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AKT2 phosphorylation of hexokinase 2 at T473 promotes tumorigenesis and metastasis in colon cancer cells via NF-κB, HIF1α, MMP2, and MMP9 upregulation

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ABSTRACT

It has been well-established that AKT2 plays an important role in the development and progression of colon cancer; however, its precise function remains unclear. In the present study, we found that AKT2 can interact with and phosphorylate hexokinase 2 (HK2), the rate-limiting enzyme in glycolysis. Moreover, threonine phosphorylation dramatically increases its catalytic activity and enhances glycolysis. Mechanistically, AKT2 phosphorylation of HK2 at T473 was found to increase hexokinase activity and lactic acid production. A mutation in the AKT2 phosphorylation site of HK2 substantially reduced the stimulating effects of AKT2 on glycolysis, cellular apoptosis, invasion, tumorigenesis, and metastasis. In addition, AKT2 regulated NF-κB, HIF1A, MMP2, and MMP9 via the phosphorylation of HK2 at the T473 site. Taken together, AKT2 increases the invasion, tumorigenesis, and metastasis of colon cancer cells in vitro and promotes lung metastasis in nude mice in vivo through the phosphorylation of the T473 site of HK2 by upregulating NF-κB, HIF1α, MMP2, and MMP9. In conclusion, our findings highlight a novel mechanism for the AKT2-HK2-NF-κB/HIF1α/MMP2/MMP9 axis in the regulation of colon cancer progression. Moreover, our results suggest that both AKT2 and HK2 may be potential targets for the treatment of colon cancer.

1. Introduction

Colon cancer is the third most common malignancy worldwide, and is associated with a high mortality rate [1]. Since an early diagnosis of colon cancer is rare, the presence of invasive or distant metastases of colon cancer cells into the surrounding tissues and the existing treatment methods (e.g., surgical resection and/or chemotherapy) cannot completely inhibit the recurrence and metastasis of colon cancer [2]. Epidemiological investigation has revealed that the occurrence and development of colon cancer is closely related to genetics, diet, living environment, intestinal flora, and other factors [3]. Recently, a large number of studies have shown that the dysregulation of various cell signaling pathways play an important role in the occurrence, development, and metastasis of colon cancer [4]. Therefore, in-depth studies of the molecular mechanism of colon cancer cell-related signal transduction pathways and the development of therapeutic drugs for key molecules have been the primary focus of colon cancer research.

Protein kinase B (AKT) is at the core of the phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin (P13K/AKT/mTOR) signal transduction pathway. A variety of growth factors, insulin, cytokines, and other mediators can stimulate AKT activation via the P13K/AKT/mTOR pathway. Subsequently, activated AKT can promote the growth and reproduction of tumor cells, inhibit cell apoptosis, promote cancer cell invasion and metastasis, and promote angiogenesis [5]. Currently, three subtypes of AKT that have been identified (i.e., PKBα [AKT1], PKBβ [AKT2], and PKBγ [AKT3]), which play an important role in tumor occurrence and development. Among these three subtypes, AKT2 is closely associated with cancer cell invasion, metastasis, angiogenesis, survival, and drug resistance [6]. Moreover, studies have shown that AKT2 is often overexpressed in colon cancer tissues and cells [7]. Although recent studies on the development of activated AKT and tumors have made significant progress [8], a large number of

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molecular mechanisms involved in the development of AKT2 in colon cancer remain unknown.

The most prominent metabolic feature of tumors is efficient aerobic glycolysis, known as the Warburg effect. In addition, tumor aerobic glycolysis activity is closely related to the tumor growth rate and invasiveness [9]. Hexokinase (HK) is the first rate-limiting enzyme in the glycolytic pathway and catalyzes the production of glucose-6-phosphate by glucose, which both produces ATP by oxidative phosphorylation or glycolysis, and also participates in the synthesis of important substances (e.g., nucleotides) through the pentose phosphate pathway [10]. HK contains four subtypes (i.e., HK1, HK2, HK3, and HK4), each of which has a particular tissue specificity. Among several subtypes, HK2 was found to be significantly up-regulated in various malignant tumors, including breast cancer, malignant pleural mesothelioma, myeloma, colon cancer, pancreatic cancer, and glioblastoma [11]. Recent studies have shown that HK2 in tumor cells not only mediates the Warburg effect, but also inhibits tumor cell apoptosis and regulates autophagy to promote tumor proliferation and metastasis [12]. HK2 deletion inhibits glycolysis and oxidative phosphorylation in human hepatocarcinoma and sensitizes cells to metformin [13]. Furthermore, it has been confirmed that blocking the expression of the *hk2* gene and using small molecule inhibitors of HK2 can kill various tumor cells [14]. Therefore, HK2 may be a potential target for exploring tumor diagnosis and treatment.

Several studies have also shown that HK2 may be an important downstream effector of the PI3K/AKT/mTOR signaling pathway and may contribute to the development of cancer [15]. In our previous study, we found AKT2 expression was positively correlated with HK2 expression in primary colon cancer specimens (Spearman's $R = 0.711$, $p < .01$). However, how HK2 promotes the development and progression of colon cancer and whether it interacts directly with AKT2 remains poorly understood. In the present study, we aimed to elucidate how AKT2 mediates colon cancer invasion, tumorigenesis, and metastasis both in vitro and in vivo, and whether these effects are related to HK2.

2. Material and methods

2.1. Cells and mice

Human colon cancer HCT-116 cell line (CCL-247™) and HT-29 cell line (HTB-38™) were purchased from the American Type Culture Collection (ATCC, USA). The cells were cultured in McCoy's 5a medium with 10% fetal bovine serum (FBS) at 37 °C in a humidified incubator with 5% CO_2 according to the instructions. Multiplex PCR was used to text negative for mycoplasma contamination. Nude BALB/c mice (5-weeks old) were purchased from Shanghai Slaccas Laboratory Animals Co., Ltd., China, and maintained under standard conventional conditions. All work performed on animals was approved by the Animal Care and Use Committee of the Zhejiang Academy of Medical Sciences.

2.2. Immunoprecipitation of endogenous AKT2 and HK2

Co-immunoprecipitation of proteins of interest was carried out as previously described [16]. Briefly, the supernatant containing the protein extract was incubated with protein A/G beads (Thermo, USA) overnight at 4 °C to pre-clarify the lysate bound to the A/G beads. Pre-clarified lysates were incubated with the primary antibody (Normal Rabbit IgG #2729, Akt2 Rabbit mAb #2964, Hexokinase I Rabbit mAb #2024 or Hexokinase II Rabbit mAb #2867; CST, USA) and protein A/G beads overnight at 4 °C according to the manufacturer's instructions. The protein A/G-antibody-antigen complex was concentrated by centrifugation at 1000 x *g* for 10 min at 4 °C, followed by washing three times with PBS. The immunoprecipitated protein was then detected by electrophoresis and Western blot.

2.3. siRNA-mediated AKT1, AKT2, and AKT3 silencing

As described previously [17], small interfering RNAs (siRNA) specifically targeting AKT1, AKT2, or AKT3 were purchased from RUIBIO Co., Guangzhou, China. The siRNA sequences are presented in Table S1. The specificity of these siRNA sequences was evaluated by the online tool, BLAST, and did not display homology with other known genes. The control siRNA did not match any mammalian genes. Lipofectamine 3000 (Thermo, USA) was used to intracellularly transfect these siRNAs and silence the related genes according to the manufacturer's protocol.

2.4. Stable overexpression of AKT2 (OV AKT2)

AKT2 cDNA was inserted into a pLVX-Neo vector to construct the AKT2 overexpressing vector, pLVX-Neo-AKT2. After sequencing, the target lentiviruses were achieved by transfecting either the pLVX-Neo-AKT2 or pLVX-Neo empty vector with the lentivirus packaging plasmids into 293 T cells according to the manufacturer's protocol. HCT-116 and HT-29 cells were then transfected with the resulting lentiviruses and G418 was used to screen for cell lines stably overexpressing AKT2. The cellular expression of AKT2 was confirmed by Western blot.

2.5. HK2-deficient in AKT2 overexpressing cell lines (OV AKT2Δhk2)

Based on the successful construction of AKT2 overexpressing colon cancer cells, the CRISPR/CAS9 system was used to knock out the *hk2* gene. A single guide RNA (sgRNA: ACTGGTCAACCTTCTGCACT) targeting HK2 was designed using the online CRISPR design tool (crispr.mit.edu). The sgRNA oligonucleotide was cloned into the LentiGuide-Puro vector (Addgene #52962) and co-transfected into AKT2 overexpressing cells using LentiCas9-Blast (Addgene #52963). Following puromycin screening, sequencing was performed to identify the efficiency of the HK2-deficient cells.

2.6. Site-directed HK2 mutagenesis and pcDNA3.1-Myc/Zeo(+)-HK2^{T473A} construction

A Q5® Site-Directed Mutagenesis Kit (NEB, USA) was used to construct mutations according to the instruction manual. HK2 Thr-473 was mutated to alanine (T473A) using the following mutant primer pairs: 5′- CAACACCGTGCCCGCCAGAAGGCCTTAGAGC -3′ and 5′- GCTCAG CTGC AGATGCTCTAAGGCCTTCTGG -3′, with pcDNA3.1-Myc/Zeo (+)-HK2 (laboratory preservation) used as a template. After successful site-directed mutagenesis (T473A), HK2 was used to construct a eukaryotic overexpression vector pcDNA3.1-Myc/Zeo(+)-HK2^{T473A} for use in subsequent rescue experiments with the unmutated HK2 eukaryotic overexpression vector, pcDNA3.1-Myc/Zeo(+)-HK2.

