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CDK2 positively regulates aerobic glycolysis by suppressing SIRT5 in gastric cancer

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Although significant progress has been made in the diagnosis and treatment of gastric cancer, the overall survival rate of the disease remains unchanged at approximately 20%-25%. Thus, there is an urgent need for a better understanding of the molecular biology aspects of the disease in the hope of discovering novel diagnosis and treatment strategies. Recent years have witnessed decisive roles of aberrant cancer cell metabolism in the maintenance of malignant hallmarks of cancers, and cancer cell metabolism has been regarded as a novel target for the treatment of cancer. CDK2, a cell cycle-dependent kinase that usually regulates cell cycle progression and the DNA damage response, is reported to be upregulated in many cancers. However, little is known about its role in cancer cell metabolism. In the present study, we showed that silencing CDK2 inhibited the aerobic glycolytic capacity of gastric cancer cell lines. Mechanism explorations showed that silencing CDK2 increased expression of the SIRT5 tumor suppressor. In addition, the physiological roles of SIRT5 in the regulation of proliferation and glycolysis were studied in gastric cancer cells. Taken together, the present study uncovered novel roles of the CDK2/SIRT5 axis in gastric cancer and suggests future studies concerning gastric cancer cell metabolism.

KEYWORDS
CDK2, CDK2/SIRT5, gastric cancer, glycolysis, SIRT5

1 | INTRODUCTION

Gastric cancer is one of the most common types of cancer and ranks second in cancer-related deaths worldwide.[1] Although significant progress has been made in the diagnosis and treatment of this cancer, only modest progress has been made in improving gastric cancer-related mortality.[2] Furthermore, estimates suggest that the gastric cancer-related death rate will continue to increase.[3] Thus, there is an urgent need to identify novel biomarkers that could be used to predict prognosis and elucidate the underlying molecular mechanisms.[4]

Ninety years ago, Otto Warburg published a series of works linking metabolism and cancer through enhanced aerobic glycolysis (also known as the Warburg effect) that distinguishes cancer from normal tissues.[5] With further studies, aberrant cancer cell metabolism came to be regarded as one of the hallmarks of cancer.[6] It is known that solid tumors reside in a microenvironment with limited oxygen and nutrient supplies and that to survive in such a hostile microenvironment, tumor cells must shift their metabolism pattern.[7] The best characterized cancer cell metabolism process is glucose metabolism. Under hypoxia conditions, cancer cells shift their metabolism to glycolysis. From the ATP generation aspect, the process of glycolysis is not highly efficient because only 2 ATP are generated. However, through glycolysis,

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cancer cells use glucose to form building blocks for macromolecule synthesis.[8] Furthermore, lactic acid can be produced by glycolysis, and accumulated lactic acid can cause an acidic microenvironment. The extracellular matrix becomes quite unstable under acidic conditions, facilitating the metastasis of cancer cells.[9,10] This information led to the hypothesis that targeting cancer cell metabolism might aid in the discovery of novel strategies against cancer.[11]

It is well accepted that nearly all cancers bear mutations that disrupt cell cycle control, leading to uncontrolled proliferation of cancerous cells. Moreover, targeting cell cycle regulators has shown promise in the treatment of cancers. For example, targetingCDK4/6 in the treatment of cancer, such as breast cancer, has been shown to be successful.[12,13] However, attempts to target CDK2, an important gene that controls DNA replication, DNA damage and quiescence, have been less successful[14,15]. Thus, there exists the need for a better understanding of malignant properties that are under the control of CDK2 in the hope of discovering novel and efficient treatment strategies. As described above, constitutively proliferating cancer cells require a sustained supply of metabolites. Thus, it is perceivable that CDK2 might regulate cancer cell metabolism to meet this demand. However, few relevant studies have been reported. Thus, in the present study, we sought to uncover the roles of CDK2 in gastric cancer cell metabolic reprogramming. Our results showed that CDK2 positively regulates aerobic glycolysis in gastric cancer cells. Additionally, mechanism studies indicated that CDK2 might regulate SIRT5, a tumor suppressor and negative regulator of glycolysis. Collectively, our results uncovered novel mechanisms connecting a cancer cell proliferation regulator and metabolism in gastric cancer and may point the way towards the discovery of novel treatment strategies for gastric cancer.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Human gastric cancer cell lines MGC-803 and SCG-7901 (the Cell Bank of the Chinese Academy of Sciences, Shanghai, China). The cells were maintained according to standard protocols provided by the manufacturer. In brief, SGC-7901 cells were cultured in RPMI-1640 medium containing FBS at a final concentration of 10%. MGC-803 cells were cultured in DMEM medium, with an FBS concentration of 10% and horse serum at a concentration of 2.5%. The cells were cultured in a humid incubator containing 5% CO_2 at 37°C.

