HepaCAM Regulates Warburg Effect of Renal Cell Carcinoma via HIF-1α/NF-κB Signaling Pathway

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OBJECTIVE To investigate how hepatocyte cell adhesion molecule (hepaCAM) regulates cancer energy metabolism through hypoxia-inducible factor (HIF-1α) in renal cell carcinoma (RCC).

MATERIALS AND METHODS The expression of hepaCAM and HIF-1α in RCC tissue samples was examined by immunohistochemistry. Glucose consumption and lactate production assays were used to detect metabolic activity in RCC cell lines. P65 and IκB kinase (IKKβ) mRNA and protein expression were detected using quantitative real-time polymerase chain reaction and western blotting, respectively. Nuclear translocation of P65 was observed by immunofluorescence staining after re-expressing hepaCAM. The luciferase reporter assay was applied to validate the transcriptional activity of HIF-1α.

RESULTS HIF-1α expression was elevated and hepaCAM suppressed in RCC compared with adjacent normal tissues. Furthermore, hepaCAM re-expression significantly decreased glycolytic metabolism in RCC cell lines, and reduced HIF-1α, IKKβ, and P65 expression. The expression of HIF-1α, GLUT1, LDHA, and PKM2 were further reduced with combined hepaCAM overexpression and treatment with the NF-κB inhibitor BAY11-7082, compared to hepaCAM overexpression alone. Additionally, hepaCAM decreased the transcriptional activity of HIF-1α and blocked P65 nuclear translocation by the NF-κB pathway.

CONCLUSION Our data suggest that hepaCAM suppresses the Warburg effect via the HIF-1α/NF-κB pathway in RCC, which is a facilitating factor in hepaCAM-reduced tumorigenesis. UROLOGY 127: 61–67, 2019. © 2018 Elsevier Inc.

Renal cell carcinoma (RCC) is one of the most common malignancies of the urinary system, representing about 4% of all adult malignancies and ranking second in urologic cancer mortality.[1] Clear cell RCCs (ccRCCs) accounts for 70%-80% of adult RCCs.[2] It is characterized by resistance to chemotherapy and traditional radiotherapy, and has a high metastatic potential.[3] Successful treatment of ccRCC patients is hampered by the lack of knowledge about the specific molecular mechanisms underlying carcinoma development, including its origin, acceleration, and progression.

Hepatocyte cell adhesion molecule (hepaCAM, or glialCAM in glial cells), a member of the immunoglobulin superfamily, is also a novel tumor suppressor that affects growth, invasion, metastasis, and apoptosis.[4,5] In RCC, hepaCAM arrests the cell cycle of 786-O cells at the G_0/G_1 stage, decreases c-myc, and prevents protein kinase Cε translocation from the cytoplasm to the plasma membrane.[6] Despite its association with several cancers,[7,8] the mechanisms underlying hepaCAM-suppressed tumor development remain largely unclear.

Most cancer cells, even in the presence of ample oxygen, dominantly produce energy by glycolysis, whereas most normal cells produce energy by mitochondrial oxidative phosphorylation. This tumor-specific Warburg effect provides the energy and biosynthetic materials needed to promote tumor progression.[9,10] This effect is promoted by oncogenic signals such as those elicited by activation of hypoxia-inducible factor (HIF)-1α, a key molecule for regulating the cellular response.[11] Upon activation and stabilization during tumor hypoxia, HIF-1α upregulates target genes involved in angiogenesis, tumor invasion and metastasis, energy metabolism, and adaptive survival.[12,13]

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Ethical Approval: *In our study, all of the assays association with human participants conformed, respectively, to the ethical standards of the institutional and national research committee, to the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The Ethics Committee of Chongqing Medical University consented to our study.*

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Although there is cross-talk between HIF-1α and NF-κB,[14] whether hepaCAM regulates the NF-κB pathway is unresolved. In the resting stage of the canonical NF-kB pathway, the heterodimeric complex of RelA (p65) and p50 is sequestered in the cytoplasm in association with IκBα, and is inactive. Once activated by external stimuli such as cytokines, lipopolysaccharide, or viruses, IκB kinases (IKKβ) phosphorylate IκBα, which is degraded by the proteasome.[15] This cascade allows translocation of the p65/p50 complex to the nucleus where it regulates transcription of its target genes.

