

Recombinant Viral Capsid Protein L2 (rVL2) of HPV 16 Suppresses Cell Proliferation and Glucose Metabolism via ITGB7/C/EBP β Signaling Pathway in Cervical Cancer Cell Lines

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Purpose: Capsid protein L2 is the minor capsid protein of human papillomavirus 16 (HPV16). Although L2-based vaccines were developed, the therapeutic effect of recombinant viral capsid protein L2 (rVL2) was still to be illustrated.

Methods: We used glucose uptake and lactate production assay to verify the inhibitory effect of rVL2 on the glucose metabolism in cervical cancer cells. Secondly, we performed gene-chip assay, RT-PCR, and Western blot to determine the role of ITGB7/C/EBP β signaling pathway in rVL2-mediated glucose metabolism in vitro. Finally, we used an animal model to verify the function of rVL2 in cervical cancer.

Results: We found that rVL2 reduced glucose uptake and lactate production levels in cervical cancer cells, which caused the inhibition of cell proliferation. rVL2 decreased the expression levels of key metabolic enzymes, including GLUT1, LDHA, and ALDOA, to affect cell metabolism in cervical cancer cells by inhibiting ITGB7/C/EBP β signaling pathway in vitro and in vivo.

Conclusion: These results demonstrated the vital role of rVL2 in the glycolysis-induced cell growth and proliferation via suppressing ITGB7/C/EBP β signaling axis.

Keywords: recombinant viral capsid protein L2, rVL2, C/EBP β , glucose metabolism, cervical cancer

Introduction

Human papillomavirus (HPV)-induced cervical cancer is one of the most frequent cancers in women of underdeveloped countries.¹ Nearly about one-third of the patients with cervical cancer experience recurrence,² and recurrence mostly occurs within 2 years of therapy completion.³ Hence, new therapeutic targets and treatment strategies remain needed.

HPV 16 is a kind of papillomavirus involved in approximately 60% of all cervical cancers worldwide.⁴ HPV is an enormous family, which is composed of the non-enveloped dsDNA viruses.⁵ Papillomaviruses have an 8Kd DNA genome, which contained 360 copies of the major capsid protein L1(VL1) and 12–36 copies of the minor capsid protein L2(VL2).^{6,7} VL1 forms pentamers that take for the majority of the capsid surface, while VL2 is mainly buried in the capsid, exposing the N-terminal residues on the surface.⁸ VL2 protects the viral genome and

promotes the cell attachment, internalization, and transport of the viral genome to the host cell nucleus to initiate viral replication.⁹ Previous studies have shown that VL2 had impact on several different cellular factors, including cellular proteins, nucleic acids, and membranes in different cellular compartments.^{10–14} Although VL2-based vaccines have been developed, the therapeutic effect of recombinant viral capsid protein L2 (rVL2) still remains illustrated.

Enhanced glucose uptake and aerobic glycolysis was one of the fundamental phenotypes of malignant tumors¹⁵ and was important for tumor relapse and chemoresistance.¹⁶ In the previous study, it has been reported that integrin $\beta 4$ (ITGB4) could promote lymph node metastasis and glucose metabolism of cervical cancer by activating the SNAI1/SIRT3 signaling pathway.¹⁷ In this study, we also found that rVL2 inactivated the integrin $\beta 7$ (ITGB7)/FAK signaling pathway to suppress the proliferation of cervical cancer cells. ITGB7 was expressed primarily by leukocytes¹⁸ and participated in various cell functions.¹⁹ ITGB7 has been reported to participate in the pathogenesis of several kinds of lymphoid malignancies, such as lymphomatous polyposis in mantle cell lymphoma,²⁰ thymic lymphoma,²¹ and mucosa-associated T- and B-cell non-Hodgkin lymphomas.²² However, its regulatory functions in cervical cancer remain investigated.

CCAAT/enhancer binding protein-beta (C/EBP β) is one member of the C/EBPs family and affected key biological processes including cell proliferation and tumor invasiveness.²³ In previous studies, C/EBP β was found to induce metastatic gene expression in prostate cancer cells.²⁴ Meanwhile, C/EBP β was also found to be a potential target for colorectal cancer treatment via suppression of miR-223.²⁵ However, the role of C/EBP β in cell metabolism remains illustrated.

In this study, we detected the inhibitory effect of rVL2 on glucose uptake and lactate production in cervical cancer cells. We demonstrated that rVL2 suppressed glucose metabolism in cervical cancer cells via inhibiting ITGB7/C/EBP β signaling axis.

Materials and Methods

Cell Lines and Cell Culture

Siha and Caski are human cervical cancer cell lines, which were purchased from the Cell Bank of the Chinese Academy of Science. Siha and Caski cells were cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone, Thermo Scientific, Waltham, MA) and 10% fetal bovine serum (Gibco, Life Technologies, Carlsbad, CA), 100 U/mL

penicillin (Biowest, Nuaillé, France), and 100 U/mL streptomycin (Biowest, Nuaillé, France) were supplemented. These cells were incubated at 37°C in a humidified atmosphere with 5% CO₂.

