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Tumor Suppressive Function of p21-activated Kinase 6 in Hepatocellular Carcinoma*

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Background: p21-activated kinase 6 (PAK6) plays ambiguous roles in tumorigenesis.
Results: PAK6, under epigenetic control of polycomb repressive complex 2 (PRC2), possessed tumor suppressive function in hepatocarcinogenesis, relying partially on its kinase activity and nuclear translocation.
Conclusion: Repression of PAK6 might contribute to hepatocarcinogenesis.
Significance: This finding provides a novel role and potential underlying mechanism of PAK6 in hepatocarcinogenesis.

Our previous studies identified the oncogenic role of p21-activated kinase 1 (PAK1) in hepatocellular carcinoma (HCC) and renal cell carcinoma (RCC). Contrarily, PAK6 was found to predict a favorable prognosis in RCC patients. Nevertheless, the ambiguous tumor suppressive function of PAK6 in hepatocarcinogenesis remains obscure. Herein, decreased PAK6 expression was found to be associated with tumor node metastasis stage progression and unfavorable overall survival in HCC patients. Additionally, overexpression and silence of PAK6 experiments showed that PAK6 inhibited xenografted tumor growth *in vivo*, and restricted cell proliferation, colony formation, migration, and invasion and promoted cell apoptosis and anoikis *in vitro*. Moreover, overexpression of kinase dead and nuclear localization signal deletion mutants of PAK6 experiments indicated the tumor suppressive function of PAK6 was partially dependent on its kinase activity and nuclear translocation. Furthermore, gain or loss of function in polycomb repressive complex 2 (PRC2) components, including EZH2, SUZ12, and EED, elucidated epigenetic control of H3K27me3-arbitrated PAK6 down-regulation in hepatoma cells. More importantly, negative correlation between PAK6 and EZH2 expression was observed in hepatoma tissues from HCC patients. These data identified the tumor suppressive role and potential underlying mechanism of PAK6 in hepatocarcinogenesis.

Hepatocellular carcinoma (HCC)[3] is a major health problem worldwide due to its high morbidity and mortality rates (1). Due to the complexity of gene interactions and diverse signaling pathways involved in HCC, effective treatment modalities are still scarce, except liver transplantation for early HCC patients (2, 3). Recently, epigenetic disorders were found to contribute to many cancers, including the initiation and progress of HCC (4). Many inhibitors targeting enzymes controlling epigenetic modifications have shown promising anti-tumorigenic effects for some malignancies, reflecting that a great potential lies in the development of epigenetic therapies (5, 6).

The p21-activated kinases (PAKs) are highly conserved serine/threonine intracellular protein kinases, classifying into two subgroups, group I comprises PAK1–3; group II comprises PAK4–6 (7). Our previous studies were focused on the oncogenic role of PAK1 in HCC, where up-regulation and activation of PAK1 by hepatitis B virus X protein conferred hepatoma cell resistance of anoikis, and in renal cell carcinoma (RCC), where PAK1 conferred RCC cell stem-like phenotype and sunitinib resistance (8, 9). PAK1 has been considered to be located in several signaling pathways contributing to oncogenesis, however, the understanding of other isoforms were much less, especially group II member PAK6, which was reported to possess ambiguous roles in cancer the progress of cancers (10).

PAK6 was first identified as a binding protein of androgen receptor (AR), disrupting AR transcriptional activity (11). In prostate cancer, the function of PAK6 was context dependent, with evidence suggesting that PAK6 either promotes or resists disease progress (12, 13). Meanwhile, PAK6 was found to be induced by INPP4B to suppress prostate cells invasion (14). In colon cancer, PAK6 conferred chemoresistance for patients undergoing 5-fluorouracil based chemotherapy (15). In clear-cell RCC, our former work indicated that PAK6 expression was decreased in the progress of clear-cell RCC and was an independent favorable clinical prognostic (16). Recently, a study (17) suggested that PAK6 was repressed by oncogenic miRNAs in HCC cells, although its expression was also down-regulated by a pan-deacetylase inhibitor. However, the detailed function

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[3] The abbreviations used are: HCC, hepatocellular carcinoma; PAK, p21-kianse activity kinase; EZH2, enhancer of zeste homolog 2; AR, androgen receptor; NLS, nuclear localization signal; IHC, immunohistochemistry; PRC2, polycomb repressive complex 2; ER, estrogen receptor; RCC, renal cell carcinoma.

and potential underlying mechanism of PAK6 in HCC remains obscure.

Thus, in this study, we integrated *in vivo* and *in vitro* experiments and clinical samples to investigate the role and potential underlying mechanism of PAK6 in hepatocarcinogenesis. In so doing, we identified PAK6, which was under epigenetic control of the polycomb repressive complex 2 (PRC2), possessed tumor suppressive function, relying on its kinase activity and nuclear translocation partially.

Materials and Methods

Cell Lines and Patient Samples—Human hepatoma cell lines Huh7, SK-Hep1, BEL-7404, PLC/PRF/5, and HepG2, and an immortalized liver cell line L02 (HL-7702) were obtained from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China), and cultured in Dulbecco's modified Eagle's medium replenished with 10% FBS at 37 °C in a humidified 5% CO_2 incubator. The cell lines have been characterized by DNA fingerprinting analysis using STR markers. HepG2.215 were derived from HepG2 cells as described previously (18). Tumor and adjacent paired non-tumor specimens were collected from HCC patients undergoing partial hepatectomy in Nantong Tumor Hospital (Nantong University, Jiangsu, China) from 2003 to 2005. Surgically removed 28 paired samples were immediately stored in TRIzol (Invitrogen) and frozen in liquid nitrogen until total RNA was extracted according to the manufacturer's instructions. 83 paired formalin-fixed, paraffin-embedded tumor specimens, along with baseline clinicopathologic characteristics and follow-up outcome, were used to construct the tissue microarray. Briefly, all specimens were reviewed histologically by hematoxylin and eosin staining. Cores were taken from each representative tumor tissue and liver tissue adjacent to the tumor within a distance of 10 mm. Duplicate 1.0-mm cylinders from two different areas, intratumoral and peritumoral, were obtained. Then, 83 paired tumors and matched peritumoral samples were used to construct tissue microarray. Informed consent was obtained from each patient, and the study protocol was conformed to the ethical guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of Fudan University.

Plasmids Construction—Expression plasmid encoding wild-type enhancer of zeste homolog 2 (EZH2) was constructed as described previously (19). Wild-type PAK6, kinase dead mutant (PAK6-DN) and nuclear localization signal (NLS) deletion (PAK6-Δ1–7) were amplified by PCR and constructed into the pcDNA3.1 (Invitrogen). The K436A mutant for kinase dead is located in the activation loop within the kinase domain, then abolishing PAK6 kinase activity (20). All plasmid constructs were confirmed by DNA sequencing. Primers used were presented as follows, wild-type PAK6, forward, 5′-GTTAAGCTTATGTTCCGCAAGAAAAAG-3′; reverse, 5′-GTGCTCGAGGCAGGTGGAGGTCT-3′; kinase dead mutant PAK6, forward, 5′-CTTCCTGAGGTCCATCATTGCGACG-3′, reverse, 5′-CCAGGTGGCCGTCGCAATGA-3′; and NLS deletion mutant PAK6, forward, GTTAAGCTTATGCGCCCTGAGATCT; reverse, GTGCTCGAGGCAGGTGGAGGTCT.

Plasmids Transfection and RNA Interference—Stable transfections with various plasmids were performed as described previously (21). PAK6 shRNA (h) lentiviral particles (Santa Cruz Biotechnology, Santa Cruz, CA), EZH2 shRNA (h) lentiviral particles, control shRNA lentiviral particles (Sigma), SUZ12 siRNAs, EED siRNAs, and control siRNA (OriGene Technologies Inc., Rockville, MD) were performed for RNA interference according to the manufacturer's instructions. PAK6-GFP (h) lentiviral particles and control lentiviral particles (Invitrogen) were performed for overexpression according to the manufacturer's instructions. Gene silence and overexpression efficiencies were confirmed by Western blot.

