WITHDRAWN: Positive feedback between slug and Akt promotes hepatocellular carcinoma growth via CDK6 expression

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Abstract

Background

Hepatocellular carcinoma (HCC) is a rapidly growing tumor associated with a propensity for vascular invasion and metastasis, but the mechanism of HCC growth is not fully understood. The objective of this study is to investigate the role of the transcriptional repressor slug in HCC growth and its detailed mechanism.

Methods

Lentivirus-mediated slug overexpression as well as CRISPR/Cas9-mediated slug knockout were conducted in hepatic or HCC cells. Then cell growth was evaluated in culture dishes as well as nude mice xenografts. Transcriptome analysis was used to explore the detailed mechanism of slug-induced HCC growth.

Results

Slug significantly facilitated HCC growth *in vitro* and *in vivo*. Mechanism dissection *via* transcriptome analysis revealed that slug transcriptionally upregulated PIK3CD and PIK3R3 expression and then activated the Akt/CDK6 signaling pathway. Administration of either the PI3K/Akt signaling inhibitor MK2206 or the CDK4/6 inhibitor PD-0332991 abrogated slug-induced HCC growth. In addition, administration of MK2206 also suppressed slug expression in HCC cells. Immunohistochemistry staining indicated that Akt activity and CDK6 expression were significantly associated with slug expression in human HCC tissues.

Conclusions

Our study determined the promoting role of slug in HCC growth and revealed that the positive feedback between slug and Akt conferred HCC growth via CDK6 expression. The CDK4/6 inhibitor PD-0332991 might be a promising drug for slug/Akt-induced HCC growth.

Background

Hepatocellular carcinoma (HCC) is a common cancer with an increasing worldwide prevalence and is the leading cause of cancer-related death [1]. HCC is a rapidly growing tumor associated with a propensity for vascular invasion and metastasis, which results in poor cancer prognosis[2]. However, the mechanism of HCC growth is not fully understood.

The transcriptional repressor slug, a member of the snail family, is known to play vital roles in cancer progression. It has been demonstrated that the expression of slug is correlated with metastasis and short survival time in HCC patients [3]. A functional study indicated that slug overexpression induces stemness and promotes HCC invasion and metastasis[2]. Aside from metastasis, slug has also been reported to be involved in human cancer growth, but the conclusions are controversial. For example, knockdown of slug inhibits the proliferation of colorectal cancer HCT116 cells, indicating a promotion effect of slug [4]. However, slug acts as a negative regulator of the proliferation of prostate cancer cells [5]. A preliminary study in HCC HepG2 cells indicated that slug induces cell proliferation [2], but the effect of slug on HCC growth needs to be further confirmed, and the detailed mechanism needs to be investigated.

In the present study, we determined the role of slug on HCC growth by lentivirus-mediated slug overexpression as well as CRISPR/Cas9-mediated slug knockout. We found that overexpression of slug in the human hepatic HL7702 cells and HCC Huh7 cells significantly induced cell growth *in vitro*. Conversely, slug knockout in HCC BEL7402 and SK-HEP-1 cells significantly inhibited cell growth. A mechanistic study with transcriptome analysis revealed that slug promoted HCC growth by activating the PI3K/Akt signaling pathway. Conversely, slug expression in HCC was also regulated by the Akt signaling pathway. The activated Akt signaling pathway promoted HCC growth by upregulating CDK6 expression. Nude mice models bearing HCC xenografts showed that slug overexpression significantly promoted tumor growth, Akt activity and CDK6 expression. In human HCC tissues, Akt activity and CDK6 expression were significantly associated with slug expression.

Methods

Chemicals and reagents

Primary antibodies against total AKT, slug, β-actin and horseradish peroxidase-conjugated secondary antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Primary antibody against p-AKT Ser473 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibody against CDK6 were purchased from Proteintech Group, Inc (Wuhan, China). MK2206 and PD-0332991 were purchased from Selleck Chemicals (Houston, TX, USA).

Cell culture

The human hepatocyte-derived cell line HL7702 and HCC Huh7, BEL7402 and SK-HEP-1 cell lines were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were propagated in Dulbecco's Modified Eagle Medium/F12 (DMEM/F12) containing 10% fetal bovine serum (FBS). Cells were maintained at 37 °C in a humidified incubator with 5% CO2 in air.

Western blot assay

Total protein was extracted from cells with RIPA lysis buffer containing protease inhibitors. The protein concentration of lysates was detected by the Bradford method. 30 μg of protein was separated by

electrophoresing on 12% SDS-polyacrylamide gel and blotted onto nitrocellulose filter membranes. The membranes were then blocked with 5% nonfat-milk for 1 hour at room temperature. The blots were then incubated with primary antibodies overnight at 4 °C and horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Immune blots were detected using the ECL detection system. Immunoblotting against β-actin was performed as an internal control.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

A total of 2500 HCC cells were plated in 96-well culture plates in 0.2 mL of medium containing 10% FBS. Then, the cells were incubated for different numbers of days, and 20 μL MTT (5 mg/mL) was added to each well and incubated for 4 hours. The supernatant was removed, and 200 μL DMSO was added to dissolve the viable cell-generated reaction products. The absorbance of well of the plates was recorded using a 96-well microplate reader at a wavelength of 490 nm.

Six-well plate colony formation assay

Five hundred HCC cells were seeded in each well of 6-well culture plates in 2 mL of medium containing 10% FBS. After culture for 2 weeks, cells were fixed in 4% paraformaldehyde and stained with crystal violet. Visible colonies in each well were counted.

Transcriptome analysis (RNA-seq)

Total RNA from each sample was extracted using TRIzol Reagent (Invitrogen). Next generation sequencing libraries were constructed according to the manufacturer's protocol (NEBNext® Ultra™ RNA Library Prep Kit for Illumina®). Poly(A) mRNA isolation was performed using the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB). mRNA fragmentation and priming were performed using NEB Next First-Strand Synthesis Reaction Buffer and NEB Next Random Primers. First-strand cDNA was synthesized using ProtoScript II Reverse Transcriptase, while second-strand cDNA was synthesized with Second-Strand Synthesis Enzyme Mix. The purified double-stranded cDNA (AxyPrep Mag PCR Clean-up [Axygen]) was treated with End Prep Enzyme Mix to repair both ends and to add dA-tailing in one reaction, followed by a T-A ligation to add adaptors to both ends. Size selection of the adaptor-ligated DNA was performed using AxyPrep Mag PCR Clean-up (Axygen), and fragments of ~360 bp (with an approximate insert size of 300 bp) were recovered. Each sample was amplified with polymerase chain reaction (PCR) for 11 cycles using P5 and P7 primers; both primers carried sequences that could anneal with the flow cell to perform bridge PCR. The P7 primer carried a six-base index that allowed multiplexing. The PCR products were cleaned up using AxyPrep Mag PCR Clean-up (Axygen), validated with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), and quantified using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). Libraries with different indices were multiplexed and loaded on an Illumina HiSeq instrument according to the manufacturer's instructions (Illumina, San Diego, CA, USA). Sequencing was carried out using a 2 ´ 150 bp paired-end (PE) configuration; image analysis and base calling were conducted with HiSeq Control Software (HCS) + OLB + GAPipeline-1.6 (Illumina) on the HiSeq instrument. The sequences were processed and analyzed by GENEWIZ Inc. (Hangzhou, China).

