PEG10 is imperative for TGF-β1-induced epithelial-mesenchymal transition in hepatocellular carcinoma

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Abstract. Substantial evidence indicates that transforming growth factor-beta 1 (TGF-β1) plays a vital role in epithelial-mesenchymal transition (EMT). PEG10 has been shown involved in invasion and metastasis of tumors. The present study investigated the role of PEG10 in TGF-β1-triggered EMT in hepatocellular carcinoma (HCC) progression. Immunohistochemistry and real-time PCR were used to measure the expression level of PEG10 in clinical HCC tissues with or without lymph node metastasis, and normal tissues. The results showed that PEG10 expression is higher in HCC tissues and associated with overall survival (OS) and lymph node metastasis. Moreover, PEG10 expression level was remarkably higher in hepatic cancer cells than the normal hepatic cell line L02. In the present study, we constructed an adenovirus vector containing the coding area of PEG10 (Ad-PEG10) and infected HepG2 cells and found that overexpression of PEG10 promoted the cell migration, invasion ability and EMT of HepG2 cells. TGF-β1 acted on HepG2 cells by enhancing cell migration, invasion, EMT and upregulating PEG10 expression level. However, cells pretreated with adenovirus vector of PEG10 shRNAs (Ad-shRNA1 and Ad-shRNA2) did not occur EMT prior to TGF-β1 stimulation. Moreover, TGF-β1 did not increase the migration and invasion of cells with PEG10 knockdown and overexpression of PEG10 confers chemoresistance to HepG2 cells. Accordingly, sufficient PEG10 expression level is essential for TGF-β1 induced EMT and associated with the chemoresistance in HepG2 cells.

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Introduction

Hepatocellular carcinoma (HCC) is a highly malignant tumor and its incidence is ranked fifth in malignant tumors (1). Epithelial-mesenchymal transition (EMT) contributes to the promotion of HCC progression and metastasis (2). In addition, chemotherapy is utilized as an aid to prevent the recurrence and metastasis of HCC (3). However, a wide range of resistance often occurs in the treatment of HCC resulting from aberrant gene expression (4). Therefore, it is necessary to identify novel therapeutic target for HCC.

PEG10 is a new genetic imprinting gene which is identified in hepatocellular carcinoma tissues (5), it contains two open reading frames (ORFs) which code two proteins (PEG10-ORF1 and PEG10-ORF2) through the mechanism of ribosomal shift. A previous study showed that PEG10-ORF1 which acts as the main functional protein is modulated by various genes, such as c-myc (6). Recent studies have proved that the expression level of PEG10 is specifically higher in HCC tissues than in normal tissues (7). Moreover, PEG10 was confirmed to play an important role in the progression, proliferation, metastasis and prognosis in human lung and breast cancer (8,9) and increase the progression of neuroendocrine prostate cancer (10). PEG10 promoted the migration of human Burkitt's lymphoma cells by increasing the expression of matrix metalloproteinase-2 and -9 (11). These results suggest that PEG10 display functional roles in many disease, especially in tumors. However, the precise roles of PEG10 in facilitating metastasis of HCC still remain unknown.

It is well accepted that aberrant EMT contributes to tumor progression, in which cells lose their epithelial cell-like characteristics and alternatively adopt a mesenchymal phenotype, thus, initiating tumor metastasis. This process is thus considered to be a vital process increasing the invasion of cancers (12). Many components in tumor microenvironment such as TGF-β1 is able to induce EMT, which has been proved to facilitate a highly invasive and metastatic phenotype in various tumors (2,13,14). In addition, TGF-β1 polymorphism has been proved to contribute to pulmonary metastasis of hepatocellular carcinoma in a mouse model (15). However, it remains to be determined whether PEG10 is involved in EMT and the TGF-β1 signaling also include the regulation of PEG10 expression in HCC metastasis.

To address these problems, we established an HCC culture model in which TGF-β1 induced migration, invasion and EMT. In the present study, we constructed a recombinant adenovirus vector of PEG10 and PEG10 shRNAs and infected them into HepG2 cells to investigate the promotive roles in HCC migration and invasion and possible mechanisms of PEG10 on TGF-β1-induced EMT. In addition, we further explored the effect of PEG10 on the chemotherapy resistance of HCC. To the best of our knowledge, the roles of PEG10 validated here have not yet been reported. Accordingly, these results might provide novel insights for ameliorating or finding new therapeutic target for the HCC progression.

Materials and methods

Patient samples. Thirty-nine HCC tissues and adjacent normal tissues were obtained from patients who underwent surgery at the Eastern Hepatobiliary Surgery Hospital from June 2014 to November 2016, and all 39 cases presented metastasis. In addition, 31 HCC tissues with lymph node metastasis were obtained at the same time. The present study was approved by the Eastern Hepatobiliary Surgery Hospital Research Ethics Committee.

Cell culture. Human normal hepatic cell line, L02 and hepatic cancer cell lines (HepG2, HCCLM3 and HUH7) were purchased from the Cell Bank in Chinese Academy of Sciences of China (Shanghai, China). Cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 100 mg/ml streptomycin, 100 IU/ml penicillin and 10% fetal bovine serum (FBS; Gibco) at 37˚C under humidified air with 5% CO_2.

Adenovirus vectors construction. We thank Hanbio, Inc., (Shanghai, China) for constructing the adenovirus vector containing the coding area of PEG10 (Ad-PEG10) and adenovirus vector of PEG10 shRNAs (Ad-shRNA1 and Ad-shRNA2), which was verified by DNA sequencing.