In this study, we determined the connection between hepaCAM and HIF-1α signaling in tissues from patients with RCC and cell lines. We also assessed representative molecules involved in the Warburg effect in cancer cells, and found that hepaCAM downregulated NF-κB activity, thus suppressing HIF-1α activation, glucose uptake, and lactate production. The results provide insightful information concerning RCC progression and a novel hepaCAM/HIF-1α mechanism that inhibits RCC glycolysis.

MATERIALS AND METHODS

Tissue Samples

We obtained a total of 43 cases of ccRCCs and tumor-adjacent normal tissues in pair, which were diagnosed using histologic examination between 2011 and 2017 in the Department of Urinary Surgery, the First Affiliated Hospital of Chongqing Medical University. Of all clinical samples, 11 RCC samples were recurrence tumors after partial nephrectomy and 32 RCC samples were primary tumors. The histologic grade and stage were determined according to the Union for International Cancer Control (UICC) guidelines, and the tissue specimens were stages T1a-T2b in 25 cases and stages T3a-T4 in 18 cases. All patients supplied informed consent. The study was approved by the Ethics Committee of Chongqing Medical University, and was conducted in accordance with the Helsinki Declaration.

Cell Culture and Transfection

Cell lines (786-O, A-498, and Caki-1) were maintained in RPMI1640 medium from Gibco, supplemented with 10% of fetal bovine serum (Gibco) at hypoxic conditions, cells were placed in incubator flushing with a gas mixture containing 1% O_2, 5% CO_2, and balance N_2. Adenoviruses overexpression hepaCAM (Ad-hepaCAM) or control (Ad-HK) was constructed by our group as previously described.[16] Other reagents in our study were as follows: HRE-Luc (Addgene) and BAY-11-7082 (MCE).

Reverse Transcription and Quantitative Real-Time PCR

Total RNAs were isolated using TRIzol (Invitrogen) and reverse transcription was implemented using PrimeScript RT reagent kits (Takara). Real-time PCR was implemented with the SYBR Premix Ex Taq II kit (Takara). Primer sequences were shown in Supplementary Table1. The comparative $2^{-\Delta\Delta Ct}$ method with β-actin serving as a regulator was used to calculate the expressions of gene.

Western Blots

Total protein extracts were prepared using reagents involved in RIPA lysis buffer, phosphatase inhibitors NaF and Na_3VO_4, and protease inhibitor PMSF (Roche). Western blot assay was performed as previously described.[5] Nuclear and cytoplasmic proteins were extracted using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Institute of Biotechnology, Jiangsu, China) as previously described.[5] Anti-hepaCAM (1:1000), anti-histone H3 (1:1000), and anti-β-actin (1:1000) were from ProteinTech; anti-HIF-1α (1:1000), anti-PKM2 (pyruvate kinase M2, 1:1000), anti-Glut1 (glucose transporter 1, 1:1000), and anti-NF-κB P65 (1:1000) were from Abcam; IKKβ (1:1000) was from cell signaling technology; LDHA (lactate dehydrogenase A, 1:1000) was from Santa Cruz Biotechnology.

Immunofluorescence

Approximately 1×10^5 cells per well were grown on coverslips in 24-well plates. After 24 hours, the cells were transfected with different treatments for 48 hours. Immunofluorescence staining was performed as previously described.[6] Anti-HIF-1α (1:1000) antibody (Abcam) was incubated. Immunofluorescent images were captured using a Nikon Eclipse 80i microscope (Eclipse 80i, Tokyo, Japan) at 400× magnification.

Immunohistochemistry

All tissue samples were fixed in 10% neutral formalin, embedded in paraffin, and cut into 5-μm-thick sections. Immunohistochemical staining was performed as previously described.[5] The protein expression levels of hepaCAM and HIF-1α were assessed with the corresponding anti- hepaCAM (1:200, ProteinTech) and anti- HIF-1α (1:200, Abcam) antibody. The total numbers of immunohistochemical images were observed by Image Pro Plus (IPP, version 6.0; Media Cybernetics, Silver Spring, MD). The stained sections were scored by the staining intensity and the immunoreactivity ratio. The staining intensity was defined as follows: 0 (no staining), 1 (light yellow), 2 (light brown), and 3 (brown), and the immunoreactivity ratio was 0 (0% immunoreactive cells), 1 (<5% immunoreactive cells), 2 (5%-50% immunoreactive cells), and 3 (>50% immunoreactive cells). The final immunoreactivity score was defined as the sum of 2 indexes. Scores of hepaCAM and HIF-1α were divided into 2 classifications: negative (final score $\leq$2) and positive (final scores >2).