2.7. Rescue of HK or HK2^{T473A} in AKT2 overexpressing HK2-deficient cell lines (OV AKT2Δhk2 + HK2^{T473A} and OV AKT2Δhk2 + HK2)

The same methods as described above were used to construct stably overexpressing AKT2 colon cancer cell lines. pcDNA3.1-Myc/Zeo (+)-HK2 and pcDNA3.1-Myc/Zeo(+)-HK2^{T473A} were transfected into colon cancer cell lines that overexpressed AKT2 while knocking out the *hk2* gene, respectively. Stable rescue of cells overexpressing HK2 and mutant HK2^{T473A} were obtained after screening with Zeocin.

2.8. Hexokinase activity and lactate production assay

Hexokinase activity assay was performed using a Hexokinase Colorimetric Assay Kit (Sigma, USA), and lactate production was detected by a Lactate Assay Kit (Sigma, USA). Briefly, a density of 1×10^5 of the above constructed cells were seeded into a 96-well plate and cultured for 24 h. The medium was replaced with fresh medium and cultured for another 6 h, at which time the cells were collected. The

hexokinase activity and amount of lactic acid production were measured according to the instructions provided by the manufacturer.

2.9. Flow cytometric cell death assay

The rate of cellular apoptosis was detected by an Annexin V-FITC Apoptosis Detection Kit (Sigma, USA). After a density of 1×10^5 cells constructed for each group were seeded into a six-well plate for 24 h, the original medium was removed and replaced with serum-free medium, and the culture was continued for an additional 48 h. After the above treatment, the cells were digested with trypsin and collected by centrifugation at 1000 $\times g$ for 5 min, washed twice with PBS, and resuspended. The cells were then sequentially stained with Annexin V-FITC and PI for 10 min at 37 °C in the dark. Finally, the rate of apoptosis was measured by flow cytometry.

2.10. Western blot

The expression of PI3K, phosphorylated (p)-PI3K, AKT2, p-AKT2, mTOR, p-mTOR, HK1, HK2, NF-κB p65, p-STAT3, Bcl-2, Bcl-XL, HIF1α, MMP2, MMP9, myc, Phospho-Akt Substrate (RXRXXS*/T*) and internal reference (β-actin, H2B Histone) in HCT-116 and HT-29 cells as well as in the nude mice lung metastasis nodules were analyzed by Western blot. The following primary antibodies were purchased from Cell Signaling Technology, Inc. (USA) with the corresponding catalog numbers: #4249 for PI3K, #4228 for p-PI3K, #2964 for AKT2, #8599 for p-AKT2, #2983 for mTOR, #2971 for p-mTOR, #2024 for HK1, #2867 for HK2, #8242 for NF-κB p65, #9145 for p-STAT3, #15071 for Bcl-2, #2764 for Bcl-XL, #36169 for HIF1α, #40994 for MMP2, #13667 for MMP9, #2276 for myc, #10001 for Phospho-Akt Substrate (RXRXXS*/T*), #4970 for β-actin and #12364 for H2B Histone. Rabbit anti-pThr473-HK2 antibodies generated by a small peptide (RARQKTpLEHC) (a gift from Professor Yu). The secondary antibodies including goat anti-rabbit IgG and goat anti-mouse IgG-horseradish peroxidase (HRP) (Catalog no., ab6721 and ab6789; Abcam, UK). The dilution of each antibody is carried out according to the instructions.

2.11. Matrigel invasion assay

Corning Matrigel Invasion Chambers (Corning, USA) were used to detect tumor cell invasion in vitro. A density of 1×10^5 cells were seeded into the upper chamber of the coated with Matrigel (BD, USA), and the lower chamber was filled with medium containing 20% FBS. After incubating for 36 h at 37 °C in a 5% CO_2 humidified incubator, the cells that did not invade the pores of the upper chamber were removed. Cells on the lower side of the filter were stained with 1% crystal violet for 10 min, and then counted under a light microscope. The experiment was repeated three times.

2.12. Xenograft tumor and in vivo metastasis assay

The nude mice xenograft tumor and lung metastasis experiments were performed largely in line with the protocols described by Yan et al. [18]. We established a total of five groups (10 mice per group) consisting of: 1) untransfected group; 2) OV AKT2 group; 3) OV AKT2*Δhk2* group; 4) OV AKT2*Δhk2* + $HK2^{T473A}$ group; and 5) OV AKT2*Δhk2* + HK2 group.

In the xenograft tumor experiment, 1×10^7 cells from individually constructed HT-29 cell lines were resuspended in PBS and injected into the left axilla of each group of BALB/c nude mice. Mice were sacrificed six weeks later, and the weight and volume of the xenograft tumors from each group were calculated. In the lung metastasis experiment, 5×10^6 of the individually constructed HT-29 cell lines were injected into each group of BALB/c nude mice through the tail vein. Mice were sacrificed 45 days later, and the number of metastatic nodules on the surface of the lungs was calculated. Finally, these nodules were confirmed by HE staining and Western blot. In all of these animal experiments, the mice were randomly assigned to the experimental group.

2.13. Statistical analysis

The differences in hexokinase activity, lactate production, apoptosis, transwell invasion, and nude mouse metastasis assays between the different groups were evaluated using one-way ANOVA test. All statistical analyses were performed using GraphPad PRISM. v6.0 software (San Diego, CA, USA). Data were presented as mean ± SD (standard deviation). p-Values < .05 were defined as statistically significant.

3. Results

3.1. Internal interaction between AKT2 and HK2

The overexpression and/or constitutive activation of AKT2 has been well documented in the involvement of multiple cancers, including colon cancer [19]. In addition, in some studies, HK2 has been implicated as a potential downstream molecule of the PI3K/AKT signaling pathway [20] and is often found to be overexpressed in colon cancer [21]. In this study, we first used AKT2 as a capture antibody for co-immunoprecipitation in HCT-116 and HT-29 cells, and then used an anti-HK2 antibody to perform a Western blot. A separate HK2 band was detected, indicating the presence of AKT2 and an internal interaction with HK2 (Fig. 1A and B). To further confirm this result, we used HK2 as a capture antibody for reverse co-immunoprecipitation, followed by a Western blot with an anti-AKT2 antibody, which also detected a specific AKT2 band. The ability of AKT2 to interact with HK2 was shown again (Fig. 1A and B). A very small number of reported studies have found that HK1 plays a role in tumor development. To verify whether AKT2 interacts with HK1, a Western blot with an anti-HK1 antibody was performed. The Western blot did not detect a HK1-specific band, indicating that AKT2 does not interact with HK1 (Fig. 1A and B). In addition, in HCT-116 and HT-29 cells, there was no interaction between HK2 and HK1, which is consistent with previous reports [22,23]. Furthermore, using immunoprecipitation, we also found no interaction between AKT1\3 and HK2 (Fig. 1C and D).

3.2. Silencing AKT2 reduces HK2 expression

In HCT-116 and HT-29 cells, to explore the upstream and downstream relationship between AKT2 and HK2, we used RNA interference technology to silence AKT1, AKT2, and AKT3 and observe any changes in HK2 expression. As shown in Fig. 1E - J, the use of siAKT1 interference significantly reduced the expression of AKT1 in both HCT-116 and HT-29 cells but did not affect the expression of HK2. Similarly, the use of siAKT3 also significantly reduced the expression of AKT3 but did not affect the level of HK2 expression. Moreover, changes in AKT1 or AKT3 expression did not affect the expression of HK2, suggesting that AKT1 and AKT3 may not be upstream molecules of HK2. The knockdown of siAKT2 significantly reduced the expression of AKT2 in both HCT-116 and HT-29 cells and also significantly decreased the level of HK2 expression, indicating that AKT2 may be a molecule located upstream of HK2.

3.3. Knocking out the hk2 gene does not affect AKT2 expression and AKT2 may phosphorylate Thr-473 of HK-2

To further verify the potential upstream and downstream relationship between AKT2 and HK2, we used lentiviruses to construct both HCT-116 and HT-29 cell lines that stably overexpress AKT2. As shown in Fig. 2A and B, the level of AKT2 expression was significantly increased in stably overexpressing AKT2 cells (OV AKT2) compared to the control group. In addition, AKT2 overexpression was associated with