Purification of Recombinant L2 Protein

HPV16 capsid protein L2 is localized to punctate nuclear regions identified as promonocytic leukemia protein oncogenic domains (PODs). Recombinant viral capsid protein L2 was manufactured as peptide synthetically and purchased from Yunmai Company (Shanghai, China), with a concentration of 5 μ g/uL. The recombinant viral capsid protein L2 is diluted with PBS at a proportion of 1:1.

Plasmid Construction and Cell Transfection

pLKO/shC/EBP β -1 or 2 was constructed according to the previously reported method.²⁶ The control vector was similarly constructed by directly inserting oligonucleotides encoding short hairpin RNA against green fluorescence protein mRNA (shGFP) into the pLKO.1 vector. The cDNA sequences of ITGB7 was sub-cloned into pcDNA3.1 (–) expression vector (Invitrogen, Carlsbad, CA, USA) to construct recombinant plasmids pcDNA3.1 (–)-ITGB7, which was transfected into Siha and Caski cells according to the manufacturer's instructions.²⁶

RNA-seq Data Analysis

RNA-seq was performed as described previously.²⁶ Gene Set Enrichment Analysis (GSEA) was used for gene functional annotation.

Glycolysis Analysis

Glycolysis analysis was performed as previously described.²⁷

Immunohistochemistry Assay

The tissue microarray (TMA) was obtained from FUSCC Tissue Bank. Searches involving human participants in this study were approved by the Ethics Committee of FUSCC. Written informed consent was also approved and obtained from each participant, and each clinical investigation was conducted according to the principles expressed in the Declaration of Helsinki consent. IHC was performed on 7- μ m-thick TMA sections using the antibody against ITGB7 (ab95816, rabbit polyclonal antibody, Abcam, 1:100 dilution), p-FAK (Tyr 925) (ab38512, rabbit polyclonal antibody, Abcam, 1:100 dilution),

p-AKT1 (Ser 473) (ab81283, rabbit polyclonal antibody, Abcam, 1:100 dilution), and p-C/EBP β (#3084, rabbit polyclonal antibody, Cell Signal Technology, 1:100 dilution). Each case contained two cores made from separate sources to preclude the heterogeneity of tumors. A known positive case sample was regarded as a positive control, and the primary antibody used non-immune mouse/rabbit serum for negative control. The immunoreactive score (IRS) was multiplicity of the staining intensity and positive cancer percentage. Finally, the assessment of the protein expression was defined as negative ($\leq 1+$) and positive (>2 to $\leq 3+$).

Cell Viability and Colony Formation Assay

Cell line sensitivity to the indicated treatment was determined through Cell Viability Assay described previously.²⁶

Western Blot Analysis

The expression levels of various proteins in Siha and Caski cells were determined by Western blot analysis as previously described.²⁶ Antibodies to GLUT1(21829-1-AP), LDHA (19987-1-AP), and ALDOA (11217-1-AP) were acquired from Proteintech TM. Antibodies to ITGB7, FAK, p-FAK (Tyr 925), AKT1, p-AKT1 (Ser 473), C/EBP β , and p-C/EBP β (Thr 235) were from Abcam. The antibody to β -actin (A5441) was obtained from Sigma-Aldrich.

In vivo Tumor Growth Assay

Animal experiments were permitted by the Ethics Committee at FUSCC and performed as previously described.²⁶ All animal experiments were approved by the Institutional Animal Care and Use Committee of Fudan University and performed according to institutional guidelines. When an average tumor volume reached 100 mm³, mice were randomized into groups (n = 5). Then, they were intraperitoneally treated with rVL2 (10 mg/kg) or PBS as the control thereafter. Administration of vehicle or agents and measurement of tumor growth with a digital caliper were performed once every other day. After treated with or without rVL2, all the mice were subjected to perform positron emission tomography/computed tomography (PET/CT) scan and were evaluated by the standard uptake value (SUV). At last, the xenograft tumors were dissected and weighed 1 week after the last rVL2 injection. The expression levels of the genes in xenograft tumor were detected by qRT-PCR assay.

Statistical Analysis

SPSS software (version 18.0) was used for statistical analysis. Comparing quantitative data used with Student's *t*-test or ANOVA analysis. $P < 0.05$ (two-tailed) was regarded as statistically significant.

Results

rVL2 Inhibits Cell Proliferation and Glycolysis in Cervical Cancer Cell Lines

Siha and Caski cells were treated with different concentrations of rVL2 for 24 hours (Figure 1A). In each cell lines, rVL2 treatment significantly reduced cell viability in a dose-dependent manner ($P < 0.05$, Figure 1A). The half-maximal inhibitory concentration (IC₅₀) values of rVL2 on Siha and Caski cells were 0.065 μ M and 0.081 μ M, respectively.