Luciferase Assay

The 5×10^3 786-O cells were transfected using Lipofectamine 2000 with 0.2 μg of HRE-Luc plasmids. Renilla luciferase plasmid was cotransfected as a transfection efficiency control. Cells were harvested after 24 hours and luciferase activity was examined by the Dual-Luciferase Reporter Assay System (Promega).

Glucose Consumption and Lactate Production

RCC cells were grown in 12-well plates at appropriate concentrations to evaluate the glucose and lactate concentration. The culture medium was replaced by fresh culture medium when cell density was 60%-70%. After different treatment, the supernatant of culture medium was collected to measure the concentrations of glucose and lactate. The levels of glucose and lactate were assessed, respectively, by the Glucose Assay Kit and the Lactate Assay Kit under microplate reader (BioTek, Winooski, VT) according to their respective manufacturer's protocols. In the meantime, the number of cells in each well was counted. All the measurements were normalized to cell numbers.

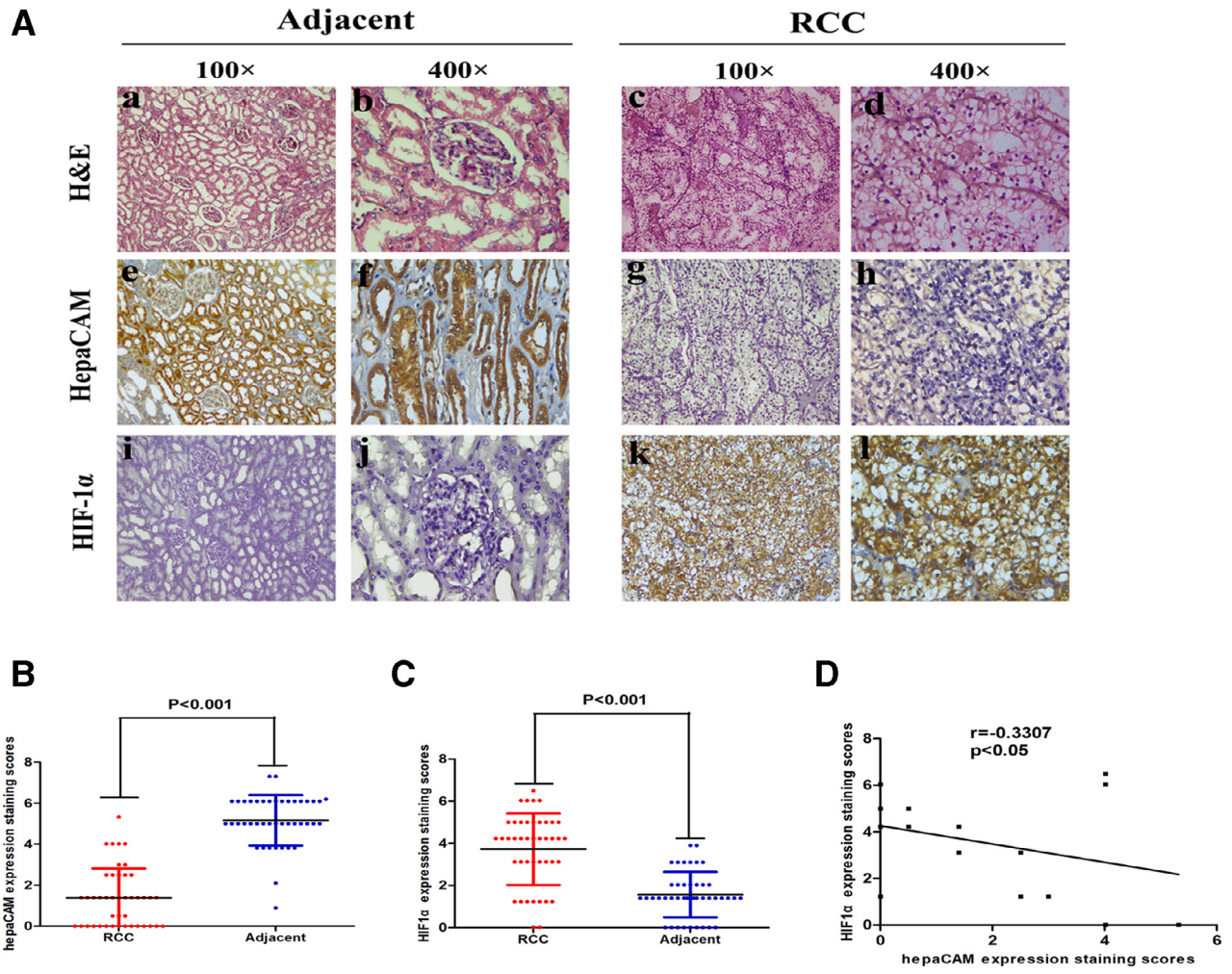

Figure 1. Low hepaCAM and high HIF-1α protein expression in RCC tissue. (**A**) Representative hematoxylin and eosin (H&E) staining and immunohistochemistry staining in 43 RCC tissues and tumor-adjacent nonmalignant tissues: H&E staining (a-d); hepaCAM expression (e-h); HIF-1α expression (i-l). (**B**) Average staining scores for hepaCAM expression in adjacent tissues and RCC tissues. (**C**) Average staining scores for HIF-1α expression in adjacent tissues and RCC tissues. RCC, renal cell carcinoma. (Color version available online.)

Statistical Analysis

