) of the E2F3a. Differential expression of 11 genes was further confirmed by real-time PCR. Eight of these 11 genes, including XAF1, CEACAM1, STAT1, ATF3, TNFSF10, KLF6, CLDN1, and TAP1, were confirmed to be upregulated by more than twofold. Functional enrichments of differentially expressed genes retrieved 21 apoptosis-related genes and 32 transcriptional regulation-related genes. These results suggest that E2F3a induces apoptosis in HepG2 cells and plays important roles in regulating transcription. Finally, positive correlation was found between E2F3a and CEACAM1 mRNA levels in clinically well-differentiated human HCC specimens. J. Cell. Biochem. 111: 1244–1251, 2010.

KEY WORDS: APOPTOSIS; CELL CYCLE; E2F3a; HepG2; HCC; TRANSCRIPTIONAL REGULATION

Cancer is a diverse class of devastating diseases that causes millions of death each year. Disruption of cell cycle control and abnormal cell proliferation is the hallmark of cancer cells [Hanahan and Weinberg, 2000]. The "retinoblastoma (Rb) pathway" is one of the most important pathways in normal cell cycle control in animal development as well as aberrant cell proliferation in carcinogenesis [Weinberg, 1995; Sherr, 1996; Mulligan and Jacks, 1998]. The Rb protein has been demonstrated to bind and regulate a large number of transcriptional regulators, including members of the E2F family [Lees et al., 1993; Dyson, 1998; Mulligan and Jacks, 1998]. Binding of members of the Rb family with E2F transcription factors causes the repression of E2F target genes [Dynlacht, 1997]. Deregulation of E2F activity has been implicated in tumorigenesis [Field et al., 1996; Yamasaki et al., 1996; Chen et al., 2009b].

Eight members of the E2F family (E2F1 through E2F8) have been identified [Bracken et al., 2004; Maiti et al., 2005; Li et al., 2008; Chen et al., 2009b]. These members act individually or synergistically to regulate cell proliferation, apoptosis as well as other cellular processes [Trimarchi and Lees, 2002; Dimova and Dyson, 2005; Iaquinta and Lees, 2007; Li et al., 2008; Tsai et al., 2008]. Traditionally, E2F proteins were artificially divided into transcriptional activators (E2F1-3) and repressors (E2F4-8) [Chen et al., 2009b]. Members of the E2F family have dual roles as oncogenes as well as tumor suppressor genes [Chen et al., 2009b]. For example, the first and best-characterized member, E2F1, has both oncogenic and tumor-suppressive activities [Pierce et al., 1999].

The E2F3 gene was originally identified as an E2F1 paralog through low-stringency hybridization experiments [Lees et al., 1993]. It was later found to encode two protein isoforms, E2F3a and E2F3b, through an alternative-promoter usage [He et al., 2000; Leone et al., 2000]. The expression of these two E2F3 isoforms is tightly regulated [Adams et al., 2000]. E2F3 has both overlapping

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*Correspondence to: Qiang Wu, Key Laboratory of Systems Biomedicine, Ministry of Education, Shanghai Center for Systems Biomedicine, Shanghai Jiao Tong University, Shanghai 200240, China. E-mail: qwu123@gmail.com

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and distinct roles with E2F1 in regulating cell proliferation and apoptosis [DeGregori et al., 1997; Kong et al., 2007]. E2F3a promotes, while E2F3b inhibits, transcriptional expression of their target genes [He et al., 2000; Aslanian et al., 2004]. E2F3 plays important roles in animal development [Tsai et al., 2008; Asp et al., 2009; Chen et al., 2009a; Chong et al., 2009a]. Finally, accumulating evidence suggests that E2F3a gene is amplified and/or over-expressed in various human tumors including bladder, breast, ovarian, lung, and prostate cancers, which indicates that E2F3a plays important roles in tumor initiation and progression [Feber et al., 2004; Foster et al., 2004; Oeggerli et al., 2004; Olsson et al., 2007; Hurst et al., 2008; De Meyer et al., 2009].

E2F proteins regulate transcription of a large number of genes important in cell proliferation, differentiation, apoptosis, metabolism as well as animal development [Chen et al., 2009b]. However, the specific target genes of each E2F family members have not been investigated in detail. Hepatoma is the third death-causing cancers worldwide. E2F3a gene is overexpressed in most liver cancer biopsies as compared with adjacent normal tissues [Liu et al., 2003; Xu et al., 2009]. Although E2F1 has been shown to be actively involved in liver carcinogenesis using a transgenic mouse model [Conner et al., 2000], no functional study of E2F3a in human liver cells has been performed. In this study, we sought to characterize the effects of overexpressing E2F3a on cell proliferation, apoptosis, and changes of gene expression pattern in HepG2, a well differentiated human liver cancer cell line.

MATERIALS AND METHODS

MATERIALS

The human hepatocellular carcinoma HepG2 cell line was kindly provided by Shanghai Cancer Institute. RPMI-1640 media, Opti-MEM, fetal bovine serum (FBS), lipofectamine^{TM} 2000, and TRIZOL reagents were purchased from Invitrogen (Carlsbad, CA). pEGFP-N2 vector was purchased from BD Biosciences (Palo Alto, CA). E2F3a expression plasmid pCMV6-XL5-E2F3a was purchased from Origene (Rockville, MD). PE Annexin V plus 7-AAD Apoptosis Detection kit was purchased from BD biosciences. QIAGEN Miniprep Kit, QIAGEN Plasmid Midi Kit, RNeasy Mini Kit, and RNeasy Micro Kit are Qiagen (Hilden, Germany) products. cDNA reverse transcription kit, TaqMan probes, primers, and Mastermix were ordered from Applied Biosystems (Carlsbad, CA). Cyber Green Q-PCR kit was from Takara (Dalian, China). E2F3 antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All primers were synthesized by Sangon Shanghai (Shanghai, China). Chemical reagents were analytical grade and were purchased from Sigma (St. Louis, MO).