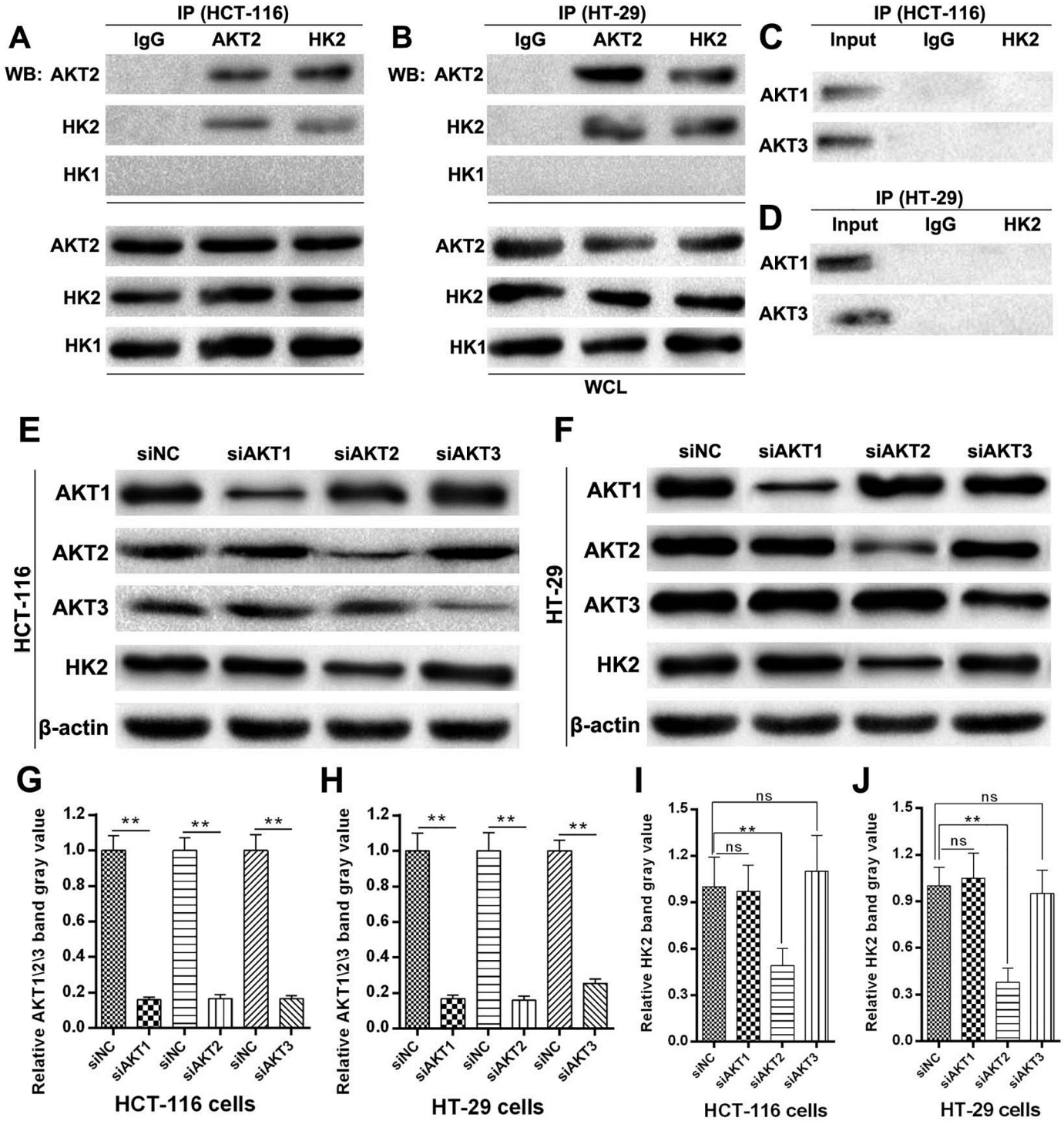

Fig. 1. AKT2 interacts with HK2, and AKT2 silencing is able to downregulate HK2 expression.
Endogenous HK2 and AKT2 in (A) HCT-116 and (B) HT-29 cell lysates were precipitated with anti-AKT2 and anti-HK2, followed by a Western blot to detect AKT2, HK2. Endogenous HK2 and AKT1/3 in (C) HCT-116 and (D) HT-29 cell lysates were precipitated with anti-HK2, followed by a Western blot to detect AKT2. AKT2 silencing, but not AKT1 or AKT3 silencing is able to downregulate HK2 expression in (E, G, I) HCT-116 cells and (F, H, J) HT-29 cells. All assays were performed in triplicate and showed representative blots.

increased HK2 expression, which demonstrates that AKT2 may be a molecule situated upstream from HK2. Subsequently, in both HCT-116 and HT-29 cells, after knocking out the *hk2* gene in OV AKT2 cells using the CRISPR/CAS9 technique (OV AKT2Δ*hk2*), the level of HK2 protein expression could not be detected with a Western blot. We also found that knocking out the *hk2* gene did not affect the expression of AKT2, further demonstrating that HK2 may be a potential molecule downstream from AKT2.

AKT is known to phosphorylate proteins on the consensus sequence RXRXX (S/T). Using the bioinformatics method described by Vandermoere et al. [24] to search for the AKT phosphorylation site in HK2, we found that HK2 contains the typical AKT phosphorylation sequence, QHRARQKT473. Moreover, another study confirmed that total AKT can phosphorylate Thr-473 of HK2 and increase the binding

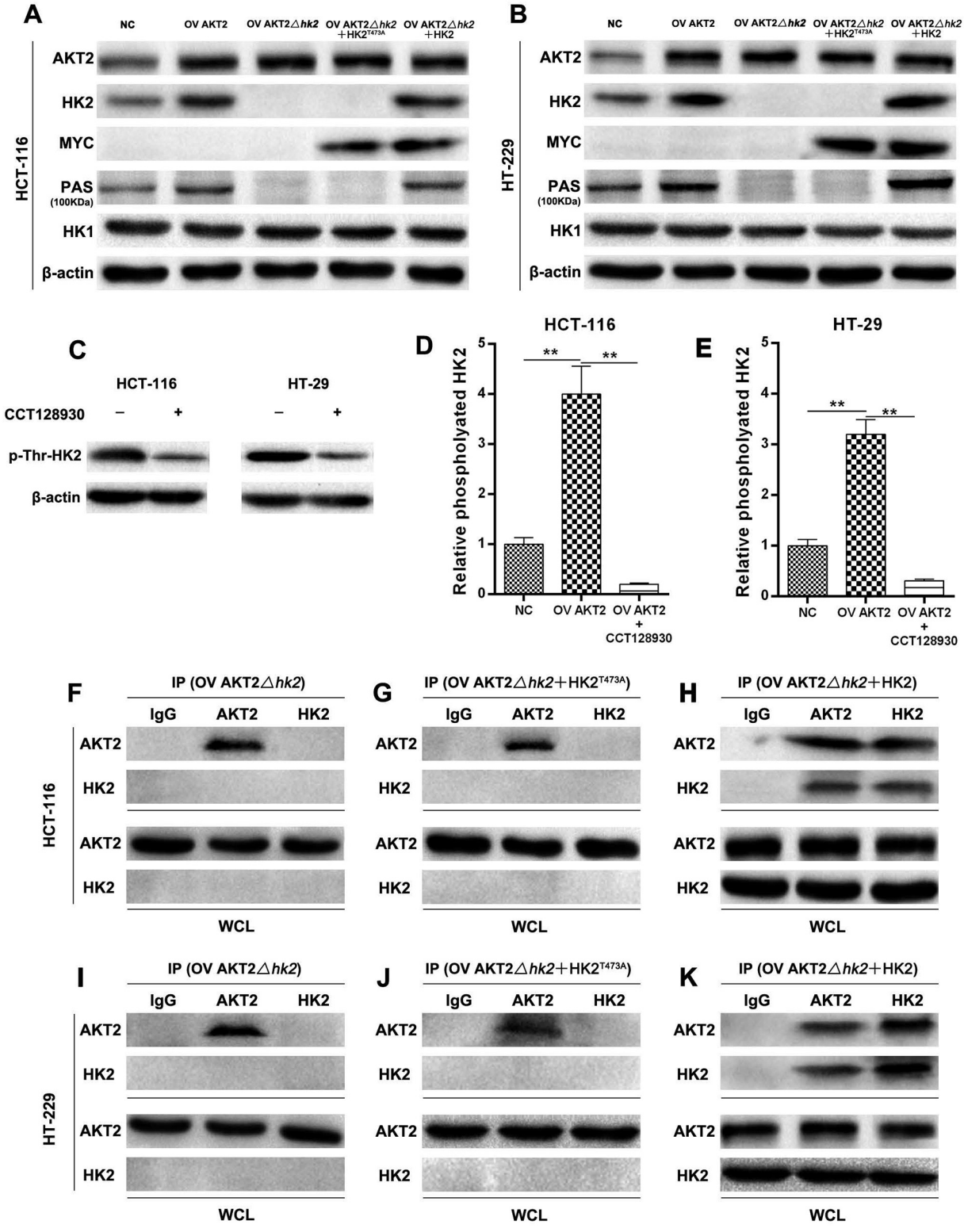

Fig. 2. AKT2 may phosphorylate Thr-473 of HK-2.

Overexpression of AKT2 led to elevate HK2 expression. However, knocking out *hk2* gene did not affect the expression of AKT2 in (A) HCT-116 cells and (B) HT-29 cells. (C) Phosphorylation levels of HK2 were significantly reduced after treatment with AKT2-specific inhibitor CCT128930 (10 nmol/L for 24 h) in wild-type colon cancer cells. AKT2 overexpression could increase the phosphorylation level of HK2 in (D) HCT-116 cells and (E) HT-29 cells. Endogenous HK2 and AKT2 in the lysates of successfully constructed (F, G, H) HCT-116 cell lines and (I, J, K) HT-29 cell lines were precipitated with anti-AKT2 or anti-HK2, followed by a Western blot to detect AKT2 and HK2.

NC, Untransduced cells; OV AKT2, Cells transduced with pLVX-Neo-AKT2 lentivirus (AKT2-overexpressing cells); OV AKT2*Δhk2*, Knocking out *hk2* gene in AKT2-overexpressing cells; OV AKT2*Δhk2* + $HK2^{T473A}$, The rescue of overexpressed $HK2^{T473A}$ mutants in AKT2-overexpressing HK2-deficient cells; OV AKT2*Δhk2* + HK2, The rescue of overexpressed HK2 in AKT2-overexpressing HK2-deficient cells. All assays were performed in triplicate and showed representative blots.

of mitochondria and HK2 to protect cardiomyocytes; however, the authors did not identify which subtype of AKT plays a role in this process [25]. In addition, in colon cancer cells, there is currently no research on whether AKT2 can phosphorylate Thr-473 of HK2. To determine whether HK2 is indeed phosphorylated by AKT2, we first constructed a eukaryotic expression plasmid for the $HK2^{T473A}$ mutant using site-directed mutagenesis. As shown in Fig. 2A and B, in OV AKT2Δ*hk2* HCT-116 and HT-29 cell lines, we rescued the overexpression of HK2 or mutant $HK2^{T473A}$ (OV AKT2Δ*hk2* + HK2 and OV AKT2Δ*hk2* + $HK2^{T473A}$, respectively). A Western blot was performed using the anti-myc tag as the primary antibody, and specific protein bands were detected in both cells. However, when anti-PAS was used as the primary antibody in the Western blot, a specific band with a molecular weight of approximately 100 kDa was detected in only OV AKT2Δ*hk2* + HK2 cells, and the band could not be detected in the OV AKT2Δ*hk2* + $HK2^{T473A}$ mutant cells. These findings suggest that AKT2 may phosphorylate HK2 at Thr-473 in HCT-116 and HT-29 cells. To rule out whether HK2 phosphorylation was caused by overexpression of AKT2, we treated wild-type HCT-116 and HT-29 colon cancer cells using the AKT2-specific inhibitor CCT128930 (10 nmol/L for 24 h) (Selleck, Houston, TX), respectively. As shown in Fig. 2C, we found a significant decrease in the expression of p-Thr-HK2 in two wild-type colon cancer cells, suggesting that AKT2 can participate in HK2 phosphorylation in wild-type HCT-116 and HT-29 cells, not only in HCT-116 and HT-29 cells overexpressing AKT2. In addition, AKT2 overexpression could increase the phosphorylation level of HK2 detected by direct phosphorylation experiments using [γ-^{32}P] ATP (Fig. 2D and E).