By the gene-chip analysis, we found that the genes related to oxidative phosphorylation and glycolysis were enriched in cells treated with rVL2 (Figure 1B) and were suppressed by rVL2 treatment (Figure 1C and D), which were confirmed by the results of qRT-PCR assay shown in Figure 1E and F. Subsequently, we used glucose uptake and lactate production assays to explore the impact of rVL2 on cell glycolysis in cervical cell lines. We found that rVL2 effectively reduced the levels of glucose uptake and lactate production in Siha and Caski cells, which were consistent with the results of colony formation assays (Figure 2G and H). The results of Western blotting assay showed that the expression levels of GLUT1, LDHA, ALDOA were downregulated by rVL2 (Figure 2I).

rVL2 Inhibits Glucose Metabolism by Inducing the Expression of ITGB7/FAK/AKT1 Signaling Pathway

We further found that the genes participated in the integrin pathway were enriched in cells treated with rVL2 (Figure 3A), and the results of Western blotting indicated that rVL2 decreased the expression levels of ITGB7, FAK, p-FAK (Tyr 925), AKT1, p-AKT1 (Ser 473), C/EBP β , and p-C/EBP β (Thr 235) in Siha and Caski cells (Figure 3B). To confirm the role of ITGB7 in rVL2-induced glycolysis inhibition, we introduced ITGB7 cDNA into Siha and Caski and treated with rVL2 (50 μ M). As shown in Figure 3C–F, the over-expression of ITGB7 rescued the function of rVL2 on

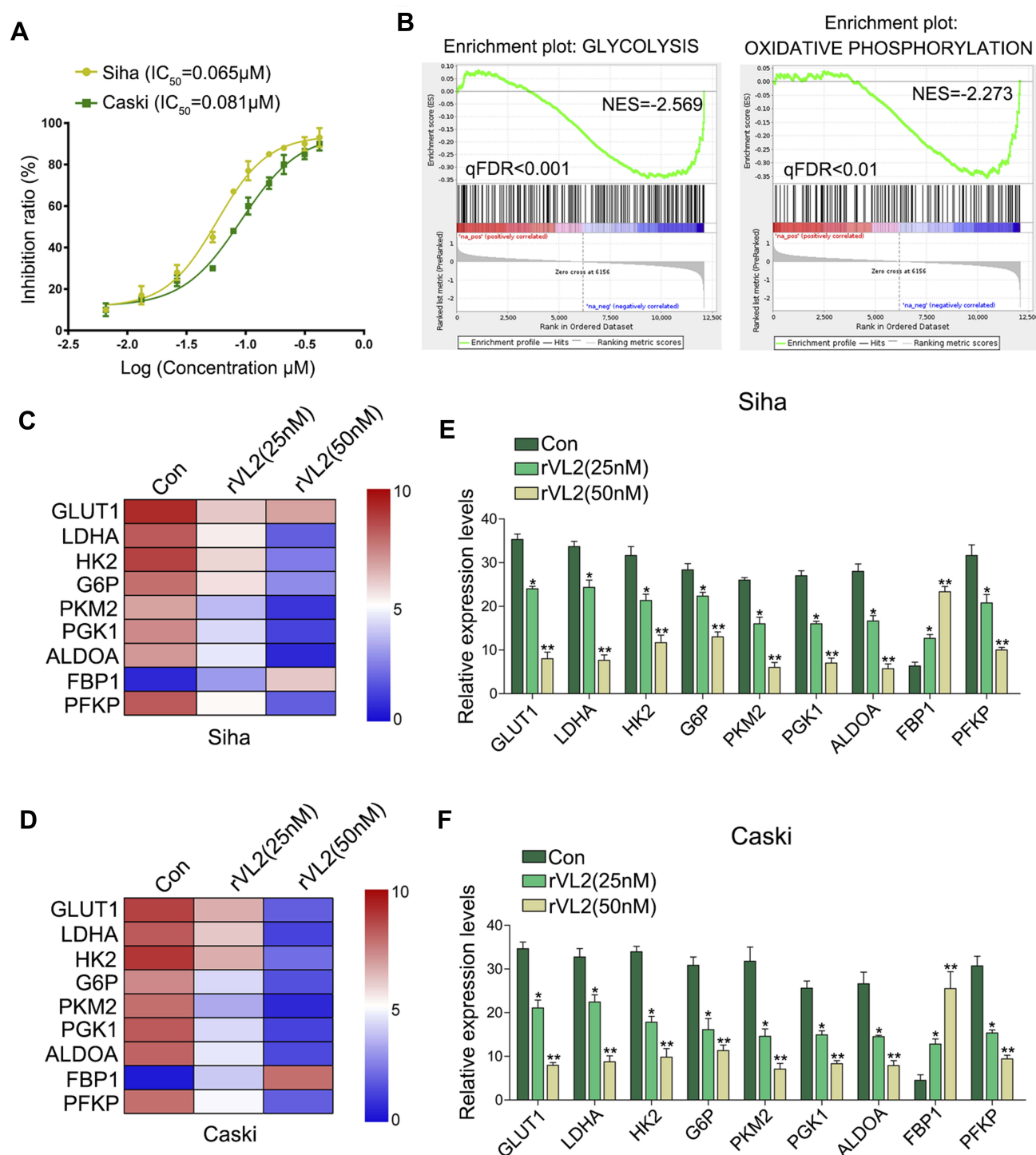


Figure 1 Recombinant viral capsid protein L2 (rVL2) suppresses cell proliferation by inhibiting cell glycolysis. **(A)** rVL2 inhibited cell viability in a dose-dependent manner in Siha and Caski cells detected by CCK-8 kit. **(B–D)** Analysis of cell function regulated by gene-chip assay. **(E and F)** rVL2 decreased the mRNA levels of GLUT1, LDHA, HK2, G6P, PKM2, PGK1, ALDOA, and PFKP detected by qRT-PCR in Siha and Caski cells treated by rVL2 (* $P < 0.05$, ** $P < 0.01$).

