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PEG10 directly regulated by E2Fs might have a role in the development of hepatocellular carcinoma

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Abstract PEG10 is an imprinted gene which is up-regulated in hepatocelluar carcinoma (HCC). However, the mechanism of PEG10 regulation remains to be elucidated. In this work the transcription factors E2F-1 and -4 were demonstrated to bind directly to the promoter of PEG10 and thereby regulate its expression. The expression profile of HCC tissues also suggested E2Fs were involved in PEG10 regulation. Further functional analysis showed that PEG10 was involved in the repression of apoptosis induced by serum deprivation and chemotherapeutic drugs. These findings link cancer genetics and epigenetics by showing that E2F acts directly upstream of an anti-apoptosis imprinted gene, PEG10.

Keywords: PEG10; E2F; Hepatocelluar carcinoma; Apoptosis

1. Introduction

The PEG10 gene was identified based on its location in an imprinted domain on human chromosome 7q21 and characterized as paternally expressed/maternally silenced [1]. The PEG10 mRNA encodes two protein isoforms (RF1 and RF2) via translational frameshifting [2]. Overexpression of PEG10 in HCC has been reported by several groups, indicating this gene has growth-promoting activity [3–6]. However it may also be involved in hepatocarcinogenesis by interacting with SIAH1, a mediator of apoptosis [7]. A variety of experiments showed that the expression of PEG10 can be regulated by androgen and it is a target of c-MYC in cancer cells [8,9]. However, the regulated mechanism of PEG10 is not elucidated.

E2F consists of a transcriptional factor family including six distinct E2F members and at least two heterodimer partners, DP1 and DP2 [10]. E2F family members regulate many genes through their interaction with other cellular proteins. Included among these so-called nuclear pocket proteins are Rb family members, RB1, p107, and p130. Interaction between pocket protein and E2F normally diminishes E2F's *trans*-activating capacity and inhibits target genes [11]. Although the pathway to cell cycle progression seems straightforward with a number of growth-promoting E2F target genes having been described, the pathways to apoptosis are less well defined and more complex [10,12].

Among E2F family, E2F-4 is conservatively expressed in normal liver tissue, while E2F-1 is not expressed in HCC tissues [13]. On the other hand, microsatellite instability and mutations of E2F-4 frequently occurred in HCC [14]. We show here PEG10 in HCC, is promoted by a mechanism mediated by E2F-1 and -4.

2. Materials and methods

2.1. Cell culture and transfection

Human hepatocyte L02 cells [15], hepatocellular carcinoma cell BEL-7404 and human embryonic kidney cell HEK293 (purchased from Type Culture Collection of Chinese Academy of Sciences, Shanghai and ATCC, respectively) were maintained in Dulbecoo's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 μg/ml streptomycin sulfate, and 100 μg/ml penicillin at 37 °C in 5% CO_2. Transient and stable transfection was performed using Lipofectamine™ (Invitrogen) according to the manufacturer's protocol.

2.2. Plasmid construction

Full-length human PEG10RF1, E2F-4, Cyclin D1 and CDK4 were cloned into pcDNA3.1A (Invitrogen), respectively, and PEG10RF1 was also cloned into pEGFP-N1 (Clontech) to construct plasmid expressing PEG10RF1-GFP fusion protein. The PEG10 promoter fragment, corresponding to nucleotides 19,515,091–19,515,997 of GenBank accession NT_079595.2, were cloned into pGL3-Basic vector (Promega) to construct the full length reporter plasmid p906, from that a series of deletions in the promoter fragment were then constructed, respectively (Fig. 1B). E2F binding site mutant m-p150 was constructed by point-mutation of p150 using QuikChange II XL site-directed mutagenesis kit (Strategene). Plasmid pcDNA-HA-E2F-1 is a kind gift from Prof. Wu (University of Science and Technology of China, Hefei, Anhui).

2.3. Cell cycle and subG1 hypodiploid FACS analysis

After serum deprivation and re-stimulation, L02 cells were collected at indicated time point. Cell cycle analysis was performed as described before [16].

To test serum deprivation induced apoptosis in cells expressing GFP fusion protein, cells were transfected with plasmids for 48 h, serum deprived for 48 h. Then all cells were collected, fixed, permeabilized and analyzed as previously described [16].

2.4. Cell viability measurement

Cells were seeded in 96-well plates and then treated with serum starvation, cisplatin or doxorubicin (Sigma). At indicated time point cell viability was measured by an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] method as described previously [16].