To further validate the results, we performed a co-immunoprecipitation assay to analyze the successful construction of OV AKT2Δ*hk2,* OV AKT2Δ*hk2* + $HK2^{T473A}$, and OV AKT2Δ*hk2* + HK2 colon cancer cell lines. As shown in Fig. 2F - K, AKT2 was used as a capture antibody for the co-immunoprecipitation assay, and an anti-HK2 antibody was used for the Western blot. Only a specific band for HK2 was detected in the OV AKT2Δ*hk2* + HK2 cells. At the same time, HK2 was used as the capture antibody for reverse co-immunoprecipitation, and only the specific AKT2 band was detected in OV AKT2Δ*hk2* + HK2 cells compared to the OV AKT2Δ*hk2* + $HK2^{T473A}$ mutant cells. These findings suggest that AKT2 may interact with HK2 at Thr-473.

3.4. AKT2 phosphorylation of HK2 can increase cellular hexokinase activity

We next sought to determine whether the phosphorylation of the T473 site of HK2 by AKT2 affects cellular hexokinase activity. As shown in Fig. 3A and B, the hexokinase activity in OV AKT2 HCT-116 and HT-29 cells was significantly increased compared with the control group ($p < .05$), which was consistent with the previous finding that AKT2 could increase the level of HK2 expression. After the *hk2* gene was knocked out in OV AKT2 cells, hexokinase activity was significantly reduced ($p < .01$), which was lower than that of the control group ($p < .01$). This finding indicated that knocking out the *hk2* gene deleted all HK2-type hexokinase activity, including the increased hexokinase activity of HK2 by AKT2 overexpression. After knocking out the *hk2* gene, we were also able to detect low levels of hexokinase activity, which may be due to contributions from other hexokinase subtypes, such as HK1\3. The stable rescue of HK2 over-expression in OV AKT2Δ*hk2* cells significantly increased the hexokinase activity ($p < .01$). However, the rescue of the overexpressed $HK2^{T473A}$ mutant in OV AKT2Δ*hk2* cells did not alter the level of cellular hexokinase activity ($p > .05$). These results indicate that HK2 phosphorylation by AKT2 can increase the hexokinase activity of colon cancer cells.

3.5. AKT2 phosphorylation of HK2 can increase lactic acid production

During the aerobic glycolysis of tumor cells, elevated glucose consumption is always accompanied by excessive lactic acid production [26]. As observed in Fig. 3C and D, AKT2 overexpression could enhance lactate production resulting from the overexpression of HK2 ($p < .01$). Knockout of the *hk2* gene in OV AKT2 cells decreased lactate production, which was rescued by the re-expression of the wild type form, rather than the mutation of Thr-473 to Ala. This suggests that AKT2 phosphorylation of HK2 at T473 can increase the production of lactic acid in colon cancer cells.

3.6. AKT2 phosphorylation of HK2 can reduce apoptosis induced by serum starvation

Following the treatment of HCT-116 and HT-29 cells with serum starvation for 48 h, we found that the rate of OV AKT2 cell apoptosis was significantly lower than that of normal control cells (Fig. 3E and F; $p < .01$). Knocking out the *hk2* gene in OV AKT2 cells increased the rate of apoptosis induced by serum starvation ($p < .05$); however, the level of apoptosis was still lower than that of the control group ($p < .05$). This finding indicates that knocking out the *hk2* gene in OV AKT2 cells did not completely reduce the ability of these cells to resist apoptosis induced by AKT2 overexpression. The stable rescue of HK2 overexpression in OV AKT2Δ*hk2* cells significantly increased the resistance to apoptosis induced by serum starvation ($p < .01$). However, the rescue of overexpressed $HK2^{T473A}$ mutants in OV AKT2Δ*hk2* cells did not alter the ability of cells to resist apoptosis ($p > .05$). These results suggest that AKT2 phosphorylation of HK2 at T473 can increase cell resistance to apoptosis induced by serum starvation.

3.7. AKT2 mediates HK2 to increase invasion ability of colon cancer cells

It has been well-established that AKT2 plays a very important role in tumor cell invasion and metastasis [27]. However, there is currently no study on whether AKT2 can mediate HK2 to increase cellular invasion. Using a transwell experiment as shown in Fig. 4A - D, in HCT-116 and HT-29 cells, AKT2 overexpression significantly increased cellular invasiveness compared to the control group ($p < .01$). Knocking out the *hk2* gene in OV AKT2 cells can reduce a certain level of cellular invasion ($p < .05$). However, the ability of AKT2 overexpression to promote cellular invasion was not completely reduced. It is suggested that AKT2 may also mediate cell invasion through other downstream molecules. The rescued overexpression of HK2 in OV AKT2Δ*hk2* cells significantly increased the invasive ability of the cells ($p < .05$); however, the rescue of overexpressed $HK2^{T473A}$ mutants in OV AKT2Δ*hk2* cells did not alter the invasive ability of the cells ($p > .05$). These results indicate that AKT2 phosphorylation of HK2 at T473 can increase the invasive capacity of these cells.

3.8. AKT2-stimulated tumorigenesis is dependent on HK2

We next explored the biological significance of AKT2-induced HK2 activation in colon cancer HT-29 cells. As shown in Fig. 4E and F, AKT2 overexpression significantly increased xenograft tumor growth in BABL/c nude mice compared with the control group ($p < .01$). Knocking out the *hk2* gene in OV AKT2 cells reduced the growth of xenograft tumors at a certain level ($p < .01$); however, the tumor weight was significantly higher than that of the control group ($p < .01$), indicating that knocking out the *hk2* gene did not completely inhibit the growth of the xenograft tumor induced by AKT2 overexpression. This outcome may be related to AKT2 through other downstream molecules that mediate colon cancer xenograft tumor growth. Stably rescuing the overexpression of HK2 in OV AKT2Δ*hk2* cells significantly increased the growth of the xenograft tumor ($p < .01$), rather than the $HK2^{T473A}$ mutant cells ($p > .05$). These results indicate that AKT2 phosphorylation of HK2 at T473 is required for colon cancer xenograft tumor growth. In these groups of xenograft tumor tissues, we also detected hexokinase activity and lactic acid

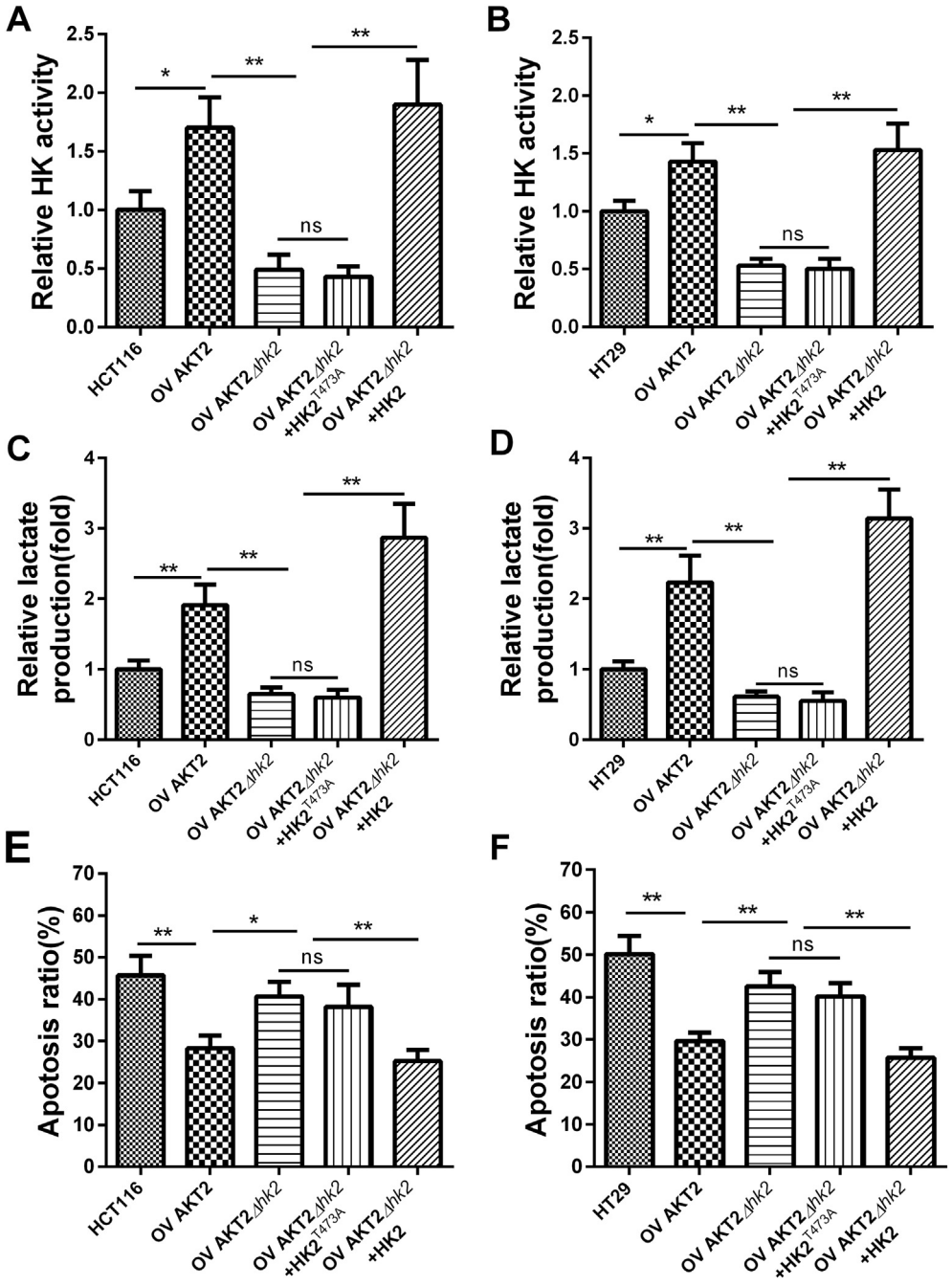

Fig. 3. AKT2 phosphorylates HK2 at T473 can increase hexokinase activity and production of lactic acid and reduce apoptosis induced by serum starvation in colon cancer cells.