CELL CULTURE AND TRANSFECTION

Expression plasmid pEGFP-E2F3a was generated by inserting the opening reading frame of E2F3a cDNA into the EcoRI/BamHI site of pEGFP-N2 vector. HepG2 cells were grown in RPMI-1640 media supplemented with 10% FBS. Exponentially proliferating cells were maintained in 5% CO_2 at 37°C in an incubator. Cells at 70–80% confluence were seeded into 6-cm culture dishes and were transfected with 12 μg of pEGFP-N2 or pEGFP-E2F3a plasmids by Lipofectamine^{TM}2000. Twenty-four hours after transfection, cells were trypsinized and subjected to semi-quantitative RT-PCR, real-time PCR, and flow cytometry analyses. The primer sets for E2F3a and GAPDH genes are shown in the Table I.

WESTERN BLOT ANALYSES

Equal amounts of proteins (20 μg) were resolved on a 10% SDS–polyacrylamide gel. Proteins were transferred to a 0.2 μm PVDF membrane at 4°C overnight (BioRad, Hercules, CA). The membrane was blocked in 5% milk in TBST (10 mM Tris, pH 7.4, 0.9% (w/v) NaCl, 0.1% Tween-20) at room temperature overnight with rotation. The membrane was then probed with an E2F3-specific antibody (1:1,000; Santa Cruz Biotechnology, Inc.) for 1 h. After washing, the membrane was incubated with an anti-mouse antibody conjugated with horseradish peroxidase (1:5,000; KPL, Gaithersburg, MD) for 1 h and then washed. Detection of E2F3 was visualized using ECL-plus kit (GE Healthcare, Buckinghamshire, UK) and exposed to X-ray films.

TABLE I. Primers and Amplicons for Cyber Green-Based Q-PCR and Semi-Quantitative RT-PCR

Gene	Gene ID	Orientation	Primer sequence	Product size (bp)
GAPDH	2597	F	CATGAGAAGTATGACAACAGCCT	113
		R	AGTCCTTCCACGATACCAAAGT	
E2F3a	1871	F	CGGTCATCAGTACCTCTCAGA	124
		R	AGACGTATCATACCGCGTTTTT	
EGR1	1958	F	ACCTGACCGCAGAGTCTTTTC	110
		R	GCCAGTATAGGTGATGGGGG	
MXD1	4084	F	TGTGAGCGACTCTGACGAG	108
		R	CACGCCTTGTGACTGTCCTG	
CLDN1	9076	F	TCTGGCTATTTTAGTTGCCACAG	102
		R	GCCTGACCAAATTCGTACCTG	
TAP1	6890	F	GGCTGCTTTGAAGCCATTAGC	152
		R	AACTGACAACGAAGGCGGTAG	
TNFSF10	8743	F	AGTGAGAGAAAGAGGTCCTCAG	104
		R	CCAGAGCCTTTTCATTCTTGGA	
E2F3a[a]	1871	F	TGGGCTGAGTCTGTCTGAGGA	422
		R	GTTGAAGCCAAGTCTTTGGAAG	
GAPDH[a]	2597	F	TGATGACATCAAGAAGGTGGTGAAG	236
		R	TCCTTGGAGGCCATGTGGGCCAT	

[a]Primer pairs used for gel-based semi-quantitative RT-PCR.

CELL CYCLE AND APOPTOSIS ANALYSES

Cell cycle or apoptosis analyses were performed using propidium iodide (PI) staining and phycoerythrin (PE) conjugated annexin V plus 7-AAD double-labeling kit (BD, San Jose, CA) according to the manufacture's protocol. Briefly, 24 h after transfection, cells were trypsinized and approximately 4×10^5 cells were suspended in the staining buffer. GFP was used as a marker for transfected cells in FACS analyses. Five thousand GFP-positive cells were gated. Their cellular DNA content or annexin V-PE staining was determined by FACSCalibur™ (BD Biosciences). The distributions of cell cycle phases and apoptotic cell population were analyzed using Cell Analyst software. All experiments were performed in duplicates.

RNA EXTRACTION AND MICROARRAY ANALYSES

For the microarray experiments, total RNA was extracted using TRIzol reagent (Invitrogen) from cells transfected with pCMV6-XL5 or pCMV6-XL5-E2F3a, and purified with RNeasy mini kit (Qiagen) following the manufacturer's specifications. The RNA quality was assessed by agarose gel electrophoresis. The RNA quantity was measured using a spectrophotometer. Subsequent cDNA synthesis, cRNA labeling, hybridization, and scanning procedures were conducted according to the standard Affymetrix protocols (www.affymetrix.com). Two separate hybridization experiments were performed for two independent transfection experiments. Human genome U133 Plus 2.0 oligonucleotide arrays representing over 47,000 transcripts and variants were used in these microarray experiments. Small samples from each transfection experiment were collected for Western blot experiments.

Microarrays were scanned using Affymetrix GeneChip Scanner 3000. Quantification and initial analyses were performed using Gene Chip Operating Software (GCOS v1, Affymetrix, Santa Clara, CA). The comparisons of gene expression profiles between E2F3a-overexpressed cells (E2F3) and control (C) samples were performed using a standard significance analysis program (MAS 5.0 software, Affymetrix). For selecting differentially expressed genes, we eliminated probe sets with signals under 100. We then selected probe sets with a "signal log2 ratio" of E2F3/C equal or more than 1, corresponding to a difference in expression levels of at least twofold. Finally, lists obtained from two separate hybridization sets were combined, and differentially expressed genes in both hybridizations were selected. This led to 162 differentially expressed genes. Functional annotations were performed using the David functional annotation clustering software (http://david.abcc.ncifcrf.gov). Only functional groups with P values equal or lower than 0.05 were reported.