2.2 | RNA extraction and quantitative PCR analysis

Total RNA was obtained by using TRIzol reagent (Invitrogen, Waltham, MA, USA). TaKaRa's PrimeScript RT Reagent Kit (TaKaRa, Tokyo, Japan) was used for reverse transcription to obtain cDNA. Expression of designated genes was determined by quantitative real-time PCR using the ABI-7500 real-time PCR system (Applied Biosystems, Waltham, MA, USA). All reactions were run in triplicate. The primer sequences are listed in Table 1.[16]

2.3 | Protein extraction and western blot analysis

Total cell lysates were obtained using RIPA buffer. In brief, cells were washed twice with ice-cold PBS and lysed in RIPA buffer supplemented with protease and phosphatase inhibitors for 10 minutes. Cell debris was removed by centrifugation at 33 984 g for 10 minutes at 4°C. Then, 20 μg whole cell lysate was subjected to electrophoresis in denaturing 10% SDS-polyacrylamide gel and then transferred to a membrane for subsequent blotting with specific antibodies. CDK2 and SIRT5 antibodies were obtained from Abcam, Cambridge, UK (ab32147 and ab108968).

2.4 | Lentivirus production and stable cell line selection

In order to silence CDK2 expression, the pLKO.1 TRC cloning vector (Addgene plasmid: 10878, Shanghai Sunrise Company, Shanghai, China) was used. Targets against CDK2 (21 bp) were 5′-CATTC-CAATCTATTGCTTCAC-3′ and 5′-TACTTCTATGCCTGATTACAA-3′. To overexpress SIRT5, pCDH-CMV-MCS-EF1-Puro (System Biosciences, California Bay Area, San Francisco, CA, USA) was used. Lentiviral particles were produced by cotransfection of pLKO.1-shCDK2 or pCDH-CMV-SIRT5-EF1-Puro expression constructs with lentivirus packaging vectors psPAX2 and pMD2.G into HEK-293T cells in a ratio of 4:3:1. Stable shRNA-expressing cell lines were obtained by infection of gastric cancer cells with lentivirus particles followed by puromycin selection.

TABLE 1 Primer sequences of genes examined in the present study

CDK2 forward	5′-GAGCCTGGGCTGCATCTTTGCTGA-3′
CDK2 reverse	5′-AGGGTCGTAGTGCAGCATTTGCG-3′
SIRT3 forward	5′-GGCGGCAGGGACGATTATT-3′
SIRT3 reverse	5′- CCCGAATCAGCTCAGCTACA-3′
SIRT4 forward	5′- CCGTAGAGCTGTGAGAGAATGA-3′
SIRT4 reverse	5′- ACCCAATGGAGGCTTTCGAG-3′
SIRT5 forward	5′-AGAGAGCTCGCCCACTGTGATTTA-3′
SIRT5 reverse	5′-AGGGTCCCTGGAAATGAAACCTGA-3′
GLUT1 forward	5′-TGTCGTGTCGCTGTTTGTGGTGGA -3′
GLUT1 reverse	5′-TGAAGAACAGAACCAGGAGCACAG -3′
HK2 forward	5′- TGATGTGGCTGTGGATGAGCT-3′
HK2 reverse	5′-GCCAGGCAGTCACTCTCAATCTG-3′
LDHA forward	5′- AGGCTGGGAGTTCACCCATTAAGC -3′
LDHA reverse	5′-GAGTCCAATAGCCCAGGATGTG -3′
PDK1 forward	5′-CTGCACCAAGACCTCGTGTTGAGA-3′
PDK1 reverse	5′-GGCAGCTTTGTTATACACTGGGAG-3′
β-actin forward	5′-CTACGTCGCCCTGGACTTCGAGC-3′
β-actin reverse	5′-GATGGAGCCGCCGATCCACACGG-3′

Expressions of designated genes were determined by quantitative real-time PCR. All reactions were run in triplicate.
GLUT1, glucose transporter 1; HK2, hexokinase 2; LDHA, lactate dehydrogenase A; PDK1, pyruvate dehydrogenase kinase 1.

2.5 | 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester cell proliferation assay

In order to measure the impact of CDK2 on cell viability and the proliferation of gastric cancer cells, the 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE) cell proliferation assay was carried out. In brief, cells cultured in 6-well plates were labeled using CFSE (Sigma-Aldrich, St Louis, MO, USA) at a final concentration of 5 μmol/L. Stained cells were measured using a flow cytometer (BD Biosciences, San Jose, CA, USA) at 488 nm excitation after 1, 2, 3, and 4 days separately. Data were analyzed using Flowing Software (University of Turku, Finland).

2.6 | Colony formation assay

Gastric cancer cells (5×10^2) stably expressing shRNA targets against CDK2, and the relevant control cells were seeded into 6-well plates. The cells were cultivated for 14 days, and then 4% paraformaldehyde was used to fix the cells. Cells were then stained with 1% crystal violet. Colonies were counted using bright field microscopy.