HK activity assay in (A) HCT-116 cells and (B) HT-29 cells; Lactate production assay in (C) HCT-116 cells and (D) HT-29 cells; Cellular apoptosis assay in (E) HCT-116 cells and (F) HT-29 cells; Values in each panel are means ± SD of three independent experiments. *$p < .05$, **$p < .01$, ns refers to no significant difference.

production similar to those obtained from these cellular experiments (Fig. 4G and H).

3.9. HK2 activity is required for AKT2-stimulated tumor metastasis

As shown in Fig. 4I and J, we found that AKT2 overexpression significantly increased the number of nodules in lung metastases ($p < .01$). A knockout of the *hk2* gene after the overexpression of AKT2 significantly reduced the number of lung metastasis nodules in nude mice ($p < .05$); however, the number of metastatic nodules was significantly higher than that of the control group ($p < .05$), indicating that knocking out the *hk2* gene did not completely reduce the lung metastasis increased due to AKT2 overexpression. Rescuing the overexpression of HK2 in OV AKT2Δ*hk2* cells significantly increased the number of lung metastasis nodules ($p < .01$). However, rescuing the overexpressed $HK2^{T473A}$ mutants in OV AKT2Δ*hk2* cells did not alter the number of lung metastases ($p > .05$). These findings indicate that AKT2 phosphorylates HK2 to increase the capacity for lung metastases by colon cancer cells.

3.10. Upstream molecules regulate the AKT2/HK2-mediated progression of colon cancer

AKT can be activated by upstream kinases. In particular, it has been

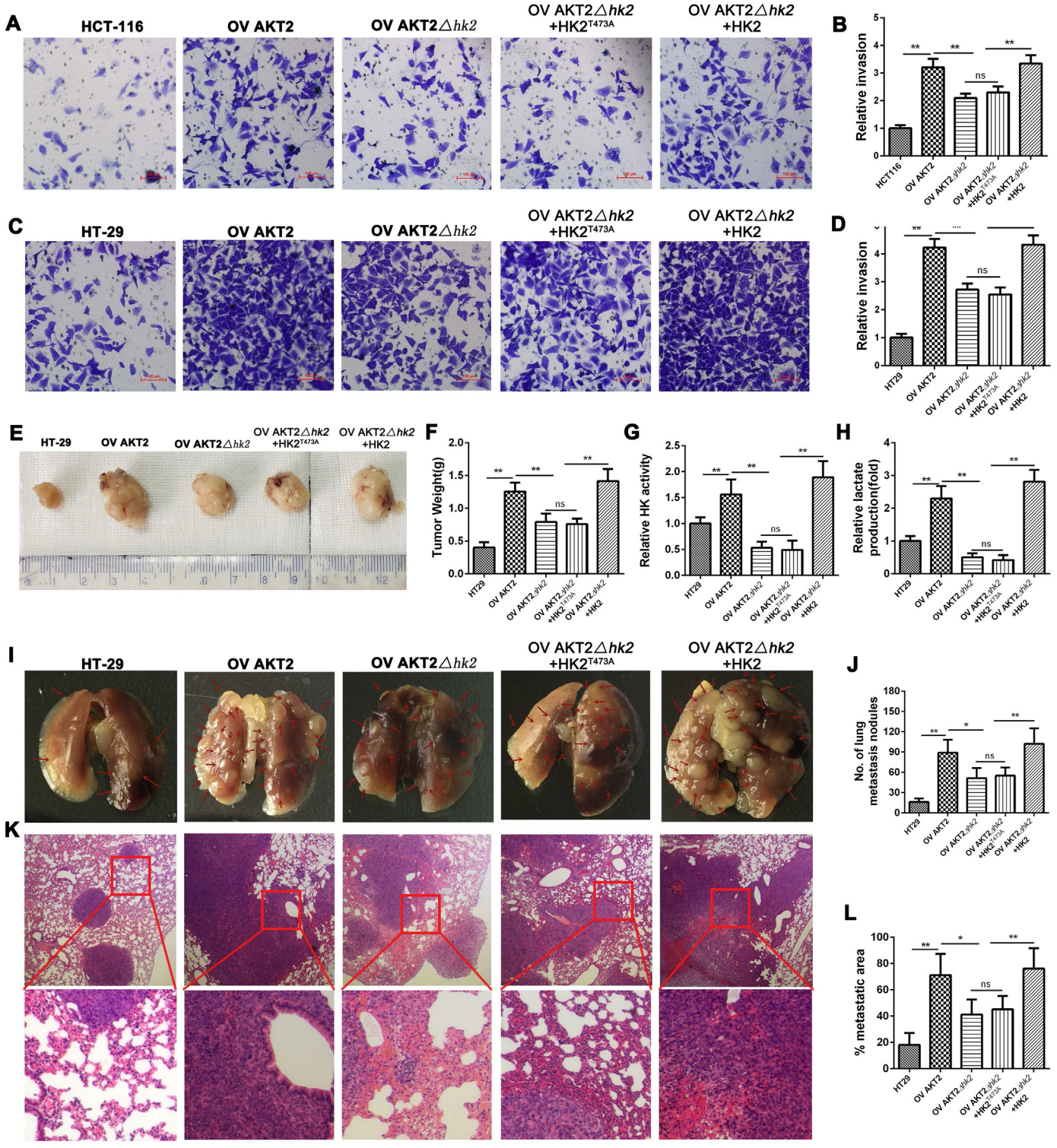

Fig. 4. HK2 activity is required for AKT2-stimulated tumor invasion, tumorigenesis and metastasis.
A and B, HCT-116 cell migration assay; C and D, HT-29 cell migration assay; E, HK2 activity is required for AKT2-stimulated xenograft tumor growth. Tumors were collected and examined six weeks after inoculation of HT-29 cells, respectively. Pictures of isolated tumors were taken. F, Tumor weights were determined. G and H, Tumors shown in E were measured for HK activity and lactate production. I, Lung metastatic burden in BALB/c nude mice 45 days after tail vein injection of HT-29 cells, as determined by counting the number of micrometastases per section (arrows point to metastatic areas). J, The lung metastatic nodule numbers in BALB/c nude mice 45 days after tail vein injection of HT-29 cells. K, Representative HE staining of metastatic foci in the lung tissues. L, Similar changes in the lung metastatic nodule numbers were also detected in the ratio of metastatic tumor area/total lung area.
Data are represented as mean ± SD ($n = 6$). * indicates $p < .05$, ** indicates $p < .01$ vs. control, ns refers to no significant difference.