RNA from each group was extracted and analyzed in triplicate. Fragments per kilobase million (FPKM) was used for statistical analysis. Differentially expressed genes (DEGs) with a p value < 0.05 by Student's t-test and an average fold change > 1.25 were considered significant.

Real-time quantitative PCR analysis

In brief, 1 μg of total RNA was subjected to reverse transcription using Superscript III transcriptase (Invitrogen, Carlsbad, CA, USA). Real-time quantitative PCR was performed on a Bio-Rad CFX96 system with SYBR Green to determine the mRNA expression level. The reaction conditions used for PCR were as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. The mRNA expression level was normalized to that of human β-actin. Relative fold changes in mRNA expression were calculated using the formula $2-\Delta\Delta Cq$ [6]. Primer sequences are listed in Supplementary Table 1.

Xenograft animal model

12 four-week-old male BALB/c nude mice weighing 18-23.5 g were obtained from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China) and housed in sterile laminar flow rooms with 12-h light and dark cycles at a temperature range of 19-23˚C and a humidity of 40-60% in the Laboratory Animal Centre of Xi'an Jiaotong University. All experimental procedures were conducted in accordance with the institutional guidelines for conduct and animal welfare. The animals were divided into two groups randomly (six mice for each group). Then they were inoculated subcutaneously into the right dorsal portion with 2×10^6 Huh7 cells infected with vector or slug overexpression lentivirus. Tumor diameters were measured once a weak. Tumor volumes (V) were calculated with the formula: V $A\times B^2/2$ (A: axial diameter; B: rotational diameter). Mice were killed 4 weeks after injection. Tumor weight was measured, and tumor specimens were analyzed for *he*matoxylin-eosin *(*HE*)* staining and immunohistochemistry staining. Experiments were conducted in accordance with relevant institutional and national guidelines for the care and use of laboratory animals. The mice were raised in 12
hour light/dark cycle at room temperature in a SPF laboratory animal room.

HCC tissues and immunohistochemistry staining Sixty-eight human HCC tissues were collected from patients who underwent partial hepatectomy for HCC at the Department of Hepatobiliary Surgery, the First Affiliated Hospital of Xi'an Jiaotong University, between August 2017 and July 2019. The approval of the institutional review board of the First Affiliated Hospital of Xi'an Jiaotong University was obtained before the samples were collected, which was performed with the permission of the patients. Tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Five-micrometer-thick sections were prepared, and immunohistochemistry staining against slug, p-Akt Ser473 and CDK6 was performed. Immunohistochemistry was performed with the DAKO EnVision™+ System. Evaluation of immunohistochemistry staining was judged by the intensity of staining. Each section was examined under a high-power field (400´) in a double-blinded manner by a pathologist to define the staining of the samples as strong or weak. Clinical and pathological information for HCC tissues is listed in supplementary table 2.

Statistical Analyses

Statistical analyses were carried out using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Quantitative data are presented as the mean ± SD. Differences among 2 groups were compared using Student's t test. Analysis of the association between slug and p-Akt or CDK6 expression was carried out using $\chi 2$ tests. Significance was assumed for p values < 0.05.

Results

Slug facilitates HCC growth in vitro

To determine whether slug could affect HCC growth, lentivirus-mediated slug overexpression was performed in HL7702 and Huh7 cells (Fig. 1A). Six-well plate colony formation assays revealed that slug overexpression significantly increased the number of colonies (Fig. 1B). Similarly, the MTT assay also indicated enhanced cell growth after slug overexpression (Fig. 1C). Next, CRISPR/Cas9-mediated slug knockout was performed in HCC BEL7402 and SK-HEP-1 cells (Fig. 1D). Six-well plate colony formation assays revealed that slug knockout significantly decreased the number of colonies (Fig. 1E). In addition, the MTT assay also indicated impaired cell growth after slug knockout (Fig. 1F).

Reciprocal activation between slug and PI3K/Akt signaling pathway

To investigate the mechanism of slug-mediated HCC cell growth, a transcriptome assay was performed by RNA-seq in Huh7 cells with slug overexpression and BEL7402 cells with slug knockout (Fig. 2A). A total of 1432 DEGs were obtained after overlapping the data in both cell models. Among these DEGs, 1083 genes were downregulated by slug, and 349 genes were upregulated by slug. Then, KEGG analysis of the downregulated genes and upregulated genes was conducted to gain insight into these DEGs. The results showed that 10 terms were enriched in the downregulated genes and 14 terms were enriched in the upregulated genes (supplementary Fig. 1). Next, we focused on the PI3K-Akt pathway (Fig. 2B) because Akt is considered a central node of many signaling pathways and signaling molecules involved in cell growth and differentiation[7]. Western blot assay was then conducted to evaluate Akt signaling activity by detecting its phosphorylation on the serine 473 residue, which is considered as the most important regulator of Akt activity. We found that slug overexpression in HL7702 and Huh7 cells significantly enhanced Akt phosphorylation (Fig. 2C). Conversely, slug knockout in BEL7402 and SK-HEP-1 cells significantly suppressed Akt phosphorylation (Fig. 2D). However, slug did not affect the expression of total Akt (Fig. 2C-D). As shown in Fig. 2B, PIK3CD and PIK3R3, 2 PI3K subunits, were upregulated after slug overexpression in Huh7 cells and downregulated after slug knockdown in BEL7402 cells. Next, the expression of these 2 genes was confirmed by qPCR, and the results showed that slug could upregulate PIK3CD and PIK3R3 expression (Fig. 2E-F). Thus, we speculated that slug might activate Akt signaling by regulating PIK3CD and PIK3R3.

It has been reported that slug is a downstream target of Akt in some human cancer types such as melanoma [8]. To investigate whether this phenomenon exists in HCC, BEL7402 and SK-HEP-1 cells were

treated with MK2206 and then subjected to western blot assay. The results revealed a suppressive effect of MK2206 on slug expression (Fig. 8G-H). Thus, we speculated that there was a positive feedback loop between slug and Akt in HCC and that reciprocal activation between slug and Akt could promote HCC growth through Akt-mediated CDK6 expression (Fig. 8C).