RNA Extraction and Quantitative RT-PCR—Total RNA from cultured cells and liquid nitrogen frozen tissues were extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. The quantitative RT-PCR assays were performed as described previously (21). *GAPDH* was used as an internal control. Experiments were repeated at least three times. Primers used were presented as follows: PAK6, forward, 5′-GCTCTCGGACTTCGGATTCT-3′, reverse, 5′-GGCATACAAAGACCTGGAGAT-3′; GAPDH, forward, 5′-AAGGTCGGAGTCAACGGATTTG-3′, reverse, 5′-CCATGGGTGGAATCATATTGGAA-3′.

Tumor Xenograft Experiment—Four- to five-week-old male athymic nude mice were purchased from the Shanghai Laboratory Animal Center (Chinese Academy of Sciences, Shanghai, China). Mice were housed in a specific pathogen-free room under controlled temperature and humidity. All animal procedures were performed accordingly to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by National Academy of Sciences and published by the National Institutes of Health. Mice aged 5–6 weeks were injected subcutaneously in the flank on each side with 1×10^7 viable cells accompanied with PBS as injection medium. 3 mice in each group in a total of 6 tumor sites were used for tumor volumes and weight analysis, and 10 mice in each group were used for survival analysis. Tumor volumes were measured once a week after injection with a caliper and calculated using the following formula: volume $= (L \times W^2)/2$, where L is length at the widest point of the tumor and W is the maximum width perpendicular to L.

Nuclear and Cytoplasmic Extraction and Western Blot—Nuclear and cytoplasmic extraction kit (Thermo Scientific, Rockford, IL) was preformed according to the manufacturer's instructions for isolating nuclear and cytoplasmic protein of cultured cells with leptomycin B (Sigma) treatment at 10 nM for 6 h or without leptomycin B treatment as control. Western blot was performed as described previously (21). Primary antibodies used included those against PAK6, Histone 3, EED, SUZ12, histone acetyllysine (Abcam, Cambridge, MA), GAPDH, Lamin B (Santa Cruz Biotechnology), EZH2 (Cell Signaling Technology, Beverly, MA), and H3K27me3 (Millipore, Billerica, MA).

Cell Proliferation, Annexin V Staining, Migration Assay, Invasion Assay, and Colony Formation Assay—Cell proliferation was calculated using the Cell Counting Kit-8 (Dojindo, Kamimashiki-gun Kumamoto, Japan) according to the manufacturer's instructions. Colony formation assay, cell migration assay (Corning Inc., NY), cell invasion assay (Millipore, Billerica, MA), and Annexin V assay (BD Biosciences) were per-

formed as described previously (22). Cell apoptosis was induced by serum-free medium for 48 h. After starvation, the cells were harvested, stained by Annexin V assay, and assessed using a flow cytometer. The *in vitro* anoikis experiment was performed according to previous studies (8, 23–25). Briefly, cells in normal culture were harvested by trypsinization and suspended. Suspended cells were plated on poly-HEMA-coated dishes (Corning Inc.) for 48 h with 10% serum before analyzing the apoptosis rate using Annexin V assay via flow cytometer. The apoptosis and anoikis rates were scored as Annexin V+ cells. Flow cytometer data were analyzed by FlowJo software (TreeStar, Ashland, OR). The absorbance was assessed by Universal Microplate Reader (BIO-TEK Instruments, Minneapolis, MN).

Chromatin Immunoprecipitation (ChIP) Assay—ChIP was performed using EZ-ChIP chromatin immunoprecipitation kit (Millipore, Billerica, MA) according to the manufacturer's protocol. Immunoprecipitation complexes were immunoprecipitated by antibody of EZH2 (Cell Signaling Technology, Beverly, MA), H3K27me3, and IgG (Millipore, Billerica, MA) overnight at 4 °C. The captured genomic DNA was obtained and used for quantitative PCR analysis. Primers for two sites in PAK6 promoter and one site in GAPDH promoter as negative control were designed. Amplification efficiency was calculated and the data were illustrated as enrichment related to IgG. Primers used were presented as follows, P2, forward, 5′-AGTTGCTTACTTTAGTTTCTGCAT-3′, reverse, 5′-GGCTAGGACACCTTCATGCA-3′; P1, forward, 5′-ACTCTGGAAGGAAGAATCTAAAC-3′, reverse, 5′-CTGTCAGTGTATAAATCATTGCA-3′; and GAPDH promoter, forward, 5′-AACTTTCCCGCCTCTCAGC-3′, reverse, 5′-CAGGAGGACTTTGGGAACGA-3′.

Immunohistochemistry (IHC) Staining—The tissue microarray was immunohistochemically analyzed as described previously (22). Primary antibodies against PAK6 (Abcam) and EZH2 (Cell Signaling Technology, Beverly, MA) were used. A semi-quantitative H-score ranged from 0 to 300 was calculated for PAK6 staining by multiplying the distribution areas (0–100) at each staining intensity level (0: negative, 1: weak staining, 2: moderate staining, 3: strong staining). EZH2 straining was calculated by the percentage of nuclear positive cells (0–100). Optimal cutoff values were estimated by X-tile plots v3.6.1 (Yale University, New Haven, CT) to achieve a lowest p value (26).

Statistical Analysis—Experimental data were presented as mean ± S.E. Student's t test was used to compare continuous variables, and the χ-square test was used to compare qualitative variables. Overall survival was estimated by Kaplan-Meier method and analyzed using a log-rank test. The correlation between PAK6 and EZH2 staining was calculated by a Spearman correlation test. Statistical analysis was preformed with MedCalc software (version 12.7.0.0; MedCalc, Mariakerke, Belgium) and GraphPad Prism5 (GraphPad Software, La Jolla, CA). All tests were two sided and p values < 0.05 were considered statistically significant.

Results

Decreased PAK6 Expression Predicts Poor Survival in HCC Patients—To investigate the role of PAK6 in HCC, the PAK6 mRNA level was first evaluated in tumor and adjacent paired non-tumor tissues. In most cases (20/28), the PAK6 mRNA level was down-regulated, compared with non-tumor tissues (Fig. 1*A*). Further analysis was preformed through immunohistochemical analysis of tissue microarray (Fig. 1*B*). PAK6 staining in the tumor decreased significantly, compared with adjacent paired non-tumor (Fig. 1*C*). Meanwhile, in tumor node metastasis stage progression, PAK6 expression was steadily declining (Fig. 1*D*). Then, the correlation between PAK6 expression and clinic characteristics and outcome were evaluated. As shown in Table 1, patients with low PAK6 expression were prone to have larger tumor size ($p = 0.005$), higher tumor node metastasis stage ($p = 0.008$), and microsatellite nodules ($p = 0.017$). Moreover, Kaplan-Meier analysis illustrated patients with low PAK6 expression were more likely to suffer death (log-rank $p = 0.001$) (Fig. 1*E*). Taken together, PAK6 expression decreased in the progress of HCC and was positively related to HCC patient survival.

PAK6 Inhibits Xenografted Hepatoma Cell Growth in Vivo—To further substantiate the role of PAK6 observed in HCC specimens, the impact on xenografted tumor growth through overexpression and silence of PAK6 was evaluated in nude mice. First, endogenous PAK6 expression was assessed in six human hepaoma cell lines and one immortalized liver cell line by Western blot, where Huh7 and SK-Hep1 were chosen to stably silence and overexpress PAK6, respectively (Fig. 2*A*). The silence and overexpression efficiency were confirmed by Western blot (Fig. 2*B*). In nude mice, each inoculation containing Huh7 cells with PAK6 silence developed into a rapidly growing and larger tumor than controls ($n = 6$). In contrast, each inoculation including SK-Hep1 cells with PAK6 overexpression developed into a slowly growing and small tumor ($n = 6$) (Fig. 2, *C* and *D*). Furthermore, the nude mice ($n = 10$) inoculated with PAK6 knockdown cells had a worse survival than control mice (log rank $p = 0.005$). Conversely, mice ($n = 10$) inoculated with PAK6 overexpression cells survived longer (log rank $p = 0.001$) (Fig. 2*E*). These overexpression and silence *in vivo* results suggested PAK6 might function as tumor suppressor in hepatocarcinogenesis.