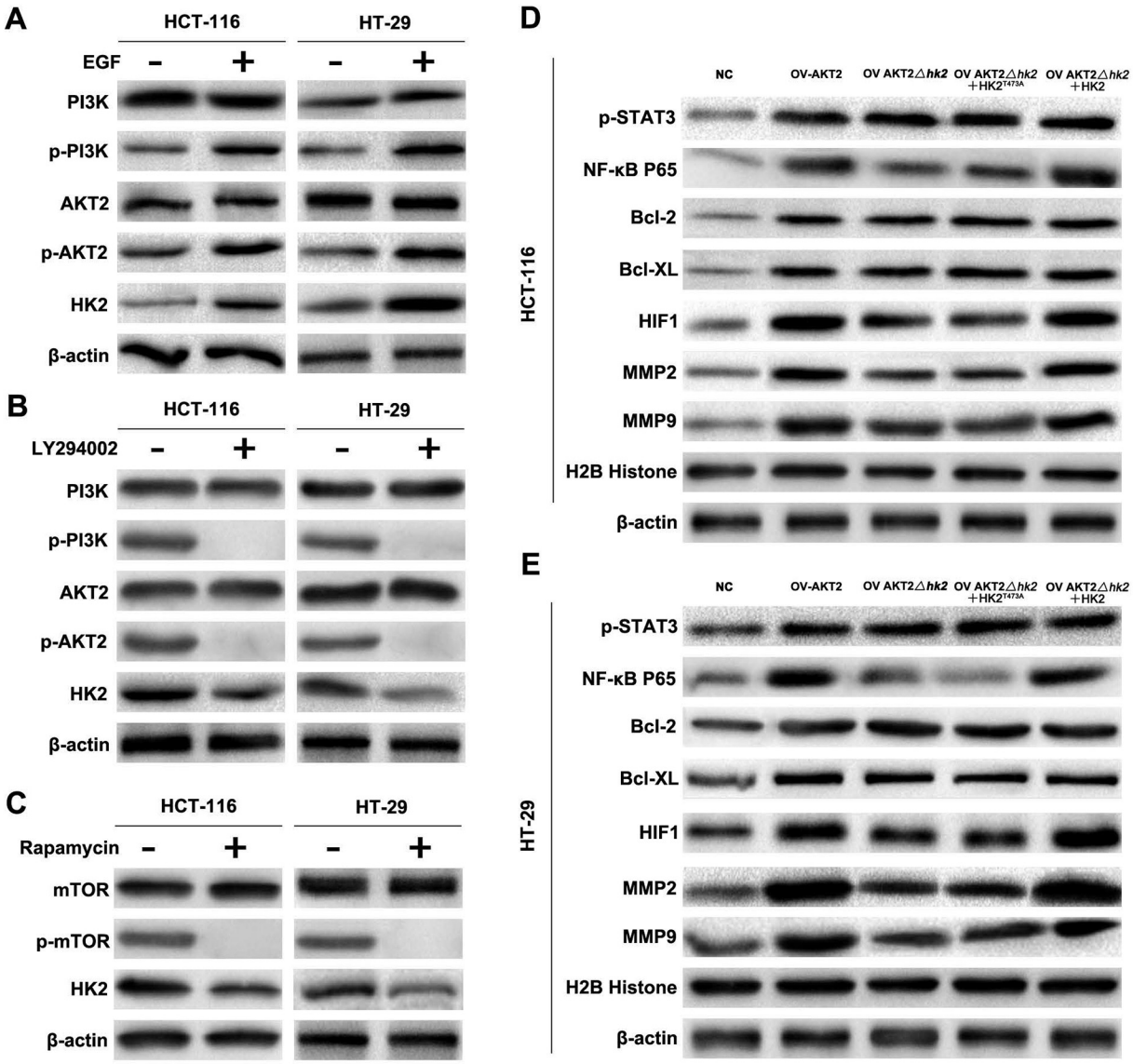

Fig. 5. Potential upstream regulators and downstream effector molecules associated with AKT2- and/or HK2-mediated promotion of colon cancer cell progression. A, HCT-116 and HT-29 cells treated with EGF (100 ng/mL for 24 h); B, HCT-116 and HT-29 cells treated with the P13K/AKT inhibitor LY294002 (10 nM for 24 h). C, HCT-116 and HT-29 cells treated with the mTOR inhibitor rapamycin (100 nM for 24 h). Western-blot for p-STAT3, NF-κB p65 (nuclear), Bcl-2, Bcl-XL, HIF1α, MMP2 and MMP9 expression in (D) HCT-116 cells and (E) HT-29 cells. H2B Histone was used as the nuclear loading control whereas β-actin served as a total protein loading control. All assays were performed in triplicate and showed representative blots. Quantification of blots and statistical analysis were showed in supplemental fig. S1 and S2.

well-established that epidermal growth factor (EGF) activates the AKT signaling pathway. Therefore, we use EGF to stimulate colon cancer cells and increase the activity of AKT2. As shown Fig. 5A, following the treatment of HCT-116 and HT-29 cells with 100 ng/mL of EGF for 24 h, the level of p-PI3K, p-AKT2, and HK2 expression was significantly increased. There was no significant change in the level of total PI3K and AKT2 expression. These results indicate that EGF can effectively activate AKT and promote HK2 expression.

After treating HCT-116 and HT-29 cells with 10 nM of the PI3K inhibitor, LY294002, for 24 h, the expression of p-PI3K and p-AKT2 protein could not be detected and the expression of HK2 protein was significantly reduced. There was also no significant down-regulation of the total level of PI3K and AKT2 expression (Fig. 5B). These results indicate that the inhibitor of PI3K, LY294002, is able to inhibit HK2 expression. Taken together, the above data confirmed that PI3K is the upstream kinase of AKT2, and HK2 is a downstream molecule of AKT2. The level of HK2 expression in colon cancer cells is regulated by activated PI3K and AKT2.

3.11. Other downstream AKT2/HK2 molecules involved in colon cancer progression

mTOR has been shown to be one of the downstream molecules of AKT kinase. As shown in Fig. 5C, after treating HCT-116 and HT-29 cells for 24 h with 100 nM rapamycin, an mTOR inhibitor, the level of both mTOR and p-mTOR expression were decreased. The level of HK2 expression was also significantly reduced. These results indicate that the inhibition of mTOR was able to reduce the expression of HK2, which may be a downstream molecular target of mTOR.

We next examined other potential downstream molecules involved in the AKT2/HK2-mediated progression of colon cancer. As shown in Fig. 5D and E, the level of expression of the transcription factor, phosphorylated signal transducers and activators of transcription 3 (p-STAT3), was significantly increased in OV AKT2 cells compared to

normal cells. However, knocking out the *hk2* gene in OV AKT2 cells did not reduce the level of p-STAT3 expression. In addition, the stable rescue of HK2 or HK2^{T473A} mutant overexpression in OV AKT2Δ*hk2* cells did not alter the level of p-STAT3 expression. These results indicate that although the overexpression of AKT2 can increase HK2 expression, the up-regulation of p-STAT3 expression is mediated by AKT2, not through HK2.

We also tested whether the transcription factor, nuclear factor-κB (NF-κB), is a downstream molecular target of AKT2 or HK2. It has been well-established that the nuclear translocation of NF-κB p65 is an effective active form. The overexpression of AKT2 can significantly increase the level of NF-κB p65 expression. The knockout of the *hk2* gene after overexpressing AKT2 has significantly reduced the level of NF-κB p65 expression. Moreover, rescuing the stable overexpression of HK2 in OV AKT2Δ*hk2* cells significantly increased the expression of NF-κB p65, rather than the HK2^{T473A} mutant. Therefore, we hypothesized that HK2 can directly regulate the expression of NF-κB p65, and AKT2 can affect NF-κB p65 expression through HK2.

It has been well-established that HK2 is associated with the cellular inhibition of apoptosis. As shown in Fig. 5D and E, although the overexpression of AKT2 increased the level of the anti-apoptosis-related protein (B-cell lymphoma-2 and B-cell lymphoma-XL, Bcl-2 and Bcl-XL) expression, knocking out the *hk2* gene in OV AKT2 cells did not alter the level of Bcl-2 and Bcl-XL expression. In addition, rescue of the stable overexpression of HK2 or the HK2^{T473A} mutant in OV AKT2Δ*hk2* cells did not alter the level of Bcl2 and Bcl-XL expression. These results indicate that although the overexpression of AKT2 can increase the level of HK2 expression, and the up-regulation of the level of expression of the anti-apoptosis-related proteins, Bcl-2 and Bcl-XL, are mediated by AKT2 not HK2.

Hypoxia inducible factor-1 (HIF-1) plays a crucial role in the process of aerobic glycolysis in tumor cells. The overexpression of AKT2 significantly increased the expression of HIF1α compared to normal control cells. Knocking out the *hk2* gene based on OV AKT2 cells significantly reduced the level of HIF1α expression. Moreover, rescue of the stable overexpression of HK2 in OV AKT2Δ*hk2* cells significantly increased the expression of HIF1α, rather than the mutant HK2^{T473A}. These results indicate that HK2 can directly regulate the expression of HIF1α, and AKT2 can affect the expression of HIF1α through HK2.

Finally, we examined two molecules matrix metalloproteinase-2 (MMP2) and matrix metalloproteinase-9 (MMP9) that are closely related to tumor invasion and metastasis. The results were found to be similar to that of the HIF1α test. Thus, AKT2 can regulate the expression of MMP2 and MMP9 through HK2, thereby improving the ability of tumor cells to invade and metastasize.

3.12. Involvement of AKT2 and/or HK2-mediated upstream and downstream molecules in the lung metastasis of colon cancer in nude mice

To further confirm the potential upstream and downstream molecules involved in HK2 in the process of colon cancer, we used a Western blot to detect the level of AKT2, p-AKT2, mTOR, p-mTOR, HK2, p-STAT3, NF-κB, Bcl-2, Bcl-XL, HIF1α, MMP2, and MMP9 protein expression in lung metastasis nodules of nude mice. As shown in Fig. 6, similar to the results found in our in vitro experiments, in the lung metastasis nodules formed by HT-29 cells overexpressing AKT2, the level of AKT2, p-AKT2, mTOR, p-mTOR, HK2, p-STAT3, NF-κB, Bcl-2, Bcl-XL, HIF1α, MMP2, and MMP9 expression was significantly increased. However, the level of NF-κB, HIF1α, MMP2, and MMP9 expression was significantly reduced in the lung metastases nodules of nude mice from the OV AKT2Δ*hk2* group. The level of NF-κB, HIF1α, MMP2, and MMP9 expression was significantly increased in the lung metastasis nodules of the OV AKT2Δ*hk2* + HK2 group, rather than the mutant OV AKT2Δ*hk2* + HK2^{T473A} group. These results indicate that AKT2 is able to regulate nuclear NF-κB, HIF1α, MMP2, and MMP9 expression via HK2.