CONFIRMATION OF DIFFERENTIALLY EXPRESSED GENES BY REAL-TIME PCR

Transfected cells were trypsinized and filtered through a cell strainer (40-μm mesh) to obtain single cell suspensions. Cell density was adjusted to approximately 5×10^6 cells per milliliter for FACS sorting. EGFP- or EGFP-E2F3a- expressing cells were sorted and collected by using a BD FACSAria™ II cell sorter (BD Biosciences). The purity of sorted cells was reanalyzed using FACSCalibur™. Total RNA was isolated from approximately 2×10^5 sorted cells and reverse transcribed using oligo-dT primer in a two-step quantitative real-time PCR (Q-PCR). Briefly, first-strand cDNA syntheses were conducted on 1 μg total RNA using SuperScript™-II RNase H− Reverse Transcriptase (Invitrogen). Q-PCR was performed using either TaqMan or Cyber Green-based methods. Custom-designed primer sequences and amplicon sizes used in Cyber Green-based methods were summarized in Table I. The mRNA levels were quantified relative to endogenous GAPDH controls. The reactions were monitored using the real-time instrument (ABI 7300). The PCR conditions were: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 95°C for 15 s, 60°C for 60 s and 95°C for 15 s. Delta CT values (CT value for genes of interest minus CT value for controls) were determined. Comparisons of the CT values were used for the quantification of gene expression.

QUANTIFICATION OF GENE EXPRESSION IN HUMAN HCC TISSUES

Clinical specimens of the human HCC and paired non-cirrhotic liver tissues were provided by Jinan Zhongshan Liver Disease Hospital with informed consent from the patients. The protocols for using these specimens have been approved by the ethics committee of the Jinan Zhongshan Liver Disease Hospital as well as the review board of Shanghai Jiao Tong University. Six specimens diagnosed by the hospital as well-differentiated HCC based on histopathology were chosen for analyses. Total RNAs were prepared from microdissected tissues of the cryosectioned slices using the RNA Micro Kit according to the manufacture's instruction. mRNAs were reverse transcribed using oligo-dT primers and quantified by real-time PCR as described above. Expression levels of E2F3a and CEACAM1 in HCC and adjacent normal tissues were compared. The expression level of GAPDH gene was used as an internal control.

RESULTS

E2F3a SIGNIFICANTLY INDUCES APOPTOSIS

To investigate the effects of E2F3a overexpression on apoptosis, we performed flow cytometry analyses (Fig. 1) on HepG2 cells transfected with pEGFP-E2F3a (Fig. 1C,D) or control pEGFP plasmids (Fig. 1A,B). Based on analyses of GFP-positive cells, we found that the E2F3a-overexpressing HepG2 cells had a significant higher percentage of cells (34.46% in Fig. 1D) than that of control cells (1.25% in Fig. 1B) in late apoptotic stage. Thus, E2F3a proteins induce apoptosis in HepG2 cells.

E2F3a HAS MINOR EFFECTS ON CELL CYCLE CONTROL

Twenty-four hours after EGFP-E2F3a overexpression, HepG2 cells were trypsinized and subjected to flow cytometry analyses to investigate the effects of E2F3a overexpression on the cell cycle. Our data based on DNA content analyses of GFP-positive cells showed that E2F3a-tansfected HepG2 cells had a slightly higher percentage of cells (14.3% vs. 10.2%) in S phase and lower percentage of cells (63.7% vs. 69.6%) in G1 phase when compared to controls (Fig. 2). The results suggest that overexpression of E2F3a has minor effects on cell cycle progression in HepG2 liver cancer cells.

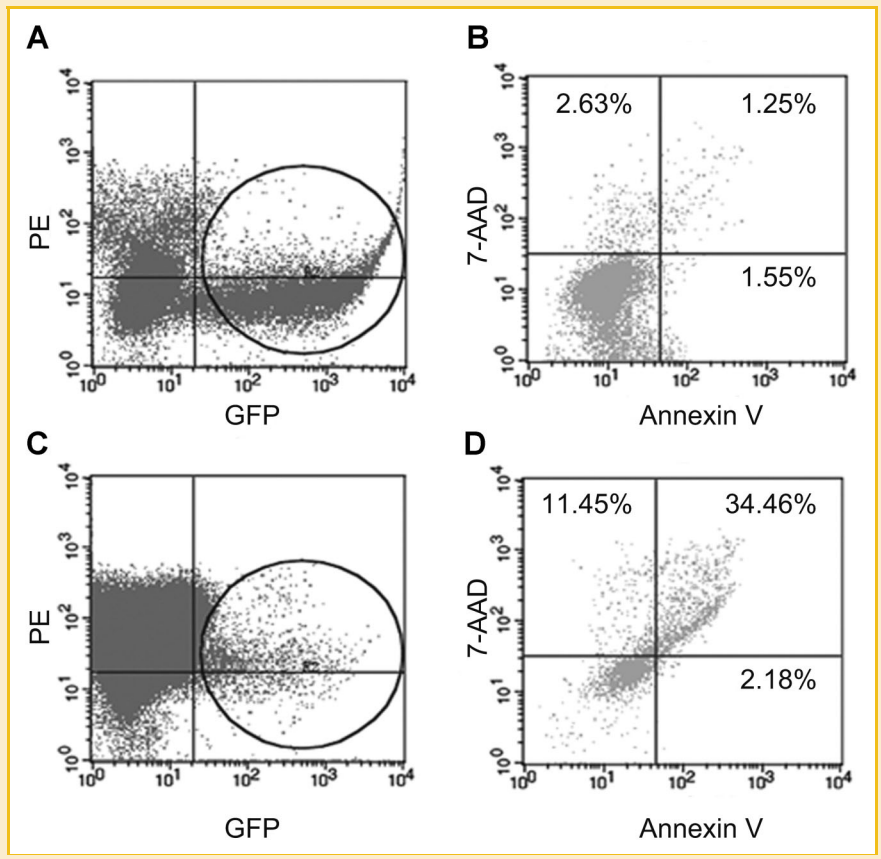

Fig. 1. FACS analyses of apoptotic cells induced by E2F3a overexpression. A: Selection of GFP positive cells upon transfection with the EGFP control plasmid. B: Selected HepG2 cells from the panel A were analyzed for apoptotic cells. C: Selection of GFP positive cells upon transfection with EGFP-E2F3a. D: Selected HepG2 cells from the panel C were analyzed for apoptotic cells. Transfection experiments were performed in duplicates. Apoptotic cells are stained by Annexin V—phycoerythrin (PE). Dead cells, including late-stage apoptotic cells, are stained by the vital dye 7-amino-actinomycin D (7-AAD).