All the statistical analyses were performed by the SPSS 16.0. Student's *t* test, one-way ANOVA, chi-square test, and Pearson correlation analysis were used to evaluate the remarkable connections among categorical variables. All data were expressed as the mean ± standard deviation and all experiments were repeated at least 3 times. Value of $P < .05$ was considered to be statistically significant.

RESULTS

Low HepaCAM and High HIF-1α Protein Expression in RCC Tissue

We evaluated 43 ccRCC tissues and 43 adjacent normal specimens from patients treated with total or partial nephrectomy. Figure 1A shows hematoxylin and eosin staining of these tissues. Immunohistochemical staining shows that approximately 79% (34 of 43) of the RCC specimens were positive for HIF-1α, vs 21% (9 of 43) of the adjacent tissues (Fig. 1C). Only 28% (12 of 43) of the ccRCC specimens stained positively for hepaCAM, vs 72% (31 of 43) of the adjacent tissues (Fig. 1B). These results indicate significantly decreased hepaCAM and increased HIF-1α expression in RCC compared with that in adjacent tissues ($P < .001$).

There was a significant correlation between high HIF-1α and low hepaCAM expression (Fig. 1D, $r = -0.3307$, $P < .05$). Thus, the correlation between hepaCAM and HIF-1α expression, and various clinicopathologic parameters in RCC tissues were evaluated. The results revealed a significant relationship between HIF-1α expression and the RCC tumor histologic stage (Supplementary Table 2, $P = .035$).

HepaCAM Suppresses the Warburg Effect and HIF-1α Targets in RCC Cells

Previous studies indicate that hepaCAM is expressed at a low level in ccRCC tumors and cell lines.[6] After hepaCAM was transfected into 786-O, A498, and Caki-1 RCC cells using an adenovirus (Ad-hepaCAM), the efficiency of hepaCAM