glucose uptake, lactate production, ATP production, and NADPH production levels in both cell lines. The expression levels of ITGB7, p-FAK (Tyr 925), p-AKT1 (Ser 473), and p-C/EBP β (Thr 235) were rescued by

the introduction of ITGB7 cDNA in Siha and Caski cells treated by rVL2 (Figure 3G). Taken together, rVL2 might regulate the glucose metabolism through ITGB7/FAK/AKT1/C/EBP β signaling pathway.

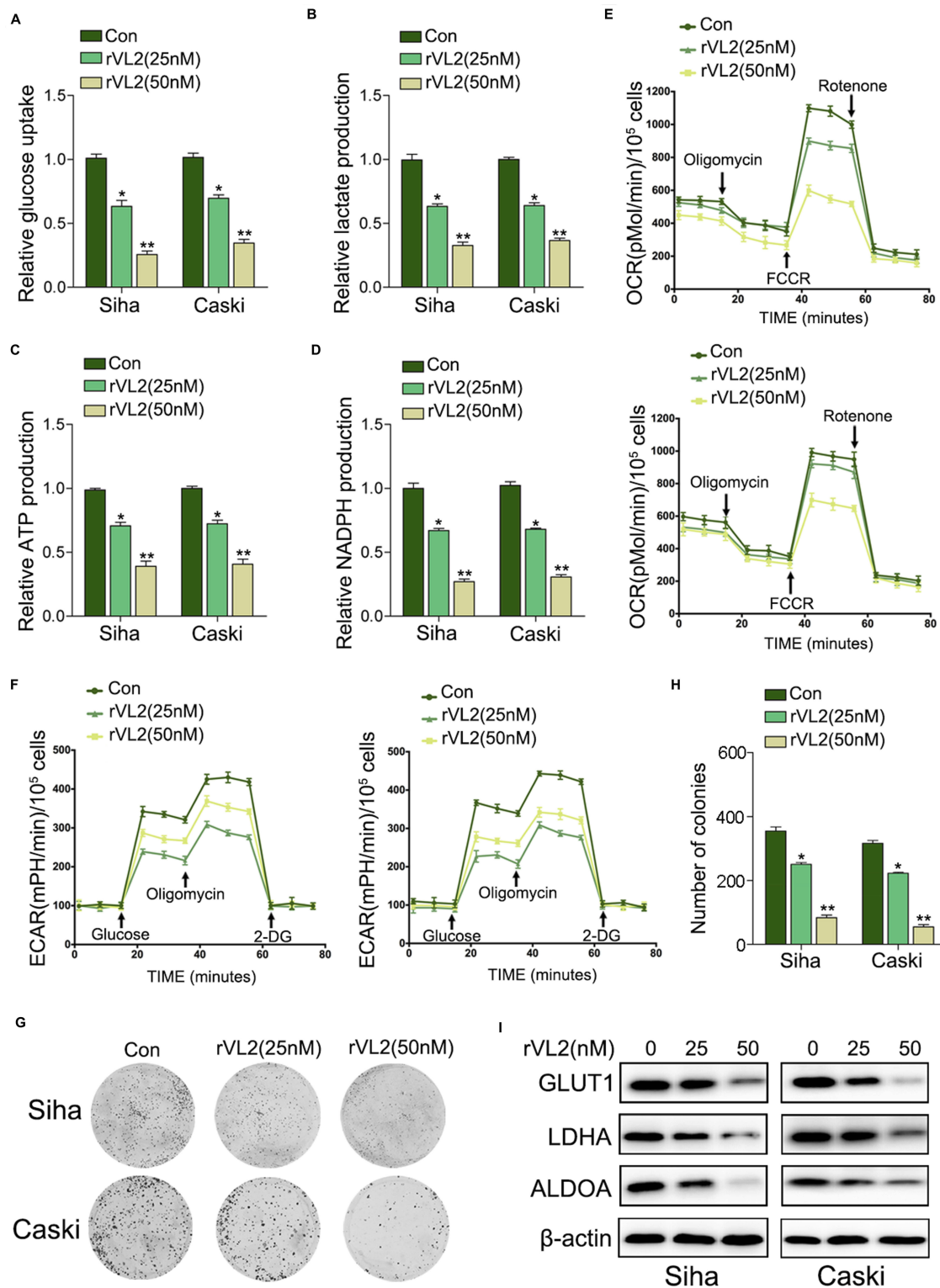


Figure 2 rVL2 suppresses cell proliferation by inhibiting cell glycolysis. **(A)** The treatment of rVL2 reduced the glucose uptake levels in Siha and Caski cells, compared with the corresponding controls (* $P < 0.05$, ** $P < 0.01$). **(B)** Lactate production was reduced by the treatment of rVL2 in Siha and Caski cells when compared to controls (* $P < 0.05$, ** $P < 0.01$). **(C)** The treatment of rVL2 decreased the ATP production in Siha and Caski cells, compared with their controls (* $P < 0.05$, ** $P < 0.01$). **(D)** NADPH production was reduced by the treatment of rVL2 in Siha and Caski cells when compared to controls (* $P < 0.05$, ** $P < 0.01$). **(E)** OCR analysis in Siha and Caski cells treated with rVL2 compared with controls. **(F)** ECAR analysis in Siha and Caski cells treated with rVL2 compared with controls. **(G–H)** The results of colony formation assay exhibited that rVL2 inhibitory effect on colony formation ability was obviously weakened by the treatment of rVL2 when compared to controls (* $P < 0.05$, ** $P < 0.01$). **(I)** The results of Western blot exhibited that protein expression level GLUT1, LDHA, and ALDOA were downregulated by treatment of rVL2 (* $P < 0.05$, ** $P < 0.01$).

rVL2 Inhibits the Promoter Activity of GLUT1, LDHA, and ALDOA by Suppressing the ITGB7/C/EBP β Signaling Pathway in Cervical Cancer Cells

As C/EBP β was an important transcriptional factor in tumorigenesis, we inferred that C/EBP β might regulate the promoter activity of the genes involved in the glucose metabolism. To verify our hypothesis, we knocked down the expression level of C/EBP β in Siha/ITGB7 OE and Caski/ITGB7 OE cell lines. Silencing of C/EBP β decreased mRNA expression level (Figure 4A) and promoter activity (Figure 4B) of GLUT1, LDHA, and ALDOA in Siha/ITGB7 OE and Caski/ITGB7 OE cell lines treated by rVL2, compared with their controls. Furthermore, we found that the knockdown of C/EBP β directly reduced the mRNA expression levels (Figure 4C) and promoter activity (Figure 4D) of GLUT1, LDHA, and ALDOA in Siha cells.

rVL2 Regulates Cell Proliferation and Glycolysis of Cervical Cancer Cells *in vivo*