2.5. RNA interference

Plasmids RNAi-1 and RNAi-2 which encoded shRNAs directed against human PEG10, were constructed by cloning oligonucleotides targeting nucleotides 1185–1203 (CACATTGAGAGAAGGCTGG)

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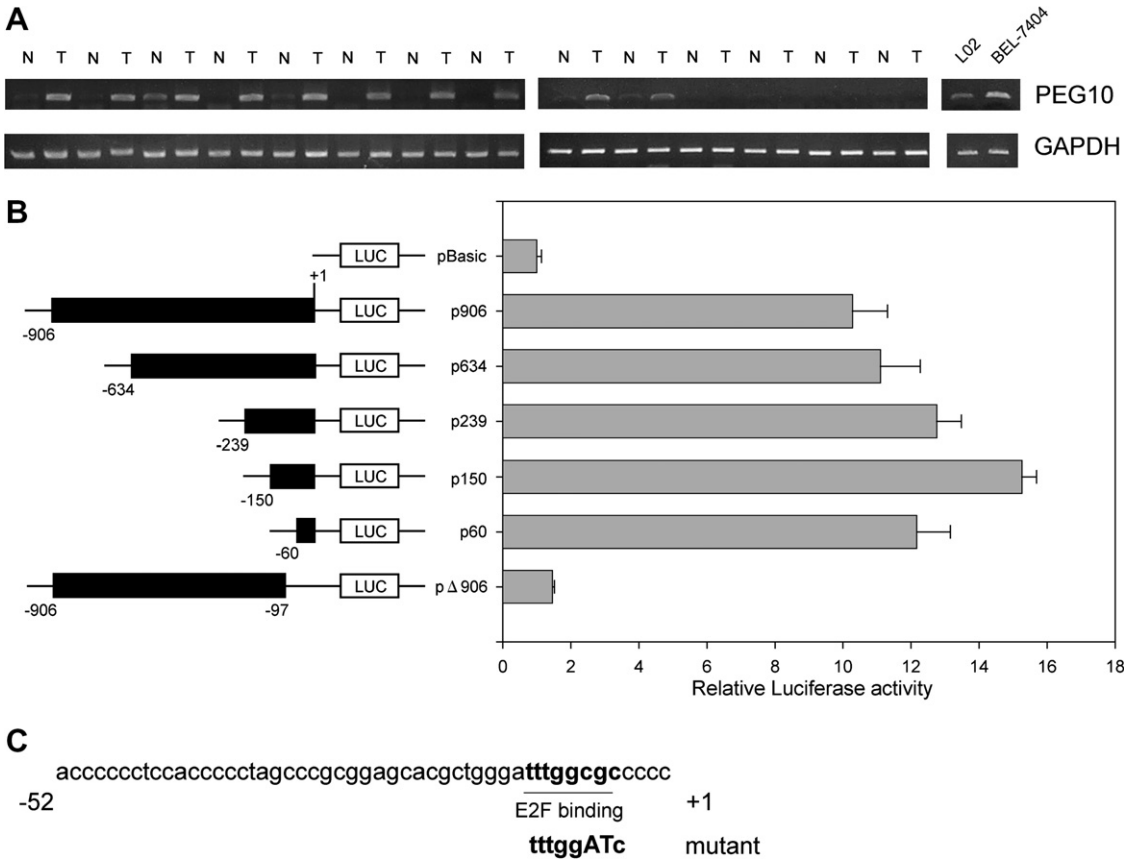

Fig. 1. Function analysis of PEG10 promoter. (A) The mRNA levels of PEG10 were tested in 14 HCC (T) and paired normal liver tissues (N), L02 cells and BEL-7404 cells by half quantitative RT-PCR. GAPDH was used as an internal control for quantification. (B) On the left, a schematic representation of 906 bp PEG10 promoter and its deleted variants inserted upstream of the luciferase gene in the reporter plasmid pGL3-Basic. The numbers indicate the 5′ and the 3′ end of the promoter DNA inserts, in relation to the transcription start site (+1). Twenty four hours after transfection, the luciferase activity was measured. Data are shown as mean ± S.D. of three independent experiments. (C) A putative E2F binding site was found in the PEG10 promoter as indicated and site-directed mutagenesis of the site is also shown.

and 1154–1172 (GTCGCTGTCTGCTCTGATT) of PEG10 sequence (NM_001040152) in pSuper.gfp (OligoEngine). The empty vector pSuper.gfp was used as control.