Quantitative real-time PCR (qRT-PCR). Firstly, total RNA was prepared from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Secondly, total RNA was reverse transcribed into cDNA using M-MLV (Promega, Madison, WI, USA) according to standard protocols. To examine the mRNA expression levels, following the protocols of SYBR Premix Ex Taq™ kit (Takara Bio, Inc., Dalian, China), qRT-PCR was carried out on an ABI Prism 7500 detection system (Applied Biosystems, Inc., Foster City, CA, USA). The primers for EMT markers and GAPDH were previously described (12). The primers for PEG10 are: PEG10: forward, 5'-CCAACGACAAGAACGATTA-3' and reverse, 5'-TTTTGCCAGTTTGAAAAAC-3'. The specificity of the amplified products was confirmed by using melting curve analysis. GAPDH served as an endogenous control. Finally, the $2^{-\Delta\Delta ct}$ method was utilized for analyzing relative gene expression.

Immunohistochemistry. The detailed procedures were described previously (16). Immunohistochemistry of hepatic, normal and hepatic tissues with lymph node metastasis for PEG10 was performed manually using an anti-PEG antibody (cat. no. ab181249; Abcam).

Western blot analysis. The whole HepG2 cell extracts were obtained using RIPA buffer (Beyotime Institute of Biotechnology, Haimen, China) following the standard protocols. The concentration of the total protein was detected by Bradford assay. A total of 30 μg samples of protein extracts were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF; Millipore, Billerica, MA, USA) membranes for western blotting. The membranes were blocked with 5% milk solution in TBST (Tris-HCl-buffered saline supplemented with 0.5% Tween-20), and incubated with the primary antibodies against PEG10, E-cadherin (ab1416; Abcam), vimentin (ab8978; Abcam), Bcl-2 (ab32124; Abcam) Bax (ab32503; Abcam) and β-actin (ab8226; Abcam) in 5% bovine serum albumin (BSA) in TBST at 4˚C overnight, followed by incubation with the corresponding horseradish peroxidase-linked secondary antibodies (Beyotime Institute of Biotechnology). Blots were washed for 15 min, three times and the signals were detected using an enhanced chemiluminescence detecting reagent (Thermo Fisher Scientific, Waltham, MA, USA).

Would healing assay. Detailed procedure was described elsewhere (17).

Transwell migration and invasion. Transwell migration and invasion assays were performed via using 24-well Millicell Hanging Cell Culture inserts (8 mm) PET (Millipore), and the PETs coated with Matrigel matrix gel (BD Biosciences, San Jose, CA, USA) were used for invasion assays. Briefly, 24 h after infection with adenovirus vectors, $8x10^4$ cells in 200 μl serum-free medium were plated onto the upper chamber, while complete medium containing 10% FBS was added to lower chamber, which was used as a chemoattractant. The time for migration assays was 36 h and 48 h for invasion assays. Then cells on the upper chamber were removed with a cotton swab, and migrated cells on the lower chamber were fixed using methanol for 30 min, following staining with crystal violet solution for 30 min and washed with phosphate-buffer solution (PBS) several times. Six random fields from each of the triplicate migration and invasion assays were counted by using phase contrast microscopy. The dye was dissolved with acetic acid, and crystal violet absorbance was measured at 570 nm using a microplate reader.

Cell adhesion assays. The detailed procedure was described previously (17). Briefly, 96-wells plate was coated with matrix gel at 37˚C for 3.5 h and blocked with 5% BSA for another 1.5 h. Cells infected with adenovirus vectors were suspended at a final concentration of 10^5 cells/well in serum-free medium. Non-adherent cells were washed away with PBS for 1 h adhesion. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT)-assay was used to examine the number of adherent cells.

MTT assays. MTT assays were used to determine the cell viability. Cells ($4x10^3$/well) were planted in 96-well plates and