We further established subcutaneous tumor formation by injecting Siha cells into nude mice. We found that rVL2 obviously retarded the growth of xenograft tumor, compared to control group (Figure 5A–C). We also found that rVL2 effectively decreased the glucose uptake levels in xenograft tumors (Figure 5D). The results of immunohistochemical staining performed that the expression levels of ITGB7, p-FAK (Tyr 925), p-AKT1 (Ser 473), and p-C/EBP β (Thr 235) in xenograft models were decreased (Figure 5E). Furthermore, we found the expression of GLUT1, LDHA, HK2, G6P, PKM2, PGK1, ALDOA, and PFKP were reduced in xenograft tumors treated by rVL2, while the expression level of FBP1 was enhanced (Figure 5F).

Discussion

In this study, we demonstrated that rVL2 decreased glucose metabolism via ITGB7/C/EBP β signaling pathway to inhibit cervical cancer cell proliferation, which supplied the new evidence on the treatment of cervical cancer. The growing studies have determined the vital role of rVP1 in the tumorigenesis in various cancers. For example, rVP1 was found to suppress the progression of hepatocellular carcinoma^{28,29} and cell metastasis of ovarian cancer cells.³⁰ Nowadays, the virus-like particles (VLPs) based on L2 proteins have been regarded as a potential candidate

for vaccine development against HPV infections.³¹ In the present study, we demonstrated that rVL2 had the anti-cancer effects on cervical cancer cells in a dose-dependent manner and further found that rVL2 reduced cell glycolysis level in cervical cancer cells by inhibiting ITGB7/C/EBP β signaling pathway, which explored the new insight of rVL2 in the cancer treatment.

Integrins are principal cell surface receptors responsible for cell extracellular matrix interactions, which are formed by associations of α and β dimers. Integrins are involved in a variety of biological processes associated with cancer development, such as migration, invasion, differentiation, proliferation, and survival. Taken example, integrin β 4 (ITGB4) was detected as a novel prognostic factor in colon cancer,³² integrin β 1 (ITGB1) functioned on the cell stemness in head and neck squamous cell carcinoma (HNSCC),³³ and integrin α 7 (ITGA7) promoted cell proliferation and was related to poor prognosis and survival profiles in non-small-cell lung cancer (NSCLC).³⁴ Our results identified that rVL2 exerted its anti-cancer effects through the suppression of ITGB7-induced signaling pathway, which further confirmed the key role of integrins in the tumorigenesis in various cancers.

C/EBP β is a kind of transcription factor regulating critical biological processes including cellular growth and tumor invasiveness.²⁴ C/EBP β upregulation promoted tumor cell invasion in an MMP3-dependent manner and was associated with metastasis in esophageal squamous cell carcinoma (ESCC).³⁵ Compared with previous studies, our results revealed the new evidence that C/EBP β may be a downstream target for ITGB7, which further affected the expression of proteins linked to glycolysis, such as GLUT1, LDHA, and ALDOA, which further determined that the regulatory function of rVL2 on cell proliferation by suppressing glycolysis in cervical cancer. Therefore, our results provided new data to clarify the therapeutic mechanism of rVL2 in cancer cells.