2.6. Luciferase reporter gene assay

To analyze PEG10 promoter function, PEG10 promoter constructs were transfected in L02 cells. To measure responses to E2F, p150 and mutant m-p150 were co-transfected with E2F expressing plasmid or pcDNA3 in HEK293 cells, respectively. Plasmid pTK-RL was co-transfected as a transfection efficiency control. Cells were cultured in 10% FBS for another 24 h after transfection. The luciferase activity in cell lysates was measured by Dual luciferase assay system (Promega) following the manufacturer's protocol.

2.7. ChIP assay

A total 1×10^7 L02 cells were transfected with pcDNA-HA-E2F-1 or not, then crosslinked with 1% formaldehyde for 15 min at room temperature. ChIP assay was performed by using a ChIP Assay Kit (Upstate) following the manufacturer's protocol. Antibody against HA (Sigma) and E2F-4 (Santa Cruz) was used. For covering E2F binding site in PEG10 promoter primers were designed as follows:
Forward: 5′-CGGAGCACGCTGGGATTTG-3′
Reverse: 5′-AGGCGCGGTCTCCACTG-3′

2.8. Quantitative real-time RT-PCR assay and half quantitative RT-PCR

Total RNA extraction from cells and tissues and reverse transcription were performed as described previously [17]. To measure mRNA level, real-time PCR assays were performed on a DNA Engine Opticon 2 (MJ Research) using the DyNAmo SYBR Green qPCR kit (FINNZYMES). A threshold cycle value for each mRNA was determined using the Opticon Monitor software (version 2.02). All data were normalized to the GAPDH mRNA level and expressed as mRNA relative change. The mRNA levels of E2F-1, E2F-4 and PEG10 in tissues were detected by half quantified PCR assays and then measured using TotalLab (Non-linear Dynamics). Expression changes between tumor and normal were calculated as Log_2 (Tumor/Normal). Primers used in PCR were designed as follows:
PEG10-forward: 5′-CCCCGGGCGCTGGTGTTG-3′
PEG10-reverse: 5′-AGCGGGGCCGGGGAGTTTC-3′
BCL-xL-forward: 5′-CTGTGCGTGGAAAGCGTAGA-3′
BCL-xL-reverse: 5′-TGCTGCATTGTTCCCATAGAG-3′
GAPDH-forward: 5′-GGGGAGCCAAAAGGGTCATCATCT-3′
GAPDH-reverse: 5′-GAGGGGCCATCCACAGTCTTCT-3′
E2F-1-forward: 5′-CCGCCATCCAGGAAAAGGT-3′
E2F-1-reverse: 5′-GCCCTCAAGGACGTTGGTG-3′
E2F-4-forward: 5′-TGTTTGGCCTACGTCACTCAT-3′
E2F-4-reverse: 5′-ACACTCTTCAGGTGAATCTGGTA-3′

2.9. Clinical samples

Clinical HCC samples at stages II and III by pathological examination were collected from Zhongshan Hospital immediately after surgery. The paired normal liver samples were 3 cm away from the edge of HCC lesions from the same patients. The 14 HCC patients (male/female, 9/5) were aged 31–71. Access to human tissues complied with both Chinese laws and the guidelines of the Ethics Committee.