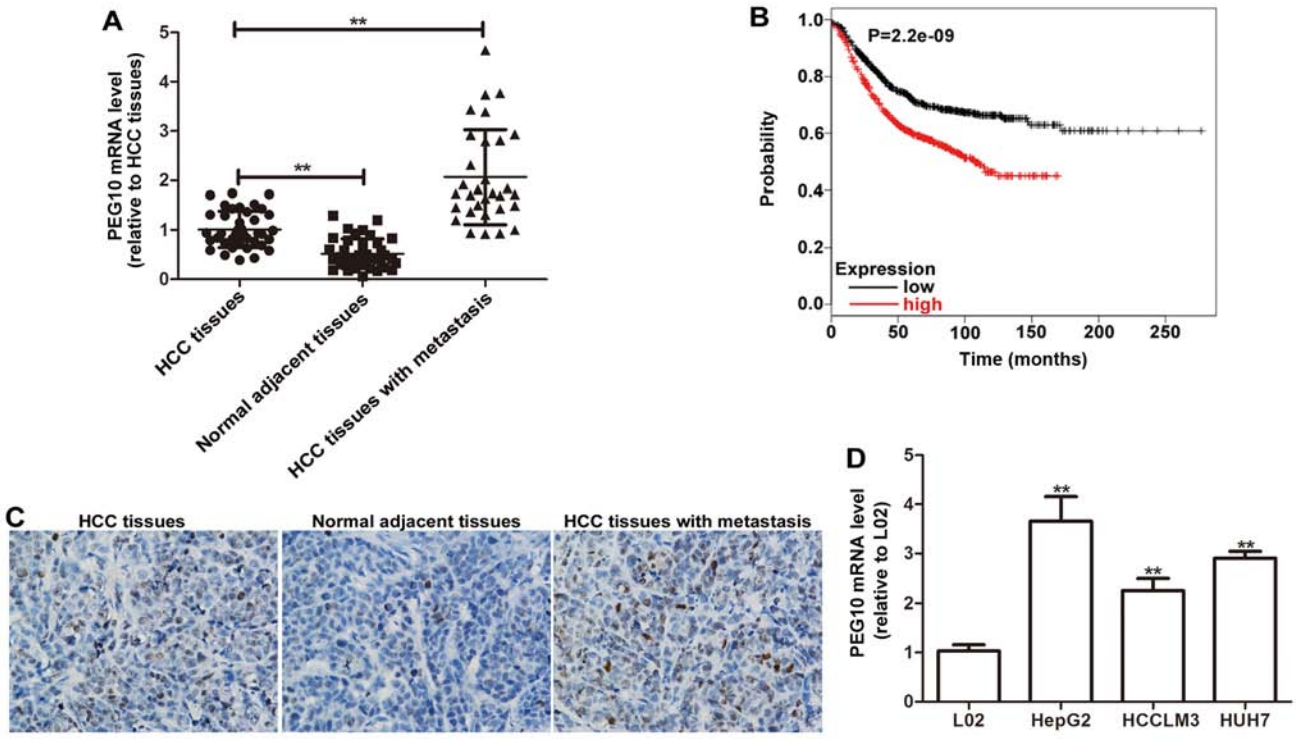

Figure 1. PEG10 is downregulated in normal adjacent tissues and cells. (A) PEG10 mRNA level was detected in hepatic cancer tissues with or without lymph node metastasis and normal adjacent tissues. (B) PEG10 expression level was considered and PEG10-high expression patients had a significant shorter overall survival time (P=2.2e-09). (C) PEG10 protein expression level was examined in hepatic cancer tissues with or without lymph node metastasis and normal adjacent tissues via immunohistochemical analyses. The results are consistent with the qRT-PCR results (A). (D) The mRNA level of PEG10 was further detected in hepatic tumor cells and normal hepatic cells through qRT-PCR analyses. PEG10 mRNA level is significantly higher in hepatic tumor cells than in normal hepatic cells. **P<0.01.

were treated with the IC_{50} of doxorubicin after the adhesion, followed by 24, 48 and 72 h of observation. MTT was added in the medium at 0.25 mg/ml concentration and the absorbance at 570 nm was measured using a microplate reader.

Cell cycle analysis. HepG2 cells were infected with Ad-PEG10 or Ad-shRNAs and then treated with doxorubicin for 48 h. The detailed procedure for cell cycle analysis ws described previously (18).

Apoptosis assay. HepG2 cells were infected with Ad-PEG10 construct or Ad-shRNAs and then doxorubicin was added in the medium. After culturing for 48 h apoptotic cells were analyzed by flow cytometry (BD Biosciences) using Annexin V-FITC apoptosis detection kit (Nanjing KeyGen Biotech, Co., Ltd., Nanjing, China) following the manufacturer's protocol.

Statistical analysis. All data were obtained from at least three independent experiments (n≥3) and presented as mean ± SD. The differences between the groups were analyzed with the Student's t-test except for qRT-PCR in which one-way analysis of variance (ANOVA) was used, and P≤0.05 were considered to indicate a statistically significant result.

Results

PEG10 expression associates with the lymph node metastasis and OS of HCC. To determine the relationship between PEG10 expression and HCC OS or metastasis PEG10 expression was detected in 39 HCC tissues with or without lymph node metastasis and adjacent normal tissues by qRT-PCR analyses. As shown in Fig. 1A, PEG10 mRNA level is significantly higher in HCC tissues than in the adjacent normal tissues. Moreover, compared to the HCC tissues without lymph node metastasis, PEG10 mRNA level is much higher in HCC tissues with lymph node metastasis (Fig. 1A) and high PEG10 expression is associated with poorer OS of the patients (Fig. 1B). PEG10 protein expression was further examined using immunohistochemical analyses, as expected, the results were consistent with the qRT-PCR results (Fig. 1C). In addition, the mRNA level of PEG10 was also detected in hepatic tumor cell lines (HepG2, HCCLM3 and HUH7) and the normal hepatic cell line L02 (Fig. 1D). The results showed that PEG10 expression level is remarkably higher in hepatic cell lines than in L02 cells, especially in HepG2 cells which were used for additional study. Therefore, our results suggest that PEG10 associates with the lymph node metastasis and OS of HCC.

Ectopic expression of PEG10 confers mesenchymal-like phenotypes in HepG2 cells and promotes cell migration and invasion. Based on the above observations, we speculated that ectopic expression of PEG10 could enhance the migration, invasion and EMT in hepatic tumor cells. We constructed the adenovirus vectors with PEG10 coding area (Ad-PEG10) or shRNAs against PEG10 (Ad-shRNA1 and Ad-shRNA2) which were further infected into HepG2 cells. The efficiency of infection was examined via qRT-PCR and western blot assays.

