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ORIGINAL ARTICLE

The oncoprotein p28^{GANK} establishes a positive feedback loop in β-catenin signaling

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p28^{GANK} (also known as PSMD10 or gankyrin) is a novel oncoprotein that is highly expressed in hepatocellular carcinoma (HCC). Through its interaction with various proteins, p28^{GANK} mediates the degradation of the tumor suppressor proteins Rb and p53. Although p53 was reported to downregulate β-catenin, whether p28^{GANK} is involved in the regulation of β-catenin remains uncertain. Here we report that both growth factors and Ras upregulate p28^{GANK} expression through the activation of the phosphoinositide 3-kinase-AKT pathway. Upregulation of p28^{GANK} expression subsequently enhanced the transcription activity of β-catenin. This effect was observed in p53-deficient cells, suggesting a p53-independent mechanism for the p28^{GANK}-mediated activation of β-catenin. p28^{GANK} overexpression also reduced E-cadherin protein levels, leading to increased release of free β-catenin into the cytoplasm from the cadherin-bound pool. Interestingly, exogenous expression of p28^{GANK} resulted in elevated expression of the endogenous protein. We also observed that both β-catenin and c-Myc were transcriptional activators of p28^{GANK}, and a correlation between p28^{GANK} overexpression and c-Myc, cyclin D1 and β-catenin activation in primary human HCC. Together, these results suggest that p28^{GANK} expression is regulated by a positive feedback loop involving β-catenin, which may play a critical role in tumorigenesis and the progression of HCC.

Keywords**:** p28^{GANK}; β-catenin; E-cadherin; positive feedback loop; hepatocellular carcinoma
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Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world and is a major cause of death in many countries [1]. HCCs display great genomic heterogeneity and may involve at least three carcinogenesis-related pathways: the p53, Rb and Wnt/β-catenin signaling pathways [2]. Three decades of basic cancer research have revealed that mutations in the components of signaling pathways that control cell growth are the basis of tumor initiation in mammals. The Ras, extracellular-signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K) signaling pathways form an intersecting biochemical network that, when mutated, drives cell growth in a manner unrestricted by environmental cues. These pathways drive tumorigenesis through coordinated protein phosphorylation events that directly regulate protein synthesis, cell-cycle progression and activity of transcription factors that regulate the expression of genes involved in these processes [3, 4].

Most human tumors harbor mutations that activate these master regulators or inactivate negative regulators of these regulators [3-5]. Each of these proteins activates a number of downstream effectors. The activation of PI3K mediates the recruitment and activation of signaling proteins with pleckstrin homology domains including the serine-threonine kinase Akt [6]. Akt phosphorylates several downstream targets, including the constitutively active glycogen synthase kinase-3 (GSK3)-β (Ser9), resulting in its inhibition [7]. GSK-3β is a critical component in β-catenin signaling and provides a mechanistic

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link between growth factor stimulation or Ras activation and β-catenin stabilization.

The β-catenin signaling pathway plays a critical role in determining cell fate, tissue homeostasis and tumorigenesis [8]. In addition to its function in the Wnt signaling pathway, β-catenin also binds to the cytoplasmic domain of type I cadherins and plays an essential role in the structural organization and function of cadherins by linking cadherins to the actin cytoskeleton via α-catenin [9]. The cadherin-bound pool of β-catenin can be released and made available for nuclear signaling. Binding partners of β-catenin can modify β-catenin/TCF-dependent transcription, thus regulating the expression of target genes [10]. Many cancers are initiated by inappropriate activation of the Wnt pathway, which is characterized by the accumulation of nuclear β-catenin and the constitutive transcriptional activity of the β-catenin/TCF complex [11, 12]. Approximately 50%-70% of all HCCs have an abnormal accumulation of β-catenin protein in the cytoplasm and the nucleus [13-15]. However, in HCC, the β-catenin mutation rate is 13%-26% [13, 15] and the axin mutation rate is 5%-10% [16, 17]. Few mutations of APC have been reported so far. These facts led us to speculate that other unknown factors might be involved in regulating β-catenin in HCC.

A novel oncogene named *gankyrin* was cloned from HCC by cDNA subtractive hybridization [18]. Its sequence is identical to that of the *p28* gene, whose product is one of the non-ATPase subunits of PA700 (19S), a regulatory complex of the human 26S proteasome [19]. The product of this novel gene ($p28^{GANK}$) forms complexes with multiple proteins. $p28^{GANK}$ overexpression accelerates the hyperphosphorylation and degradation of Rb, releasing the transcription factor E2F-1 from the Rb repressor complex [18]. Furthermore, the binding of $p28^{GANK}$ to Cdk4 prevents Cdk4 from binding to $p16^{INK4a}$ [20], which accelerates cell cycle progression. $p28^{GANK}$ also binds to the E3 ubiquitin ligase MDM2, facilitating the ubiquitylation and subsequent proteasomal degradation of p53 [21]. Recently, an additional target of $p28^{GANK}$, C/EBPα, was identified, and the degradation of C/EBPα by the ubiquitin-proteasome system was shown to contribute to the development of liver cancer [22]. In the present study, we demonstrate that activation of β-catenin signaling regulates $p28^{GANK}$ expression, which subsequently enhances the activation of β-catenin/TCF-mediated transcription. The oncoprotein $p28^{GANK}$ thus establishes a positive feedback loop that regulates β-catenin signaling and plays an important role in tumorigenesis and progression of HCC.

Results

Growth factor stimulation and Ras activation upregulate $p28^{GANK}$ expression

To investigate factors that may influence $p28^{GANK}$ expression, we cloned a 1226 bp fragment of the 5'-flanking region of the human *$p28^{GANK}$* gene into a pGL3 luciferase reporter vector, after analysis using the Gene2Promoter (Genomatix) online software. To determine whether $p28^{GANK}$ upregulation was correlated with cell cycle progression in normal and HCC cell lines, we examined the potential cell cycle dependence of $p28^{GANK}$ expression in HepG2 and HEK293 cells after mitogenic stimulation. Starvation has been previously shown to induce cell cycle arrest, but starved cells were able to enter S phase after stimulation with growth factors, such as epidermal growth factor (EGF) or hepatocyte growth factor (HGF) [23, 24]. We thus used reporter gene assays to measure the effect of growth factor stimulation on $p28^{GANK}$ expression. As shown in Figure 1A and 1B, $p28^{GANK}$ reporter gene activity was significantly increased after stimulation with different doses of EGF or HGF. Endogenous $p28^{GANK}$ expression was strongly induced in liver cells after 6 to 48 h of stimulation with EGF (Figure 1C, 1D and Supplementary information, Figure S1A). EGF treatment also increased c-Myc protein levels (Figure 1D, Supplementary information, Figure S1A).

Ras is a key signal transduction component downstream of growth factors; therefore, we aimed to determine whether Ras is involved in regulating $p28^{GANK}$ expression. As shown in Figure 1E, the relative luciferase activity of $p28^{GANK}$ reporter increased in a dose-dependent manner after Ras transfection. Quantitative RT-PCR and western blot analysis also showed that $p28^{GANK}$ expression was upregulated at both the mRNA and the protein levels (Figure 1F and 1G). The results also demonstrated that Ras increased the levels of c-Myc protein, indicating that Ras activation regulated c-Myc and $p28^{GANK}$ levels simultaneously.