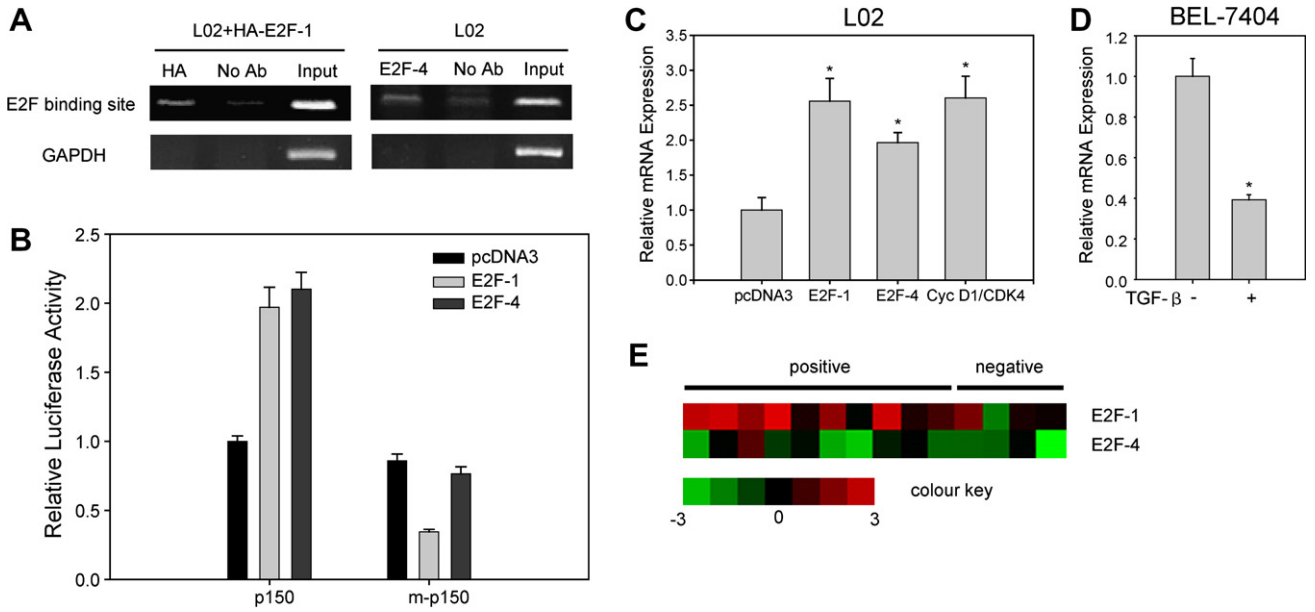

Fig. 2. PEG10 is a direct target of E2F. (A) ChIP assay showed E2F-1 and E2F-4 binded to the PEG10 promoter region containing the putative site. GAPDH was used as a negative control. (B) A 150 bp fragment containing E2F-like binding element of the PEG10 promoter has E2F-response activity in the luciferase report assay which is abrogated by a point-mutation in the element. Details were described in Section 2. (C) Effects of the E2F-1, -4 and Cyclin D/CDK4 complex overexpression on the PEG10 mRNA expression in L02 cells. Forty eight hours after transfection of indicated plasmids, mRNA level of PEG10 gene was measured by quantitative real-time PCR, normalized mRNA level of GAPDH housekeeping gene. Cells transfected with control plasmid pcDNA3 were defined as 1. (D) Effects of TGFβ stimulation on the PEG10 mRNA expression in BEL-7404 cells. Cells were treated with 2 ng/ml TGF-β for 48 h. The mRNA level of PEG10 gene was measured as above. Cells without stimulation were defined as 1. Data are shown as mean ± S.D. of three independent experiments. $^{*}P < 0.01$. (E) Expression of E2F in HCC clinical samples. Expression patterns of E2F-1 and E2F-4 in 14 pairs of clinical samples. Samples were divided into two groups, positive and negative, according to the expression of PEG10 in tumor. Each column represented a pair of HCC samples. Graduated colour patterns correspond to the degrees of expression changes (Tumor vs. Normal).

3. Results and discussion

3.1. PEG10 is up-regulated in HCC

We examined the mRNA expression levels of PEG10 in clinic HCC samples and paired normal liver tissues. Ten out of 14 samples showed elevated mRNA levels of PEG10 in tumors compared to paired normal tissues (Fig. 1A). Consistently, the mRNA level of PEG10 was low in normal hepatic cell L02 and high in hepatocellular carcinoma cell BEL-7404 (Fig. 1A). These data verified previous reports that PEG10 was up-regulated in HCC [6].

3.2. Confirmation of the E2F regulatory element inPEG10 promoter

In order to investigate transcriptional regulation of PEG10 expression, the promoter fragment between −906 and +1 of *PEG10* gene was subcloned into luciferase reporter vector. A series of reporter plasmids containing various deletions in the promoter fragment were also constructed. All these reporters were transiently transfected into L02 cells, to quantify promoter activities. The luciferase activity of empty vector was used as the reference for normalizing the activities of the other constructs (Fig. 1B). Deletion analyses of PEG10 promoter revealed that the p150 exhibited a highest level of activity. Similar high levels of activity were observed for the other plasmids except *p*Δ906 that lacked the region −96/+1 of PEG10 promoter and exhibited much lower activity (about 10% of activity of p150) (Fig. 1B). These data indicated that a key regulatory element was located in the region between −96 and +1. Then we investigated putative transcriptional factor-binding sites in −96/+1 region using the MatInspector database. As shown in Fig. 1C, within the region of −50/+1, there was one putative E2F binding site. Thus, E2F was probably a key transcriptional factor responsible for PEG10 regulation.