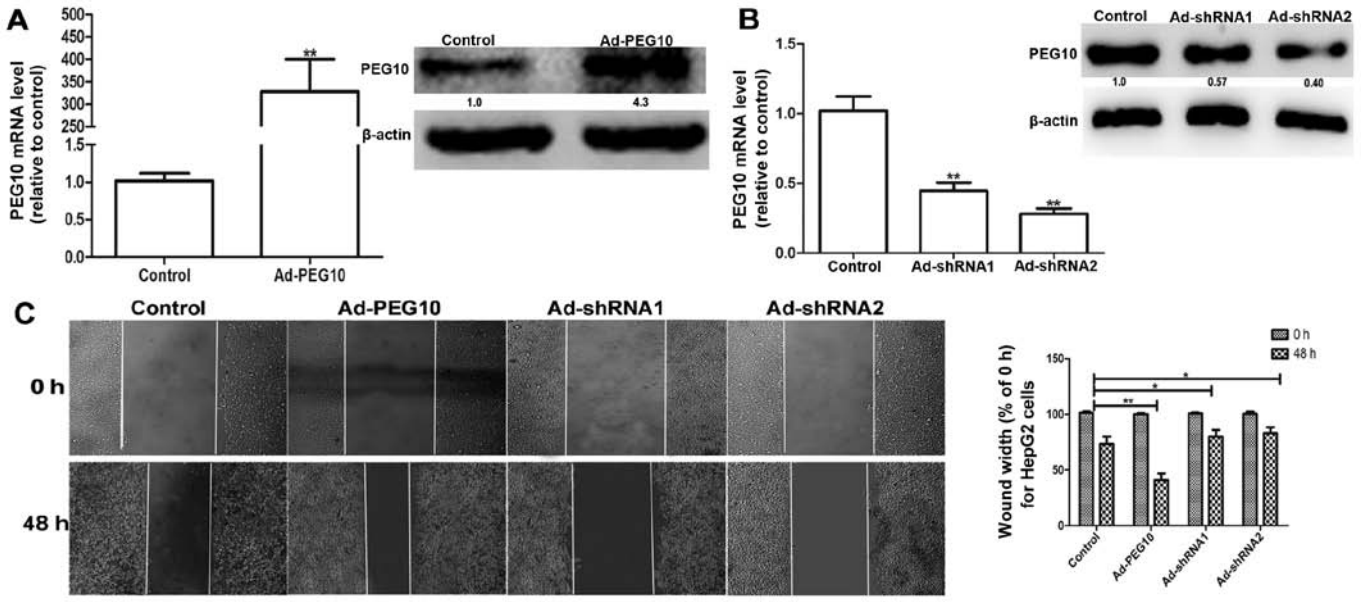

Figure 2. Overexpression of PEG10 promotes migration in HepG2 cells. (A and B) The infection efficiency of Ad-PEG10 and Ad-shRNAs was examined via qRT-PCR and western blot analyses. Cells treated with Ad-PEG10 significantly increased the mRNA and protein level of PEG10 (A), while treatment with Ad-shRNAs remarkably decreased the levels of PEG10 (B). (C) Wound-healing assay was performed to examine the migration ability of HepG2 cells. **P<0.01.

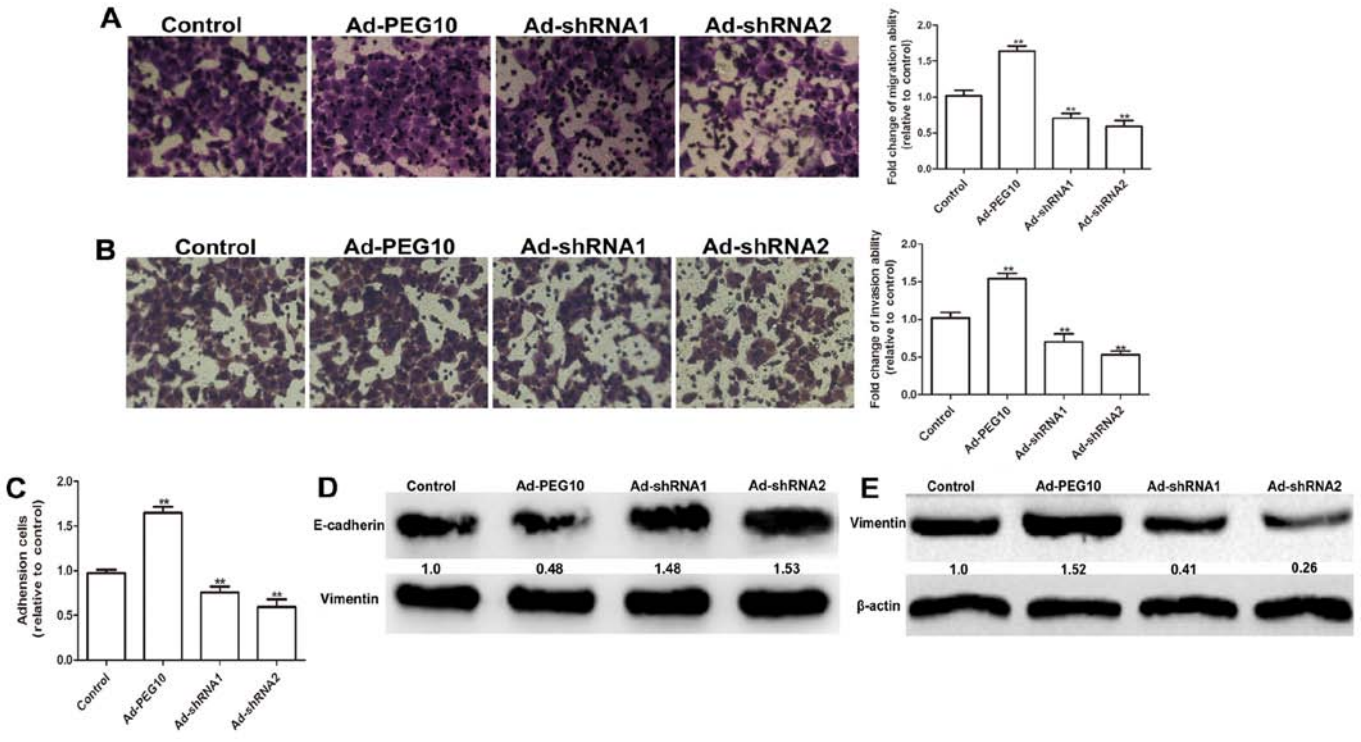

Figure 3. Overexpression of PEG10 promotes migration, invasion and EMT in HepG2 cells. (A and B) Transwell migration and invasion assays were used to detect the migration and invasion of HepG2 cells. Overexpression of PEG10 could increase the migration (A) and invasion (B), while knockdown of PEG10 could attenuate the ability in HepG2 cells. (C) Cell adhesion assay detection of HepG2 cells treated with Ad-PEG10 or Ad-shRNAs. (D and E) Western blot detection of EMT marker protein levels in HepG2 cells treated with Ad-PEG10 or Ad-shRNAs. **P<0.01.