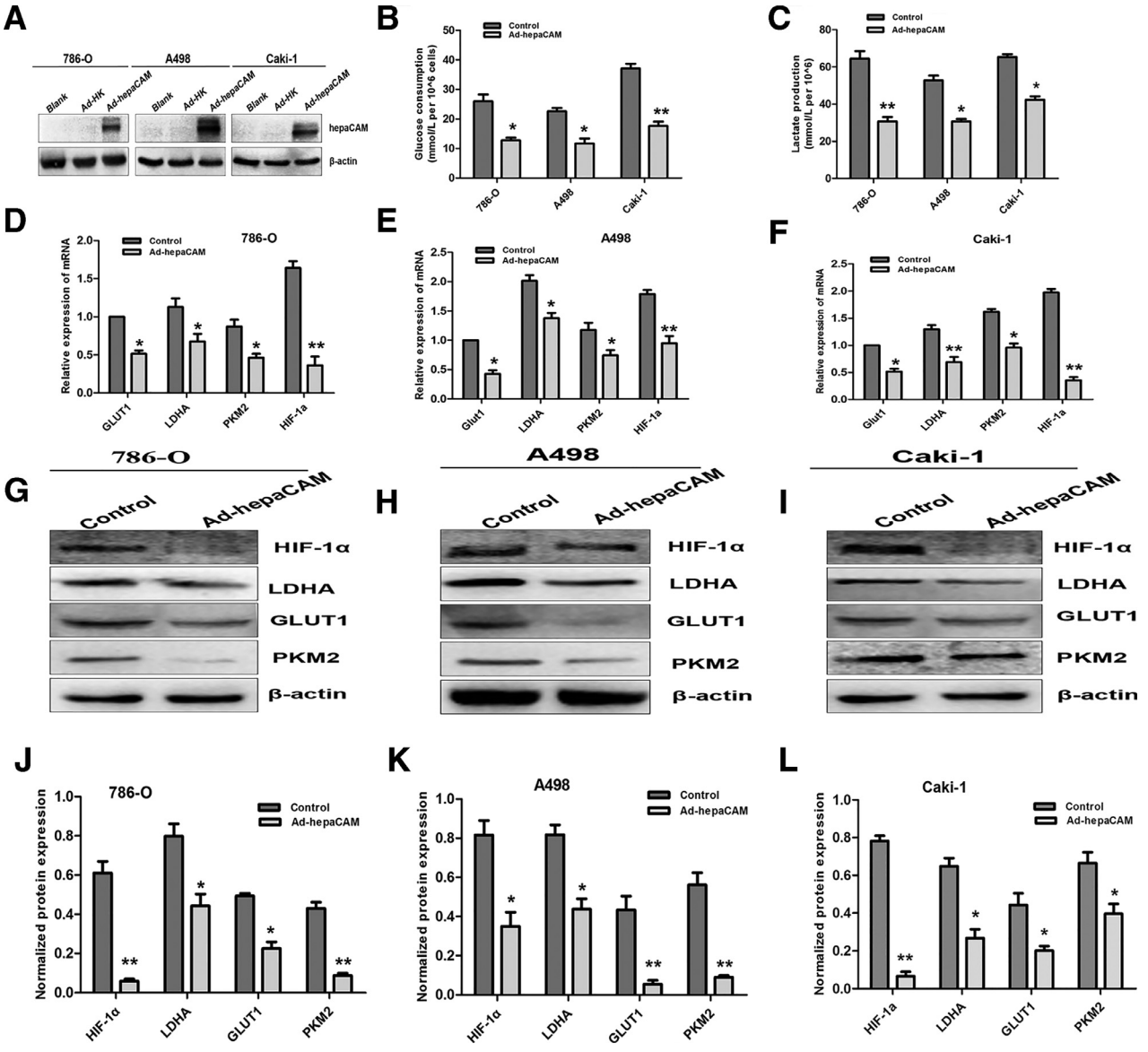

Figure 2. HepaCAM suppressed the Warburg effect and HIF-1α targets in RCC cells. (**A**) 786-O, A498, and Caki-1 cells were infected by Ad-HK or Ad-hepaCAM under hypoxia for 48 hours and analyzed by western blotting. (**B**) HepaCAM significantly decreased the glucose consumption in RCC cells using glucose consumption assay. (**C**) Overexpression of hepaCAM robustly decreased the lactate production in RCC cells using lactate production assay. (**D-F**) The qRT-PCR analysis demonstrated that hepaCAM overexpressing decreased the mRNA expression of HIF1α and its target genes (GLUT1, PKM2, and LDHA) in all RCC cells maintained under hypoxia condition for 48 hours. β-Actin was used as internal control. (**G-L**) Western blot detected the protein expression of HIF1α, GLUT1, LDHA, and PKM2. β-Actin was used as internal control. Ad-hepaCAM group vs control: ** P <.01; * P <.05. qRT-PCR, quantitative real-time polymerase chain reaction; RCC, renal cell carcinoma.

overexpression was detected by western blot (Fig. 2A). The cells were then incubated with 1% O_2 for 48 hours. HepaCAM overexpressing cells had significantly decreased glucose consumption (Fig. 2B) and lactate production (Fig. 2C) in all 3 cell lines under hypoxia.

An increasing number of studies indicate that HIF-1α regulates glycolysis by a few proteins including GLUT1, PKM2, LDHA, and pyruvate dehydrogenase lipoamide kinase 1.[11] The mRNA and protein expression levels of these genes were reduced in hepaCAM overexpressing RCC cell lines (Fig. 2D-L). Collectively, these results suggest that overexpressing hepaCAM suppresses glucose metabolism, most probably by regulating the HIF-1α cascade in RCC cells.

HepaCAM Inhibits P65 and IKKβ Expression

In our previous study, we demonstrated that hepaCAM inhibited the proliferation of RCC cells, and that NF-κB might regulate the HIF-1α level and contribute to the Warburg effect.[17] Thus, we hypothesized that hepaCAM could reduce the Warburg effect in RCC by regulating NF-κB signaling. qRT-PCR showed that hepaCAM overexpression significantly decreased P65 mRNA levels compared to those of control RCC cells (Fig. 3A). Additionally, western blots demonstrated that hepaCAM re-expression in RCC cells reduced P65 and IKKβ levels compared to the levels in control cells (Fig. 3B-E). Taken together, these results demonstrate that hepaCAM decreases the levels of HIF-1α, at least partly by altering NF-κB signaling.