3.3. E2F-1 and E2F-4 bind to the promoter and regulate the transcription of PEG10

Among E2F family, E2F-4 was expressed in normal liver while E2F-1 was expressed in HCC. Thus, we investigated whether PEG10 was regulated by them. The results of CHIP showed that both exogenous E2F-1 and endogenous E2F-4 could bind to PEG10 promoter (Fig. 2A). To determine whether the putative binding site is essential for E2F to regulate the transcription of PEG10, luciferase plasmids with wild type or mutated E2F binding sequence were constructed (Fig. 1C). Luciferase report assay showed that both exogenous E2F-1 and -4 up-regulated the promoter activity of the wild type p150 but not mutant m-p150 (Fig. 2B). Interestingly, E2F-1 even repressed that of m-p150. Consistently, the overexpression of E2F-1 and -4 in L02 cells could up-regulate the expression of PEG10 at mRNA level (Fig. 2C). These findings indicated that both E2F-1 and -4 could positively regulate the transcriptional expression of PEG10 by directly binding to its promoter.

PEG10 is a target of E2F, so it is reasonable to expect PEG10 can be regulated by E2F/Rb pathway. Cyclin D/CDK4 complex, in this pathway, can phosphorylate pocket proteins and release E2F [18], and TGF-β can repress expression

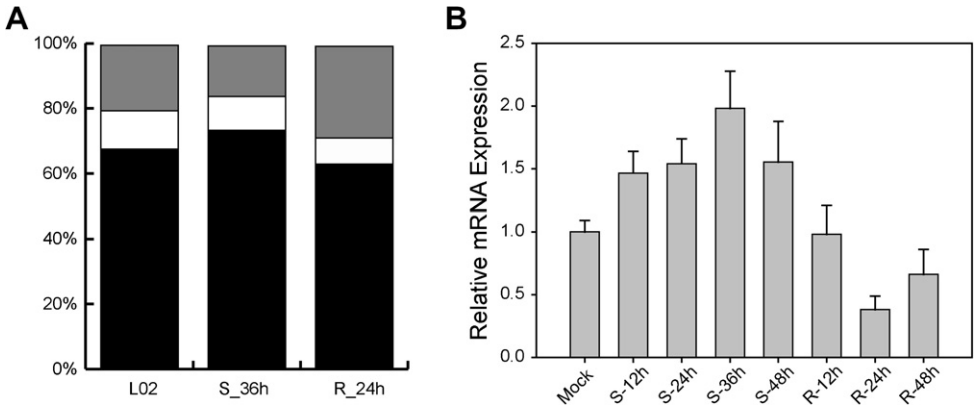

Fig. 3. PEG10 expression varies during cell cycle. L02 cells were seeded, serum-starved (S) and then re-treated with 10% serum (R). (A) Cell cycle was analyzed at indicated time point. Black: G1 phase; white: S phase; grey: G2/M phase. (B) At indicated time point the mRNA level of PEG10 was measured by quantitative real-time PCR.

of E2F target gene by keeping Rb dephosphorylated and inducing the formation of E2F-4/p107 or E2F-4/Rb complexes [19]. We found that overexpression of Cyclin D/CDK4 complex, as well as E2F-1 and -4, caused elevated mRNA level of PEG10 in L02 cells (Fig. 2C). On the other hand, mRNA level of PEG10 was significantly decreased when we treated BEL-7404 cells with TGF-β (Fig. 2D). These results also supported indirectly that PEG10 was a target of E2F.

In support to our results, previous microarray studies showed that PEG10 (KIAA1051) was up-regulated on overexpression of E2F-1, -2 or -3 in osteosarcoma cells U2OS [20], as well as on knockdown of Rb1 in non-small lung carcinoma cells H1299 [21]. And cDNA array data from Tsou et al. suggested that in HCC clinical samples the expression profile of PEG10 was similar to that of many E2F target genes, such as CDK4, CDC45, CDC20 [5].