Despite the significant developments in detection and therapy of cervical cancer during the past decades, cervical cancer still remains a high rate of mortality due to the drug resistance.² In the past years, chemoresistance and tumor relapse have been found to be related to the enhanced glucose uptake and aerobic glycolysis.¹⁶ For instance, glucose transporter 1 (GLUT1) has been determined to promote the malignant phenotype of non-small cell lung cancer through Integrin β 1/Src/FAK Signaling pathway.³⁶ In the present study, the expression levels of GLUT1, LDHA, and ALDOA were decreased by the treatment of

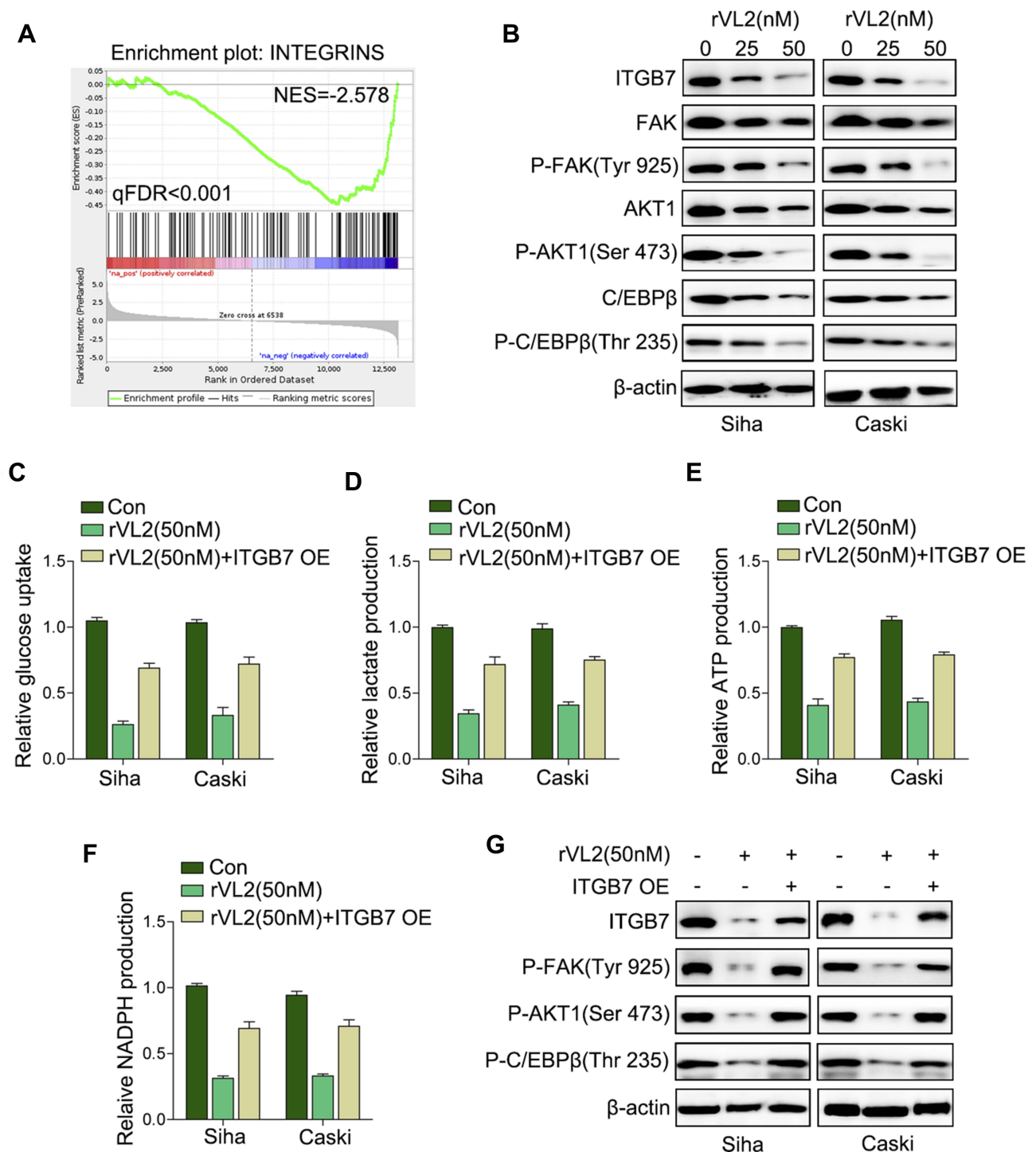


Figure 3 rVL2 regulates glucose metabolism by suppressing ITGB7/C/EBP β signaling pathway. **(A)** Analysis of cell function regulated by gene-chip assay. **(B)** The results of Western blot exhibited that ITGB7, p-FAK, AKT1, p-AKT1, C/EBP β and p-C/EBP β protein expression levels were downregulated by treatment of rVL2. **(C–G)** ITGB7 overexpression rescued the induction of rVL2 on the glucose uptake, lactate production, ATP production, and NADPH production detected by real-time PCR and Western blot.

rVL2. rVL2 could inhibit the transcription of GLUT1, LDHA, and ALDOA by suppressing the ITGB7/C/EBP β signaling pathway in cervical cancer cells. These findings revealed the new effect of rVL2 on cell proliferation and drug resistance in cancer cells.