2.7 | Cell apoptosis measurement

To examine the impact of SIRT5 on apoptosis of gastric cancer cells, the Annexin V-FITC Apoptosis Detection Kit (Yeason, Shenzhen, Guangdong, China) was used. The assay was carried out according to protocols provided by the supplier.

2.8 | Glycolysis assessment

To examine the effect of CDK2 on aerobic glycolysis of gastric cancer cells, the Seahorse Bioscience XF96 Extracellular Flux Analyzer was used (Seahorse Bioxcience, Billerica, MA, USA). Cellular glycolysis capacity was measured by testing the extracellular acidification rate (ECAR). Mitochondrial respiration capacity was measured by testing the oxygen consumption rate (OCR). All the assays were carried out according to the manufacturer's protocols.

2.9 | Statistical analyses

Statistical analyses were carried out using SPSS software (version 17.0; IBM Corp., Armonk, NY, USA) using independent t tests (for continuous variables) and Pearson's χ^2 tests (for categorical variables). Statistical significance was based on 2-sided P-values of < 0.05.

3 | RESULTS

3.1 | CDK2 positively regulated aerobic glycolysis in gastric cancer

To observe the role of CDK2 in gastric cancer cell metabolism, we first silenced CDK2 expression in the gastric cancer cell lines SGC-7901 and MGC-803. Efficacy of the silencing effect was confirmed by western blot analysis (Figure 1A,B). Then, we assessed the impact of CDK2 silencing on the glycolysis rate. As shown, silencing CDK2 expression significantly inhibited ECAR levels, indicating that CDK2 is a positive regulator of aerobic glycolysis (Figure 1C,D). During the process of glucose metabolic reprogramming, mitochondrial respiration is impaired, as measured by OCR examination. In CDK2-silenced SGC-7901 and MGC-803 cells, we observed an increase in OCR values, which further suggested the positive role of CDK2 in aerobic glycolysis reprogramming (Figure 1E,F). In addition, we examined the impact of CDK2 knockdown on the expression of key glycolytic genes, including glucose transporter 1 (GLUT1), hexokinase 2 (HK2), lactate dehydrogenase A (LDHA) and pyruvate dehydrogenase kinase 1 (PDK1). Our quantitative real-time PCR results indicated that CDK2 knockdown resulted in a decrease in the expression of these genes (Figure 1G,H).

## 3.2 | CDK2 negatively regulated SIRT5 expression in gastric cancer cells

SIRT3, SIRT4 and SIRT5 are mitochondrially localized tumor suppressors and play negative roles in metabolic reprogramming. Thus, we assessed the impact of CDK2 on the expression status of SIRT3, SIRT4 and SIRT5. Quantitative PCR results suggested that CDK2 knockdown increased SIRT5 mRNA expression levels in SGC-7901 and MGC-803 cells (Figure 2A,B). Next, we measured the levels of SIRT5 in CDK2-silenced gastric cancer cells. Immunoblotting results showed that decreased CDK2 expression increased SIRT5 protein levels in SGC-7901 and MGC-803 cells (Figure 2C,D).

## 3.3 | SIRT5 inhibited gastric cancer cell proliferation

Although SIRT5 has been reported to play tumor-suppressive roles, little has been reported on its impact on gastric cancer cell proliferation. Thus, we first overexpressed SIRT5 in SGC-7901 and MGC-803 cells. Then, we carried out the CFSE proliferation assay, and the results indicated that SIRT5 negatively regulated cell viability in gastric cancer cells (Figure 3A,B). Next, we examined the influence of SIRT5 on colony formation capacity in gastric cancer cells. Overexpression of SIRT5 attenuated the colony formation capacity of SGC-7901 and MGC-803 cells (Figure 3E,G). We also tested the impact of SIRT5 on apoptosis of gastric cancer cells. Annexin V apoptosis experiment results indicated that overexpression of SIRT5 increased cell apoptosis of SGC-7901 and MGC-803 cells (Figure 3D,F).