Growth factor stimulation and Ras activation increase $p28^{GANK}$ expression through the activation of PI3K signaling

The ability of growth factors and Ras to activate the PI3K-AKT and Raf-MEK-ERK kinase cascades prompted us to ask which pathway is involved in regulating $p28^{GANK}$ expression. LY294002, a PI3K inhibitor, significantly suppressed EGF- or HGF-induced $p28^{GANK}$ reporter gene activity, and conversely, the ERK inhibitors PD98059 and U0126 enhanced reporter gene activity (Figure 2A and data not shown). In Ras-transfected cells, these inhibitors evoked the same effects (Figure 2B). Moreover, inhibiting PI3K signaling in unstimulated cells also reduced $p28^{GANK}$ reporter expression, indicat-

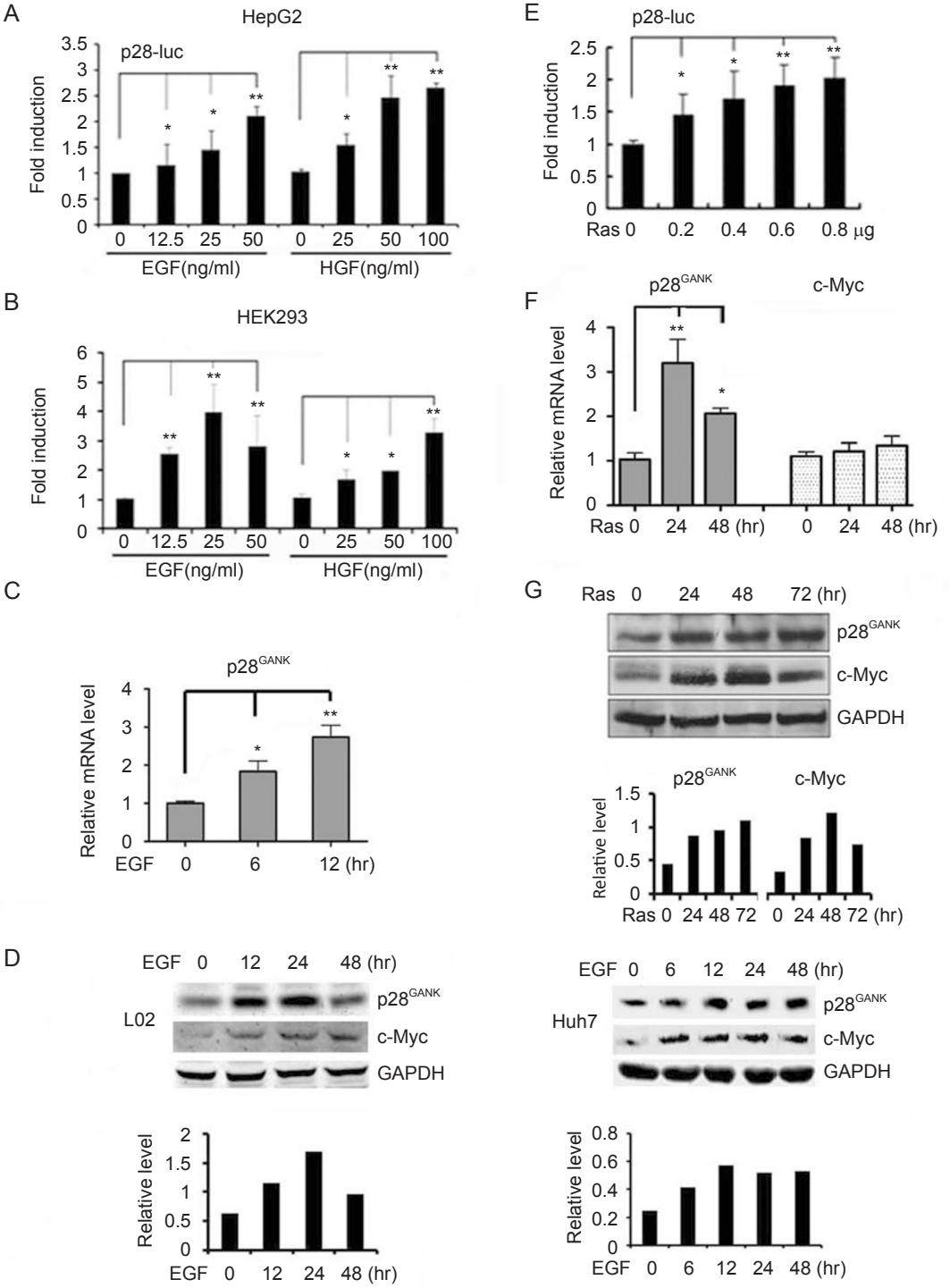

Figure 1 Growth factor stimulation and Ras activation upregulated p28^{GANK} expression. Growth factor stimulation upregulated p28^{GANK} expression in **(A)** HepG2 and **(B)** HEK293 cells. Cells were transiently transfected with p28^{GANK} reporter plasmid and the control plasmid pRL-TK. After treatment with EGF or HGF for 12 h, p28^{GANK} reporter activities were determined. **(C)** EGF upregulated p28^{GANK} expression. HepG2 cells were deprived of FBS for 12 h and then stimulated with EGF (20 ng/ml) for 0-12 h. **(D)** Upregulation of p28^{GANK} by EGF was detected by western blotting. p28^{GANK} protein levels were detected in EGF-treated L02 and Huh7 cells. The protein levels were quantified relative to the loading control. **(E)** Upregulation of p28^{GANK} was detected by gene reporter analysis in Ras-transfected cells. HEK293 cells were transiently transfected with a p28^{GANK} reporter plasmid and 0-0.8 μg Ras plasmid. After 24 h, the luciferase activities were measured. **(F)** Upregulation of p28^{GANK} expression was detected by qPCR in Ras-activated cells. HEK293 cells were transiently transfected with a Ras expression plasmid for 0-24 h, and qPCR was used to analyze *p28^{GANK}* and *c-Myc* expression. **(G)** Upregulation of p28^{GANK} was detected by western blotting in Ras-transfected HEK293 cells (*P < 0.05; **P < 0.01).