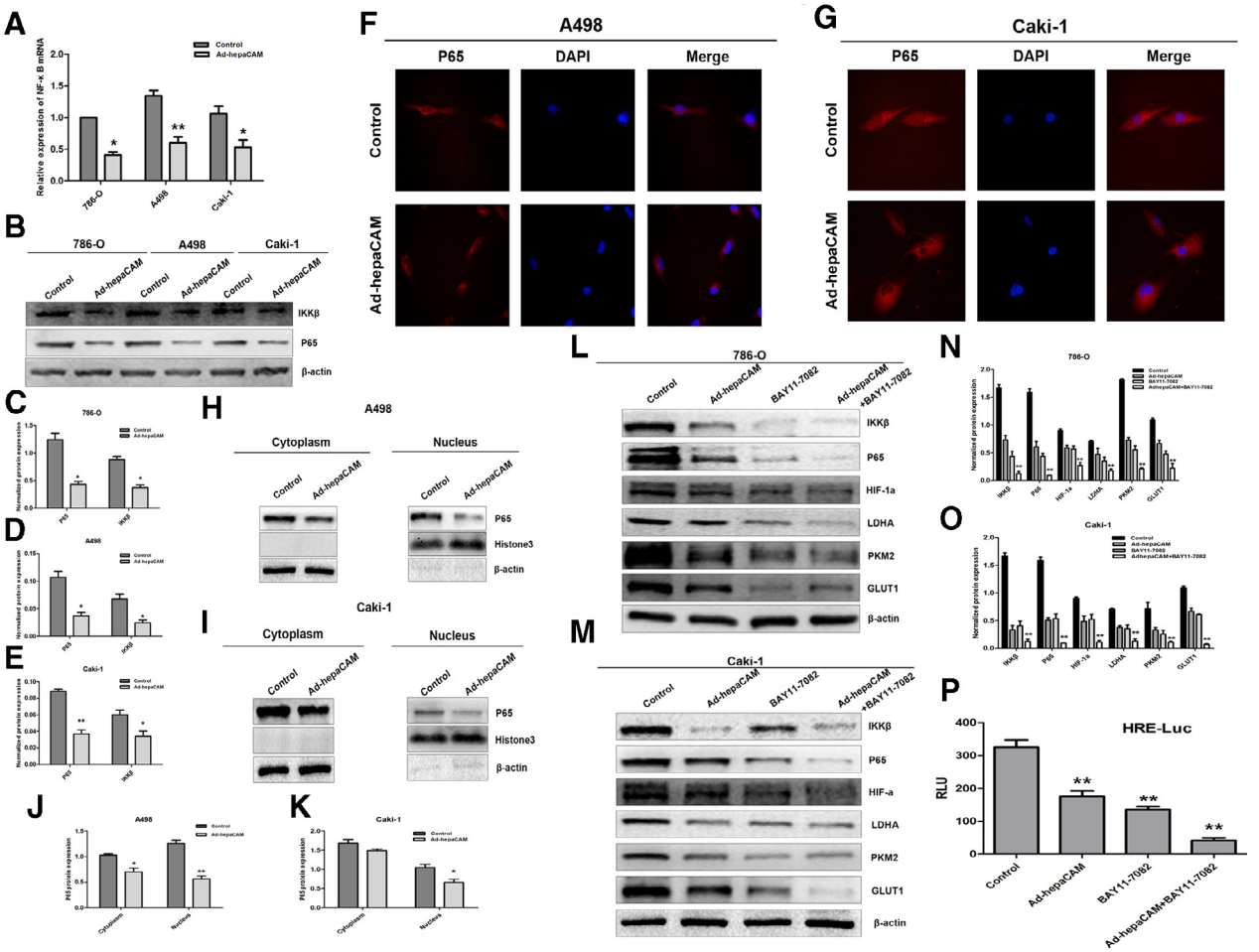

Figure 3. HepaCAM suppressed HIF-1α by NF-κB signaling pathway. (**A**) The qRT-PCR analysis showed hepaCAM overexpressing decreased the mRNA expression of NF-κB. β-Actin was used as internal control. (**B-E**) Western blot detected the expression of P65 (RELA) and IKKβ in all RCC cells maintained under hypoxia condition for 48 hours. β-Actin was used as internal control. Date represented mean $\pm$ SD ($n = 3$). Ad-hepaCAM group vs control: ** P <.01; * P <.05. (**F, G**) Immunofluorescence staining demonstrated the expression of P65 was reduced in the nucleus after infection with Ad-hepaCAM in A498 and Caki-1 cells under hypoxia for 48 hours; magnification 400×. (**H-K**) Western blot demonstrated that hepaCAM significantly downregulated P65 expression in the nucleus. Histone 3 and β-actin were used as internal controls. Date represented mean $\pm$ SD ($n = 3$). Ad-hepaCAM group vs control: ** P <.01; * P <.05. (**L-O**) Western blotting was used to detect the level of HIF-1a, IKKβ, P65, GLUT1, PKM2, and LDHA in cells treated with BAY11-7082 under hypoxia. β-Actin was used as internal control. Date represented mean $\pm$ SD ($n = 3$). * P <.05; ** P <.01 vs BAY11-7082. (**P**) Luciferase reporter assay measured that luciferase activity was significantly repressed with Ad-hepaCAM or BAY11-7082 under hypoxia. (** P <.01 Ad-hepaCAM or BAY11-7082 vs control; ** P <.01 Ad-hepaCAM + BAY11-7082 vs Ad-hepaCAM or BAY11-7082, respectively.) qRT-PCR, quantitative real-time polymerase chain reaction; RCC, renal cell carcinoma; SD, standard deviation. (Color version available online.)

HepaCAM Suppresses P65 Nuclear Translocation