Slug promotes HCC growth via Akt signaling

To determine whether slug promotes HCC growth *via* the Akt signaling pathway, MK2206, a PI3K/Akt signaling inhibitor, was used in the following study. MK2206 suppressed Akt phosphorylation in a concentration-dependent manner in BEL7402 and SK-HEP-1 cells (Fig. 3A and D). Colony formation assay revealed that MK2206 significantly inhibited the growth of BEL7402 and SK-HEP-1 cells (Fig. 3B-C and E-F). More importantly, administration of MK2206 also inhibited slug-mediated Huh7 cell growth (Fig. 3G-I).

The slug/Akt pathway promotes HCC growth by regulating CDK6 expression

In recent years, cyclin-dependent kinase (CDK) 4 and CDK6 have attracted much interest in the field of cancer research, and CDK4/6 inhibitors have made great achievements in breast cancer therapy[9]. In our study, the RNA-seq data indicated that slug could induce CDK6 expression in both Huh7 and BEL7402 cells (Fig. 2B). Thus, we focused on CDK6 to investigate whether the slug/Akt pathway promotes HCC growth by regulating CDK6 expression. qPCR revealed that slug overexpression in HL7702 and Huh7 cells upregulated CDK6 mRNA expression (Fig. 4A), while slug knockout in BEL7402 and SK-HEP-1 cells downregulated CDK6 mRNA expression (Fig. 4B). Consistently, western blot analysis also showed an enhancement of CDK6 expression induced by slug (Fig. 4C-D). More importantly, administration of MK2206 significantly impaired slug-induced CDK6 expression in Huh7 cells, indicating that slug promotes CDK6 expression *via* Akt (Fig. 4E). Then, PD-0332991, a CDK4/6 inhibitor, was used in our study to determine whether slug/Akt promotes HCC growth *via* CDK6. As shown in Fig. 4F, PD-0332991 significantly inhibited slug/Akt-induced Huh7 cell growth in a dose-dependent manner.

Slug promotes HCC growth, Akt activity and CDK6 expression in vivo

To determine whether slug could promote HCC growth *in vivo*, a total of 12 nude mice were subcutaneously injected with vector control or slug-overexpressing Huh7 cells. No mice died during the experimental period. There is no difference of the body weight between the two groups (data not shown). Growing curves of the xenografts showed that slug overexpression significantly facilitated tumor growth *in vivo* (Fig. 5B). The mice were sacrificed after 4 weeks, and the tumors were obtained (Fig. 5A). HE staining confirmed that the tumors obtained from xenografts were HCC (Fig. 5C). In addition, the slug overexpression group had a higher tumor weight than the vector control group (Fig. 5D). Furthermore, immunohistochemistry staining revealed that slug overexpression upregulated the expression of p-Akt Ser473 and CDK6 (Fig. 5E).

Association between slug expression, Akt activity and CDK6 expression in human HCC tissues.

In this section, 68 human HCC tissues were immunohistochemically stained for slug, p-Akt Ser473 and CDK6 expression (Fig. 6A). Thirty-eight samples (55.9%) had strong slug expression, 40 samples (58.8%) had strong p-Akt Ser473 expression (Fig. 6B), and 43 samples (63.2%) had strong CDK6 expression (Fig. 6C). Correlation analysis with $\chi 2$ tests indicated that expression of both p-Akt Ser473 (Fig. 7B) and CDK6 (Fig. 6C) in human HCC tissues was associated with slug expression.

Discussion

Cell growth induced by sustaining proliferative signaling is one of the hallmarks of cancer[10]. In situ growth of HCC results in vascular invasion and accelerates intrahepatic spread and extrahepatic metastasis [11, 12]. Thus, therapeutics targeting growth are an important approach for HCC treatment. In the present study, we determined that the transcription repressor slug could promote HCC growth *in vitro* and *in vivo*. Mechanistic dissection by transcriptome analysis revealed that slug promoted HCC growth by activating the Akt signaling pathway. Inversely, the Akt signaling pathway also upregulated slug expression. The positive feedback between slug and Akt consequently promoted HCC growth *via* Akt-induced CDK6 expression. In addition, we found that a CDK6 inhibitor could effectively block slug/Akt-induced HCC growth.

In recent decades, aberrant slug expression has been reported in several types of human cancers. For example, in breast cancer, slug expression is elevated with increasing tumor grade and prognostic indices, and an increased level of slug is associated with metastatic disease or disease recurrence [13]. In HCC, slug expression is correlated with metastasis and poor prognosis in patients [3]. Slug has multiple functions in HCC progression. Slug acts downstream of several oncogenic proteins, such as USP5 and integrin β4, and promotes epithelial-mesenchymal transition (EMT) and therefore participates in HCC invasion and metastasis [14, 15]. Slug also promotes HCC progression by promoting stem cell-like behaviors [16] and vasculogenic mimicry [17]. Consistent with other reports in HepG2 cells [2], we found that slug could promote HCC growth in multiple cell models in the present study. More importantly, our mechanistic study revealed a positive feedback loop between slug and Akt in HCC cells and also revealed that blocking Akt activity with MK2206 significantly inhibits slug-induced HCC growth.

Akt kinase, also known as protein kinase B (PKB), is a signaling molecule of cell growth and differentiation[7]. Cellular Akt is mainly activated by PI3K in response to various growth factors and cytokines [18]. The Akt signaling pathway is one of the most frequently altered signaling networks in human cancers, and its deregulation plays a crucial role in the pathogenesis of many human cancers [7, 19]. The Akt signaling pathway is more significantly activated in high-grade HCC tumors and is associated with poor prognosis in HCC patients, indicating that it might be an attractive target in HCC therapy [20]. However, the adverse effects of PI3K/Akt inhibitors restrict their clinical application [19]. In prostate cancer, PI3K/Akt signaling pathway could be activated by slug because slug is a direct transcriptional repressor of PTEN tumor suppressor [21]. In the present study, slug did not alter PTEN expression in HCC cells (data not shown). However, we found that slug could transcriptionally upregulate

the PI3K subunits PIK3CD and PIK3R3 in HCC cells, indicating a novel mechanism of slug-induced PI3K/Akt signaling pathway.

The cyclin-dependent kinases CDK4 and CDK6 play a crucial role in the G1S phase transition. PD-0332991, a CDK4/6 inhibitor, was approved by the FDA in 2015 for the treatment of patients with estrogen receptor-positive, HER2-negative metastatic breast cancer [22]. A preclinical study showed that PD-0332991 significantly suppressed HCC growth [23]. In the present study, we found that CDK6 is a downstream effector of slug/Akt feedback and a contributor of HCC growth, indicating that PD-0332991 might be a promising drug for combating slug/Akt-induced HCC growth.