Overexpression of PAK6 Restricts Hepatoma Cell Tumorigenecity in Vitro—Given the aforementioned results, *in vitro* function of SK-Hep1 cells with PAK6 overexpression were investigated. As PAK6 expression was related with tumor size and tumor node metastasis stage (Table 1), the impact on cell proliferation and survival were first investigated. The proliferation and apoptosis induced by serum starvation experiments indicated PAK6 overexpression inhibited cell proliferation and enhanced cell apoptosis (Fig. 3, *A* and *B*). The colony formation was also inhibited by PAK6 overexpression (Fig. 3*C*). Moreover, PAKs were well characterized by regulation of cytoskeletal organization mediating alterations in cell adhesion and motility, thus, cell migration and invasion assays were preformed to investigate the impacts of PAK6 on migration and invasion (7). The experiments indicated PAK6 overexpression resisted cell migration and invasion (Fig. 3, *D* and *E*). Finally, as our previous study about PAK1 suggested, PAKs might regulate cell anoikis, a specialized form of apoptosis that happens due to inadequate or inappropriate cell-matrix interactions, thus, the role of PAK6 in anoikis was assessed. Using a Annexin V assay, the anoikis rate of SK-Hep1 cells with PAK6 overexpression was

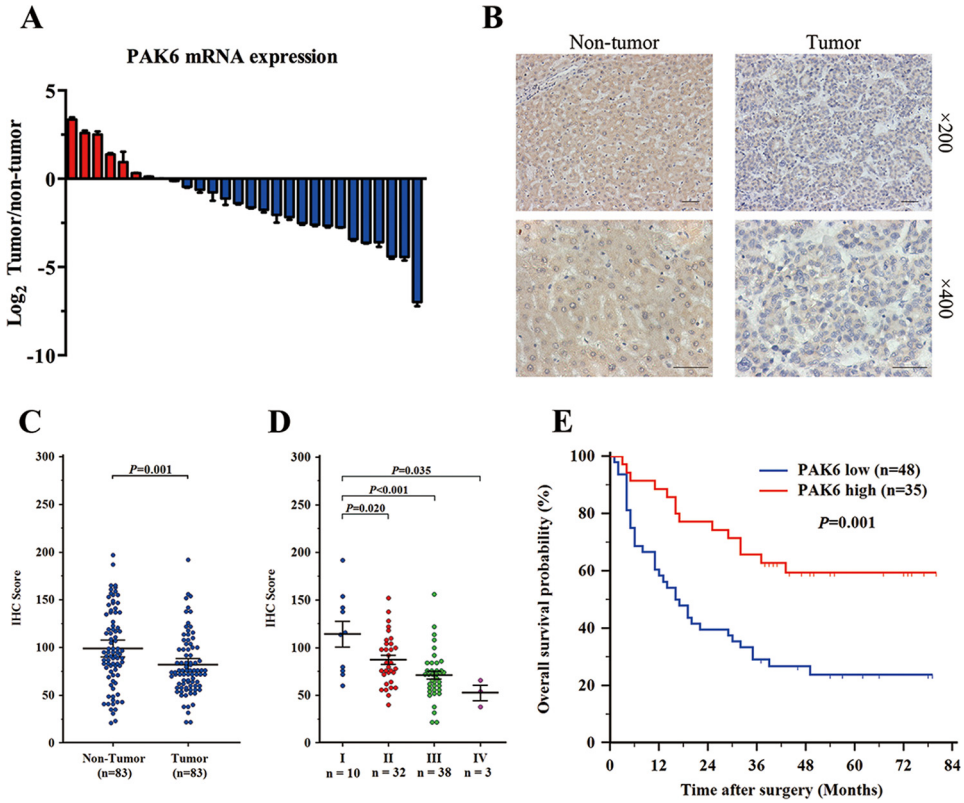

FIGURE 1. **Decreased PAK6 expression predicts poor survival in HCC patients.** *A,* waterfall plot of the PAK6 mRNA level in HCC tissues of patients compared with adjacent paired non-tumor tissues ($n = 28$). *Red bar* represents the patients with a higher PAK6 expression in tumor than paired non-tumor; *blue bar* represents the patients with a lower PAK6 expression in tumor than paired non-tumor. *B,* representative IHC images of PAK6 expression in non-tumor tissue and paired tumor tissue (original magnification ×200 and 400). *C,* scatter plots for IHC staining score in paired non-tumors and tumors ($n = 83$). *D,* scatter plots for PAK6 IHC staining score from tumor node metastasis stage I to IV. *E,* Kaplan-Meier analysis of the overall survival dichotomized by PAK6 expression. *p* value is determined by log-rank test. The *scale bar* is 50 μm.

higher than controls, suggesting PAK6 overexpression promoted cell anoikis (Fig. 3*F*). Collectively, PAK6 overexpression may resist cell proliferation, colony formation, migration and invasion, and promote cell apoptosis and anoikis *in vitro*.

Silence of PAK6 Promotes Hepatoma Cell Tumorigenecity in Vitro—Silence of PAK6 in Huh7 and SK-Hep1 cells were used to verify the functional results observed in overexpression experiments. The silence efficiencies were confirmed by Western blot (Figs. 2*B* and 4*A*). In both cell lines, PAK6 knockdown was found to promote cell proliferation (Fig. 4*B*), and to resist cell apoptosis induced by serum starvation (Fig. 4C). Meanwhile, PAK6 silence enhanced colony formation capability (Fig. 4*D*), cell migration (Fig. 4*E*), and invasion (Fig. 4*F*). Finally, the decreased anoikis rates were also caused by PAK6 knockdown (Fig. 4*G*). Taken together, these *in vitro* functional experiments further consolidated that PAK6 might execute a tumor suppressive role in the progress of HCC.

Kinase Activity and Nuclear Translocation Determine PAK6 Function—Given the nature of kinase activity and nuclear translocation, the kinase dead and NLS deletion mutant PAK6 was first generated to assess the impact of kinase activity and nuclear location on the tumor suppressive function of PAK6. The kinase dead mutant of PAK6 (PAK6-DN) was generated as described previously; this mutation (K436A) could abolish PAK6 kinase activity as its location in the activation loop within the kinase domain (20). A polylysine sequence (KKKKK) was found in the PAK6 N-terminal (amino acid 3–7), this sequence was demonstrated as a NLS for PAK5 and a similar sequence (KRKK) at the close position was also found in PAK4 (27, 28). Thus, deletion of amino acids 1–7 at the N terminus (PAK6-Δ1–7) was generated to demonstrate our hypothesis that this polylysine sequence was NLS for PAK6. The efficiencies of stable overexpression of wild-type PAK6, PAK6-DN, and PAK6-Δ1–7 were confirmed by Western blot (Fig. 5*A*). Then, a Western blot of nuclear and cytoplasmic extraction was performed in SK-Hep1 cells with wild-type PAK6 and PAK6-Δ1–7. The results indicated that mutant PAK6 lacking amino acids 1–7 failed to shuttle to the nucleus after leptomycin B treatment, suggesting the NLS of PAK6 was mapped into this polylysine sequence in N-terminal (Fig. 5*B*).

After construction of PAK6 mutants, the functional experiments were performed to investigate the importance of kinase activity and nuclear location on PAK6 tumor suppressive function. As shown in Fig. 5, the lack of kinase activity disrupted PAK6 function in inhibiting cell proliferation, survival, migration, and invasion, suggesting PAK6 function was dependent on its kinase activity (Fig. 5, *C–H*). Meanwhile, NLS lacking the mutant depleted the function in cell proliferation, migration, and invasion, but the depletion level was less than the kinase dead mutant (Fig. 5, *C–G*). However, the NLS deletion mutant failed to affect the PAK6 function in promoting cell apoptosis and anoikis, suggesting the PAK6 tumor suppressive role was partially dependent on its nuclear translocation (Fig. 5, *D* and *H*).