As shown in Fig. 2A, the mRNA and protein levels of PEG10 in HepG2 cells infected with Ad-PEG10 is significantly higher than the control group. In contrast, the Ad-shRNAs-infected cells showed lower expression levels (Fig. 2B). In addition, the wound-healing, Transwell migration and invasion assays showed that HepG2 cells infected with Ad-PEG10 exhibited greater migration and invasion ability than the control cells, while Ad-shRNAs inhibited this effects (Figs. 2C and 3A and B). The adhesion ability is essential for the invasion of tumor cells (12). As shown in Fig. 3C, cells overexpressing PEG10 markedly enhanced the adhesive ability, while infection with Ad-shRNAs remarkably decreased cell adhesion. The

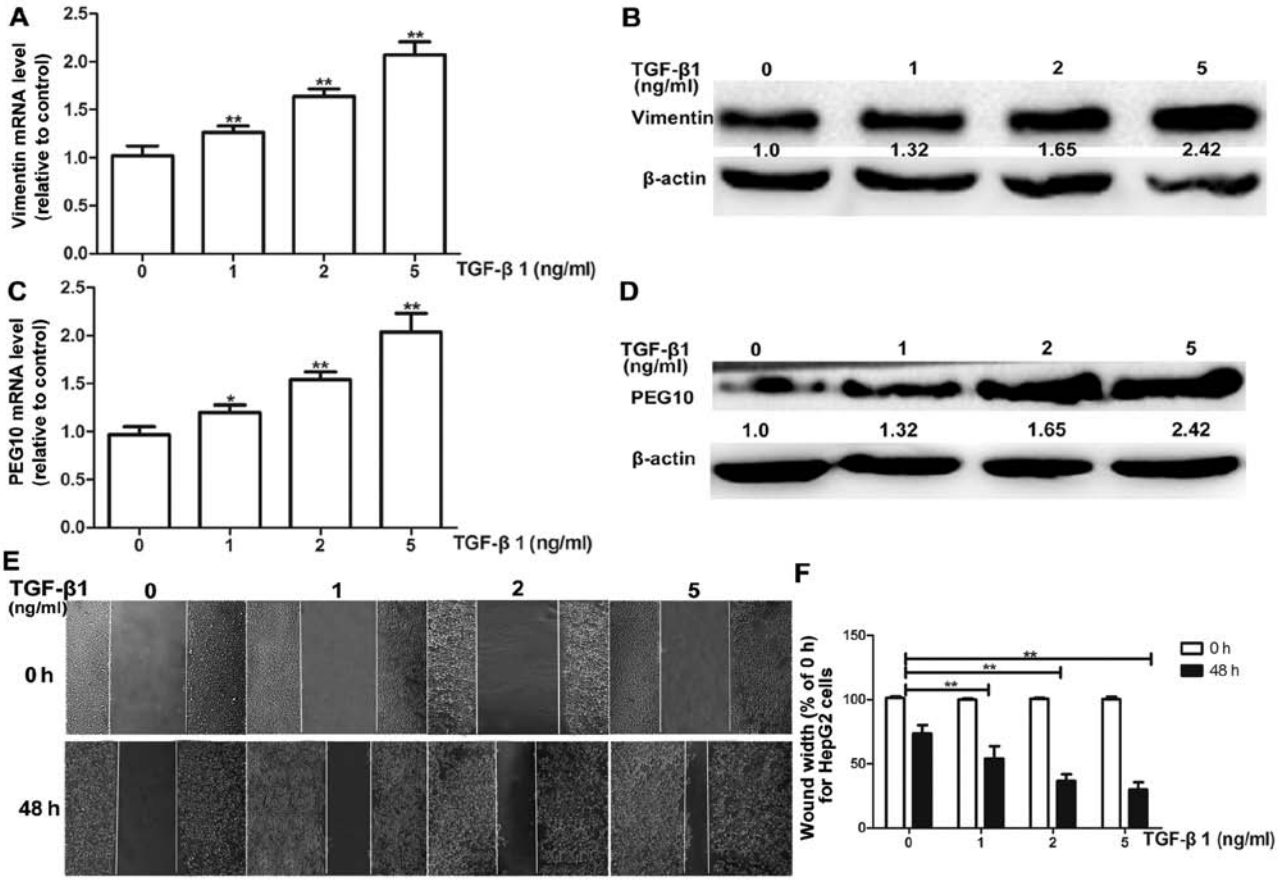

Figure 4. PEG10 plays an essential role in TGF-β1-induced migration. (A and B) The expression levels of vimentin were examined via qRT-PCR and western blot analysis with different concentrations of TGF-β1. (A) The mRNA and (B) protein levels are increased concentration-dependently on TGF-β1. (C and D) The expression levels of PEG10 were examined via qRT-PCR and western blot analysis with different concentrations of TGF-β1. The mRNA (C) and protein (D) levels are increased concentration-dependently on TGF-β1. (E and F) The migration of HepG2 cells were induced concentration-dependently on TGF-β1. *P<0.05, **P<0.01.

expression of EMT markers (E-cadherin and vimentin) were further examined in HepG2 cells infected with Ad-PEG10 or Ad-shRNAs. As shown in Fig. 3D and E, overexpression of PEG10 in HepG2 cells increased the expression levels of mesenchymal marker protein, vimentin and decreased the expression levels of epithelial marker protein, E-cadherin. In contrast, Ad-shRNAs could reduce the expression of vimentin and promote the expression of E-cadherin. Overall, our results indicate that PEG10 overexpression could promote EMT, migration and invasion of HepG2 cells.