In sum, we identified a vital role for rVL2 in glucose metabolism and cell proliferation. Our data also provided the new mechanistic insight into the effect of rVL2/ITGB7/C/EBP β signaling axis in the progression of cervical cancer cells. Therefore, rVL2 may warrant consideration as

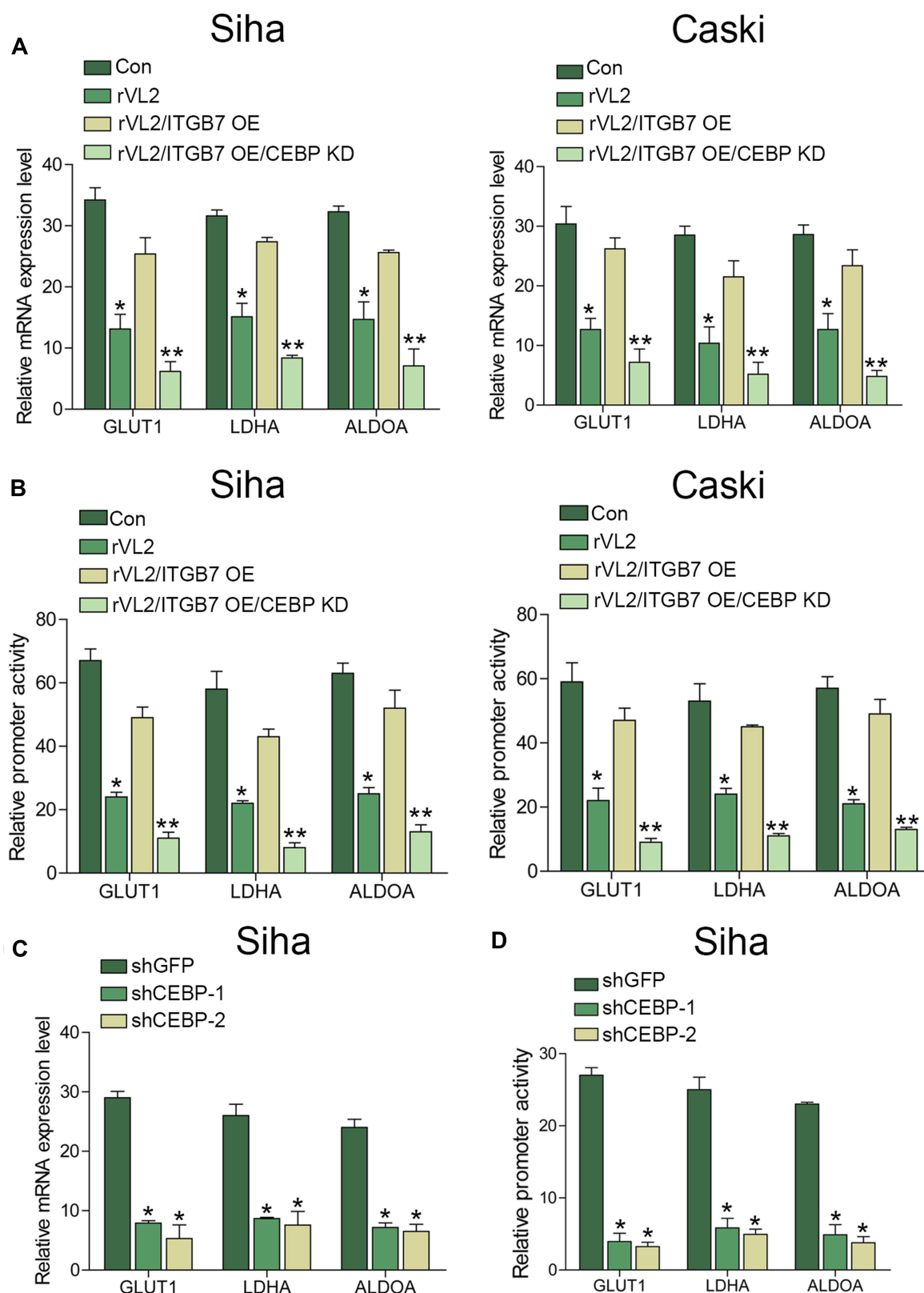


Figure 4 rVL2 inhibits the promoter activity of GLUT1, LDHA, and ALDOA by inhibiting C/EBP β in cervical cancer cells. **(A)** Knockdown of C/EBP β reduced the mRNA expression levels in Siha/ITGB7 OE and Caski/ITGB7 OE cells treated with rVL2. (* $P < 0.05$, ** $P < 0.01$). **(B)** Knockdown of C/EBP β reduced the promoter activity in Siha/ITGB7 OE and Caski/ITGB7 OE cells treated with rVL2. (* $P < 0.05$, ** $P < 0.01$). **(C and D)** Silencing effect of C/EBP β with shRNAs was detected by real-time PCR. * $P < 0.05$.