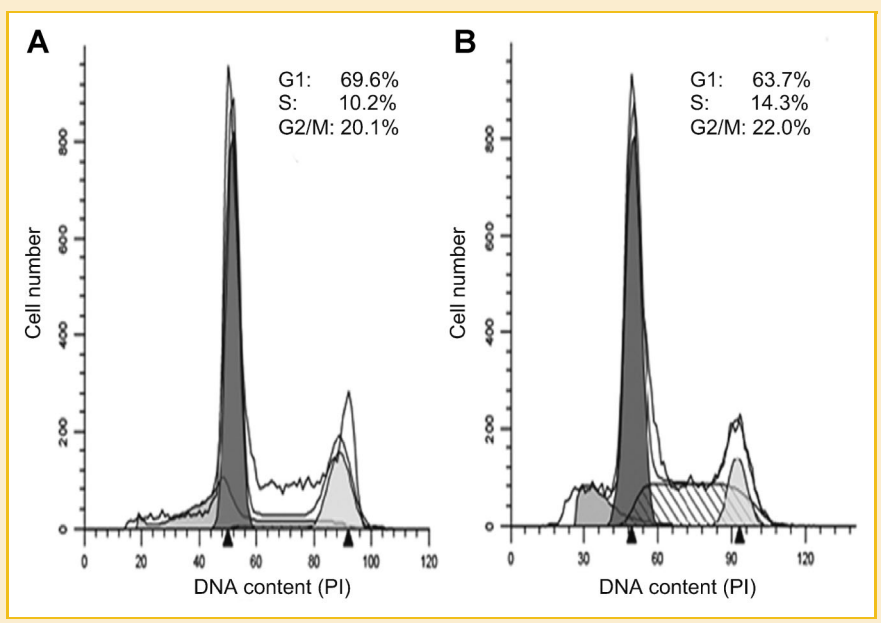

Fig. 2. Effect of E2F3a overexpression on cell cycle distribution of HepG2 cells. A: FACS analyses of HepG2 cells transfected with control EGFP plasmids. B: FACS analyses of HepG2 cells transfected with EGFP-E2F3a plasmids. The DNA content is measured by staining with propidium iodide (PI). The estimated proportion of the G1, S, and G2/M cells are also indicated. Data are obtained from the gated GFP-positive HepG2 cells. The experiments were performed in duplicates.

TRANSFECTION EFFICIENCY ASSESSED BY FLOW CYTOMETRY ANALYSES AND FLUORESCENCE IMAGING

Members of the E2F family exert their functions through regulating the transcriptions of multiple target genes. To investigate the molecular mechanisms of the E2F3a cellular functions, we sought to identify its candidate target genes through microarray analyses. We first measured green fluorescent cell ratio for the EGFP-E2F3a transfection efficiency (Fig. 3). Flow cytometry analyses showed that the average transfection efficiency was about 20% (Fig. 3A,B). This result is consistent with the percentage of GFP-positive cells observed under the green fluorescence (Fig. 3D) versus the total cell number under the brightfield (Fig. 3C).

E2F3a OVEREXPRESSION AFTER TRANSFECTION IN HepG2 CELLS

We next measured the levels of overexpression of E2F3a mRNAs and proteins by RT-PCR and Western blot analyses. To determine the E2F3a overexpression level after pCMV6-XL5-E2F3a transfection, we analyzed the E2F3a mRNA level by semi-quantitative RT-PCR. We observed a >90-fold increase in HepG2 cells compared with cells transfected with control vectors (Fig. 4A). We also observed similar folds of expression increase by Cyber Green-based real-time PCR (data not shown). In addition, Western blot analyses demonstrated that E2F3a protein expression is significantly increased at 24 h after transfection (Fig. 4B). Because E2F3a proteins are expressed at a much higher level at 24 h after transfection than at 48 h (data not shown), we harvested cells at 24 h after transfection for all of the microarray experiments.

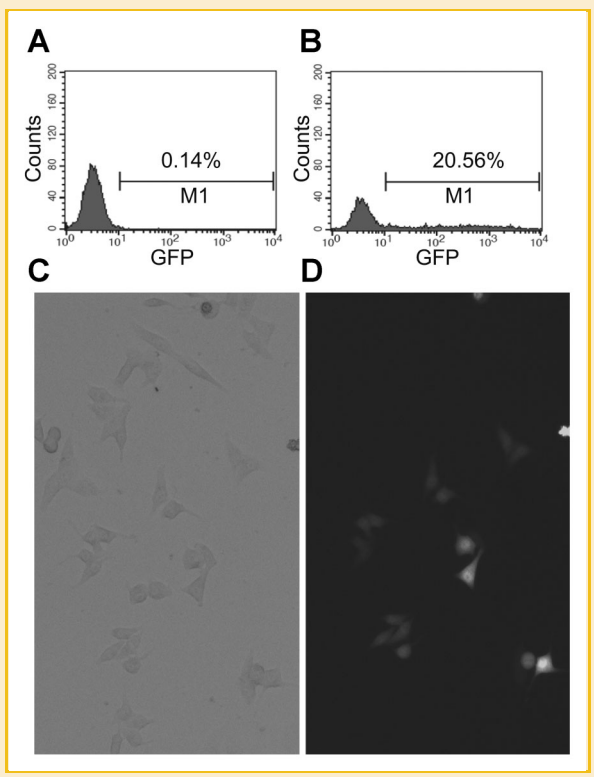

Fig. 3. Assessment of transfection efficiency in HepG2 cells by FACS analyses. A: Control untransfected log phase cells. B: HepG2 cells in log phase were transfected with pEGFP-E2F3a vector. C: Photomicrograph of transfected cells under a brightfield microscope. D: Photomicrograph of transfected cells in the same field as panel C under a fluorescent microscope. The experiments were performed in triplicates.