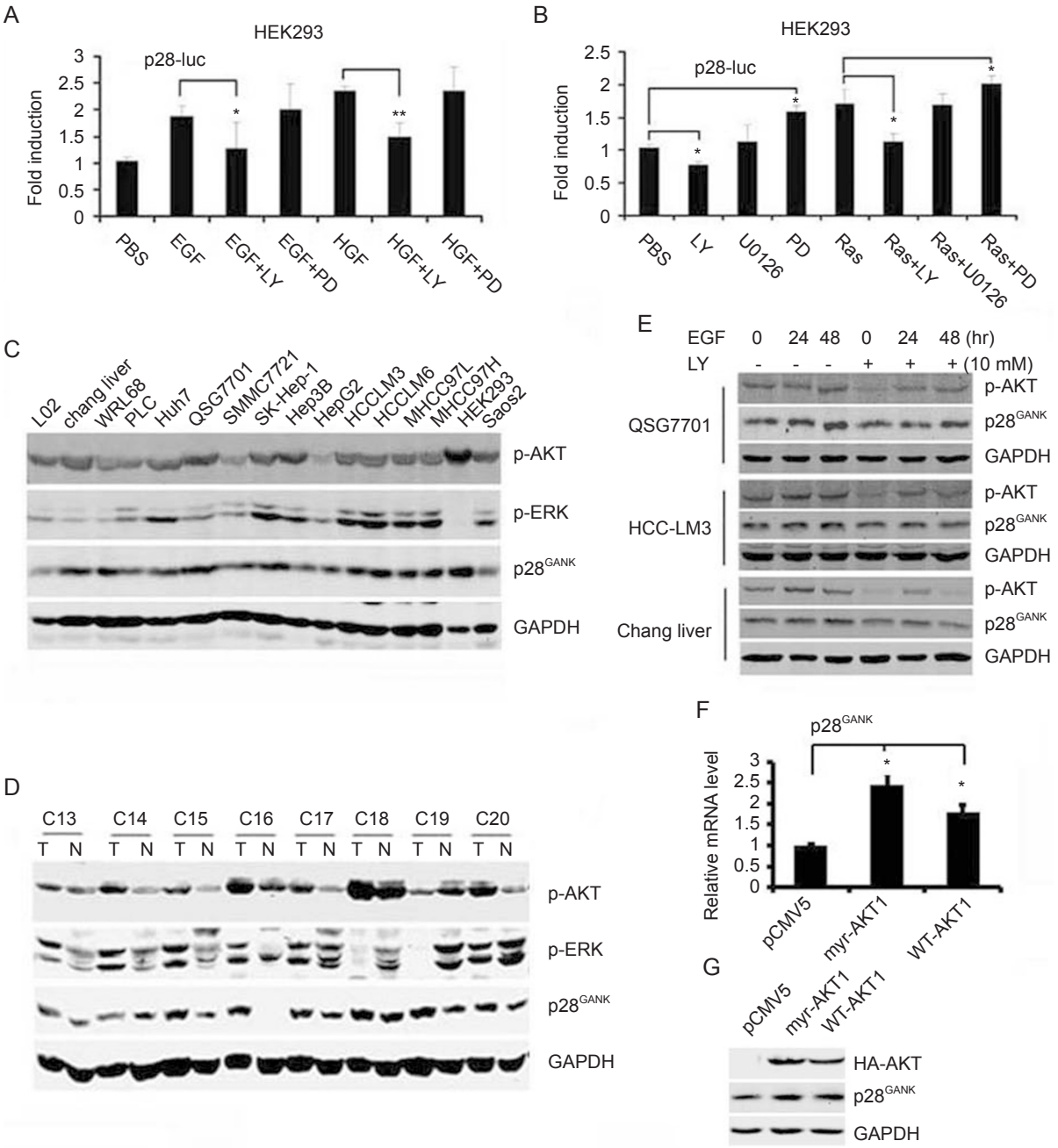

Figure 2 Growth factors and Ras increased p28^{GANK} expression through the activation of PI3K-AKT signaling. **(A)** PI3K inhibition abolished growth factor-induced p28^{GANK} upregulation. HEK293 cells were transiently transfected with a p28^{GANK} reporter plasmid. Cells were deprived of serum for 12 h, at which time PI3K inhibitor LY294002 or ERK inhibitor PD98059 was added to the medium. After 2 h, cells were stimulated for 6 h with EGF or HGF at a final concentration of 20 ng/ml or 50 μg/ml, respectively, and luciferase activities were detected. **(B)** PI3K inhibitor abrogated Ras-induced p28^{GANK} upregulation. HEK293 cells were transiently transfected with pGL3-p28 with or without the Ras plasmid. Cells were stimulated with LY294002, PD98059 or U0126 after starvation as above, and luciferase activities were detected. **(C)** p-AKT, p-ERK and p28^{GANK} expression in HCC cell lines. The HCC cell lines HEK293 and Saos2 were lysed, and endogenous p-AKT, p-ERK and p28^{GANK} expression levels were detected by western blotting. **(D)** p-AKT, p-ERK and p28^{GANK} expression in representative clinical HCC samples. Clinical HCC samples were lysed, and p-AKT, p-ERK and p28^{GANK} expression levels were detected by western blotting. **(E)** The effect of AKT activation on the expression of p28^{GANK}. QSG7701, HCCC-LM3 and Chang liver cells were stimulated with EGF at the indicated time points with or without Ly294002 pretreatment. The cells were lysed and analyzed by western blot. **(F, G)** L02 cells were transiently transfected with 0.5 μg pCMV5, myr-AKT1 or WT-AKT1 for 48 h, and p28^{GANK} expression was measured by quantitative RT-PCR and western blotting (*P < 0.05; **P < 0.01).

ing that PI3K activity had a positive effect on p28GANK expression (Figure 2B). To evaluate the correlation between the level of expression of p28GANK and the activity levels of AKT and ERK, we assessed the expression of p28GANK and the phosphorylation of AKT and ERK in several HCC cell lines and 40 primary HCC samples. p28GANK protein levels more closely correlated with AKT activation than with ERK activation (Figure 2C, 2D, Supplementary information, Figure S1D). p28GANK expression was also examined in untreated HCC cell lines and HCC cell lines treated with EGF or PI3K inhibitors. EGF significantly activated AKT and upregulated p28GANK expression. Correspondingly, the administration of LY294002 or wortmannin, inhibitors of PI3K, inhibited AKT phosphorylation and decreased p28GANK expression (Figure 2E, Supplementary information, Figure S1B). Further, we used a constitutively active AKT construct, pCMV5-myr-AKT1 and wild-type AKT1 cDNA to investigate the effects of AKT on p28GANK expression. AKT activation upregulated p28GANK expression in L02 and HEK293 cells (Figure 2F and 2G, Supplementary information, Figure S1C).

p28GANK positively regulates β-catenin signaling independent of p53

Previous studies have revealed that p28GANK can promote cell proliferation. β-catenin signaling is one of the most important cascades in regulating cell survival and proliferation. Therefore, we aimed to determine whether p28GANK expression affects β-catenin/TCF-dependent transcription. The effect of p28GANK expression on β-catenin/TCF-dependent reporter activity was assessed using the TopFlash (pGL-OT and the mutant control construct pGL-OF) reporter system in HEK293 and HepG2 cells. The expression of p28GANK induced the activation of β-catenin/TCF-mediated reporter expression in a concentration-dependent manner in HEK293 cells (Figure 3A). β-catenin/TCF-dependent reporter activity was also assessed in HepG2 cells after adenovirus delivery of siRNA against p28GANK (AdSip28). Consistent with our results in HEK293 cells, pGL-OT activity was reduced by p28GANK inhibition (Figure 3B). To determine whether knockdown of endogenous β-catenin would affect p28GANK-mediated activation of the reporter, we examined reporter activation after using an shRNA to target β-catenin. As shown in Figure 3C, knockdown of β-catenin significantly suppressed p28GANK-induced activation of this reporter. Next, we analyzed the effects of p28GANK on the distribution of β-catenin in the nucleus and cytoplasm of HepG2 and HEK293 cells. We found that p28GANK not only increased β-catenin nuclear accumulation but also moderately elevated β-catenin protein levels in the cytoplasm (Figure 3D, Supplementary information, Figure S2A). To further assess the regulation of β-catenin by p28GANK, we knocked down p28GANK in HepG2 cells and detected the nuclear protein levels of β-catenin and other related proteins. As shown in Figure 3E, nuclear β-catenin, c-Myc and cyclinD1 decreased with the loss of p28GANK.

It is known that overexpression of wild-type p53 downregulates β-catenin in a variety of cell types, and this effect is dependent on the integrity and functionality of p53 [25, 26]. Previous reports have shown that the binding of p28GANK to MDM2 facilitates p53-MDM2 interactions and increases the ubiquitylation and degradation of p53 [24]. We therefore asked whether p28GANK might regulate β-catenin through p53. We examined the activity of the pGL-OT reporter in p53-null Hep3B cells. As shown in Figure 3F, the reporter activity decreased with decreasing expression of p28GANK. The real-time PCR assay also indicated that a decrease in p28GANK resulted in the downregulation of c-Myc and cyclin D1 expression (Figure 3G). Our observations suggest a novel role for p28GANK in positively regulating β-catenin/TCF-mediated transcription.

Recently, a study revealed that p28GANK can activate AKT [27], which phosphorylates several downstream targets including GSK-3β (Ser9), resulting in its inhibition [7]. Given that GSK-3β provides a mechanistic link between growth factor stimulation or Ras activation and β-catenin stabilization, we examined the ability of p28GANK to regulate AKT and GSK-3β phosphorylation. In HepG2, L02 and Huh7 cells, the expression of p28GANK significantly increased p-AKT levels, but had no effect on p-GSK-3β levels (Supplementary information, Figure S2B-S2D). Similar results were observed when p28GANK was stably knocked down in Huh7 and LM3 cells (Supplementary information, Figure S2E). Taken together, these data indicate that the GSK-3β pathway may not be the main mechanism through which p28GANK plays its regulatory role.