3.4. Expression of E2F in HCC tissues

As described above, PEG10 was found to be a target of E2F in cultured cells. We then investigated whether there is a correlation between PEG10 and E2F expression in HCC. We examined the mRNA expression levels of E2F-1 and E2F-4 in clinic HCC samples. Coincident with results in cells, E2F-1 expression was positive correlated to PEG10 expression (Fig. 2E), suggesting the overexpression of PEG10 in HCC was caused by upregulated E2F-1. But E2F-4 expression seemed negative correlated unexpectedly (Fig. 2E). It has been considered that E2F-4 is properly expressed in normal liver and represses the expression of target genes in association with pocket proteins, p107 and p130. When E2F-4 is decreased, the repression will be abrogated and target genes can be transcribed [22]. Meanwhile, E2F-4 has *trans*-activating capability and also can transcriptionally stimulate E2F target gene promoters [23], which could explain our results on overexpression experiments of E2F-4 in cells. However, the "loss of repression" mechanism may be predominant in vivo as implied by the result from clinical samples.

3.5. Expression of PEG10 varies during cell cycle

Since a variety of E2F target genes participate in cell cycle control, especially in G1/S transition, we wonder whether PEG10 is involved in this process. L02 cells were arrested in G1 phase by serum deprivation and re-entered cell cycle after serum re-stimulation (Fig. 3A). It was observed that the mRNA level of PEG10 was increased following serum deprivation and was decreased after serum re-stimulation (Fig. 3B). However, ectopic expression of PEG10 in synchronized L02 cells or knocking down of PEG10 in BEL-7404 cells barely affected the transition from G1 phase to S phase (data not shown). These results suggested that PEG10 may be expressed during G1 phase rather than during G2 phase. But PEG10 seemed not to play a role in G1/S transition.

3.6. PEG10 protects hepatic cells from apoptosis induced by serum deprivation and chemotherapeutic drugs

Because E2F/Rb pathway is involved in not only cell cycle control but also cell fate decision including apoptosis, we then investigate the function of PEG10 in apoptosis. Ectopic expression of PEG10 rather than GFP protein in L02 cells markedly reduced cell apoptosis induced by serum deprivation (Fig. 4A). In reverse, the cell apoptosis induced by serum deprivation would significantly increase if the expression of PEG10 was knocked down with RNAi in BEL-7404 cell (Fig. 4B and C). Meanwhile, more survival cells after serum deprivation were observed in PEG10 stable transfected L02 cells compared to the cells treated with empty plasmid (Fig. 4D). Similar results were obtained when cells were treated with cisplatin or doxorubicin (Fig. 4F). Together, these results suggested that the existence of PEG10 could protect hepatic cells from apoptosis. In addition, we found that the mRNA level of BCL-xL, an important anti-apoptosis protein, was elevated in PEG10 stable transfected cells (Fig. 4E), probably underlying the mechanism for apoptosis resistance of PEG10.

Previous reports indicated that PEG10 has a positive role in cell proliferation [7,24]. Data in this study suggested that PEG10 is also involved in resistance to apoptosis induced by serum starvation and chemotherapeutic drugs. These data indicated that PEG10 may have a role in development of HCC. Two candidate pathways, inhibition of SIAH function and inhibition of TGF-β have been reported to explain how PEG10 performs anti-apoptosis activity. Our data suggested that induction of BCL-xL by PEG10 may also have contribution in this process.