To explore the mechanism underlying hepaCAM-mediated NF-κB signaling in RCC, we determined the subcellular localization of P65 in A498 and Caki-1 cells. Immunofluorescence analysis showed that hepaCAM re-expression reduced translocation of P65 to the nucleus, whereas the negative control group was unchanged (Fig. 3F and G). Western blot results showed similar hepaCAM-mediated changes in the expression of P65 in the cytoplasm and nucleus (Fig. 3H-K).

HepaCAM-Dependent HIF-1α Levels Are Regulated by NF-κB

A previous study showed that HIF-1α was upregulated by binding of NF-κB to the hypoxia response element (HRE), and hepaCAM inhibited NF-κB activity.[14] Thus, we speculated that the NF-κB pathway was involved in hepaCAM-dependent regulation of HIF-1α. To dissect whether hepaCAM regulated the Warburg effect of RCC cells via HIF-1α/NF-κB, 786-O, and Caki-1 cells were treated with 10- or 20-μM BAY11-7082, an NF-κB inhibitor, for 48 hours. Western blots revealed that expression of the HIF-1α and P65 proteins was reduced in these cells with combined treatments of Ad-hepaCAM and BAY11-7082, compared to the expression in cells treated with either agent alone (Fig. 3L-O). Additionally, the combined treatment suppressed the protein expression of GLUT1, LDHA, and PKM2, downstream small molecules of HIF-1α affecting glycolysis (Fig. 3L-O). These results indicate that hepaCAM may downregulate HIF-1α via NF-κB signaling.

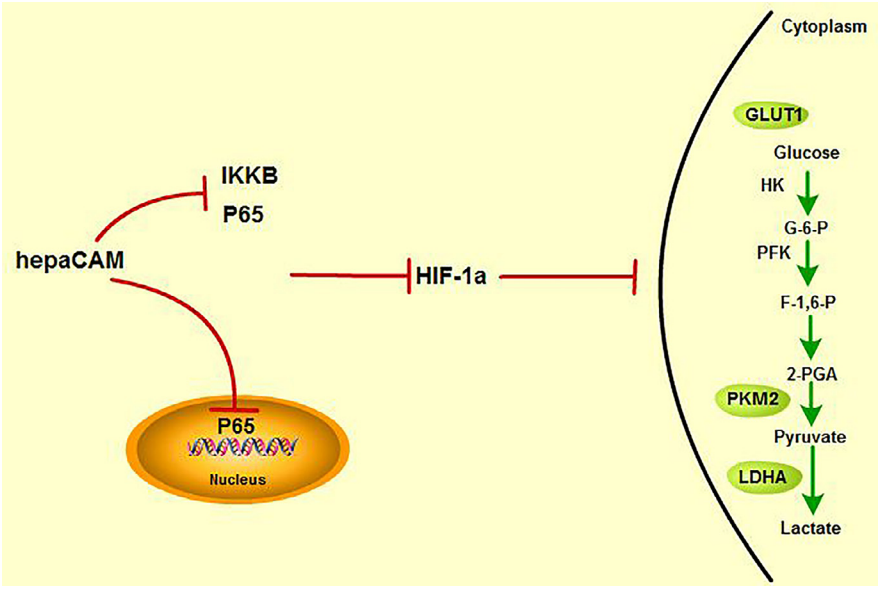

Figure 4. A model depicts the mechanism of hepaCAM-inhibiting RCC glycolysis. HepaCAM inhibits HIF-1α expression by NF-κB signaling and reduces the expression of glycolysis correlative proteins GLUT1, PKM2, and LDHA. RCC, renal cell carcinoma. (Color version available online.)