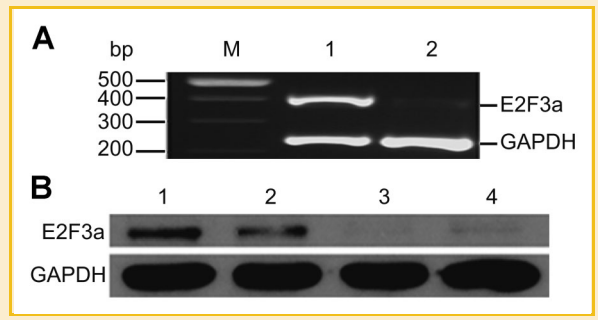

Fig. 4. Quantification of E2F3a mRNA and protein expression levels after transfection. A: RT-PCR of E2F3a with GAPDH as an internal control. Lane M: DNA ladder; Lane 1: E2F3a transfection; Lane 2: mock transfection. B: Western blots using an anti-E2F3 antibody with GAPDH as an endogenous control for protein concentration. Lanes 1 and 2: E2F3a transfections corresponding to the microarray tests 1 and 2, respectively; Lanes 3 and 4: mock transfections corresponding to the microarray tests 1 and 2, respectively.

E2F3a REGULATES EXPRESSION OF GENES IN MANY CELLULAR PROCESSES

To identify E2F3a target genes, total RNAs isolated from pCMV6-XL5-E2F3a transfected and control cells were hybridized to the Affymetrix U133 plus 2.0 array containing oligonucleotide probe pairs for ~35,000 different genes. By combining the statistically significant changes (>99% confidence) with the fold change in expression levels for any given gene, we chose a minimal fold change (cutoff) of 2 to minimize the number of false positives. Our data showed that the expression of 162 genes in HepG2 cells was altered to at least twofold in duplicate array analyses (Supplementary Material Table S1). Among these differentially expressed genes, 160 genes were upregulated to at least twofold, and two genes downregulated. In order to get a better understanding of the biological processes in which these genes are involved, we grouped them using the David software with the highest-classification-stringency enrichment algorithm. We retrieved 39 annotation clusters, including one cluster of 21 genes implicated in apoptotic processes (enrichment score of 4.71, $P < 0.001$), one cluster of 32 genes related to transcriptional regulation (enrichment score of 1.53, $P < 0.05$), and one cluster of nine genes involved in cell cycle regulation (enrichment score of 1.02, $P < 0.06$). These three clusters of genes were summarized in supplementary material (Table S2).

CONFIRMATION OF E2F3a TARGET GENES BY REAL-TIME PCR

To verify the microarray results, we selected 11 genes (TNFSF10, TAP1, CLDN1, EGR1, MXD1, XAF1, CEACAM1, STAT1, JUN, KLF6, and ATF3), which are functionally involved in cell proliferation and apoptosis, for real-time PCR analyses. Approximately 2×10^5 FACS-sorted cells expressing EGFP-E2F3a and control cells expressing EGFP were compared for the expression levels of these genes by real-time PCR. We confirmed that 8 (TNFSF10, TAP1,

CLDN1, XAF1, CEACAM1, STAT1, KLF6, and ATF3) of 11 are upregulated by more than twofold in HepG2 cells (Fig. 5). Among them, the XIAP-associated factor-1 (XAF1) and the tumor necrosis factor (ligand) superfamily, member 10 (TNFSF10), genes are induced by more than 30-fold (Fig. 5). We also performed similar experiments using pCMV6-XL5-E2F3a-transfected cells and observed 8- and 11-fold increase of TNFSF10 and XAF1 expression, respectively (data not shown). Given that the transfection efficiency is 20% (Fig. 3), this result is consistent with more than 30-fold increase of TNFSF10 and XAF1 expression in FACS-sorted EGFP-E2F3a-transfected cells (Fig. 5).

THE EXPRESSION OF E2F3a AND CEACAM1 IN HUMAN HCC TISSUES

Cell adhesion genes are known to play important roles in liver tumor. We analyzed expression levels in human liver cancers of an unknown cell adhesion gene, carcinoembryonic antigen-related cell adhesion molecule 1, which are moderately upregulated in microarray experiments. The mRNA levels of E2F3a and CEACAM1 were compared between well-differentiated HCC and paired normal tissues. E2F3a and CEACAM1 genes are upregulated by 2- to 3.5-fold in 5 out of 6 human HCC specimens compared with adjacent normal tissues (Fig. 6). This confirms that CEACAM1 is up-regulated in clinically well-differentiated human liver cancers.

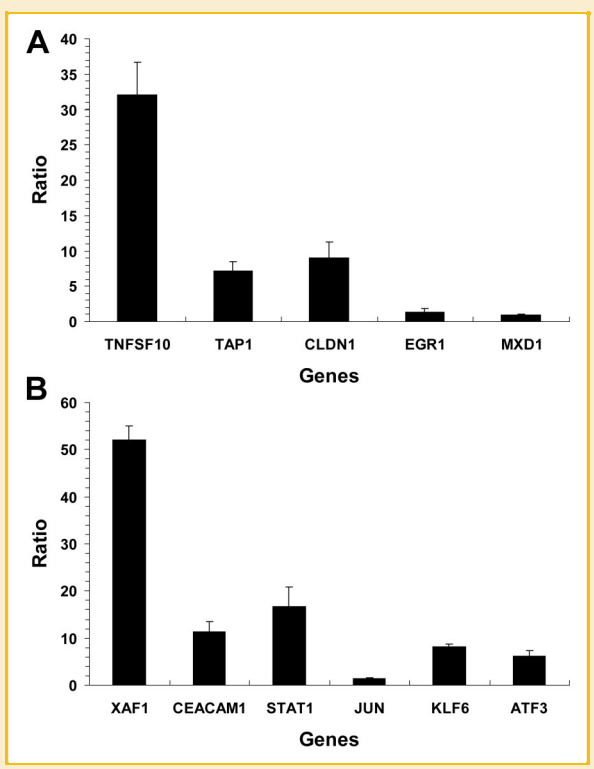

Fig. 5. Verification of E2F3a target genes by quantitative real-time PCR experiments. A: FACS-sorted cells were analyzed by using the Cyber Green-based fluorescence assay. B: FACS-sorted cells were analyzed by using the TaqMan-based assay. n = 3, data are presented as mean ± SE and represented as fold changes from controls.