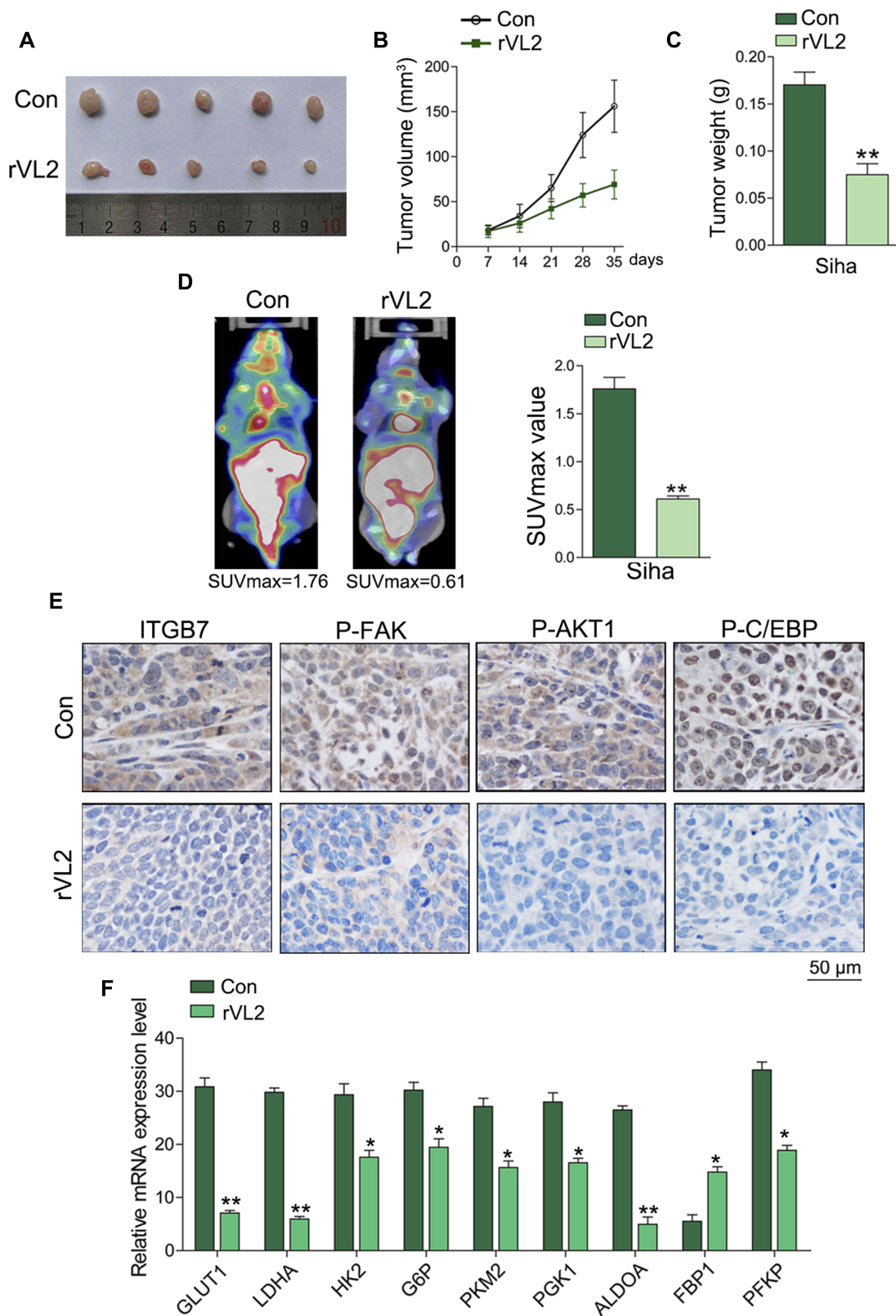


Figure 5 rVL2 inhibits cell proliferation and glycolysis of cervical cancer cells in vivo. **(A)** Treatment with rVL2 slowed the speed of tumor growth in vivo ($P < 0.05$). **(B and C)** The tumor volume and weight in the experimental group were significantly lower than those in control group ($**P < 0.01$). **(D)** rVL2 can significantly suppress the glucose uptake in cervical cancer in vivo and resulted in a lower SUVmax value ($**P < 0.01$). **(E)** Immunohistochemical analyses of ITGB7, p-FAK, p-AKT1, and p-C/EBP β expression in tissues. **(F)** The expression levels of GLUT1, LDHA, HK2, G6P, PKM2, PGK1, ALDOA, FBP1, and PFKF were detected by real-time PCR in xenograft tumors and the corresponding controls ($*P < 0.05$, $**P < 0.01$).

a predictive agent for the therapy of cervical cancer patients in prospective clinical studies.

Abbreviations

rVL2, recombinant viral capsid protein L2; C/EBP β , CCAAT/enhancer-binding protein β ; ITGB2, integrin β 2; GLUT1, glucose transporter protein-1; LDHA, lactate dehydrogenase A; HK2, hexokinase 2; G6P, glucose-6-phosphatase; PKM2, pyruvate kinase isozymes M2; PGK1, phosphoglycerate kinase 1; ALDOA, aldolase A, fructose-bisphosphate; PFKP, platelet phosphofructokinase.

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Disclosure

The authors report no conflicts of interest in this work.

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