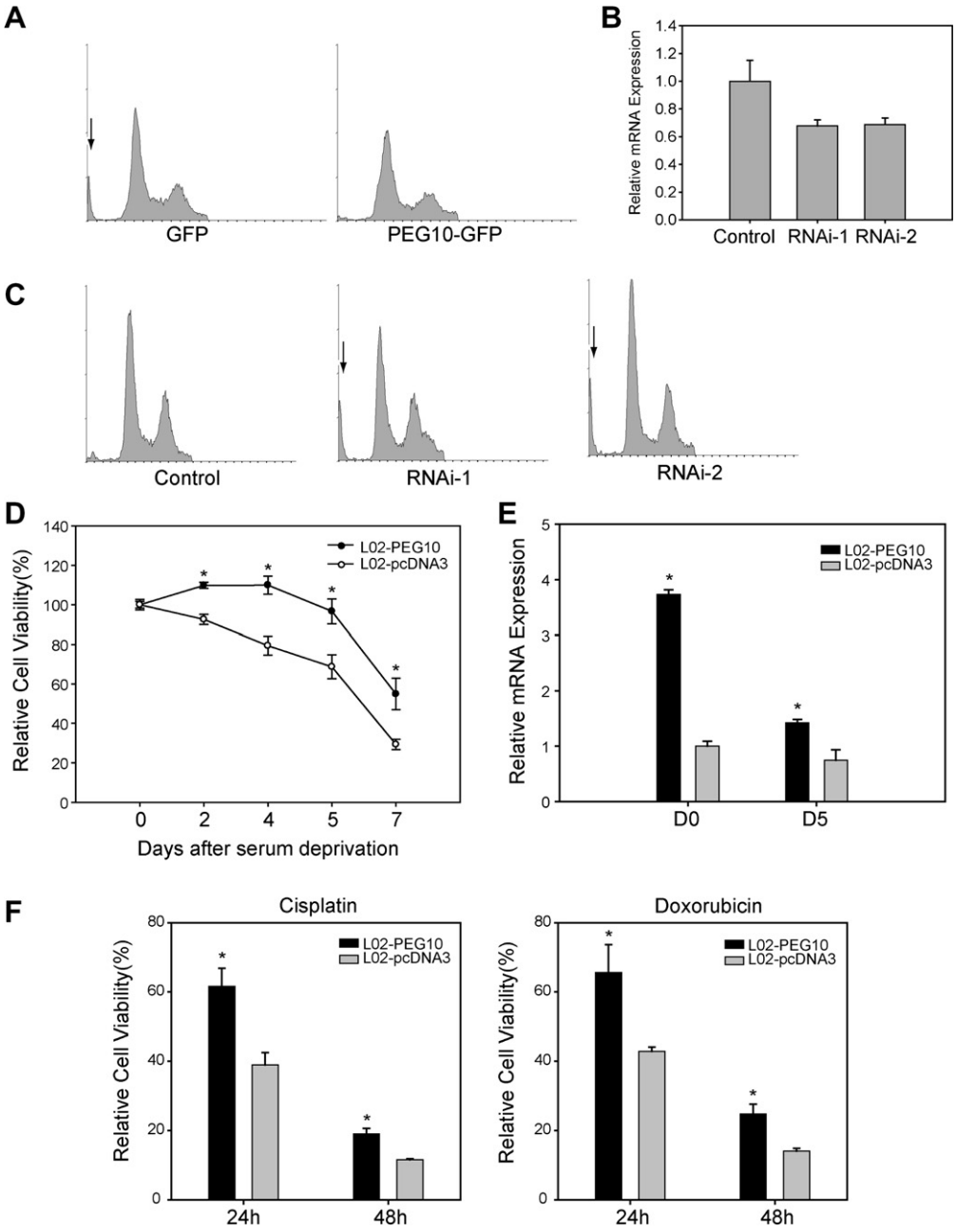

Fig. 4. PEG10 protects hepatic cell lines against apoptosis. L02 cells (A) and BEL-7404 cells (C) were transfected with indicated plasmids and deprived of serum, and then subG1 hypodiploid FACS analysis of GFP positive cells were performed. Arrows indicated the increased apoptosis cells. (B) Knocking down of PEG10 by RNAi plasmids. Forty eight hours after transfection of indicated plasmids, the mRNA level of PEG10 gene was measured. Cells transfected with control plasmid pSuper.gfp were defined as 1. (D) Growth rates of PEG10 and pcDNA3 stable transfected L02 cells in serum deprivated medium. (E) The mRNA levels of BCL-xL in the above cells were measured by quantitative real-time PCR. D0 and D5 indicated 0 and 5 days after serum deprivation. (F) PEG10 and pcDNA3 stable transfected and wild type L02 cells were treated with 10 mM cisplatin or 2 mM doxorubicin for 24 and 48 h, the viability of cells were counted by MTT assay. Data are shown as mean ± S.D. of three independent experiments. $^{*}P < 0.01$.

Epigenetic phenomena, including de novo methylation and loss of imprinting, have been well documented in preneoplastic and malignant tissues [25], but how classic genetic pathways contribute to the dysregulated expression of imprinted genes is less concerned. E2F/Rb pathway is a classic genetic pathway involved in a variety of human cancers [12], and genetic study in HCC has shown that microsatellite instability and mutations of E2F-4 occur commonly in HCC [14]. Here we reported that the imprinted gene PEG10 is a target of E2F, providing a link between genetics and epigenetics in HCC.

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