Expression of p28GANK decreased E-cadherin levels and increased free cytoplasmic β-catenin

To determine whether the induction of β-catenin signaling was due to β-catenin being released from the membrane-associated E-cadherin/β-catenin complex pool, levels of E-cadherin from HEK293 cells overexpressing p28GANK and HepG2 cells were analyzed by immunoblotting. As shown in Figure 4A, overexpression of p28GANK significantly downregulated E-cadherin expression in HEK293 cells. The use of adenovirus-delivered siRNA to suppress p28GANK expression in HepG2 cells decreased levels of p28GANK and increased

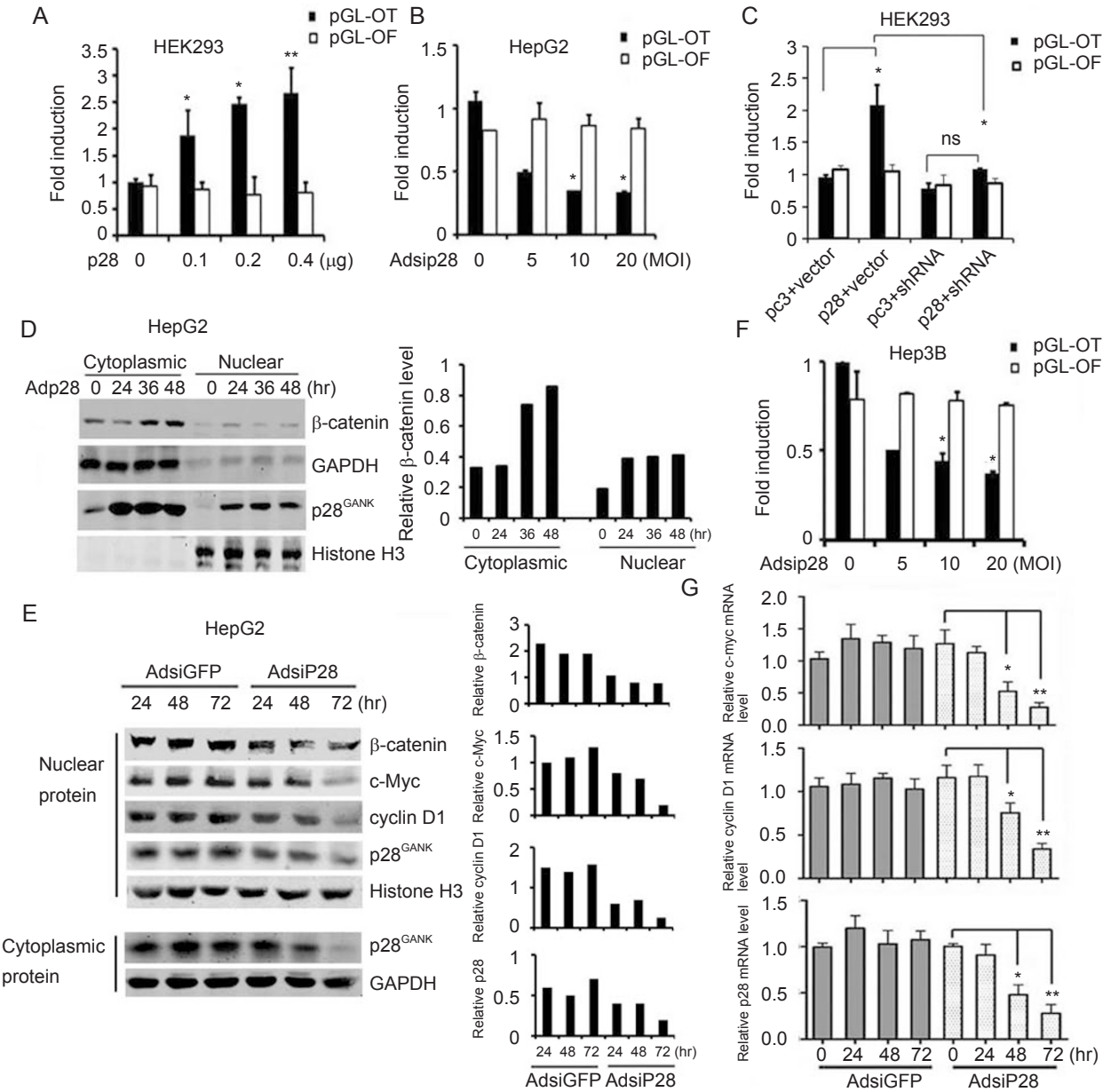

Figure 3 p28^{GANK} positively regulated β-catenin signaling. **(A)** p28^{GANK} increased β-catenin/TCF signaling activity. HEK293 cells were transferred to a 48-well plate and co-transfected with pGL-OT or pGL-OF in combination with increasing amounts of the p28^{GANK} expression construct (0-0.4 μg) or control plasmid. Luciferase activities were determined 24 h after transfection. **(B)** Suppression of p28^{GANK} decreased β-catenin/TCF transcriptional activity. HepG2 cells were co-transfected with pGL-OT or pGL-OF with the control plasmid pRL-TK. Twelve hours after transfection, the cells were infected with increasing amounts of adenovirus-delivered siRNA directed toward p28^{GANK} (AdSip28, 0, 5, 10, 20 MOI). Luciferase activity was measured 48 h after adenovirus infection. **(C)** Inhibition of endogenous β-catenin abolished p28^{GANK}-induced pGL-OT activation. HEK293 cells were co-transfected with the pGL-OT or pGL-OF together with 0.2 μg p28^{GANK} expression construct with or without β-catenin-shRNA plasmids. After 48 h, the luciferase activity was measured. **(D)** p28^{GANK} increased β-catenin nuclear localization. HepG2 cells were infected with increasing amounts of p28^{GANK} expression Advirus as indicated. The cytoplasmic and nuclear proteins were extracted and subjected to western blotting analysis. **(E)** Knockdown of p28^{GANK} reduced β-catenin, c-Myc and cyclin D1 protein levels in nucleus. HepG2 cells were infected with AdSip28 and AdSipGFP (negative control) and harvested at 24-72 h. Nuclear fractions were separated and proteins were subjected to western blot analysis using the indicated antibodies. **(F**, **G)** p53 was dispensable in the role of p28^{GANK}-mediated activation of β-catenin. **(F)** Hep3B cells were co-transfected with pGL-OT and the control plasmid. Forty-eight hours after infection with AdSip28, the luciferase activity was measured. **(G)** Hep3B cells were infected with AdSip28 (20 MOI) to knockdown p28^{GANK}, using AdSipGFP as a control. After 0-72 h, cells were harvested, and qPCR was performed to analyze *c-Myc, cyclin D1* and *p28^{GANK}* expression (*$P < 0.05$; **$P < 0.01$).

E-cadherin protein levels (Figure 4B). Further, in Huh7 and QSG7701 cells, adenovirus-mediated overexpression of p28GANK markedly decreased E-cadherin protein levels (Figure 4C and 4D). Next, we performed co-IP assays in liver cancer cells by precipitating either p28GANK or E-cadherin. Even though p28GANK and E-cadherin can both form a complex with MDM2, we could not detect a p28/E-cadherin complex (Supplementary information, Figure 3A and 3B). These data are consistent with previous studies that identified E-cadherin as an MDM2-binding protein and confirmed that E-cadherin was a substrate for the MDM2 E3 ubiquitin ligase [28].

To further characterize the effect of p28GANK, we evaluated the level of the E-cadherin/β-catenin complex by immunoprecipitation. We observed that the level of E-cadherin/β-catenin complex decreased upon expres-