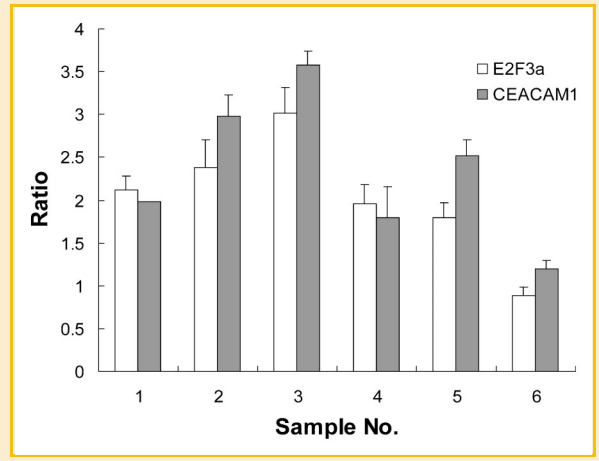

Fig. 6. Expression of E2F3a and CEACAM1 in six human HCC tissues. Total RNA was extracted from microdissected tissues from HCC foci or adjacent normal cells of frozen slices and reverse transcribed using oligo-dT primers. Quantification of mRNAs was performed using the TaqMan-based Real-time PCR analyses and GAPDH was used as an internal standard. n = 3, data are presented as mean ± SE and represented as fold changes of HCC from normal control tissues.

DISCUSSION

The RB/E2F pathway is one of the best characterized pathways involved in the majority of human cancers. To date, much work bas been focused on elucidating the function of E2F1 [Field et al., 1996; Yamasaki et al., 1996; Chen et al., 2009b], very few studies have addressed the pathophysiological functions of E2F3. Although recent studies have identified target genes of E2F proteins in several cell types [Takahashi et al., 2000; Wells et al., 2000, 2002; Müller et al., 2001; Ren et al., 2002], no report has been found to identify E2F3a target genes in liver cancer cells. Transcription factors may behave differently in specific tissues [Wells et al., 2000] or in different developmental stages [Chong et al., 2009b]. E2F3a gene expression is upregulated in the hepatocellular carcinoma (HCC) biopsies [Xu et al., 2009]. In this study, the phenotypic and molecular effects of E2F3a overexpression on HepG2 cells were characterized.

E2F1 induces apoptosis and cell proliferation in a transgenic model of mouse hepatocarcinogenesis [Conner et al., 2000]. E2F3a increases the apoptosis of epidermis through the activation of caspase-3 in a transgenic mouse model [Paulson et al., 2006]. E2F3a also contributes to the apoptosis resulting from the RB loss in mouse embryos [Ziebold et al., 2001]. Very recently, E2F3a has been shown to mediate DNA damage-induced apoptosis [Martinez et al., 2010]. Consistent with these results, overexpression of E2F3a proteins significantly increases the apoptotic events in HepG2 cells, suggesting a proapoptotic role of the E2F3a proteins in human liver cancer cells.

The RB/E2F pathway plays important roles in regulating cell cycle progression. Overexpression of E2F1, E2F2, or E2F3 is sufficient to induce S-phase entry in quiescent fibroblasts [Lukas et al., 1996]. Moreover, E2F3 deficiency impairs the cell cycle progression in

mouse embryonic fibroblasts [Humbert et al., 2000]. In addition, both E2F1 and E2F3 are required for the cell cycle entry; however, only E2F3 is required for continued proliferation [Kong et al., 2007]. In this study, by taking advantage of low levels of endogenous E2F3a expression in HepG2 cell line, E2F3a was overexpressed by transfecting an E2F3a-expressing plasmid. We found that E2F3a overexpression results in only 4% increase in S-phase entry. This suggests that E2F3a has minor effects on cell proliferation of the HepG2 cells.

Microarray analyses identified 162 E2F3a target genes in unsorted cells. We do not know how many of these genes are directly regulated by E2F3a. Many of them may be the indirect target genes of E2F3a. Nevertheless, functional classification of these genes retrieved three clusters of genes that are involved in apoptosis, transcriptional control, and cell cycle regulation. These data unveiled the potential molecular mechanisms of proapoptotic effect of E2F3a protein. Among the 11 genes verified using quantitative real-time PCR, TNFSF10, TAP1, CLDN1, XAF1, CEACAM1, STAT1, KLF6, and ATF3 were upregulated by more than twofold in HepG2 cells. XAF1 and TNFSF10 are the two most prominent genes upregulated by E2F3a. XAF1 is a pro-apoptotic protein that inhibits the X-linked inhibitor of apoptosis (XIAP) [Liston et al., 2001]. TNFSF10 can activate the extrinsic apoptotic cell death pathway upon binding to the cognate death receptors at the cell surface [Gonzalvez and Ashkenazi, 2010]. Thus, E2F3a may induce apopotosis of HepG2 cells through the XAF1 and TNFSF10 pathways.

We also examined the mRNA expression levels of E2F3a and CEACAM1 in human HCC tissues. We found that both E2F3a and CEACAM1 expressions were upregulated in five out of six HCC specimens versus the adjacent non-cirrhotic liver tissues. This is consistent with the significant induction of CEACAM1 in the HepG2 cells when E2F3a is overexpressed.