TABLE 1
Correlation between PAK6 expression and clinic characteristics

Characteristic	Patients (n = 83)	PAK6 expression: High (%) (n = 35)	PAK6 expression: Low (%) (n = 48)	p
	% number	*%*		
Age (years)[a]				0.631
Mean	55.04	54.48	55.44	
Median	55	55	54.5	
IQR[b]	50–60	51–58	49–64.5	
Gender				0.991
Male	70 (84.3)	30 (42.9)	40 (57.1)	
Female	13 (15.7)	5 (38.5)	8 (61.5)	
Tumor size (cm) [a,c]				0.005[d]
Mean	7.44	5.75	8.68	
Median	6	4.5	7	
IQR	3.5–11	3–7	4–12	
Tumor multiplicity				0.355
Single	74 (89.2)	33 (44.6)	41 (55.4)	
Multiple	9 (10.8)	2 (22.2)	7 (77.8)	
TNM stage				0.008[d]
I	10 (12.0)	7 (70.0)	3 (30.0)	
II	32 (38.6)	18 (56.3)	14 (43.7)	
III	38 (45.8)	10 (26.3)	28 (73.7)	
IV	3 (3.6)	0 (0.0)	3 (100.0)	
Tumor differentiation				0.793
1 + 2	52 (62.7)	23 (44.2)	29 (55.8)	
3 + 4	31 (37.3)	12 (38.7)	19 (61.3)	
Tumor encapsulation				0.856
Absent	21 (25.3)	8 (38.1)	13 (61.9)	
Present	62 (74.3)	27 (43.5)	35 (56.5)	
Microsatellite nodules				0.017[d]
Absent	67 (80.7)	33 (49.3)	34 (50.7)	
Present	16 (19.3)	2 (12.5)	14 (87.5)	
Venous invasion				0.241
Absent	73 (88.0)	33 (45.2)	40 (54.8)	
Present	10 (12.0)	2 (20.0)	8 (80.0)	
Liver cirrhosis				0.832
No	45 (54.2)	18 (40.0)	27 (60.0)	
Yes	38 (45.8)	17 (44.7)	21 (55.3)	

[a] The data are modeled as continuous variables.
[b] IQR, interquartile range.
[c] The results are calculated by Mann-Whitney test.
[d] $p < 0.05$ is considered statistically significant.

EZH2-mediated Epigenetic Repression Dictates PAK6 Down-regulation—Given the aforementioned results, the molecular mechanism of PAK6 repression in HCC addressed our attentions. Growing evidence showed overexpression of EZH2, a core enzymatic component of PRC2 catalyzing transcriptional repressor histone H3 lysine 27 trimethylation (H3K27me3), was a marker of advanced and metastatic disease in many tumors, including HCC (29, 30). Thus, the hypothesis that PAK6 expression was directly under epigenetic control of PRC2 in HCC was investigated. First, the expression of components of PRC2, EZH2, SUZ12, and EED were assessed in different HCC cell lines. Among them, SK-Hep1 had higher expression of PRC2 components, whereas Huh7 had lower expression of these proteins (Fig. 6*A*). Then, gain or loss function of EZH2 experiments indicated overexpression of EZH2 in Huh7 cells decreased the protein and mRNA levels of PAK6 (Fig. 6*B*), whereas silence of EZH2 or treatment with 3-deazaneplanocin A, an EZH2 inhibitor, in SK-Hep1 cells rescued the protein and mRNA level of PAK6 (Fig. 6, *C* and *D*). These experimental results suggested that PAK6 was under epigenetic regulation of EZH2. A further step was taken to investigate whether the PAK6 repression was directly due to the increasing enrichment of H3K27me3 catalyzed by EZH2. Two pairs of primers at a CpG enrichment area and transcriptional start site, respectively, and one pair of primers at the GAPDH promoter as negative control were designed to assess the enrichment of EZH2 and H3K27me3 (Fig. 6*E*). After EZH2 overexpression in Huh7 cells, the enrichment of EZH2 and H3K27me3 were increased in two sites at the PAK6 promoter (Fig. 6*F*), whereas, after knockdown of EZH2 or treatment with 3-deazaneplanocin A in SK-Hep1 cells, the enrichment of EZH2 and H3K27me3 were depleted (Fig. 6, *G* and *H*). Furthermore, silence of SUZ12 and EED, two other components of PRC2, also rescued the protein and mRNA level of PAK6 in SK-Hep1 cells, respectively (Fig. 6, *I* and *J*). Collectively, these results suggest that enrichment of the epigenetic silence of H3K27me3 mediated by PRC2 dictated the PAK6 decrease in HCC. At last, histone acetylation alteration and cooperation with H3K27me3 in gene regulation have been previously reported in HCC (31–33). Thus, the impact of histone acetylation on decreased PAK6 expression was assessed using trichostatin A, a histone deacetylase. Treating SK-Hep1 cells with trichostatin A also recovered the protein and mRNA levels of PAK6, suggesting histone hypoacetylation also contributed to the repression of PAK6 (Fig. 6*K*).

Negative Correlation between PAK6 and EZH2 in HCC Specimens—To substantiate the aforementioned results, the clinical relevance of EZH2 and PAK6 was analyzed. Using immunochemical analysis, PAK6 expression was negatively correlated with EZH2 expression in patient specimens ($r = -0.52$, $p < 0.001$) (Fig. 7*A* and 7*B*). Furthermore, a Kaplan-Meier analysis was preformed to investigate the relationship of the overall survival with four groups cataloged by different PAK6 and EZH2 expressions, where the group of patients with high PAK6 and low EZH2 expression had a longer overall survival than those with low PAK6 and high EZH2 expression ($p < 0.001$) (Fig. 7*C*).

Discussion

Historically much of the focus was directed toward group I PAK1 and group II PAK4, however, other PAK isoforms were less known. Here we demonstrated that PAK6 was decreased in the progress of HCC and its repression was associated with poor clinical outcome, suggesting PAK6 might be a tumor suppressive gene in HCC. Most PAKs were considered oncogenic genes, however, emerging evidence indicates that PAKs might have a different, even opposite function in the progress of cancer, such as opposite regulating results of estrogen receptor α (ERα) by PAK1 and PAK6 (34, 35). These differences might be caused by regulating manners, such as a different effect of Cdc42 interaction (36), optimal phosphorylation sites (37), or especially by special substrates, such as the unique substrate AR for PAK6 (13, 38). Admittedly, most functions of PAK isoforms were overlapped and studies about unique function were sporadic. Thus, studies about the unique function of different PAK isoforms were of importance to better understand the role of the PAK family in the progress of cancer.

DNA methylation analyses has proved that hypermethylation was found in the PAK6 promoter in prostate and adenocarcinoma of uterine cervical cancers (39, 40). Additionally, miRNAs were also involved in suppression of PAK6 (17, 41). Here, our work demonstrated increasing enrichment of H3K27me3 in the PAK6 promoter catalyzed by PRC2, which