PEG10 is essential in TGF-β1-induced EMT in vitro. Firstly, the suitable condition for TGF-β1 induction was tested, HepG2 cells were treated with different concentrations (1, 2 and 5 ng/ml) of TGF-β1 for 72 h. HepG2 cells exhibited a dose-dependent increase in mRNA and protein levels of vimentin (Fig. 4A and B). Most importantly, the PEG10 mRNA and protein levels were also increased concentration-dependently on TGF-β1 (Fig. 4C and D). Moreover, the TGF-β1 treatment was confirmed to promote migration and invasion ability of HepG2 cells via wound-healing and Transwell migration and invasion assays (Figs. 4E and F and 5A and B), indicating the TGF-β1 pathway was activated. To further examine whether PEG10 is involved in TGF-β1-induced EMT, HepG2 cell- infected Ad-PEG10 or Ad-shRNAs were treated with 2 ng/ml TGF-β1. As shown in Fig. 6A-C, Ad-PEG10 infected cells exhibited higher levels of migration and invasion relative to cells treated TGF-β1 alone, while decreased levels of migration and invasion were observed in Ad-shRNAs-infected cells. In addition, Ad-PEG10 infected cells exhibited higher levels of vimentin relative to cells treated TGF-β1 alone, while decreased levels of vimentin were observed in Ad-shRNA-infected cells (Fig. 6D and E). Therefore, these results suggest that PEG10 expression is essential for TGF-β1-induced EMT.

Overexpression of PEG10 confers doxorubicin resistance in HepG2 cells. A previous study has shown that EMT possessed a promotive role in chemoresistance in HCC (19). Here, we further investigated whether ectopic expression of PEG10 lead to doxorubicin resistance in HepG2 cells. MTT assays were performed to detect the cell viability, as shown in Fig. 7A, HepG2 cells overexpressing PEG10 displayed low doxorubicin sensitivity and knockdown of PEG10 increased doxorubicin sensitivity. Furthermore, HepG2 cell viability was also examined by cell cycle assays. As expected, when treated with doxorubicin, HepG2 cells showed a decrease of cells in S phase, while HepG2 cells overexpressing PEG10 showed litte change compare to the control. Noteworthy, HepG2 cells treated with doxorubicin plus shRNAs of PEG10 exhibited

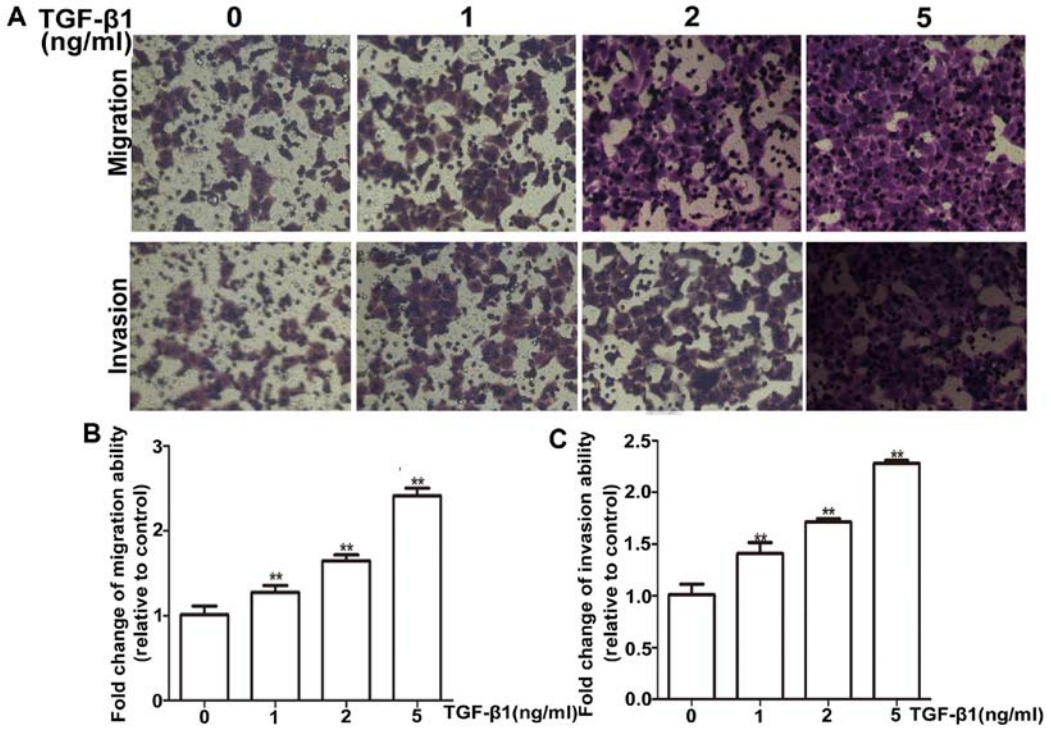

Figure 5. TGF-β1 induces cell migration and invasion concentration-dependently. (A-C) The migration and invasion of HepG2 cells were induced concentration-dependently on TGF-β. **P<0.01.