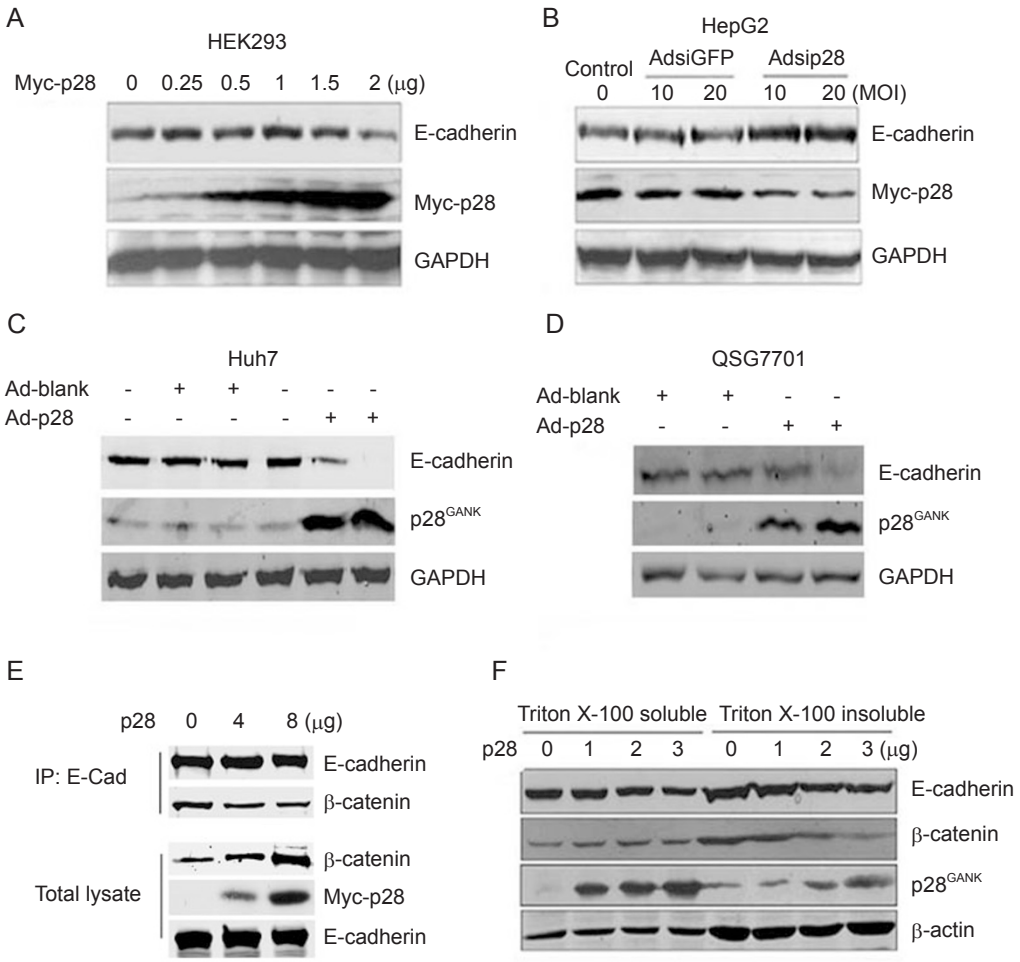

Figure 4 Expression of p28GANK led to decreased E-cadherin and increased free cytoplasmic β-catenin. **(A)** Expression of p28GANK led to decreased E-cadherin in HEK293 cells. HEK293 cells were seeded in a 12-well plate and transfected with increasing amounts of p28GANK expression constructs (0-2 μg) for 48 h; the cell lysates were subjected to western blotting analysis. **(B)** Knockdown of p28GANK increased E-cadherin protein levels in HepG2 cells. HepG2 cells were infected with Ad-Sip28 (10 or 20 MOI) to knock down p28GANK; AdSiGFP was used as a control. Cells were harvested and E-cadherin was detected by western blotting. **(C**, **D)** Overexpression of p28GANK downregulated E-cadherin protein levels in HCC cells. **(C)** Huh7 and **(D)** QSG7701 cells were infected with Adp28GANK (10 or 20 MOI) to increase p28GANK expression; Ad-blank was used as a control. After 48 h, cells were harvested and E-cadherin was detected by western blotting. **(E)** Expression of p28GANK reduced the association of E-cadherin with β-catenin. HEK293 cells were transfected with p28GANK expression constructs (0-8 μg) for 48 h; cell lysates containing equal amounts of E-cadherin were immunoprecipitated with an E-cadherin-specific antibody. Precipitated proteins and cell lysates were blotted with the indicated antibodies. **(F)** The association of the E-cadherin/catenin complex with the cytoskeleton was examined using Triton X-100-fractionated samples. HEK293 cells were transfected with p28GANK plasmids for 48 h and the cells were fractionated in a Triton X-100 solution. Equal amounts of the soluble and insoluble fractions were subjected to western blotting. β-actin was used as a loading control.

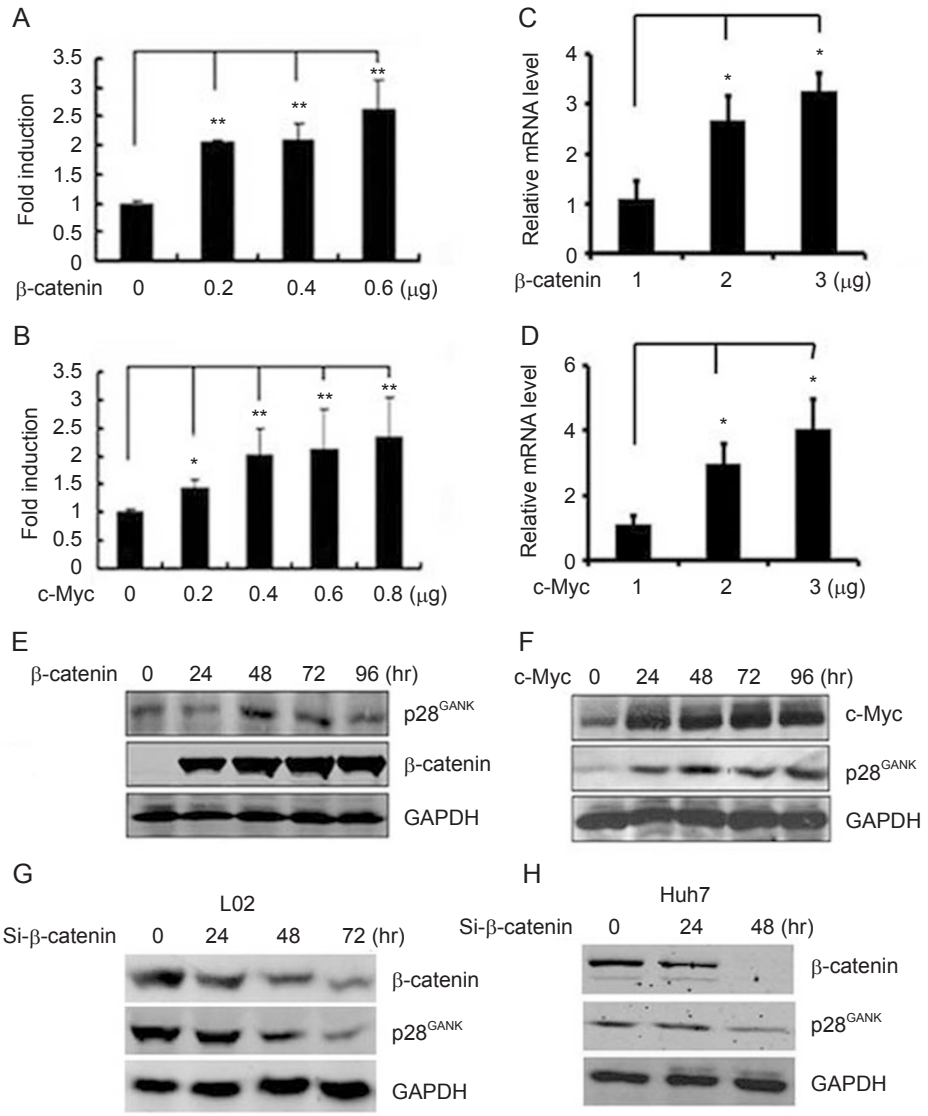

Figure 5 β-catenin and c-Myc are transcriptional activators of p28^{GANK}. **(A**, **B)** p28^{GANK} promoter activity was upregulated by β-catenin and c-Myc. HEK293 cells were transiently transfected with pGL3-p28 and increasing amounts of β-catenin (0-0.6 μg) or c-Myc (0-0.8 μg) plasmids. The cells were then lysed and luciferase activity was measured. **(C**, **D)** Upregulation of p28^{GANK} expression was detected by quantitative RT-PCR in β-catenin- and c-Myc-transfected cells. HEK293 cells were transfected with 0.5 μg β-catenin and c-Myc expression plasmids for the indicated period of time. qPCR was used to analyze *p28^{GANK}* and *c-Myc* expression. **(E**, **F)** p28^{GANK} protein levels were detected in β-catenin- and c-Myc-transfected cells. HEK293 cells were transfected with 0.5 μg of pcDNA3-β-catenin or pCMV-c-Myc expression plasmids for 0-96 h, and β-catenin, c-Myc and p28^{GANK} expression were analyzed. **(G**, **H)** Knocking down β-catenin reduced p28^{GANK} expression. siRNA targeting β-catenin was transfected into L02 and Huh7 cells. After transfection for 0-72 h, β-catenin and p28^{GANK} were detected by western blotting (**P* < 0.05; ***P* < 0.01).