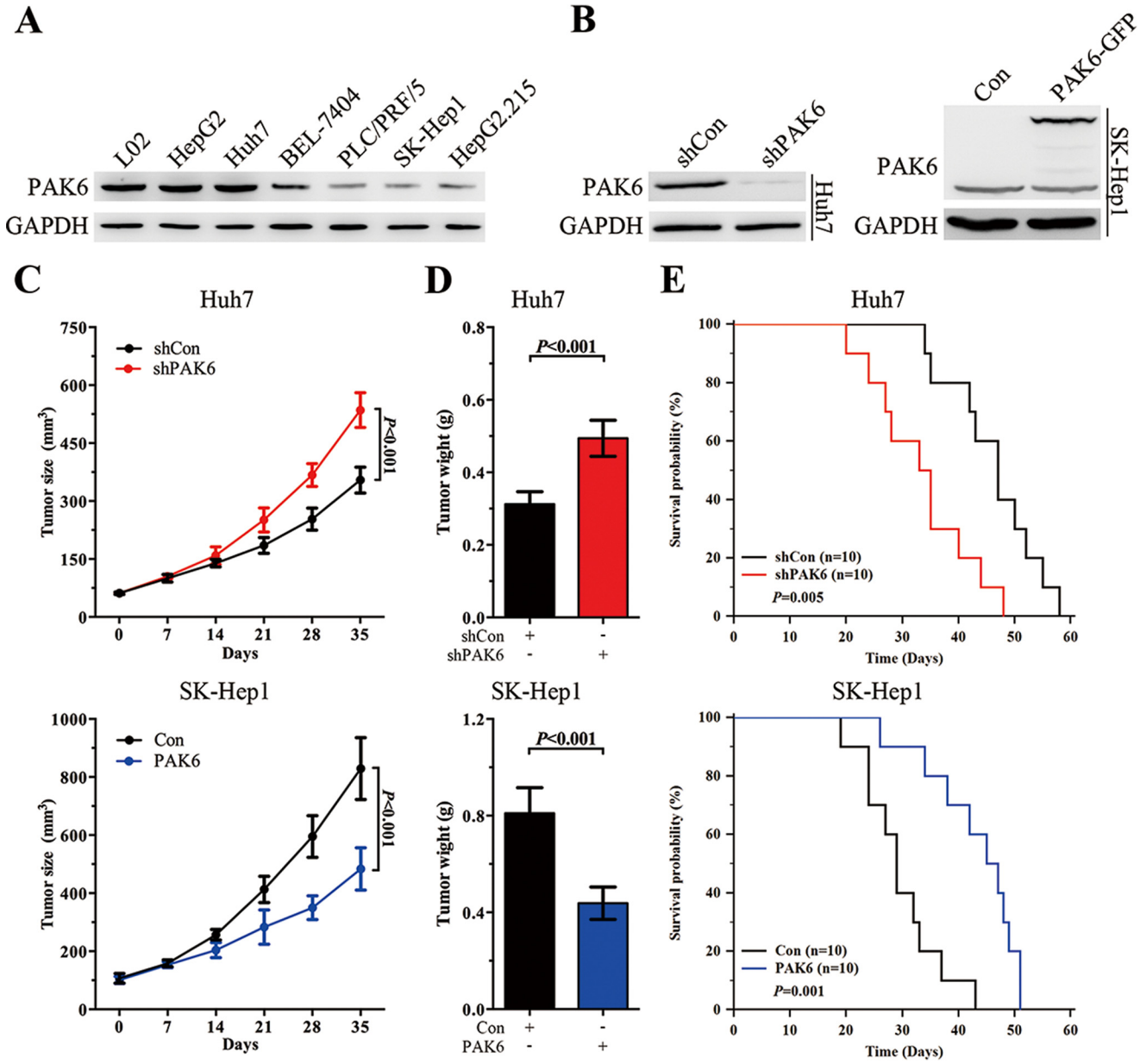

FIGURE 2. **PAK6 inhibits xenografted hepatoma cell growth *in vivo*.** *A*, Western blot for endogenous PAK6 expression in six mammalian HCC cell lines and one immortalized liver cell line. *B*, Western blot for efficiencies of Huh7 stably transfected with NS-shRNA or PAK6-shRNA and SK-Hep1 stably transfected with empty or PAK6, respectively. Tumor growth curve (*C*), tumor weight analysis (*D*), and overall survival (*E*) for groups of nude mice xenografted with Huh7 cells stably transfected with NS-shRNA or PAK6-shRNA and SK-Hep1 stably transfected with empty or PAK6, respectively, where 3 mice were used in each group of 6 tumor sites for tumor volume and weight analysis, and 10 mice in each group were used for survival analysis. *p* value of survival analysis is determined by log-rank test.

contributed to PAK6 suppression in HCC via gain or loss of function of PRC2. Functionally, EZH2 was reported to directly interact with DNA methyltransferase to consolidate the repression of targeting genes, thus, DNA hypermethylation in the PAK6 promoter might be a subsequent consequence of EZH2-mediated modification, suggesting cooperation of histone and DNA modification might contribute to PAK6 repression (42). Besides these, histone hypoacetylation was also involved in PAK6 regulation (Fig. 6*J*). Thus, these results might broaden our understanding of regulating mechanisms of PAK6 expression.

Previous studies have proved kinase activity was required for PAK6 inhibiting AR transcriptional activity (13, 20). Meanwhile, a kinase-independent mechanism of PAKs was also reported (10). In our work, the kinase dead mutant depleted the function of PAK6 in HCC, suggesting kinase activity was indispensable for PAK6. As for nuclear translocation, it was reported that PAK6 could translocate to the nucleus as part of the AR complex, although another study reported PAK6 remained primarily in the cytoplasm and inhibited nuclear translocation of AR (11, 20). Admittedly, the mechanisms of PAK6 shuttling into the nucleus and function of PAK6 in the nucleus were less known. We identified that a polylysine sequence in the N-terminal acted as NLS for PAK6. A functional analyses indicated that nuclear translocation was partially required for the PAK6 tumor sup-