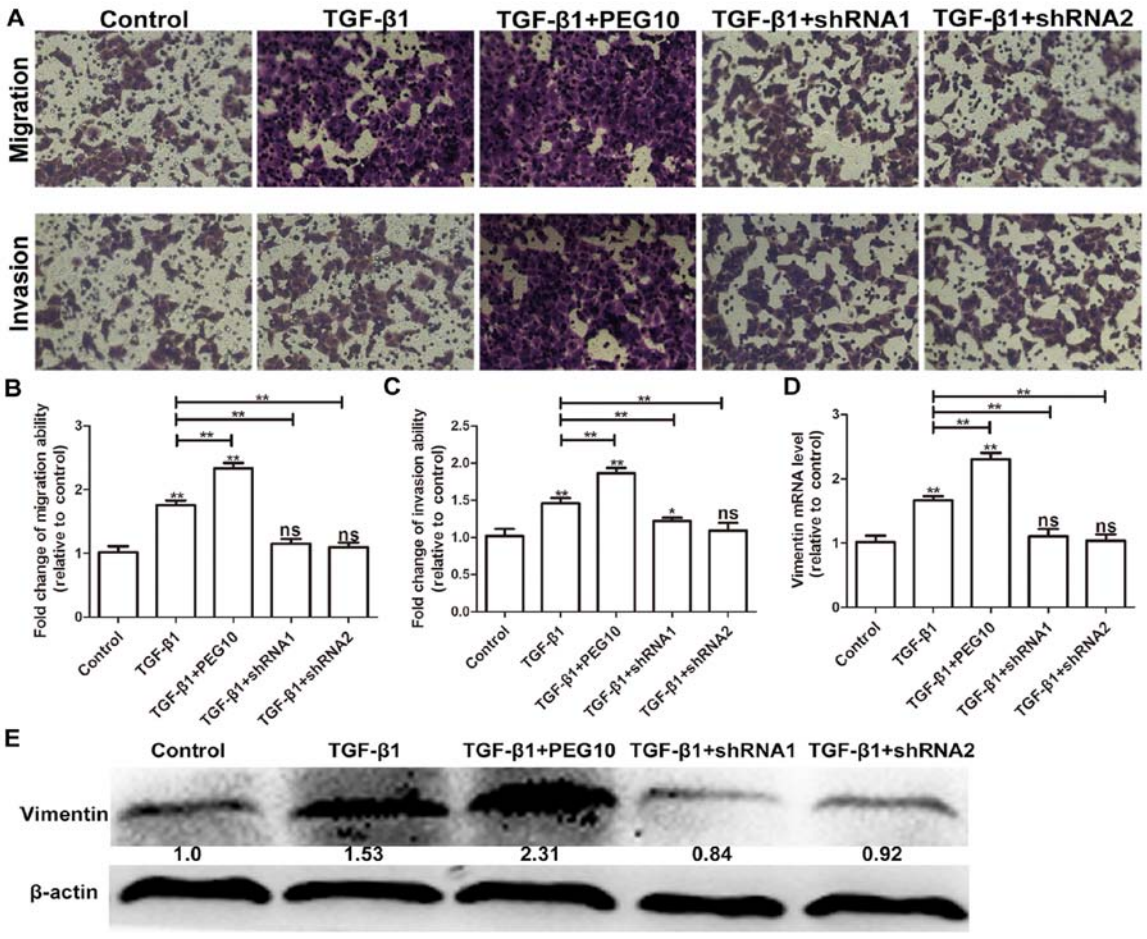

Figure 6. PEG10 plays an essential role in TGF-β1-induced EMT. 1. (A-C) Upregulation or downregulation of PEG10 prior to TGF-β1 treatment enhanced or attenuated the effects of TGF-β1 on migration and invasion of HepG2 cells. (D and E) Upregulation or downregulation of PEG10 prior to TGF-β1 treatment enhanced or attenuated the effects of TGF-β1 on EMT in HepG2 cells. *P<0.05, **P<0.01.

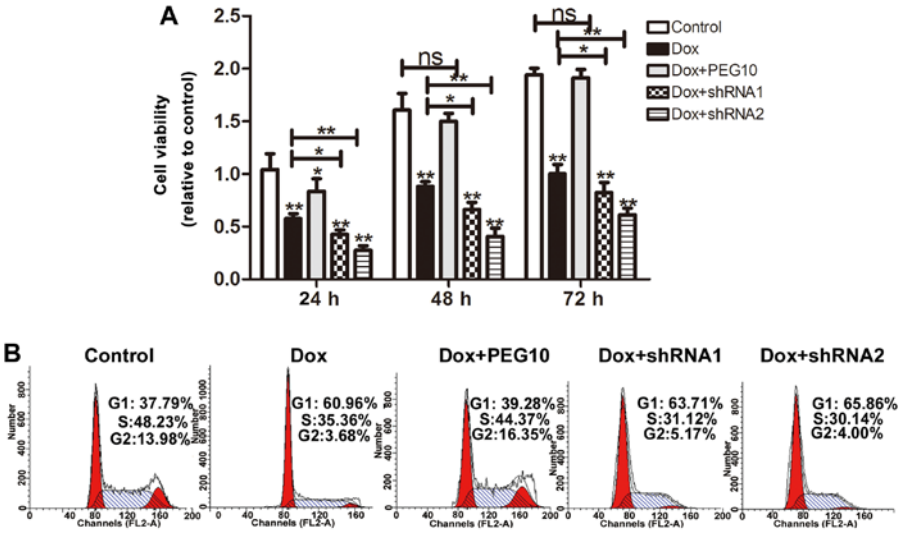

Figure 7. Overexpression of PEG10 confers doxorubicin resistance in HepG2 cells. (A) HepG2 cells were infected with Ad-PEG10 or Ad-shRNAs and followed by doxorubicin treatment, then the cell viability was examined by MTT assay. (B) Cell cycle assays were performed in cells described in A. Compared with the control group cells, HepG2 cells treated with doxorubicin significantly decreased the S phase and knockdown of PEG10 enhanced this effect, while overexpression of PEG10 reversed this effect. *P<0.05, **P<0.01.