Conclusions

Overall, in the present study, we determined a promoting role of slug on HCC growth and revealed that the positive feedback loop consisting of slug and Akt promoted HCC growth by upregulating CDK6 expression. Further preclinical and clinical studies are needed to evaluate the suppressive effect of PD-0332991 on slug/Akt-induced HCC growth.

Abbreviations

CDK cyclin-dependent kinase

DMEM/F12 Dulbecco's Modified Eagle Medium/F12

EMT epithelial-mesenchymal transition

FBS fetal bovine serum

HCC hepatocellular carcinoma

HE hematoxylin-eosin

MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

PCR polymerase chain reaction

PKB protein kinase B

Declarations

Ethics approval and consent to participate

All animal experiments in this study were granted ethical and legal approval prior to the beginning of the study. All procedures performed in this study involving animals were in accordance with the ethical standards of the Institutional Animal Care and Use Committee (IACUC) at Xi’an Jiaotong University.

Consent for publication

The human participants involved in this research consent to publish the manuscript and all the authors agree with the submission and publication of this manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no conflict of interests.

The transcriptome data are openly available in supplementary files.

Authors' contributions

XL supervised the study and revised the manuscript. SH and MZ designed the experiments. XZ and XL wrote the manuscript. GY prepared the transcriptome assays. XW conducted the immunohistochemistry staining. YL and DG performed the western blot assays. XZ and YZ conducted the animal experiments. YC conducted the MTT assay and colony formation assay. YC was responsible for purchasing all materials and reagents used in our study. All authors read and approved the final manuscript.

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Supplementary Information

Supplementary Fig. 1. KEGG analysis was conducted using the downregulated genes and upregulated genes. Transcriptome assay was performed by RNA-seq in Huh7 cells with slug overexpression and BEL7402 cells with slug knockout. DEGs were obtained after overlapping the data in both cell models. Then the DEGs upregulated or downregulated by slug were subjected to the KEGG analysis respectively.

Supplementary Fig. 2. Full-length blots of western blot analysis of slug protein expression in HL7702 and Huh7 cells with/without slug overexpression and in BEL7402 and SK-HEP-1 cells with/without slug knockout. (left) cropping gels, (right) original, full-length blots. The red lines indicated the corresponding

bands of the cropping blots of BEL7402 and SK-HEP-1 cells. The purple lines indicated the corresponding bands of the cropping blots of HL7702 and Huh7 cells.

Supplementary Fig. 3. Full-length blots of western blot analysis of total Akt and p-Akt protein expressions in HL7702 and Huh7 cells with/without slug overexpression and in BEL7402 and SK-HEP-1 cells with/without slug knockout. (left) cropping gels, (right) original, full-length blots. The red lines indicated the corresponding bands of the cropping blots of BEL7402 and SK-HEP-1 cells. The purple lines indicated the corresponding bands of the cropping blots of HL7702 and Huh7 cells.

Supplementary Fig. 4. Full-length blots of western blot analysis of slug protein expression in BEL7402 and SK-HEP-1 cells with/without MK2206 treatment. (left) cropping gels, (right) original, full-length blots. The red and purple lines indicated the corresponding bands of the cropping blots of BEL7402 and SK-HEP-1 cells, respectively.

Supplementary Fig. 5. Full-length blots of western blot analysis of total Akt and p-Akt protein expressions in BEL7402 and SK-HEP-1 cells with/without MK2206 treatment and in Huh7 cells with/without slug overexpression and MK2206 treatment. (left) cropping gels, (right) original, full-length blots. The red, purple and green lines indicated the corresponding bands of the cropping blots of BEL7402 cells, SK-HEP-1 and Huh7 cells, respectively.

Supplementary Fig. 6. Full-length blots of western blot analysis of CDK6 protein expression in HL7702 and Huh7 cells with/without slug overexpression. (left) cropping gels, (right) original, full-length blots. The red and purple lines indicated the corresponding bands of the cropping blots of HL7702 and Huh7 cells, respectively.

Supplementary Fig. 7. Full-length blots of western blot analysis of CDK6 protein expression in BEL7402 and SK-HEP-1 cells with/without slug knockout. (left) cropping gels, (right) original, full-length blots. The red and green lines indicated the corresponding bands of the cropping blots of BEL7402 and SK-HEP-1 cells, respectively.

Supplementary Fig. 8. Full-length blots of western blot analysis of p-Akt and CDK6 protein expressions in Huh7 cells with/without slug overexpression and MK2206 treatment. (left) cropping gels, (right) original, full-length blots. The red lines indicated the corresponding bands of the cropping blots.