sion of p28^{GANK} (data not shown). To further confirm this result, we loaded lysates containing equal amounts of E-cadherin protein to repeat the immunoprecipitation assay. As shown in Figure 4E, the levels of associated β-catenin protein were moderately decreased upon p28^{GANK} expression. Cell fractionation with Triton X-100 was used to evaluate the attachment of the E-cadherin/catenins complex to the cytoskeleton. As seen in Figure 4F, the Triton X-100-insoluble fraction of p28^{GANK}-expressing cells contained significantly less E-cadherin/β-catenin complex than that of control cells, indicating that p28^{GANK} attenuated the attachment of the E-cadherin/β-catenin complex to the cytoskeleton. Notably, β-catenin levels were higher in the Triton X-100 soluble fraction of p28^{GANK}-

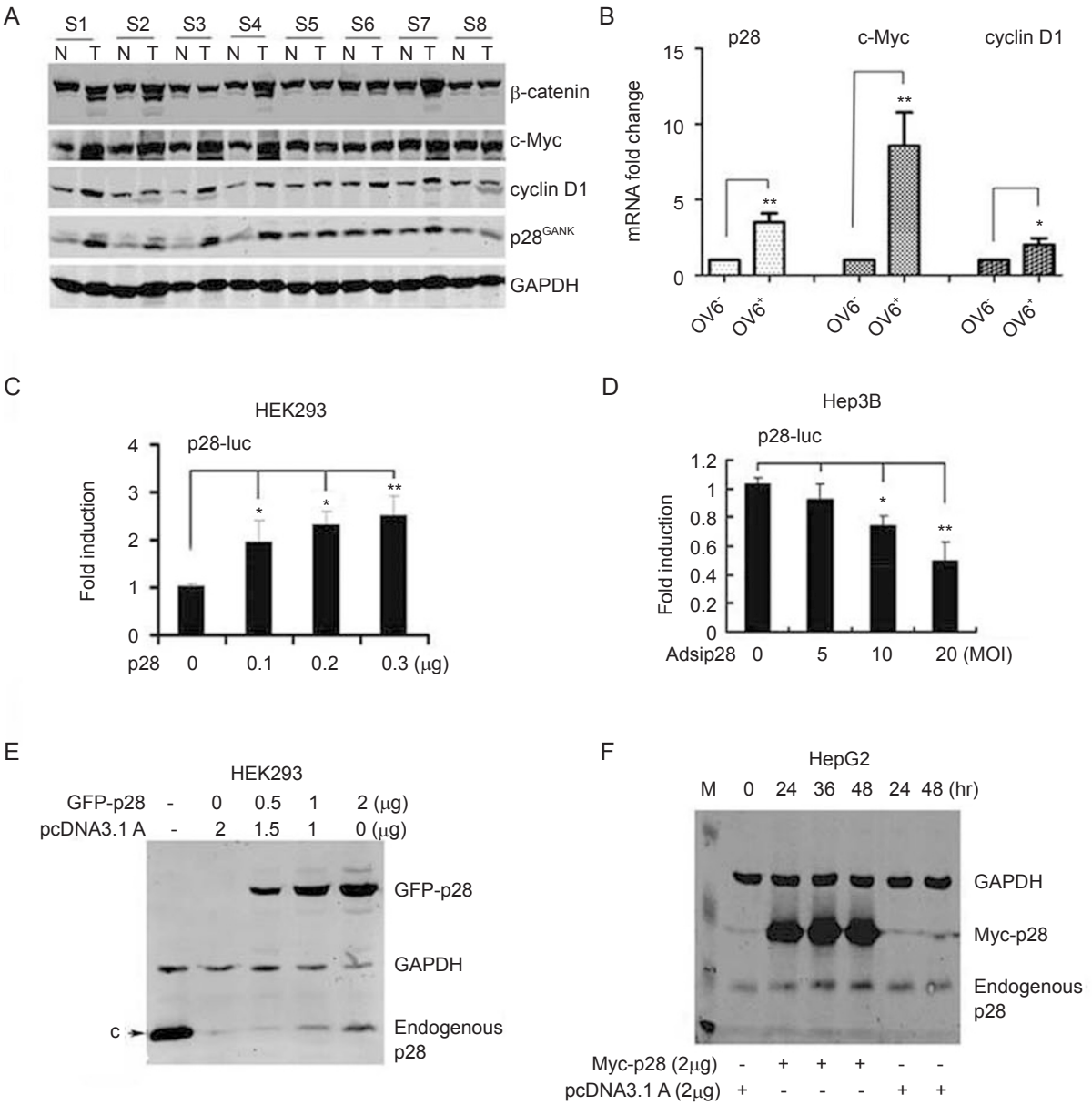

Figure 6 p28GANK expression correlated with β-catenin and established a positive feedback loop in β-catenin signaling. **(A)** Immunoblot analysis of proteins prepared from primary human hepatic tumors (T) and apparently normal liver tissues (N). **(B)** p28GANK is upregulated in OV6+ cells. OV6+ cells were sorted, and *p28GANK, c-Myc* and *cyclin D1* were detected by qPCR. **(C)** p28GANK upregulated the activity of its own promoter. HEK293 cells were transiently transfected with pGL3-p28 in combination with the control plasmid or increasing amounts of p28GANK plasmid (0-0.3 μg) for 24 h. Cells were then lysed and luciferase activities were detected. **(D)** Knockdown of endogenous p28GANK suppressed the activity of its own promoter in Hep3B cells. Cells were co-transfected with the pGL3-p28 vector and the control plasmid. After 12 h, the cells were infected with AdSip28 (0-20 MOI). Luciferase activity was measured 48 h after adenovirus infection. **(E)** p28GANK upregulated its own expression in a dose-dependent manner. HEK293 cells were transfected with increasing amounts of GFP-p28GANK expression plasmid (0-2 μg) for 48 h. Cells were then lysed and subjected to immunoblotting. c: positive control. **(F)** p28GANK upregulated its own expression in a time-dependent manner. HepG2 cells were transfected with myc-p28 plasmid or empty pcDNA3.1A vector for the time indicated. The cell lysates were subjected to immunoblotting with antibodies directed against p28GANK and GAPDH. M: marker (**P* < 0.05; ***P* < 0.01).

expressing cells than in that of control cells (Figure 4F, Supplementary information, Figure 3C). These data support the hypothesis that the expression of p28^{GANK} is correlated with reduced membrane localization of the E-cadherin/β-catenin complex, which results in increased levels of cytoplasmic β-catenin.

β-catenin and c-Myc are transcriptional activators of p28^{GANK}

β-catenin and c-Myc are two transcription factors that play important roles in regulating cell growth and cell cycle progression; therefore, we asked whether there was a relationship between p28^{GANK} and these two factors. Reporter gene assays were performed in HEK293 cells that expressed exogenous β-catenin and c-Myc. To our surprise, both β-catenin and c-Myc significantly increased p28^{GANK} promoter activity (Figure 5A and 5B). The quantitative PCR results further confirmed the positive roles of β-catenin and c-Myc in regulating the expression of p28^{GANK} (Figure 5C and 5D). In HEK293 cells, overexpression of wild-type β-catenin by transient transfection increased p28^{GANK} protein levels (Figure 5E, Supplementary information, Figure S4A). Overexpression of c-Myc also induced p28^{GANK} protein levels (Figure 5F, Supplementary information, Figure S4B). Using shRNA to inhibit β-catenin expression in L02 and Huh7 cells, we found that p28^{GANK} expression decreased with decreasing levels of β-catenin (Figure 5G and 5H).