In summary, we demonstrated that E2F3a functions as an apoptosis inducer in the HepG2 cell line. Moreover, transcription profiling based on microarray analyses identified 162 target genes. Functional annotation of these target genes revealed that apoptosis is one of the most important pathways linked to E2F3a. Furthermore, most of the E2F3a-regulated apoptotic genes can be verified by quantitative RT-PCR. These results provide significant insights into the apoptotic functions of the E2F3a and the underlying molecular mechanisms. Future studies will focus on the elucidation of the specific E2F3a target genes and their exact roles in mediating apoptosis.

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REFERENCES

Adams MR, Sears R, Nuckolls F, Leone G, Nevins JR. 2000. Complex transcriptional regulatory mechanisms control expression of the E2F3 locus. Mol Cell Biol 20:3633–3639.

Aslanian A, Iaquinta PJ, Verona R, Lees JA. 2004. Repression of the Arf tumor suppressor by E2F3 is required for normal cell cycle kinetics. Genes Dev 18:1413–1422.

Asp P, Acosta-Alvear D, Tsikitis M, van Oevelen C, Dynlacht BD. 2009. E2F3b plays an essential role in myogenic differentiation through isoform-specific gene regulation. Genes Dev 23:37–53.

Bracken AP, Ciro M, Cocito A, Helin K. 2004. E2F target genes: Unraveling the biology. Trends Biochem Sci 29:409–417.

Chen D, Pacal M, Wenzel P, Knoepfler PS, Leone G, Bremner R. 2009a. Division and apoptosis of E2F-deficient retinal progenitors. Nature 462:925–929.

Chen HZ, Tsai SY, Leone G. 2009b. Emerging roles of E2Fs in cancer: An exit from cell cycle control. Nat Rev Cancer 9:785–797.

Chong JL, Tsai SY, Sharma N, Opavsky R, Price R, Wu L, Fernandez SA, Leone G. 2009a. E2F3a and E2F3b contribute to the control of cell proliferation and mouse development. Mol Cell Biol 29:414–424.

Chong JL, Wenzel PL, Saenz-Robles MT, Nair V, Ferrey A, Hagan JP, Gomez YM, Sharma N, Chen HZ, Ouseph M, Wang SH, Trikha P, Culp B, Mezache L, Winton DJ, Sansom OJ, Chen D, Bremner R, Cantalupo PG, Robinson ML, Pipas JM, Leone G. 2009b. E2F1-3 switch from activators in progenitor cells to repressors in differentiating cells. Nature 462:930–934.

Conner EA, Lemmer ER, Omori M, Wirth PJ, Factor VM, Thorgeirsson SS. 2000. Dual functions of E2F-1 in a transgenic mouse model of liver carcinogenesis. Oncogene 19:5054–5062.

De Meyer T, Bijsmans IT, Van de Vijver KK, Bekaert S, Oosting J, Van Criekinge W, van Engeland M, Sieben NL. 2009. E2Fs mediate a fundamental cell-cycle deregulation in high-grade serous ovarian carcinomas. J Pathol 217:14–20.

DeGregori J, Leone G, Miron A, Jakoi L, Nevins JR. 1997. Distinct roles for E2F proteins in cell growth control and apoptosis. Proc Natl Acad Sci USA 94:7245–7250.

Dimova DK, Dyson NJ. 2005. The E2F transcriptional network: Old acquaintances with new faces. Oncogene 24:2810–2826.

Dynlacht BD. 1997. Regulation of transcription by proteins that control the cell cycle. Nature 389:149–152.

Dyson N. 1998. The regulation of E2F by pRB-family proteins. Genes Dev 12:2245–2262.

Feber A, Clark J, Goodwin G, Dodson AR, Smith PH, Fletcher A, Edwards S, Flohr P, Falconer A, Roe T, Kovacs G, Dennis N, Fisher C, Wooster R, Huddart R, Foster CS, Cooper CS. 2004. Amplification and overexpression of E2F3 in human bladder cancer. Oncogene 23:1627–1630.

Field SJ, Tsai FY, Kuo F, Zubiaga AM, Kaelin WG, Jr., Livingston DM, Orkin SH, Greenberg ME. 1996. E2F-1 functions in mice to promote apoptosis and suppress proliferation. Cell 85:549–561.

Foster CS, Falconer A, Dodson AR, Norman AR, Dennis N, Fletcher A, Southgate C, Dowe A, Dearnaley D, Jhavar S, Eeles R, Feber A, Cooper CS. 2004. Transcription factor E2F3 overexpressed in prostate cancer independently predicts clinical outcome. Oncogene 23:5871–5879.

Gonzalvez F, Ashkenazi A. 2010. New insights into apoptosis signaling by Apo2L/TRAIL. Oncogene 29:4752–4765.

Hanahan D, Weinberg RA. 2000. The hallmarks of cancer. Cell 100: 57–70.

He Y, Armanious MK, Thomas MJ, Cress WD. 2000. Identification of E2F-3B, an alternative form of E2F-3 lacking a conserved N-terminal region. Oncogene 19:3422–3433.

Humbert PO, Verona R, Trimarchi JM, Rogers C, Dandapani S, Lees JA. 2000. E2F3 is critical for normal cellular proliferation. Genes Dev 14:690–703.

Hurst CD, Tomlinson DC, Williams SV, Platt FM, Knowles MA. 2008. Inactivation of the Rb pathway and overexpression of both isoforms of E2F3 are obligate events in bladder tumours with 6p22 amplification. Oncogene 27:2716–2727.

Iaquinta PJ, Lees JA. 2007. Life and death decisions by the E2F transcription factors. Curr Opin Cell Biol 19:649–657.

Kong LJ, Chang JT, Bild AH, Nevins JR. 2007. Compensation and specificity of function within the E2F family. Oncogene 26:321–327.