Figures

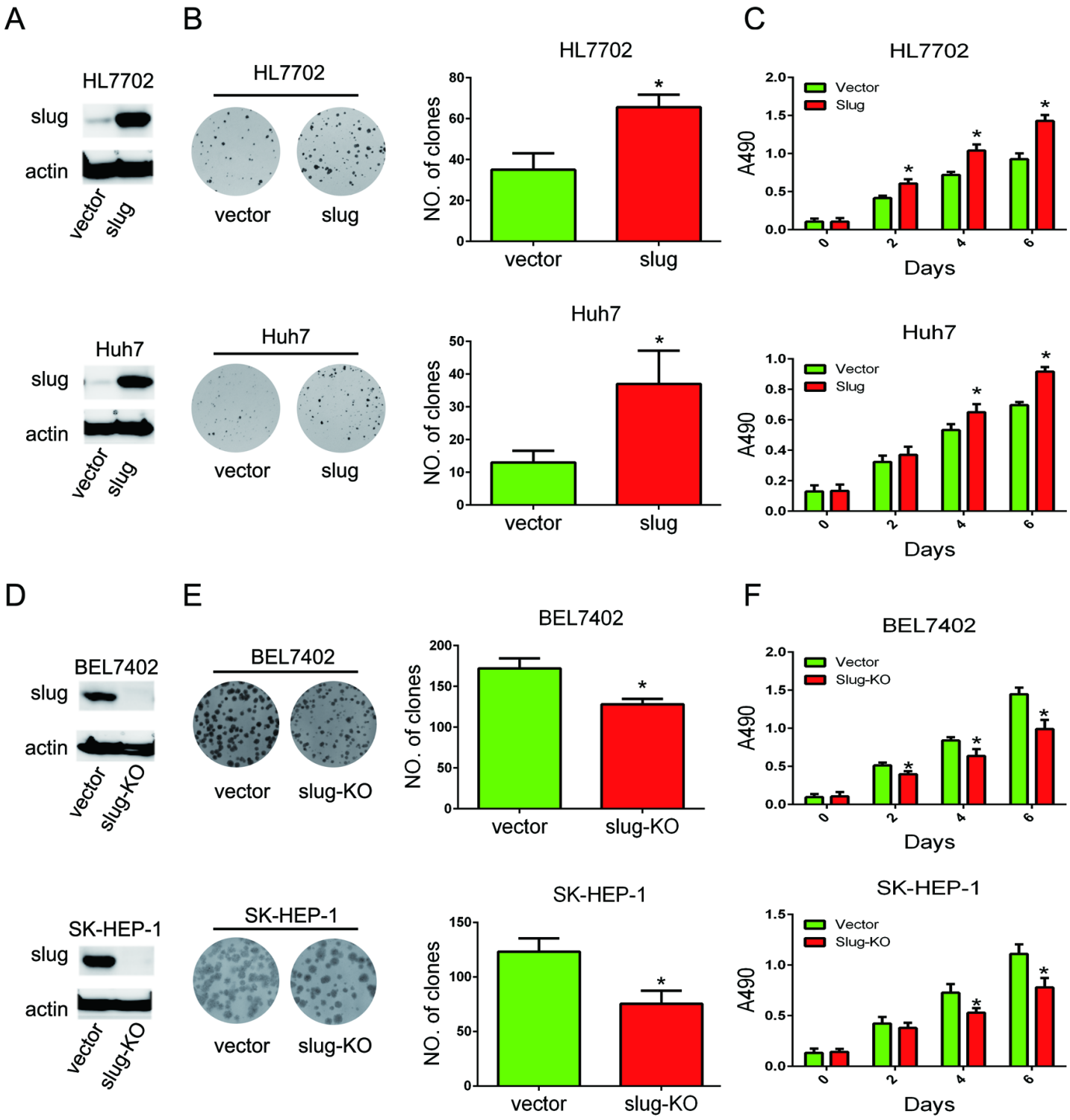


Figure 1

Slug facilitates HCC growth in vitro. A. Lentivirus-mediated slug overexpression was performed in HL7702 and Huh7 cells. Expression of slug was evaluated by western blot assay. Full-length western blots were presented in Supplementary Figure 2. Six-well plate colony formation assay for HL7702 and Huh7 cells after slug overexpression. Left, representative photos. Right, statistic data for visible colonies. C. Growth of HL7702 and Huh7 cells after slug overexpression measured by MTT assay. D. CRISPR/Cas9-mediated

slug knockout was performed in BEL7402 and SK-HEP-1 cells. Expression of slug was evaluated by western blot assay. Full-length western blots were presented in Supplementary Figure 2. E. Six-well plate colony formation assay for BEL7402 and SK-HEP-1 cells after slug knockout. Left, representative photos. Right, statistic data for visible colonies. F. Growth of BEL7402 and SK-HEP-1 cells after slug knockout measured by MTT assay. *P<0.05 vs the vector group.