To assess the role of β-catenin and c-Myc in the transcriptional activation of p28^{GANK}, we analyzed the p28^{GANK} promoter using MatInspector (Genomatix) and found putative DNA-binding sites for E2F, NFAT, STAT1, SP1, v-Myb, HIF-1, GATA and other transcription factors. Interestingly, putative binding sites for TCF/LEF and Myc/Max were also predicted. To determine whether β-catenin and Myc transcription complexes functioned through these two predicted sites, we mutated the predicted sites in the reporter construct (Supplementary information, Figure S4C). The mutation of these two predicted sites did not eliminate the activation of the reporter upon the overexpression of β-catenin or c-Myc (Supplementary information, Figure S4D and S4E). Furthermore, no direct binding of β-catenin/TCF or Myc/Max complexes to the putative transcriptional regulation sites was detected by ChIP assay (data not shown).

p28^{GANK} expression correlates with β-catenin activation and establishes a positive feedback loop to regulate β-catenin signaling in HCC

Although previous studies have reported varying expression levels and localization of wild-type and mutant β-catenin proteins, most reports indicate that the nuclear localization of β-catenin is correlated with tumor progression and tumor cell proliferation [29-31]. c-Myc and cyclin D1 are major regulators of cell cycle progression and cell proliferation. Increased β-catenin levels may promote neoplastic conversion by triggering the expression of c-Myc and cyclin D1, resulting in uncontrolled cell cycle progression. Thus, we assessed the correlation between β-catenin signaling and the expression of p28^{GANK} in human primary hepatic tumors and found a correlation between the activation of β-catenin signaling and the expression of p28^{GANK}, c-Myc and cyclin D1 (Figure 6A, Supplementary information, Figure S5A). We previously reported that β-catenin signaling was significantly activated in tumorigenic OV6-positive liver progenitor cells [32]. We thus detected the expression of *c-Myc*, *cyclin D1* and *p28^{GANK}* in sorted OV6$^+$ SMMC7721 liver carcinoma cells and found that the levels of these gene products were higher in OV6$^+$ cells than in OV6$^-$ cells (Figure 6B). These data indicate that β-catenin signaling-induced p28^{GANK} expression may be an important mechanism for promoting cell cycle activation in human liver cancers.

We further examined the effect of p28^{GANK} expression on the activity of its own promoter in HEK293 and Hep3B cells. p28^{GANK} significantly increased the expression of a reporter driven by the p28^{GANK} promoter in HEK293 cells (Figure 6C) but had no effect on the activity of a pGL3-luc control construct (Supplementary information, Figure S5B). Furthermore, knocking down endogenous p28^{GANK} in Hep3B cells resulted in a decrease in p28^{GANK} promoter activity (Figure 6D). Conversely, transient transfection of HEK293 cells with a GFP-tagged p28^{GANK} expression vector or HepG2 cells with myc-p28^{GANK} resulted in increased levels of endogenous p28^{GANK} (Figure 6E, 6F and Supplementary information, Figure S5C and S5D).

Discussion

This study demonstrates that growth factor stimulation and Ras activation upregulate p28^{GANK} expression through the activation of β-catenin signaling. Elevated expression of β-catenin, which results in the constitutive activation of numerous β-catenin/TCF target genes, has been detected in various types of cancers, including HCC. In the present study, we found a correlation between the presence of activated β-catenin and increased p28^{GANK} expression. Interestingly, elevated p28^{GANK} expression positively regulated β-catenin/TCF transcriptional activity. Growth factors and Ras activation activated β-catenin and c-Myc through the Raf-ERK and PI3K-AKT pathways; our results indicate that the PI3K

pathway positively controlled p28^{GANK} expression. Akt is a general mediator of growth factor-induced survival and in a number of cell types it has been shown to suppress apoptosis induced by growth factor withdrawal, cell-cycle discordance, loss of cell adhesion and DNA damage [6, 33]. Thus, a signaling pathway has been defined in which growth factor receptor activation leads to the sequential activation of PI3K and Akt, which then promotes cell survival and inhibits apoptosis. Our studies and others have revealed that p28^{GANK} can increase resistance to DNA damage-induced apoptosis [24, 34].

Previous studies have found that the activation of β-catenin signaling is mediated by multiple target genes, most of which are direct β-catenin/TCF-dependent transcriptional targets [11, 35]. We demonstrated that p28^{GANK} is regulated by β-catenin signaling and that the elevated expression of p28^{GANK} positively regulates β-catenin/TCF transcriptional activity. Earlier studies have shown that high levels of functional p53 can downregulate the amount and transcriptional activity of β-catenin [25, 26]. This effect was eliminated by blocking the activities of components that regulate the turnover of β-catenin, such as GSK-3β and the proteasomal system. In addition, Higashitsuji *et al.* demonstrated that p28^{GANK} can bind to MDM2 and increase the ubiquitination and degradation of p53 [21]. These reports led us to hypothesize that p28^{GANK} might affect β-catenin through the function of p53. By performing experiments using the p53-negative cell line Hep3B, we showed that the effect of p28^{GANK} on β-catenin was independent of p53.

Man *et al.* demonstrated that p28^{GANK} plays an essential role in Ras-induced tumorigenesis and promotes activation of AKT [27]. Given that GSK-3β provides a mechanistic link between growth factor stimulation or Ras activation and β-catenin stabilization, we examined whether p28^{GANK} regulates AKT and GSK-3β phosphorylation. We found that p28^{GANK} significantly enhanced the phosphorylation of AKT, but showed no effect on GSK-3β. We were unable to demonstrate a direct protein-protein interaction between p28^{GANK} and β-catenin or GSK3β, suggesting that additional proteins are likely to play roles in regulating β-catenin. p28^{GANK} is a component of the 26S proteasome, which is capable of directly or indirectly regulating turnover of most proteins. Yang *et al.* reported that MDM2 associates with E-cadherin and regulates its degradation [28]. This interaction suggests a potential role for the association of p28^{GANK} with MDM2 in regulating E-cadherin stability. Our results demonstrate that overexpression of p28^{GANK} decreased the levels of E-cadherin, which, in turn, decreased the levels of membrane-bound β-catenin, resulting in a corresponding increase in cytoplasmic β-catenin. Furthermore, we investigated whether p28^{GANK}, MDM2 and E-cadherin form a complex and found that even though p28^{GANK} and E-cadherin both formed a complex with MDM2, we were unable to detect an interaction between p28^{GNAK} and E-cadherin. Therefore, the exact mechanism by which p28^{GANK} downregulates E-cadherin remains to be elucidated. The role of p28^{GANK} in regulating the distribution of β-catenin between the membrane and the cytoplasm may partially explain the mechanism by which p28^{GANK} regulates β-catenin signaling.

In HCC, accumulation of β-catenin presents at an early stage. Most authors have reported that the prevalent type of HCC is of a moderately differentiated to well-differentiated grade with β-catenin expression at the membrane and in the cytoplasm [14, 29, 30]. These conclusions are consistent with recent results showing that p28^{GANK} plays an oncogenic role primarily at the early stages of human hepatocarcinogenesis [36]. β-catenin can enter the nucleus and regulate the transcription of target genes such as cyclin D1 and c-Myc [10, 30]. Both proteins play a role in the G1-S checkpoint of the cell cycle by affecting the activity of retinoblastoma tumor-suppressor (Rb) [37]. p28^{GANK} can accelerate the degradation of Rb and is coordinately expressed with β-catenin, c-Myc and cyclin D1, further indicating that p28^{GANK} plays a role similar to that of c-Myc and cyclin D1 in HCC progression. Interestingly, more than 50% of murine HCCs induced by transgenic expression of c-Myc contained mutations in the β-catenin regulatory sequence [13]. Similar mutations were found in HCCs of H-ras transgenic mice. These results indicate that the Ras and c-Myc cancer pathways in this tissue operate in parallel with mutant β-catenin. The function of p28^{GANK} in tumorigenesis coincides with Ras and c-Myc. β-catenin signaling contributes to the activation of tumorigenic OV6^{+} liver progenitor cells [32] and we detected p28^{GANK} expression after sorting OV6^{+} cells. Surprisingly, much higher p28^{GANK} mRNA levels were present in OV6^{+} liver cells than in OV6^{-} cells (Figure 6B), indicating a potential role of p28^{GANK} in regulating stem cell-like self-renewal.