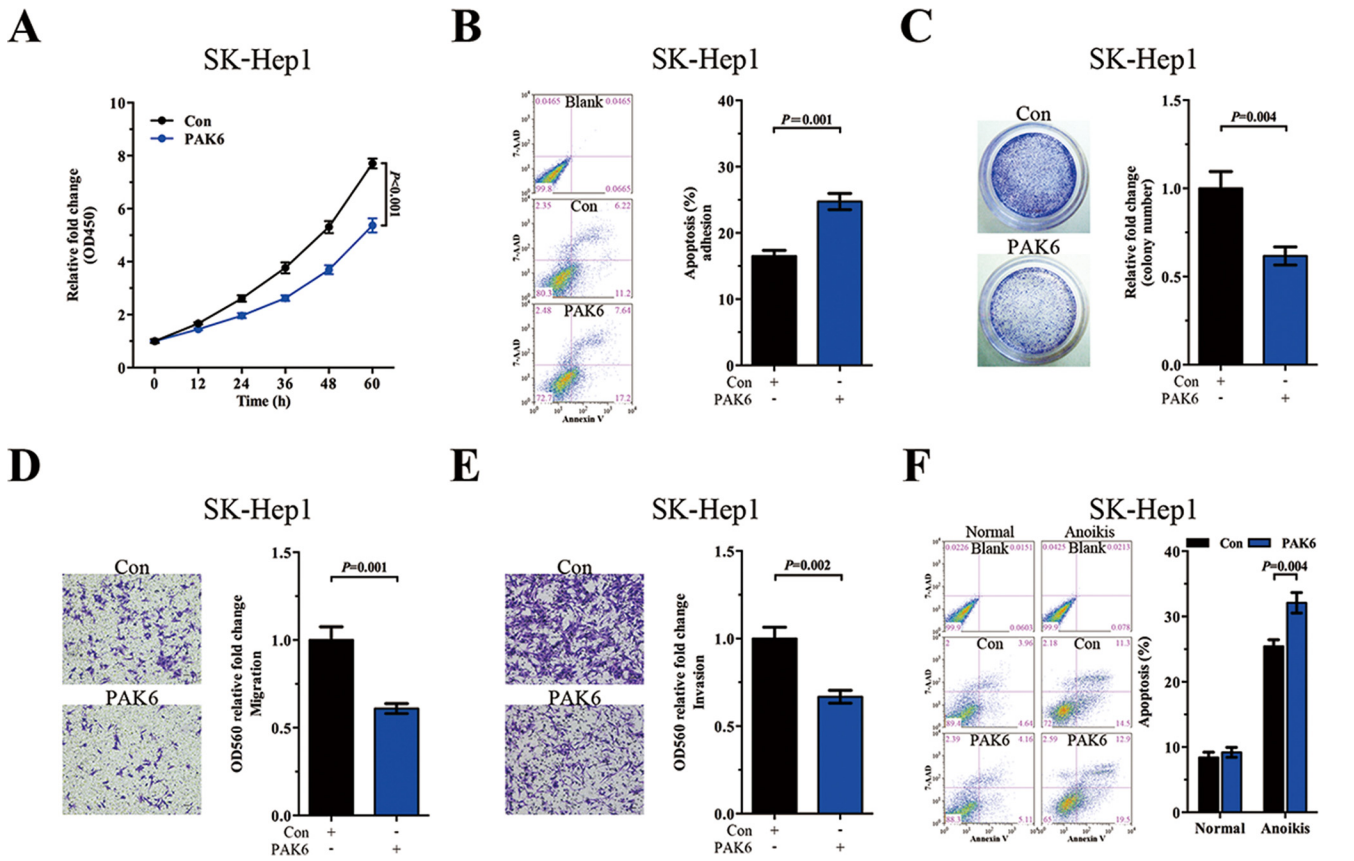

FIGURE 3. **Overexpression of PAK6 restricts hepatoma cell tumorigenecity *in vitro*.** *A,* CCK-8 assay for cell proliferation; *B,* Annexin V assay for cell apoptosis induced by serum starvation for 48 h. *C,* colony formation; *D,* migration assay (original magnification ×100); *E,* invasion assay (original magnification ×100); and *F,* Annexin V assay for cell anoikis induced by culturing on low-adhesive tissue culture plates with 10% serum and a no anoikis-induced condition as control, were performed in SK-Hep1 cells stably transfected with control or PAK6. The *scale bar* is 50 μm.

pressive function in HCC. Not considered a DNA binding domain in PAKs, PAK6 might act as a binding partner of transcriptional factors to regulate gene transcription (43). Thus, kinase activity and nuclear translocation were critical for the PAK6 tumor suppressive function, although the detailed mechanisms of PAK6 were less clarified in cancer progress.

In HCC, hepatic AR had a critical role in hepatocarcinogenesis, which could promote anchorage-dependent and -independent cell growth and suppress cell apoptosis (44). As a well known negative regulator of AR, PAK6 might regulate HCC cell proliferation and apoptosis via orchestrating the AR signaling. Meanwhile, PAK6 could phosphorylate the E3 ligase murine double minute-2 (MDM2) at two novel sites, Thr-158 and Ser-186, suggesting PAK6 might regulate protein ubiquitination mediated by MDM2 (13). Furthermore, PAK6 could interact with CCAAT/enhancer-binding protein α (C/EBPα), which was a poor prognostic factor in HCC (45–47). Presumably, PAK6 influenced C/EBPα signaling to regulate gene transcription, although the functional significance of the interaction has not been described (47). Moreover, remodeling of the cytoskeleton is one well characterized feature of the PAK family, which is a key factor in tumor cell migration and invasion. Orchestrating the cytoskeleton might be an important means of PAK6 in suppression of HCC cell migration and invasion. Thus, although the detailed substrate and mechanism were not well known, broadening PAK6-specific substrates and molecular mechanisms might improve our understanding of function of PAK6 in tumorigenesis.

Recently, a retrospective study suggested PAK6 was overexpressed in HCC and was a negative prognostic factor (48). The detailed reason of discrepant results is not yet known. The possible reason might be the tremendous variability in technically performing IHC, the intensity of the stains and interpretation of results; these would cause inconsistent oncologic outcome results of IHC. Admittedly, the context-dependent function of PAK6 has been found in prostate cancer. However, in our study, the *in vivo* and *in vitro* functional analyses support our findings in IHC analysis, suggesting PAK6 functioned as a tumor suppressor. Meanwhile, in clear-cell renal cell carcinoma, we also found PAK6 may function as a tumor suppressor (16). Thus, more studies of PAK6 function were warranted in different contexts to further explore the PAK6 function in cancer progression.

The experimental observations showed that PAK6 repression, due to epigenetic control of H3K27me3 mediated by PRC2, promoted HCC cell proliferation, survival, migration, and invasion (Fig. 7*D*). The clinical studies also suggested relevance of our finding. Together, the EZH2/PAK6 axis may contribute to hepatocarcinogenesis and PAK6 may serve as a

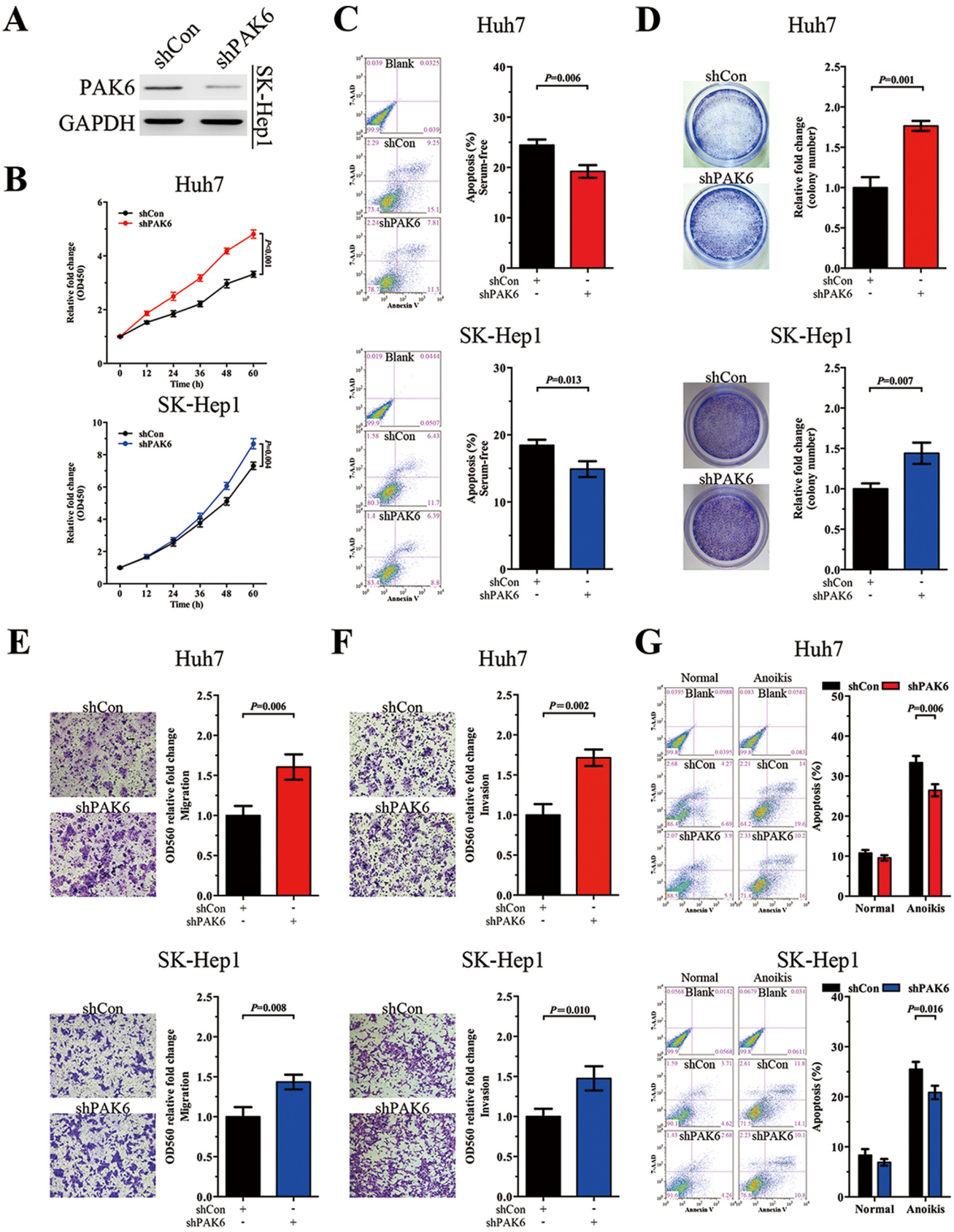

FIGURE 4. **Silence of PAK6 promotes hepatoma cell tumorigenecity *in vitro*.** *A*, Western blot for efficiency of SK-Hep1 stably transfected with NS-shRNA and PAK6-shRNA. *B*, CCK-8 assay for cell proliferation; *C*, annexin V assay for cell apoptosis induced by serum starvation; *D*, colony formation; *E*, migration assay (original magnification ×100); *F*, invasion assay (original magnification ×100); and *G*, annexin V assay for cell anoikis induced by culturing on low-adhesive tissue culture plates with 10% serum and a no anoikis-induced condition as control were performed in Huh7 and Sk-Hep1 cells stably transfected with NS-shRNA and PAK6-shRNA, respectively. The *scale bar* is 50 μm.