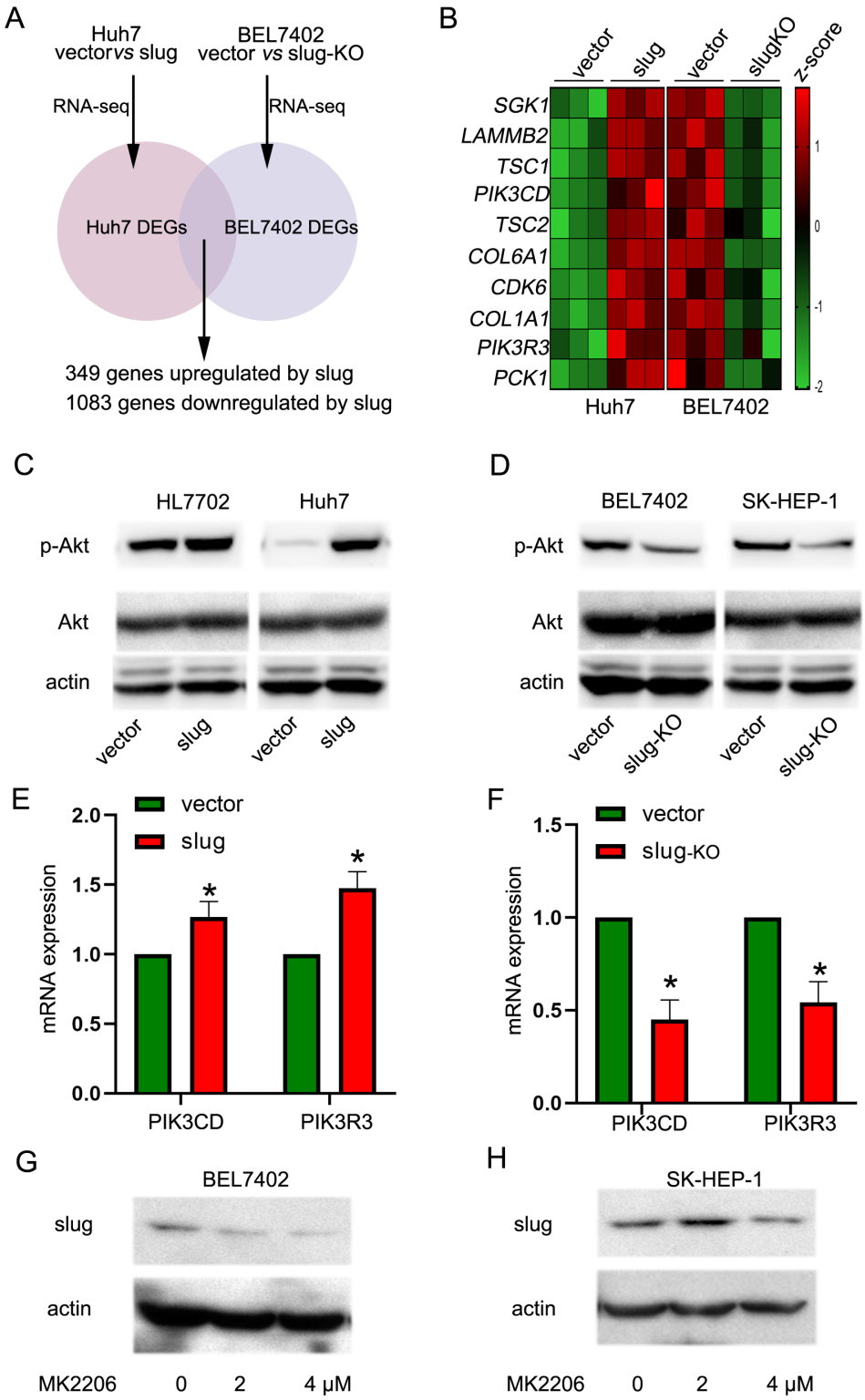


Figure 2

Reciprocal activation between slug and PI3K/Akt signaling pathway in HCC cells. A. Transcriptome assay was performed by RNA-seq in Huh7 cells with slug overexpression and BEL7402 cells with slug knockout. DEGs were obtained after overlapping the data in both cell models. Among these DEGs, 1083 genes were downregulated by slug, and 349 genes were upregulated by slug. B. Heatmap of DEGs involved in the PI3K-Akt pathway. C. Western blot assay was performed to evaluate Akt activity in HL7702 and Huh7 cells overexpressing slug. Full-length western blots were presented in Supplementary Figure 3. D. Western blot assay was performed to evaluate Akt activity in BEL7402 and SK-HEP-1 cells with slug knockout. Full-length western blots were presented in Supplementary Figure 3. E. qPCR assay was performed to confirm the expression of PIK3CD and PIK3R3 in Huh7 cells with slug overexpression. F. qPCR assay was performed to confirm the expression of PIK3CD and PIK3R3 in BEL7402 cells with slug knockout. G. BEL7402 cells were treated with MK2206 for 48 hours, and slug expression was evaluated with western blot assay. Full-length western blots were presented in Supplementary Figure 4. H. SK-HEP-1 cells were treated with MK2206 for 48 hours, and slug expression was evaluated with western blot assay. Full-length western blots were presented in Supplementary Figure 4.

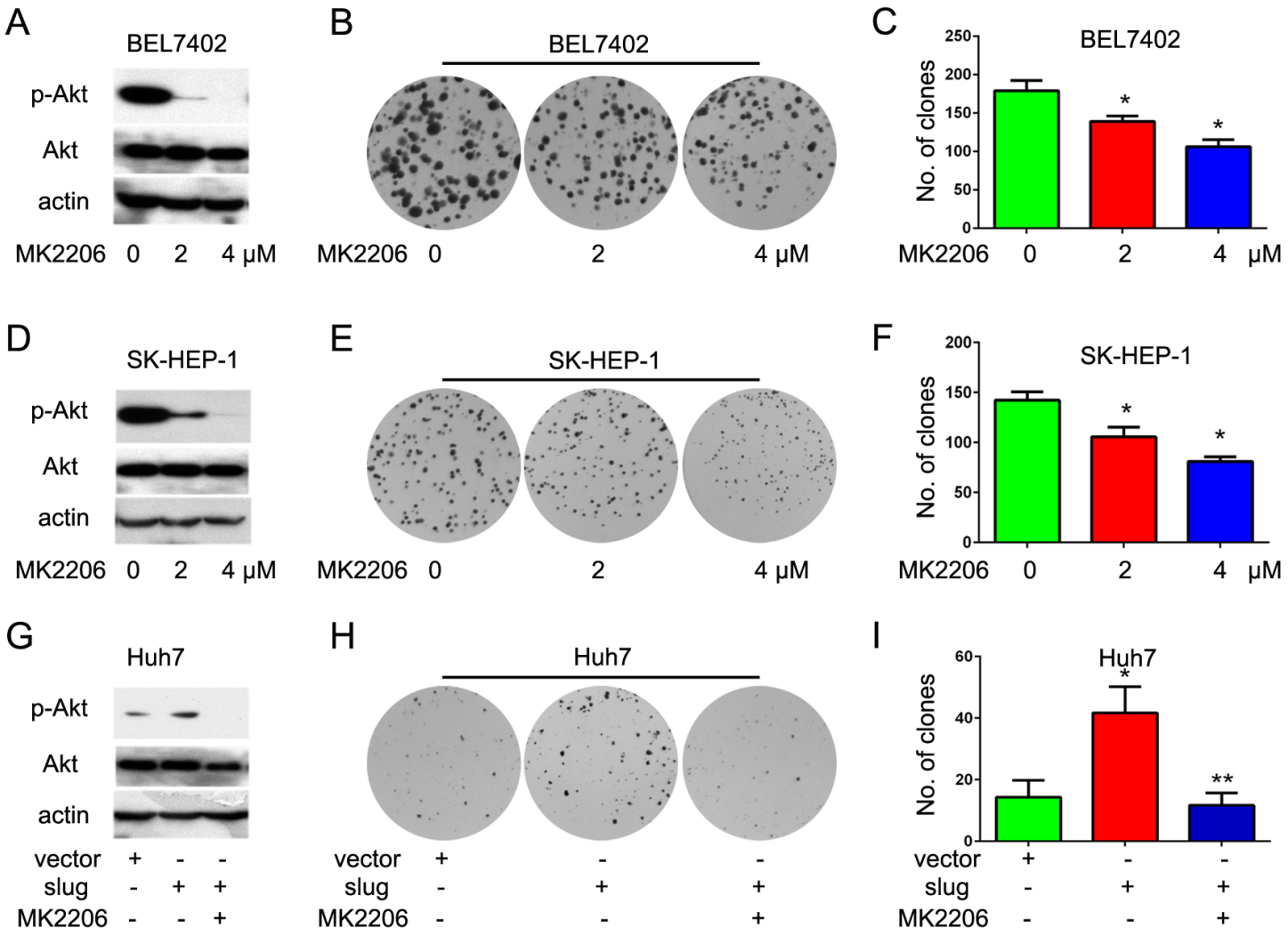


Figure 3

. Slug promotes HCC growth via Akt. A. BEL7402 cells were treated with MK2206 (0 μM, 2 μM, 4 μM) for 48 hours, and phosphorylated Akt and total Akt were detected by western blot assay. Full-length western blots were presented in Supplementary Figure 5. B-C. Colony formation assay of BEL7402 cells treated with MK2206 (0 μM, 2 μM, and 4 μM). B. Representative photos; C. Statistical data. *P<0.05 vs the 0 μM group. D. SK-HEP-1 cells were treated with MK2206 (0 μM, 2 μM, 4 μM) for 48 hours, and phosphorylated Akt and total Akt were detected by western blot assay. Full-length western blots were presented in Supplementary Figure 5. E-F. Colony formation assay of SK-HEP-1 cells treated with MK2206 (0 μM, 2 μM, and 4 μM). E. Representative photos; F. Statistical data. *P<0.05 vs the 0 μM group. G. Slug-overexpressing Huh7 cells were treated with 2 μM MK2206 for 48 hours, and phosphorylated Akt and total Akt were detected by western blot assay. Full-length western blots were presented in Supplementary Figure 5. H-I. Colony formation assay of slug-overexpressing Huh7 cells treated with MK2206 (0 μM, 2 μM, and 4 μM). H. Representative photos; I. Statistical data. *P<0.05.