Cancer cells of different origins often use the mechanisms of normal embryonic development to achieve their malignant status. The β-catenin/TCF pathway is a classic example. Similarly, p28^{GANK} was previously shown to be highly expressed during normal liver regeneration as well as in HCC [38]. This oncofetal pattern of p28^{GANK} expression could potentially be a result of β-catenin/TCF activation during tumorigenesis. Transcriptional upregulation of p28^{GANK} by β-catenin/TCF may therefore contribute to the pleiotropic effects of the β-catenin/TCF signaling pathway and could result in a significant function for p28^{GANK} in β-catenin-mediated oncogenesis. Fu-

ture studies will define the role of p28^{GANK} in β-catenin/TCF-induced HCC tumorigenesis. Our findings here outline a positive feedback loop involving β-catenin signaling and p28^{GANK} in which excess β-catenin induces the accumulation of p28^{GANK} and high p28^{GANK} levels upregulate β-catenin/TCF transcriptional activation. This feedback loop may therefore underlie the high expression of p28^{GANK} observed in HCC and disruption of the feedback loop through knockdown of p28^{GANK} may affect tumorigenesis.

Materials and Methods

Antibodies and reagents

p28^{GANK}-(sc-8991), c-Myc-(sc-40), β-catenin-(sc-7963) and GSK-3β-(sc-9166) specific antibodies were obtained from Santa Cruz Biotechnology. The HA-tag-, cyclin D1- and histone H3-specific antibodies were obtained from Cell Signaling Technology (Beverly, MA). The MDM2 (F-414) antibodies were obtained from Bioworld technology. The β-actin- and GAPDH-specific antibodies were obtained from Kangcheng Biosystem. EGF was purchased from BD Biosciences (San Diego, CA) and HGF was obtained from PeproTech (Rocky Hill, NJ). Inhibitors PD98059 and LY294002 were purchased from Sigma (St. Louis, MO) and used at a final concentration of 15 μM.

Plasmids

The human p28^{GANK} reporter vector was constructed by cloning a 1 226 bp putative p28^{GANK} promoter region into a pGL3-basic reporter vector containing the promoter driving the firefly luciferase gene (Promega). The proximal promoter region was first amplified by PCR from the human genome using primers based on the genomic DNA sequence of the p28^{GANK} gene. The forward primer (5′-ACAGCCGTTAGAGCTTCACCAATCAC-3′) contained an *Xho* I restriction enzyme site at the 5′ end and the reverse primer (5′-ACAATAGGGAACACTAATTCTGAGCC-3′) carried a *Hind* III site. The PCR product was then cloned into the pGL3-Basic reporter vector with the same sites. PSG5-PI3Kp85 was a kind gift from Dr Claudia Cosentino (Genome Stability Cancer Research Institute, London, UK); PCMV5-myr-AKT1 and pCMV5-wt-AKT1 were provided by Dr Jin Q Cheng (Department of Pathology, University of South Florida, USA); and pGL-β-catenin-OT was kindly provided by Daru Lu (Fudan University, Shanghai, China). The expression vectors containing Ras, pCMV-c-Myc and pcDNA3-β-catenin were obtained from the Chinese National Human Genome Center (Shanghai, China).

Cell lines, transfections, adenoviral infection and luciferase assays

The cell lines were obtained from the American Type Culture Collection (Manassas, VA) or the Cancer Institute of Shanghai Jiao Tong University (Shanghai, China). Cells were grown in DMEM or RPMI 1640 (Gibco BRL, Life Technologies) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained in a humidified 37 ºC incubator with 5% CO_2. Adenovirus was produced as previously described [34]. Viral infection was performed in a minimal volume of serum-free DMEM for 1.5 h. Mock cultures that did not contain virus were used as controls. After infection for 1.5 h, 2 ml of fresh growth medium was added and cells were placed in the incubator. Cells were co-transfected with a mixture of the indicated luciferase reporter plasmids, the renilla luciferase control reporter vector pRL-TK and the indicated amounts of constructs by PEI (Polyplus, AFAQ). After treatment, the cells were lysed, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's recommendations.

Reverse transcription polymerase chain reaction and real-time qPCR

Total RNA was isolated with TRIzol reagent (Invitrogen) and reverse transcribed with SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. PCR amplification was performed in a 50 μl reaction mixture containing 2 μl cDNA, 1× PCR buffer, 1.5 mmol/L $MgCl_2$, 0.8 mmol/L deoxynucleotide triphosphatase, 0.2 μmol/L each primer and 1 unit Taq DNA polymerase (Roche Molecular Systems, Inc., Pleasanton, CA). For real-time PCR, we used the specific SYBR-Fluo from TaKaRa Biotechnology Co., Ltd. PCR was performed with the following primers: *p28*-F, 5′-TGGAGGGGTGTGTGTCTAAC-3′ and *p28*-R, 5′-ACTTGCAGGGGTGTCTTTTC-3′; *c-Myc*-F, 5′-TCCAGCTTGTACCTGCAGGATCTGA-3′ and *c-Myc*-R, 5′-CCTCCAGCAGAAGGTGATCCAGACT-3′; *cyclin D1*-F, 5′-CCGATGCCAACCTCCTCAAC-3′ and *cyclin D1*-R, 5′-GCGGGCCAGGTTCCACTTGAG-3′; *β-actin*-F, 5′-GGA CTC CTA TGT GGG TGG CGA GG-3′ and *β-actin*-R, 5′-GGGAGAGCATGCCCTCGTAGAT-3′.

Primary human tissue samples

Protein extracts were prepared from tissue samples obtained from patients with primary hepatic tumors in the course of direct surgery at the Eastern Hepatobiliary Surgery Institute. Specimens obtained from 2006 to 2009 were from patients in the Eastern Hepatobiliary Surgery Institute in Shanghai, China. Informed consent was obtained from all patients for subsequent use of their resected tissues. From each patient, tissue samples were taken from non-neoplastic mucosa at the proximal surgical margin and from the primary tumor.

Cell fractionation

Cytosolic and nuclear proteins were prepared from cells using a Nuclear and Cytoplasmic Extraction Reagent Kit (Pierce, USA) as described before [39]. To obtain Triton X-100-soluble and -insoluble fractions, cells were incubated with Triton buffer (1% Triton X-100, 0.3 M sucrose, 25 mM HEPES, pH 7.4, 100 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM MgCl2, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride) for 15 min on a rocking platform. After centrifugation, the supernatant (Triton X-100-soluble fraction) was collected. The cell pellet was resuspended in SDS lysis buffer (20 mM Tris, pH 7.5, 2.5 mM EDTA, 1% SDS and 1 mM dithiothreitol) and subjected to standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis.

Immunoblotting and immunoprecipitation

Cells were lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, pH 8.0, 30 mM NaF, 1 mM Na_3VO_4, 40 mM

β-glycerophosphate, 0.1 mM PMSF, protease inhibitors, 10% glycerol and 1% Nonidet-P40) and equal volumes of cell lysates from each condition were resolved by 8%-16% SDS-PAGE. Protein phosphorylation was visualized by western blotting using antibodies directed against the indicated antigens. For coimmunoprecipitation studies, whole-cell lysates from HEK293 cells were prepared in 1 ml RIPA buffer, incubated overnight with 2 μg of the indicated antibody, and then incubated with 40 μl of a 1:1 slurry of protein A and protein G sepharose beads (Santa Cruz Biotechnology) for 3 h at 4 ºC. The beads were washed four times with lysis buffer and resuspended in equal volumes of RIPA buffer.

Statistical analysis

Results are expressed as the mean±SEM. Student's *t*-tests were used for statistical analysis and statistical significance was defined as $P < 0.05$.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)