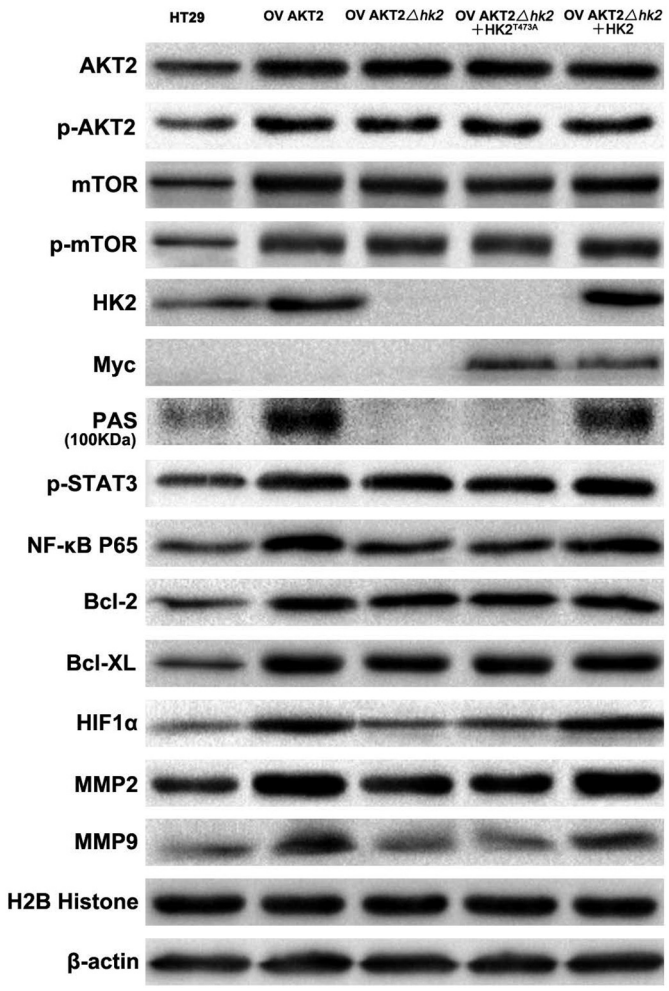

Fig. 6. Potential upstream regulators and downstream effector molecules associated with HK2-mediated promotion of colon cancer progression in vivo. Western-blot for AKT2, p-AKT2, mTOR, p-mTOR, HK2, Myc, PAS, p-STAT3, NF-κB, Bcl-2, Bcl-XL, HIF1α, MMP2 and MMP9 expression in metastatic foci found in the lungs of nude mice from the different treated groups. All assays were performed in triplicate and showed representative blots. Quantification of blots and statistical analysis were showed in supplemental fig. S3.

Therefore, our in vivo findings suggest that mTOR, NF-κB, HIF1α, MMP2 and MMP9 may be downstream molecules of the AKT2/HK2 pathway that promote colon cancer progression. In particular, mTOR may act as an upstream molecule of HK2, while NF-κB, HIF1α, MMP2 and MMP9 may be downstream molecular targets of HK2 (Fig. 7).

4. Discussion

Although the role of AKT2 and HK2 in the development and progression of human cancer has been established [24–26], their specific mechanisms in the development of colon cancer remains unknown. In the present study, we found that AKT2 phosphorylates the T473 site of HK2, activates its enzymatic activity, increases intracellular lactate production, resists serum starvation-induced apoptosis, and promotes cell invasion, xenograft tumor growth, and metastasis.

The overexpression of the AKT2 isoform is an early event and plays a predominant role in colon carcinogenesis. This capability of AKT2 has been attributed to the fact that AKT2, in addition to regulating cell migration and invasion, also inhibits apoptosis; however, the precise mechanism of AKT2-driven development of colon cancer is poorly understood. Recently, HK2 was been reported to be highly expressed in malignant tumors. HK2 may promote colon cancer progression via

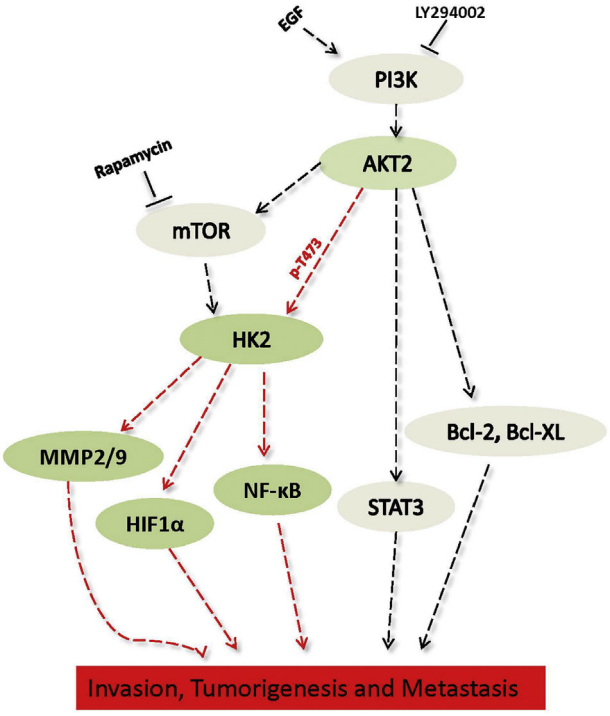

Fig. 7. Schematic representation of AKT2 contributes to increase colon cancer cell invasion, tumorigenesis and metastasis through phosphorylates HK2 at T473.

different mechanisms, of which one of the most important mechanisms may be aerobic glycolysis [28,29]. Although the correlation between HK2 and tumorigenesis has been confirmed by mouse models [30], the specific mechanism by which HK2 mediates the development of colon cancer remains unclear.

Currently, some studies suggest that there may be interactions between AKT2 and HK2. It has been reported that miR-29b negatively regulates AKT2/AKT3 expression, resulting in the down-regulation of HK2/PKM2, which leads to a decrease in the Warburg effect and a slowing of ovarian cancer progression [31]. Moreover, Zhuo et al. demonstrated that the elevation of HK2 in osteosarcoma induced by activated PI3K/Akt signaling exerts anti-apoptotic and proliferative effects by regulating the Warburg effect [32]. In addition, double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK) silencing results in decreased glioma cell viability and ATP/lactate production upon low glucose stress, which is mediated by partially blocked AKT activation and subsequent inhibition of HK2 mitochondria translocation [33]. However, how AKT2 and HK2 function together in colon cancer remains poorly understood to date.

In the present study, an interaction between AKT2 and HK2 but not AKT1 or AKT3, in colon cancer cells was identified by immunoprecipitation assays. Since AKT is known to be closely related to insulin signaling and HK2 is a key rate-limiting enzyme in glycolysis, is a major subtype in insulin-sensitive tissue and is upregulated in tumors, one of the consequences of this interaction may be to alter the aerobic glycolysis metabolism of tumor cells. In addition, HK2 up-regulation results in increased glycolysis rates. In this study, we reported that the overexpression of AKT2 can simultaneously increase the expression level and phosphorylation level of HK2, enhanced the activity of cellular hexokinase, and promoted aerobic glycolysis in HCT-116 and HT-29 cells, while producing large amounts of lactic acid. A knockout of the *hk2* gene following the overexpression of AKT2 can significantly reduce the hexokinase activity of the cells and the amount of lactic acid produced. Rescuing the stable overexpression of HK2 in OV AKT2Δ*hk2* cells significantly increased the hexokinase activity and amount of lactic acid, but the mutant $HK2^{T473A}$ did not. These results indicate that AKT2 contributes to increased levels of aerobic glycolysis through HK2 in colon cancer cells, can more efficiently obtain the energy required by these cells, and obtain a large number of intermediate products necessary for cellular proliferation, further promoting tumor development. This is similar to a previous report that c-Src can mediate HK2 to increase hexokinase activity and lactic acid production in tumor cells [34].

In addition, recent research shows that HK2 contributes to the inhibition of apoptosis through the suppression of the formation of mitochondrial permeability transition pores in association with voltage-dependent anion channel (VDAC) protein [11]. We also found that AKT2 mediates HK2 resistance to apoptosis induced by serum starvation. Since HK2 is closely related to the nutritional metabolism of tumor cells, we only tested serum starvation-induced apoptosis in the present study. In a future experiment, we will examine whether AKT2 mediates HK2 resistance to apoptosis induced by other factors.

AKT has been well-characterized as a serine/threonine protein kinase that phosphorylates the RXRXX(S/T) signature sequence [24], and HK2 exhibits the typical AKT phosphorylation sequence, QHRARQKT473. Moreover, a study has confirmed that the total AKT can phosphorylate Thr-473 HK2 and increase the binding of mitochondria and HK2 to protect cardiomyocytes [25]; however, the authors did not determine which subtype of AKT plays a role in this process. In addition, in colon cancer cells, no studies to date have assessed whether AKT2 can phosphorylate Thr-473 of HK2. Indeed, although wild type HK2 was found to be phosphorylated by AKT2, the phosphorylation did not occur when threonine 473 in HK2 was mutated.

Our further studies indicate that AKT2/HK2 mediates the promotion of colon cancer cell invasion, xenograft tumors, and metastasis that can be triggered by EGF-stimulated PI3K activation. PI3K acts as an upstream kinase for AKT2 and HK2. In addition, AKT2 has been reported to be a molecule located upstream from mTOR. Although AKT2 acts as an upstream molecule of HK2 and can directly interact and phosphorylate HK2, rapamycin, an inhibitor of mTOR, was also found to reduce the expression of HK2 in this study. This finding should be studied further to explain the specific associated molecular mechanisms in future research.

We also investigated whether AKT2 affects p-STAT3, NF-κB, Bcl-2, Bcl-XL, HIF1α, MMP2, and MMP9 via the phosphorylation of the T473 site of HK2, which are important molecules closely related to cancer cell invasion, apoptosis, and metastasis. We found that AKT2 regulates NF-κB, HIF1α, MMP2, and MMP9 via the phosphorylation of the HK2 T473 site. A previous study showed that HK2 is a downstream target of HIF1α [30]. However, in our study, we found that HK2 is the upstream molecule of HIF1α. This inconsistency may indicate that there are interactions in complex cellular signaling pathways in different cancer cells. We also found that AKT2 does not regulate the level of p-STAT3, Bcl-2, and Bcl-XL protein expression via HK2. In addition, to gain insight into the specific role of the AKT2-HK2-NF-κB/HIF1α/MMP2/MMP9 axis in colon cancer, there is a need to investigate how HK2 could modulate the expression of MMP2/9, HIF-1a and NF-κB in the future.

5. Conclusions

In conclusion, we found that AKT2 increases the invasion, tumorigenesis, and metastasis of colon cancer cells in vitro and promotes lung metastasis in nude mice in vivo through the phosphorylation of the T473 site of HK2 by upregulating NF-κB, HIF1α, MMP2, and MMP9. Our findings highlight a novel mechanism for the AKT2-HK2-NF-κB/HIF1α/MMP2/MMP9 axis in the regulation of colon cancer progression. Moreover, our results suggest that both AKT2 and HK2 may be potential targets for the treatment of colon cancer.