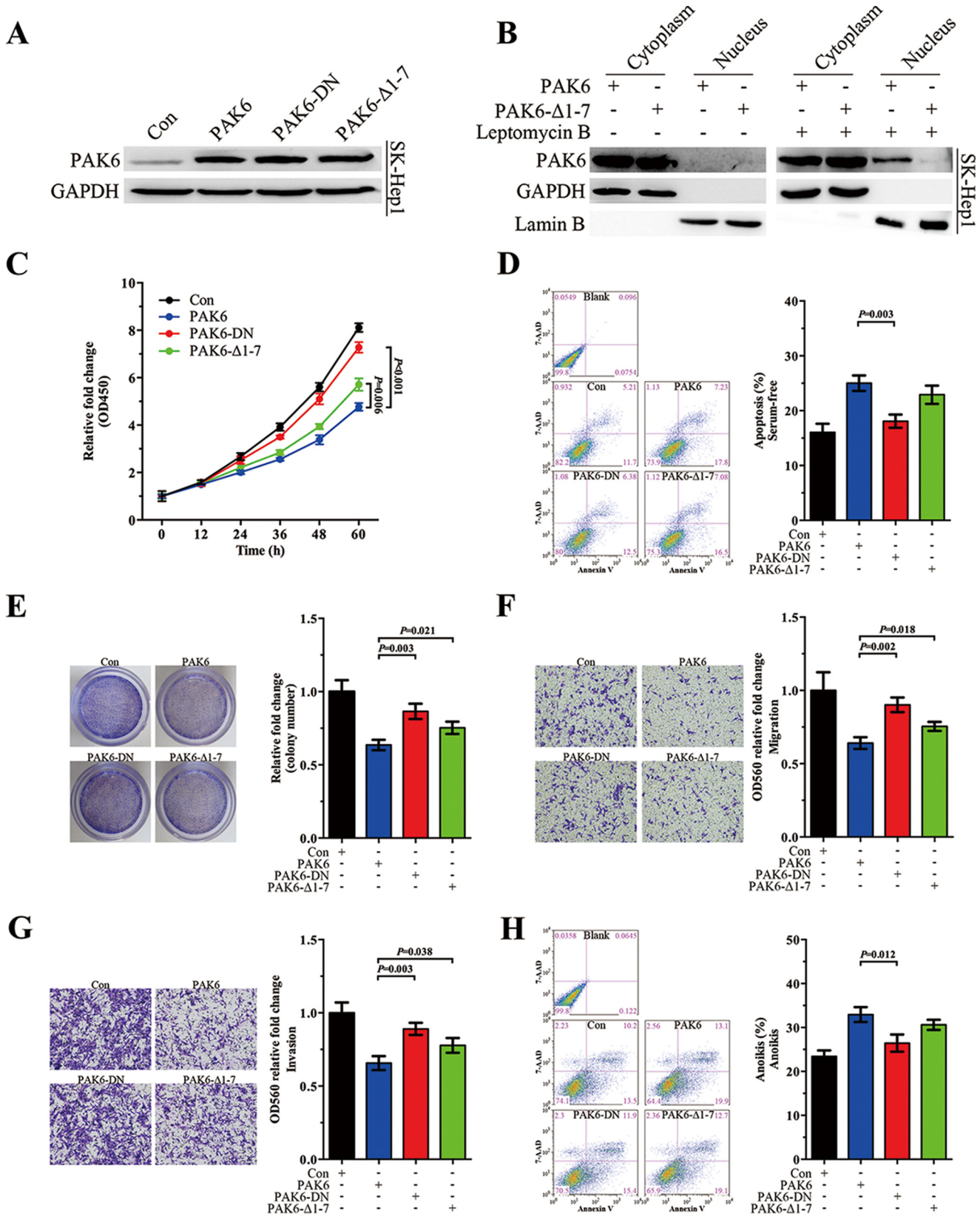

FIGURE 5. **The kinase activity and nuclear translocation determine PAK6 function.** *A,* Western blot for PAK6 expression of SK-Hep1 cells stably transfected with empty, wild-type PAK6 (*PAK6*), dead kinase mutant (*PAK6-DN*), and NLS deletion mutant (*PAK6-Δ1–7*). *B,* Western blot for PAK6 expression in the cytoplasm and nucleus of SK-Hep1 cells stably transfected with wild-type PAK6 (*PAK6*) and NLS deletion mutant (*PAK6-Δ1–7*), after treatment with leptomycin B at 10 nM for 6 h or without leptomycin B treatment as control. *C,* CCK-8 assay for cell proliferation; *D,* annexin V assay for cell apoptosis induced by serum starvation; *E,* colony formation; *F,* migration assay (original magnification ×100); *G,* invasion assay (original magnification ×100); and *H,* annexin V assay for cell anoikis induced by culturing on low-adhesive tissue culture plates with 10% serum and a no anoikis-induced condition as control were performed in SK-Hep1 cells stably transfected with empty vector, wild type PAK6 (*PAK6*), dead kinase mutant (*PAK6-DN*), and NLS deletion mutant (*PAK6-Δ1–7*). The *scale bar* is 50 μm.