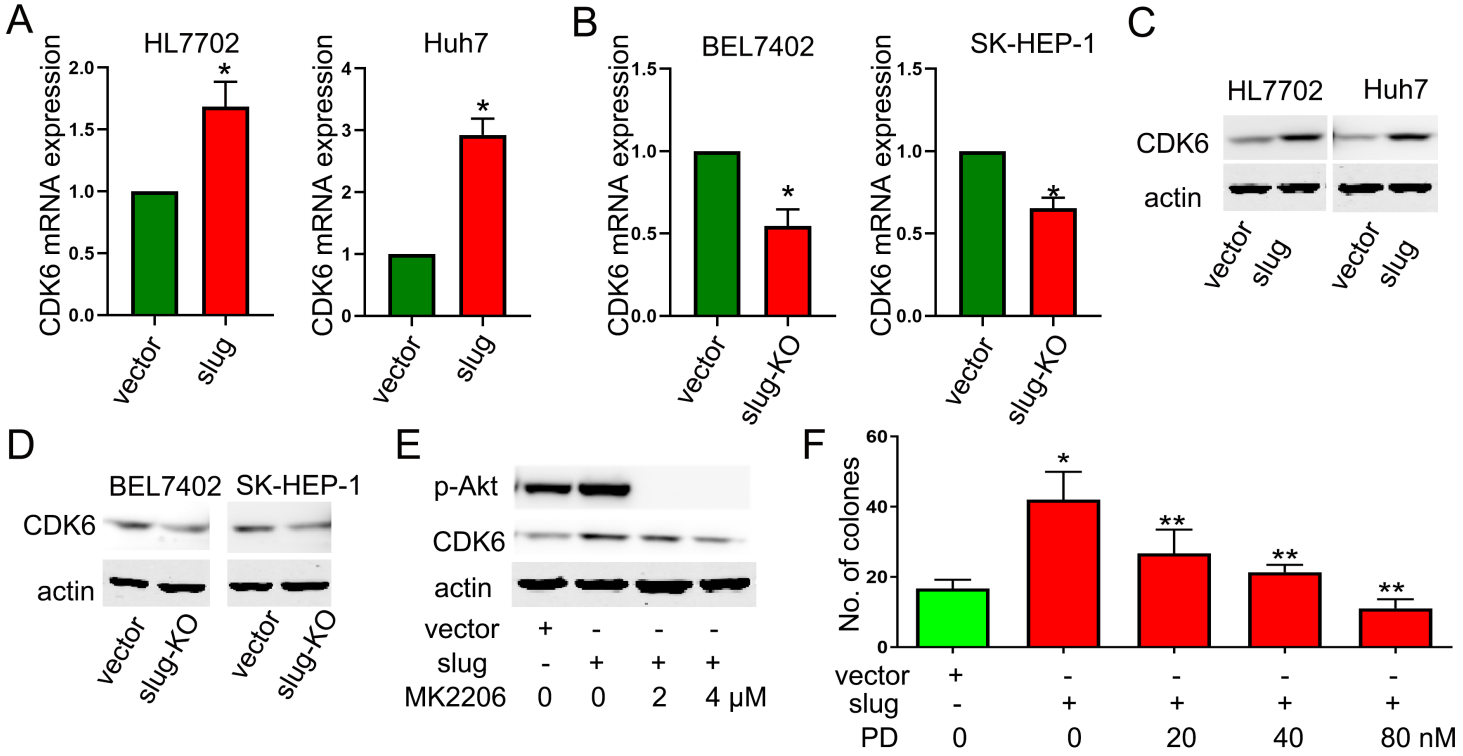


Figure 4

The Slug/Akt pathway promotes HCC growth by regulating CDK6 expression. A. CDK6 mRNA expression in HL7702 and Huh7 cells after slug overexpression. *P<0.05 vs the vector group. B. CDK6 mRNA expression in BEL7402 and SK-HEP-1 cells after slug knockout. *P<0.05 vs the vector group. C. CDK6 expression detected by western blot assay in HL7702 and Huh7 cells after slug overexpression. Full-length western blots were presented in Supplementary Figure 6. D. CDK6 expression detected by western blot assay in BEL7402 and SK-HEP-1 cells after slug knockout. Full-length western blots were presented in Supplementary Figure 7. E. Huh7 cells with slug overexpression were treated with 0, 2 and 4 μM MK2206 for 48 hours, and the expression of slug and p-Akt Ser473 was detected by western blot assay. Full-length western blots were presented in Supplementary Figure 8. F. Huh7 cells overexpressing slug

were treated with the CDK4/6 inhibitor PD-0332991, and cell growth was evaluated by 6-well plate colony formation assay. *P<0.05 vs the vector group, **P<0.05 vs the 0 nM group.