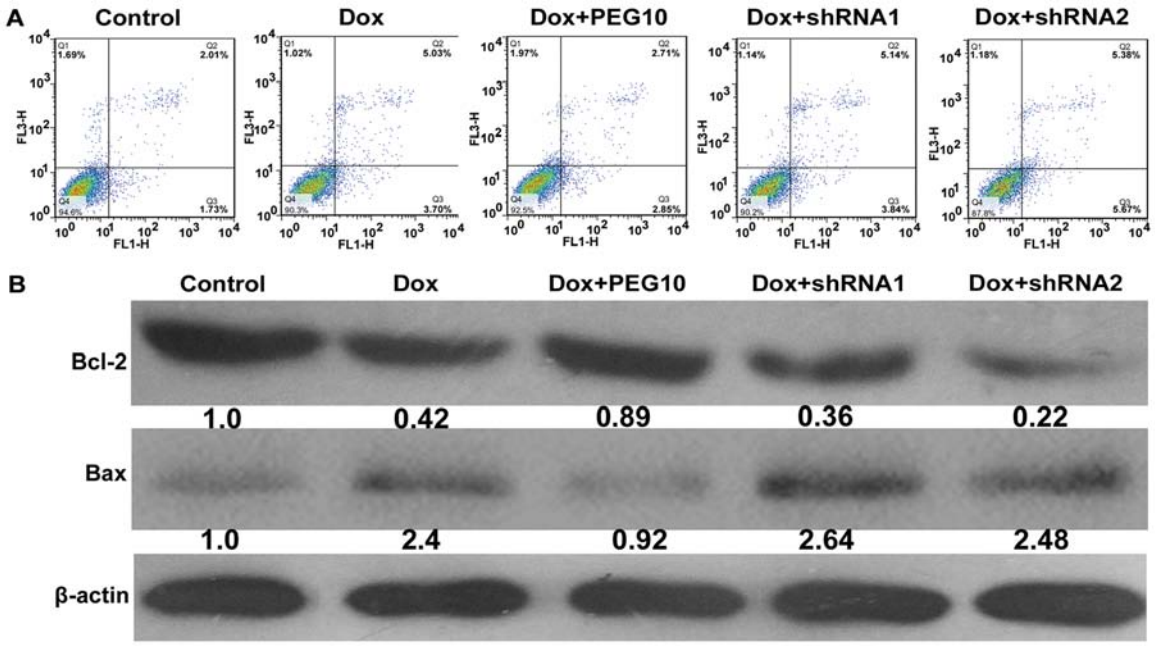

Figure 8. Overexpression of PEG10 inhibits doxorubicin-induced apoptosis in HepG2 cells. (A) Cell apoptosis assays were carried out in cells described in Fig. 7. HepG2 cells treated with doxorubicin significant promoted cell apoptosis and knockdown of PEG10 enhanced this effect, while overexpression of PEG10 reversed this effect. (B) The protein levels of Bcl-2 and Bax were detected via western blot assays.

a stronger decrease compared to the group treated with doxorubicin alone (Fig. 7B). In addition, HepG2 cells were analyzed by apoptosis assays. Results showed that upregulation of PEG10 in HepG2 cells inhibited apoptosis induced by doxorubicin treatment and knockdown of PEG10 enhanced the effect (Fig. 8A). The protein level of apoptotic markers (Bcl-2 and Bax) were examined by western blot analysis. As shown in Fig. 8B, exposure of the Ad-PEG10 infected cells to doxorubicin decreased the expression of Bax and increased Bcl-2 expression. Conversely, treatment with Ad-shRNAs promoted the expression of Bax but decreased Bcl-2 expression. Hence, these results indicate that enforced expression of PEG10 was able to confer doxorubicin resistance in HepG2 cells.

Discussion

Although previous studies have indicated that PEG10 might be a critical factor in gallbladder adenocarcinoma (20), immune response (21), breast (9) and lung cancer (8), the related studies of PEG10 on the EMT and chemoresistace of HCC are rare and the mechanism of PEG10 in the progression of HCC is still unclear. EMT has been implicated in several tumor

types, including breast (12) and human non-small cell lung cancer (22). TGF-β1 is a well-known trigger and plays a vital role in the EMT process (16). Thus, in the present study, a recombinant adenovirus vector of PEG10 and its shRNAs were constructed and transferred into HepG2 cells to explore the promotive effects on HCC migration, invasion and the possible roles in TGF-β1-induced EMT.

In the present study, 39 HCC tissues with or without lymph node metastasis and adjacent normal tissues were used to explore the relationship between the expression level of PEG10 and OS or lymph node metastasis of HCC. Our results showed that high PEG10 expression is associated with poorer OS of the patients, which is consistent with previous studies (23,24) and makes our results even more reliable. Although recent studies have suggested that EMT is not required for lung and pancreatic cancer metastasis (25,26), our results provide substantial evidence that overexpression of PEG10 enhances EMT and migration, invasion and adhesion in HCC, which is consistent with the functional roles of PEG10 in breast cancer (9). However, further studies are necessary for investigating the mechanisms of PEG10 in EMT and whether PEG10 promotes the metastasis of HCC via EMT. Most importantly, as TGF-β1 is a key factor involved in the induction of EMT, stimulation with TGF-β1 led to the transition of HCC to a mesenchymal phenotype, which is proved in this study. However, following stimulation of TGF-β1 with knockdown of PEG10 exhibited a significant abrogation in TGF-β1-induced EMT, migration and invasion in HCC and upregulation of PEG10 enhanced these effects. To the best of our knowledge, effects of PEG10 on EMT and TGF-β1-induced EMT, migration and invasion have not been previously reported.

Evidence has confirmed that EMT plays a promotive role in chemoresistance of various cancers and targeting EMT with Met inhibitors has been proved to reverse chemoresistance in small cell lung cancer (27,28). In addition, doxorubicin resistance is one reason for HCC recurrence (19). Subsequently, we further investigated whether PEG10 is associated with doxorubicin resistance in HCC. We demonstrated that overexpression of PEG10 conferred doxorubicin resistance in HepG2 cells. However, cells with knockdown of PEG10 displayed greater sensitivity to doxorubicin. These results might provide evidence for the fact that patients with high expression of PEG10 have poor survival, and tumor recurrence in HCC (7).

In conclusion, our results present that PEG10 promoted migration, invasion and EMT in HCC and enhanced TGF-β1-induced EMT. In addition, PEG10 aids in chemoresistance in HepG2 cells. These results may provide a new potential therapeutical target for HCC metastasis and chemoresistance. However, further study is needed to explain the action of PEG10 in HCC and the mechanisms of PEG10 in TGF-β1-induced EMT, and thus, resolve the issue of HCC chemoresistance.

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