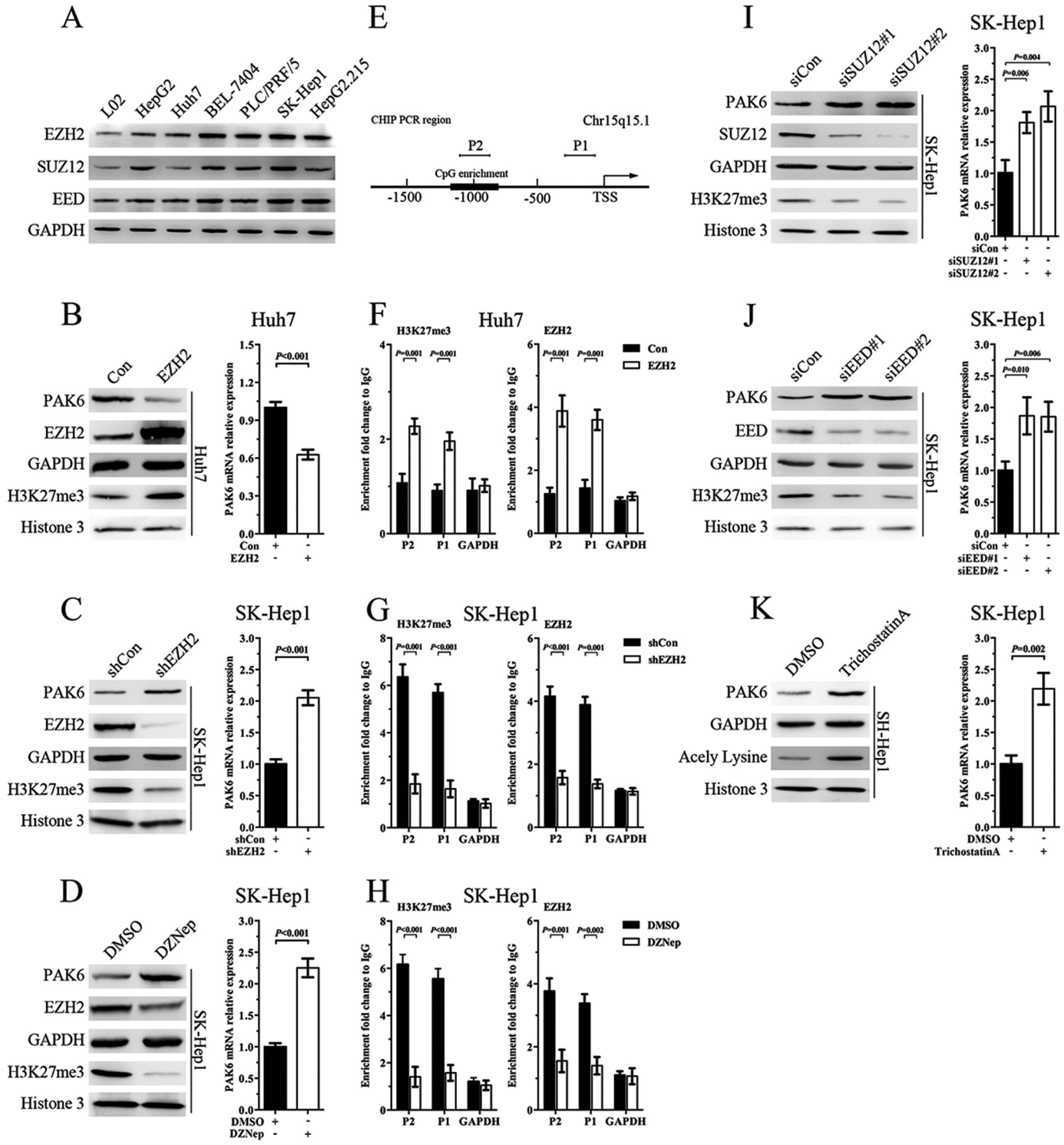

FIGURE 6. **EZH2-mediated epigenetic repression dictates PAK6 down-regulation.** *A*, Western blot analysis for EZH2, SUZ12, and EED expression in different hepatoma cell lines and one immortalized liver cell line. *B*, Western blot and RT-PCR analysis for PAK6 expression of Huh7 cells stably transfected with empty vector and EZH2. *C*, Western blot and RT-PCR analysis for PAK6 expression of SK-Hep1 cells stably transfected with NS-shRNA and EZH2-shRNA. *D*, Western blot and RT-PCR analysis for PAK6 expression of SK-Hep1 cells treated with DMSO and 3-deazaneplanocin A (10 μM). *E*, the diagram for primers set for ChIP analysis. Two pairs of primers were designed located in the CpG enrichment and transcriptional starting site at the PAK6 promoter, and one pair of primers was designed at the GAPDH promoter as a negative control. ChIP-qRCR analysis for enrichment of EZH2 and H3K27me3 in Huh7 cells stably transfected with empty and EZH2 (*F*), SK-Hep1 cells stably transfected with NS-shRNA and EZH2-shRNA (*G*), and SK-Hep1 cells treated with DMSO and 3-deazaneplanocin A (*DZNep*) (*H*) (10 μM). *I*, Western blot and RT-PCR analysis for PAK6 expression of SK-Hep1 cells transfected with NS-siRNA and two different SUZ12-siRNAs. *J*, Western blot and RT-PCR analysis for PAK6 expression of SK-Hep1 cells transfected with NS-siRNA and two different EED-siRNAs. *K*, Western blot and RT-PCR analysis for PAK6 expression of SK-Hep1 cells treated with DMSO and trichostatin A (100 nM).

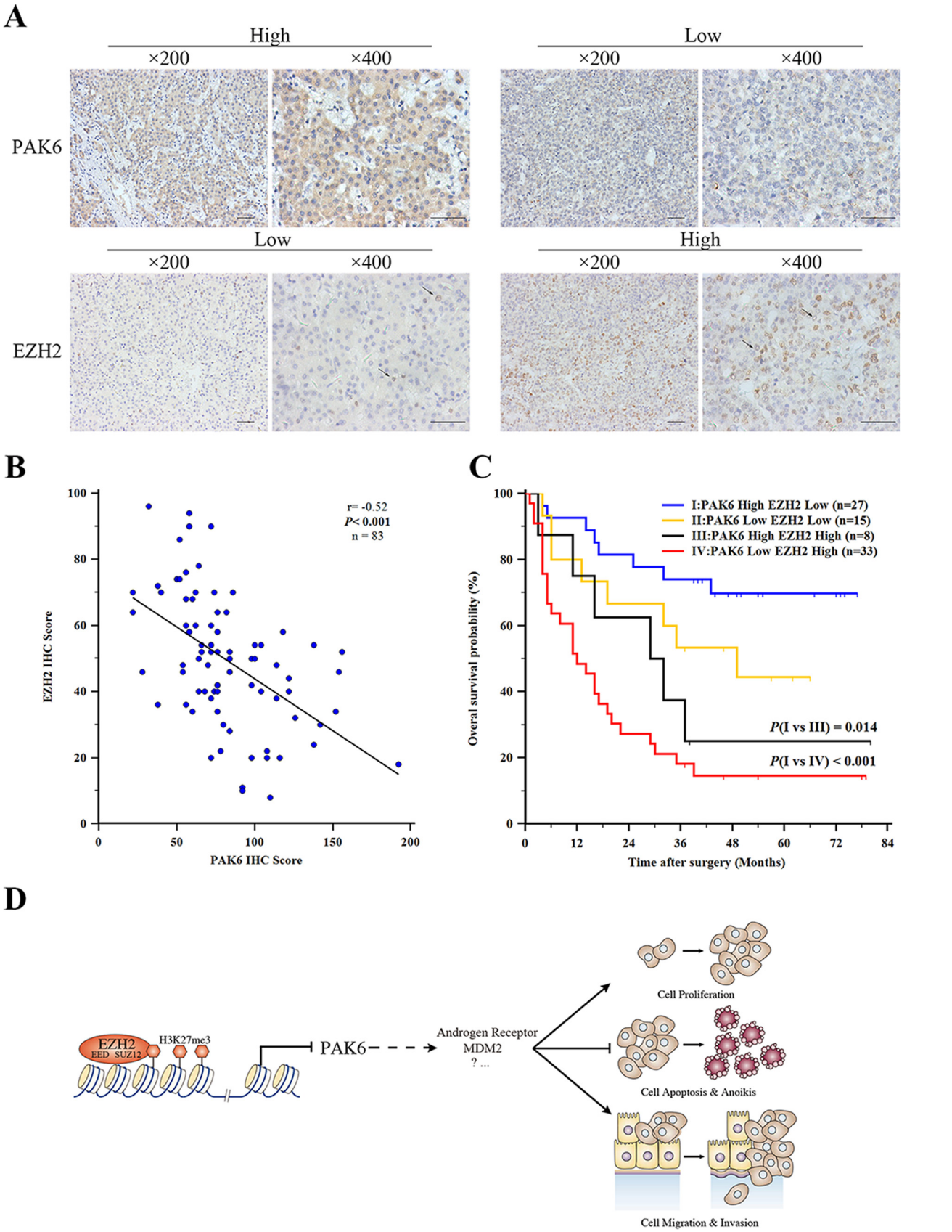

FIGURE 7. **Negative correlation between PAK6 and EZH2 in HCC specimens.** *A,* representative IHC images of different PAK6 and EZH2 staining in two HCC patients (original magnification ×200 and 400). *Black arrows* represent positive nuclear EZH2 staining. *B,* correlation analysis between PAK6 and EZH2 IHC staining in 83 patient tissues ($r = -0.52$, $p < 0.001$). *C,* Kaplan-Meier analysis for four groups cataloged by different PAK6 and EZH2 expression. *D,* a schematic model of the EZH2-PAK6 regulatory pathway in HCC. *p* value of survival analysis is determined by log-rank test. The *scale bar* is 50 μm.

potential biomarker for personalized management of HCC patients.

Author Contributions—J. J. X. and J. X. G. designed the study. W. S. L. performed and analyzed the experiments Y. D. L., H. O. L., W. J. Z., and Q. F. provided technical assistance and contributed to the preparation of the figures. All authors reviewed the results and approved the final version of the manuscript.

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