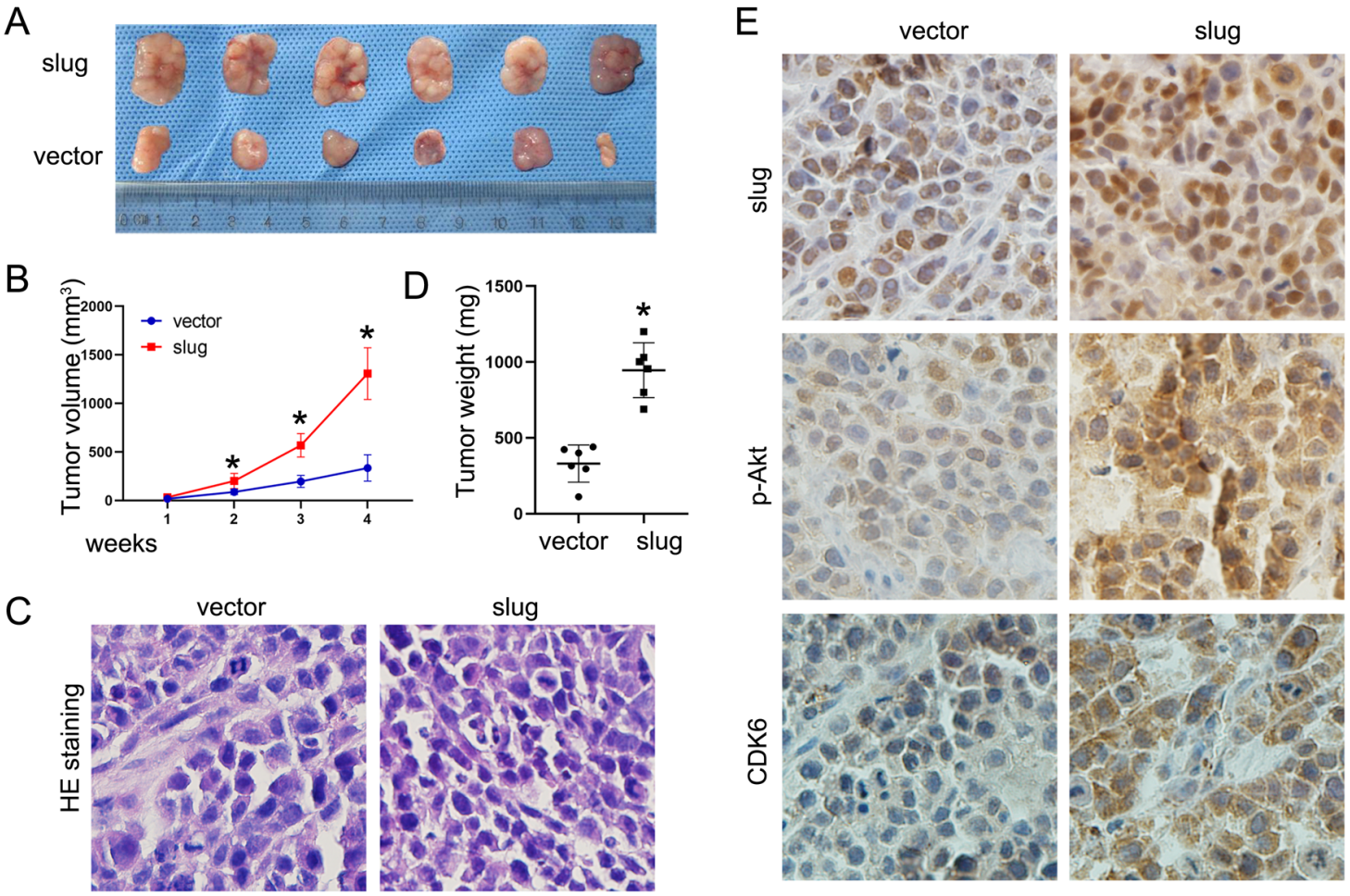
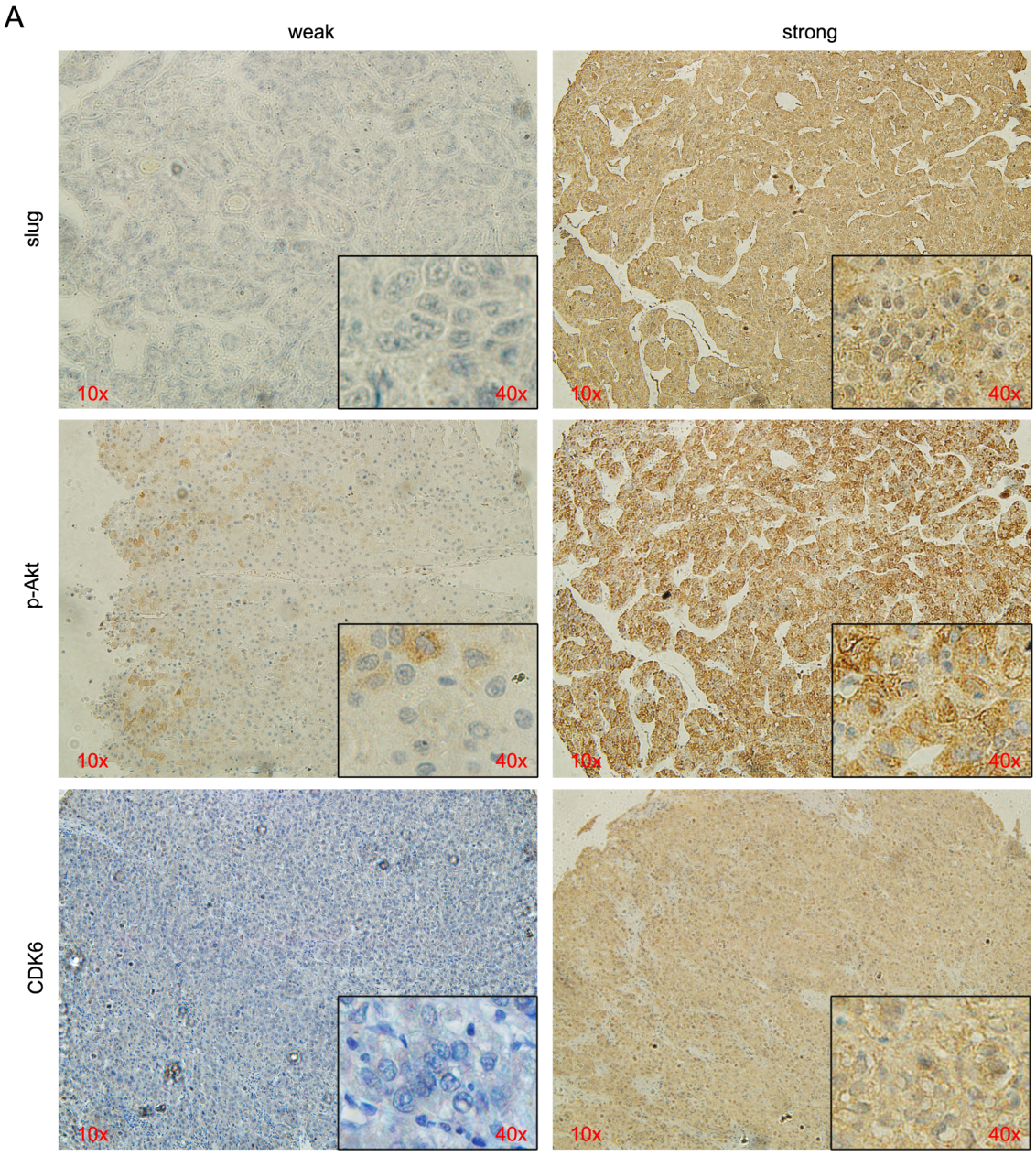


Figure 5

Slug promotes HCC growth, Akt activity and CDK6 expression in vivo. A total of 12 nude mice were subcutaneously injected with 2×106 vector control or slug-overexpressing Huh7 cells. The mice were sacrificed after 4 weeks, and the tumors were obtained. A. Representative photos of tumors. B. Growing curves of the xenografts. *P<0.05 vs the vector group. C. HE staining of the xenografts. D. Tumor weight of the xenografts. *P<0.05 vs the vector group. E. Immunohistochemistry staining for slug, p-Akt Ser473 and CDK6 expression in xenografts.



B

	p-Akt^{weak}	p-Akt^{strong}	Total
slug^{weak}	18	12	30
slug^{strong}	10	28	38
Total	28	40	68

C

	CDK6^{weak}	CDK6^{strong}	Total
slug^{weak}	16	14	30
slug^{strong}	9	29	38
Total	25	43	68

Figure 6

Association between slug expression, Akt activity and CDK6 expression in human HCC tissues. Sixty-eight human HCC tissues were immunohistochemically stained for slug, p-Akt Ser473 and CDK6 expression. A. Representative photos. B. Association between slug and p-Akt Ser473 expression in HCC tissues. C. Association between slug and CDK6 expression in HCC tissues.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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- supplementaryfigure2.jpg
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