We next analyzed whether hepaCAM affected HIF-1α transcriptional activity through the NF-κB pathway. We constructed 786-O cells stably expressing firefly luciferase with HRE-FLuc and a Renilla luciferase (internal control) reporters. As expected, dual luciferase reporter analysis showed that overexpression of hepaCAM reduced HRE-mediated transcription, which was significantly repressed by combining the Ad-hepaCAM and BAY11-7082 treatments (Fig. 3P). Collectively, these results indicate that hepaCAM can interdict HIF-1α synthesis through NF-κB signaling (Fig. 4).

DISCUSSION

Proliferating tumor cells must produce the energy needed to support rapid cell division. Thus, they reprogram their metabolic processes to meet these needs. The Warburg effect is the most representative metabolic phenotype in tumor cells.[18,16] Furthermore, glycolytic metabolism appears to have evolved as an adaptation to hypoxia during the early stage of tumor vascular development, as it allows ATP production under hypoxic conditions.

HIF-1α plays a significant role in regulating tumor metabolism. High HIF-1α expression is connected with mortality of various cancers such as bladder, breast, colon, and prostate.[19] HepaCAM expression is low or lost in various carcinomas and tumor cell lines. Our previous research demonstrated a decrease of hepaCAM in urologic malignancies.[5-8,20] The present study provides a more detailed and comprehensive account of those findings. While it is clear that hepaCAM serves as a fundamental tumor suppressor role in the progression of RCC, the mechanisms underpinning this are unclear. Our data showed higher HIF-1α expression in RCC than in adjacent normal tissues, and a significant inverse correlation between hepaCAM (decrease) and HIF-1α (increase) in RCC tissues.

HIF-1α promotes tumor survival, growth, and metastasis.[21-23] Specifically, HIF-1α provides growth advantages for tumors in the absence of oxygen by increasing the transcription of genes encoding several glycolytic enzymes, and GLUT1 and GLUT3.[11] We demonstrated that re-expression of hepaCAM led to altered energy metabolism of RCC cells and significantly reduced the mRNA and protein expression of HIF1α and all target genes regulating glycolysis in cultured RCC cell lines. Our data strongly indicate that hepaCAM inhibits glycolytic metabolism by HIF1α in RCC cells.

A considerable amount of evidence shows that AKT and mammalian target of rapamycin (mTOR) can regulate HIF-1α mRNA, protein, and activity.[24-26] Our previous studies showed that hepaCAM reduced proliferation of RCC and bladder cancer cells by inhibiting c-myc and mTOR/AMPK activation, respectively.[7,8] Other studies revealed that mTORC1 and NF-κB were capable of inducing translation and transcription of HIF-1α, respectively.[17,27,28] However, the molecular mechanism of the interaction between hepaCAM and HIF-1α remained to be clarified.

We hypothesized that hepaCAM suppressed the Warburg effect by regulating HIF-1α through the NF-κB pathway. Importantly, we found that overexpression of hepaCAM reduced IKKβ and P65 protein expression and blocked NF-κB nuclear translocation in RCC cells. Furthermore, we demonstrated that combined overexpression of hepaCAM and treatment with BAY11-7082 impaired NF-κB/HIF-1α signaling, and the transcriptional activation of HIF-1α was strongly attenuated in 786-O cells by this combined treatment. Overall, our data reveal that hepaCAM acts as a potent inhibitor of the Warburg effect, reducing glucose consumption and the production

of lactic acid in RCC cells by altering NF-κB/HIF-1α signaling pathway.

In summary, this study reveals a novel mechanism of action for hepaCAM in regulating processes involved in tumor progression, such as glycolysis, which supplies ATP for rapid tumor growth. It will be beneficial to explore the molecular mechanisms underlying tumorigenesis by further investigating the relationship among hepaCAM, NF-κB, and HIF-1α.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.1016/j.urology.2018.11.033.

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