Lees JA, Saito M, Vidal M, Valentine M, Look T, Harlow E, Dyson N, Helin K. 1993. The retinoblastoma protein binds to a family of E2F transcription factors. Mol Cell Biol 13:7813–7825.

Leone G, Nuckolls F, Ishida S, Adams M, Sears R, Jakoi L, Miron A, Nevins JR. 2000. Identification of a novel E2F3 product suggests a mechanism for determining specificity of repression by Rb proteins. Mol Cell Biol 20:3626–3632.

Li J, Ran C, Li E, Gordon F, Comstock G, Siddiqui H, Cleghorn W, Chen HZ, Kornacker K, Liu CG, Pandit SK, Khanizadeh M, Weinstein M, Leone G, de Bruin A. 2008. Synergistic function of E2F7 and E2F8 is essential for cell survival and embryonic development. Dev Cell 14:62–75.

Liston P, Fong WG, Kelly NL, Toji S, Miyazaki T, Conte D, Tamai K, Craig CG, McBurney MW, Korneluk RG. 2001. Identification of XAF1 as an antagonist of XIAP anti-Caspase activity. Nat Cell Biol 3:128–133.

Liu LX, Jiang HC, Liu ZH, Zhu AL, Zhang WH, Wu LF, Zhou J, Wang XQ, Wu M. 2003. Expression of cell cycle/growth regulator genes in human hepatocellular carcinoma and adjacent normal liver tissues. Oncol Rep 10:1771–1775.

Lukas J, Petersen BO, Holm K, Bartek J, Helin K. 1996. Deregulated expression of E2F family members induces S-phase entry and overcomes p16INK4A-mediated growth suppression. Mol Cell Biol 16:1047–1057.

Maiti B, Li J, de Bruin A, Gordon F, Timmers C, Opavsky R, Patil K, Tuttle J, Cleghorn W, Leone G. 2005. Cloning and characterization of mouse E2F8, a novel mammalian E2F family member capable of blocking cellular proliferation. J Biol Chem 280:18211–18220.

Martinez LA, Goluszko E, Chen HZ, Leone G, Post S, Lozano G, Chen Z, Chauchereau A. 2010. E2F3 is a mediator of DNA damage-induced apoptosis. Mol Cell Biol 30:524–536.

Müller H, Bracken AP, Vernell R, Moroni MC, Christians F, Grassilli E, Prosperini E, Vigo E, Oliner JD, Helin K. 2001. E2Fs regulate the expression of genes involved in differentiation, development, proliferation, and apoptosis. Genes Dev 15:267–285.

Mulligan G, Jacks T. 1998. The retinoblastoma gene family: Cousins with overlapping interests. Trends Genet 14:223–229.

Oeggerli M, Tomovska S, Schraml P, Calvano-Forte D, Schafroth S, Simon R, Gasser T, Mihatsch MJ, Sauter G. 2004. E2F3 amplification and overexpression is associated with invasive tumor growth and rapid tumor cell proliferation in urinary bladder cancer. Oncogene 23:5616–5623.

Olsson AY, Feber A, Edwards S, Te Poele R, Giddings I, Merson S, Cooper CS. 2007. Role of E2F3 expression in modulating cellular proliferation rate in human bladder and prostate cancer cells. Oncogene 26:1028–1037.

Paulson QX, McArthur MJ, Johnson DG. 2006. E2F3a stimulates proliferation, p53-independent apoptosis and carcinogenesis in a transgenic mouse model. Cell Cycle 5:184–190.

Pierce AM, Schneider-Broussard R, Gimenez-Conti IB, Russell JL, Conti CJ, Johnson DG. 1999. E2F1 has both oncogenic and tumor-suppressive properties in a transgenic model. Mol Cell Biol 19:6408–6414.

Ren B, Cam H, Takahashi Y, Volkert T, Terragni J, Young RA, Dynlacht BD. 2002. E2F integrates cell cycle progression with DNA repair, replication, and G(2)/M checkpoints. Genes Dev 16:245–256.

Sherr CJ. 1996. Cancer cell cycles. Science 274:1672–1677.

Takahashi Y, Rayman JB, Dynlacht BD. 2000. Analysis of promoter binding by the E2F and pRB families in vivo: Distinct E2F proteins mediate activation and repression. Genes Dev 14:804–816.

Trimarchi JM, Lees JA. 2002. Sibling rivalry in the E2F family. Nat Rev Mol Cell Biol 3:11–20.

Tsai SY, Opavsky R, Sharma N, Wu L, Naidu S, Nolan E, Feria-Arias E, Timmers C, Opavska J, de Bruin A, Chong JL, Trikha P, Fernandez SA, Stromberg P, Rosol TJ, Leone G. 2008. Mouse development with a single E2F activator. Nature 454:1137–1141.

Weinberg RA. 1995. The retinoblastoma protein and cell cycle control. Cell 81:323–330.

Wells J, Boyd KE, Fry CJ, Bartley SM, Farnham PJ. 2000. Target gene specificity of E2F and pocket protein family members in living cells. Mol Cell Biol 20:5797–5807.

Wells J, Graveel CR, Bartley SM, Madore SJ, Farnham PJ. 2002. The identification of E2F1-specific target genes. Proc Natl Acad Sci USA 99:3890–3895.

Xu T, Zhu Y, Xiong Y, Ge YY, Yun JP, Zhuang SM. 2009. MicroRNA-195 suppresses tumorigenicity and regulates G1/S transition of human hepatocellular carcinoma cells. Hepatology 50:113–121.

Yamasaki L, Jacks T, Bronson R, Goillot E, Harlow E, Dyson NJ. 1996. Tumor induction and tissue atrophy in mice lacking E2F-1. Cell 85:537–548.

Ziebold U, Reza T, Caron A, Lees JA. 2001. E2F3 contributes both to the inappropriate proliferation and to the apoptosis arising in Rb mutant embryos. Genes Dev 15:386–391.