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Competing interests

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2019.03.011.

References

[1] K. Tamas, A.M. Walenkamp, E.G. de Vries, M.A. van Vugt, R.G. Beets-Tan, B. van Etten, D.J. de Groot, G.A. Hospers, Rectal and colon cancer: not just a different anatomic site, Cancer Treat. Rev. 41 (8) (2015) 671–679.
[2] R. Dienstmann, R. Salazar, J. Tabernero, Personalizing colon cancer adjuvant therapy: selecting optimal treatments for individual patients, J. Clin. Oncol. 33 (16) (2015) 1787–1796.
[3] D.F. Birt, G.J. Phillips, Diet, genes, and microbes: complexities of colon cancer prevention, Toxicol. Pathol. 42 (1) (2014) 182–188.
[4] T. Hu, Z. Li, C.Y. Gao, C.H. Cho, Mechanisms of drug resistance in colon cancer and its therapeutic strategies, World J. Gastroenterol. 22 (30) (2016) 6876–6889.
[5] G. Risso, M. Blaustein, B. Pozzi, P. Mammi, A. Srebrow, Akt/PKB: one kinase, many modifications, Biochem. J. 468 (2) (2015) 203–214.
[6] I. Hers, E.E. Vincent, J.M. Tavare, Akt signalling in health and disease, Cell. Signal. 23 (10) (2011) 1515–1527.
[7] M. Martini, M.C. De Santis, L. Braccini, F. Gulluni, E. Hirsch, PI3K/AKT signaling pathway and cancer: an updated review, Ann. Med. 46 (6) (2014) 372–383.
[8] E. Agarwal, M.G. Brattain, S. Chowdhury, Cell survival and metastasis regulation by Akt signaling in colorectal cancer, Cell. Signal. 25 (8) (2013) 1711–1719.
[9] M.G. Vander Heiden, L.C. Cantley, C.B. Thompson, Understanding the Warburg effect: the metabolic requirements of cell proliferation, Science 324 (5930) (2009) 1029–1033.
[10] V.P. Tan, S. Miyamoto, HK2/hexokinase-II integrates glycolysis and autophagy to confer cellular protection, Autophagy 11 (6) (2015) 963–964.
[11] G.S. Krasnov, A.A. Dmitriev, V.A. Lakunina, A.A. Kirpiy, A.V. Kudryavtseva, Targeting VDAC-bound hexokinase II: a promising approach for concomitant anti-cancer therapy, Expert Opin. Ther. Targets 17 (10) (2013) 1221–1233.
[12] A. Peschiaroli, A. Giacobbe, A. Formosa, E.K. Markert, L. Bongiorno-Borbone, A.J. Levine, E. Candi, A. D'Alessandro, L. Zolla, A. Finazzi Agro, G. Melino, miR-143 regulates hexokinase 2 expression in cancer cells, Oncogene 32 (6) (2013) 797–802.
[13] D. DeWaal, V. Nogueira, A.R. Terry, K.C. Patra, S.M. Jeon, G. Guzman, J. Au, C.P. Long, M.R. Antoniewicz, N. Hay, Hexokinase-2 depletion inhibits glycolysis and induces oxidative phosphorylation in hepatocellular carcinoma and sensitizes to metformin, Nat. Commun. 9 (1) (2018) 446.
[14] W. Kim, J.H. Yoon, J.M. Jeong, G.J. Cheon, T.S. Lee, J.I. Yang, S.C. Park, H.S. Lee, Apoptosis-inducing antitumor efficacy of hexokinase II inhibitor in hepatocellular carcinoma, Mol. Cancer Ther. 6 (9) (2007) 2554–2562.
[15] C.L. Neary, J.G. Pastorino, Akt inhibition promotes hexokinase 2 redistribution and glucose uptake in cancer cells, J. Cell. Physiol. 228 (9) (2013) 1943–1948.
[16] S.V. Barysch, C. Dittner, A. Flotho, J. Becker, F. Melchior, Identification and analysis of endogenous SUMO1 and SUMO2/3 targets in mammalian cells and tissues using monoclonal antibodies, Nat. Protoc. 9 (4) (2014) 896–909.
[17] B. Zheng, L. Geng, L. Zeng, F. Liu, Q. Huang, AKT2 contributes to increase ovarian cancer cell migration and invasion through the AKT2-PKM2-STAT3/NF-kappaB axis, Cell. Signal. 45 (2018) 122–131.
[18] M. Yan, Q. Xu, P. Zhang, X.J. Zhou, Z.Y. Zhang, W.T. Chen, Correlation of NF-kappaB signal pathway with tumor metastasis of human head and neck squamous cell carcinoma, BMC Cancer 10 (2010) 437.
[19] S. Haggblad Sahlberg, A.C. Mortensen, J. Haglof, M.K. Engskog, T. Arvidsson, C. Pettersson, B. Glimelius, B. Stenerlow, M. Nestor, Different functions of AKT1 and AKT2 in molecular pathways, cell migration and metabolism in colon cancer cells, Int. J. Oncol. 50 (1) (2017) 5–14.
[20] L. Wang, H. Xiong, F. Wu, Y. Zhang, J. Wang, L. Zhao, X. Guo, L.J. Chang, Y. Zhang, M.J. You, S. Koochekpour, M. Saleem, H. Huang, J. Lu, Y. Deng, Hexokinase 2-mediated Warburg effect is required for PTEN- and p53-deficiency-driven prostate cancer growth, Cell Rep. 8 (5) (2014) 1461–1474.
[21] W. Zhu, Y. Huang, Q. Pan, P. Xiang, N. Xie, H. Yu, MicroRNA-98 suppress Warburg effect by targeting HK2 in colon cancer cells, Dig. Dis. Sci. 62 (3) (2017) 660–668.
[22] G.N. Masoud, W. Li, HIF-1alpha pathway: role, regulation and intervention for cancer therapy, Acta Pharm. Sin. B 5 (5) (2015) 378–389.
[23] F. Massari, C. Ciccarese, M. Santoni, R. Iacovelli, R. Mazzucchelli, F. Piva, M. Scarpelli, R. Berardi, G. Tortora, A. Lopez-Beltran, L. Cheng, R. Montironi, Metabolic phenotype of bladder cancer, Cancer Treat. Rev. 45 (2016) 46–57.
[24] F. Vandermoere, I. El Yazidi-Belkoura, Y. Demont, C. Slomianny, J. Antol, J. Lemoine, H. Hondermarck, Proteomics exploration reveals that actin is a signaling target of the kinase Akt, Mol. Cell. Proteomics 6 (1) (2007) 114–124.
[25] D.J. Roberts, V.P. Tan-Sah, J.M. Smith, S. Miyamoto, Akt phosphorylates HK-II at Thr-473 and increases mitochondrial HK-II association to protect cardiomyocytes, J. Biol. Chem. 288 (33) (2013) 23798–23806.
[26] S.Y. Lunt, M.G. Vander Heiden, Aerobic glycolysis: meeting the metabolic requirements of cell proliferation, Annu. Rev. Cell Dev. Biol. 27 (2011) 441–464.
[27] G.Z. Cheng, W. Zhang, L.H. Wang, Regulation of cancer cell survival, migration, and invasion by twist: AKT2 comes to interplay, Cancer Res. 68 (4) (2008) 957–960.
[28] S. John, J.N. Weiss, B. Ribalet, Subcellular localization of hexokinases I and II directs the metabolic fate of glucose, PLoS One 6 (3) (2011) e17674.
[29] J. Mathew, A. Loranger, S. Gilbert, R. Faure, N. Marceau, Keratin 8/18 regulation of glucose metabolism in normal versus cancerous hepatic cells through differential modulation of hexokinase status and insulin signaling, Exp. Cell Res. 319 (4) (2013) 474–486.
[30] K.C. Patra, Q. Wang, P.T. Bhaskar, L. Miller, Z. Wang, W. Wheaton, N. Chandel, M. Laakso, W.J. Muller, E.L. Allen, A.K. Jha, G.A. Smolen, M.F. Clasquin, B. Robey, N. Hay, Hexokinase 2 is required for tumor initiation and maintenance and its systemic deletion is therapeutic in mouse models of cancer, Cancer Cell 24 (2) (2013) 213–228.
[31] Y. Teng, Y. Zhang, K. Qu, X. Yang, J. Fu, W. Chen, X. Li, MicroRNA-29B (mir-29b) regulates the Warburg effect in ovarian cancer by targeting AKT2 and AKT3, Oncotarget 6 (38) (2015) 40799–40814.
[32] B. Zhuo, Y. Li, Z. Li, H. Qin, Q. Sun, F. Zhang, Y. Shen, Y. Shi, R. Wang, PI3K/Akt signaling mediated Hexokinase-2 expression inhibits cell apoptosis and promotes tumor growth in pediatric osteosarcoma, Biochem. Biophys. Res. Commun. 464 (2) (2015) 401–406.
[33] X. Hou, Y. Liu, H. Liu, X. Chen, M. Liu, H. Che, F. Guo, C. Wang, D. Zhang, J. Wu, X. Chen, C. Shen, C. Li, F. Peng, Y. Bi, Z. Yang, G. Yang, J. Ai, X. Gao, S. Zhao, PERK silence inhibits glioma cell growth under low glucose stress by blockage of p-AKT and subsequent HK2's mitochondria translocation, Sci. Rep. 5 (2015) 9065.
[34] J. Zhang, S. Wang, B. Jiang, L. Huang, Z. Ji, X. Li, H. Zhou, A. Han, A. Chen, Y. Wu, H. Ma, W. Zhao, Q. Zhao, C. Xie, X. Sun, Y. Zhou, H. Huang, M. Suleman, F. Lin, L. Zhou, F. Tian, M. Jin, Y. Cai, N. Zhang, Q. Li, c-Src phosphorylation and activation of hexokinase promotes tumorigenesis and metastasis, Nat. Commun. 8 (2017) 13732.