3.4 | SIRT5 inhibited aerobic glycolysis in gastric cancer cells

As described above, CDK2 played positive roles in aerobic glycolysis, and SIRT5 was shown to be a possible downstream effector of CDK2. Thus, we assessed the impact of SIRT5 on aerobic glycolysis in gastric cancer cells. As shown, the overexpression of SIRT5 in SGC-7901 and MGC-803 cells inhibited the glycolysis rate, as seen
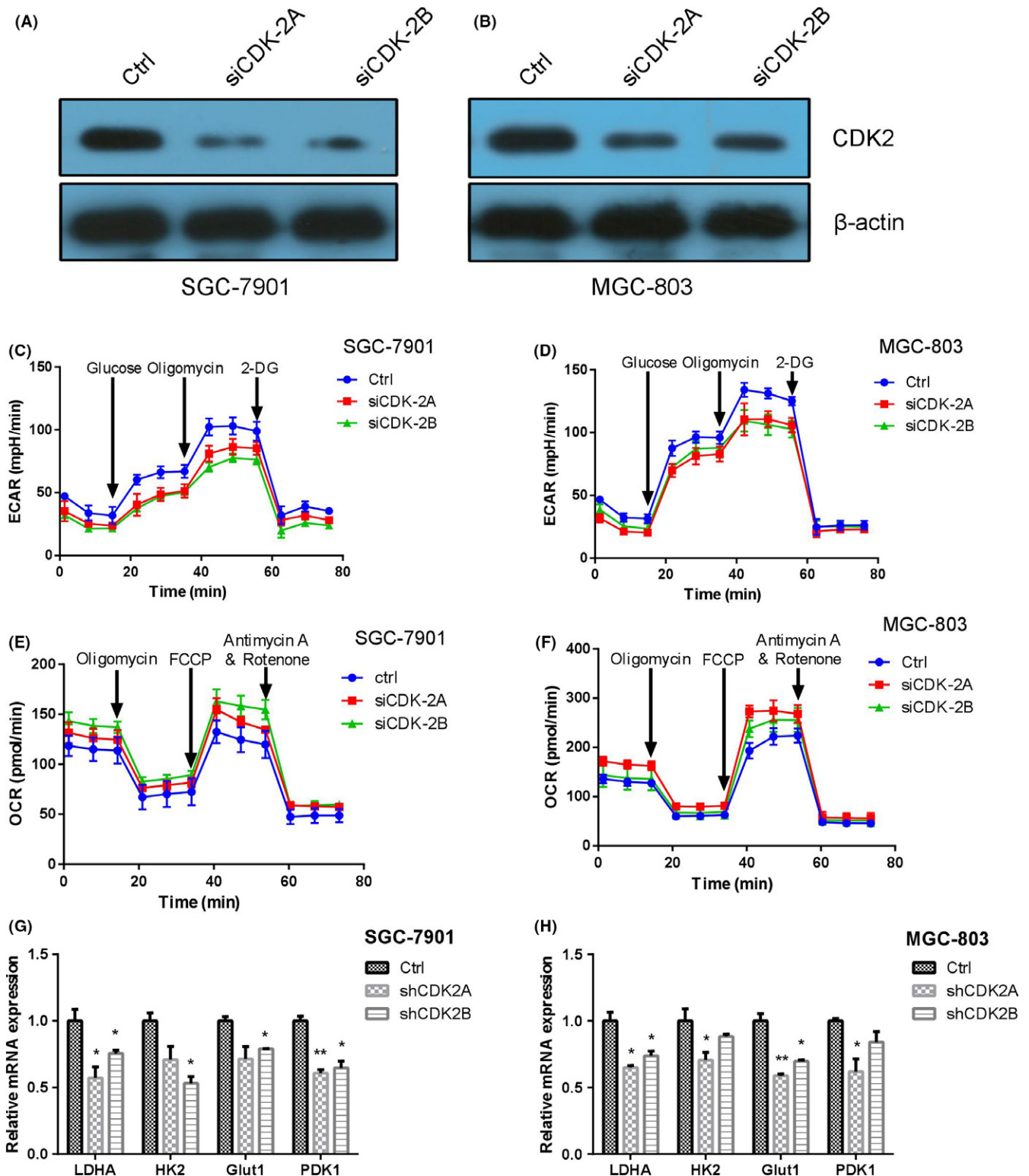
FIGURE 1   CDK2 positively regulates aerobic glycolysis in gastric cancer. A,B, CDK2 expression effectively silenced in SGC-7901 and MGC-803 cells as validated by immunoblotting analysis with CDK2 antibody. C,D, Impact of CDK2 on aerobic glycolysis was confirmed by extracellular acidification rate (ECAR) measurement using Seahorse Bioscience XF96 Extracellular Flux Analyzer (Seahorse Bioxcience), and silencing CDK2 expression inhibited ECAR values in SGC-7901 and MGC-803 cells. E,F, Oxygen consumption rate (OCR) values increased in CDK2 silenced gastric cancer cell lines, indicating that CDK2 played negative roles in mitochondrial respiration. Aerobic glycolysis is a multistep process, and glucose transporter 1 (GLUT1), hexokinase 2 (HK2), lactate dehydrogenase A (LDHA) and pyruvate dehydrogenase kinase 1 (PDK1) are key glycolytic genes. G,H, In CDK2 silenced SGC-7901 and MGC-803 cells, the expression status of these genes decreased. *$P < 0.05$; **$P < 0.01$
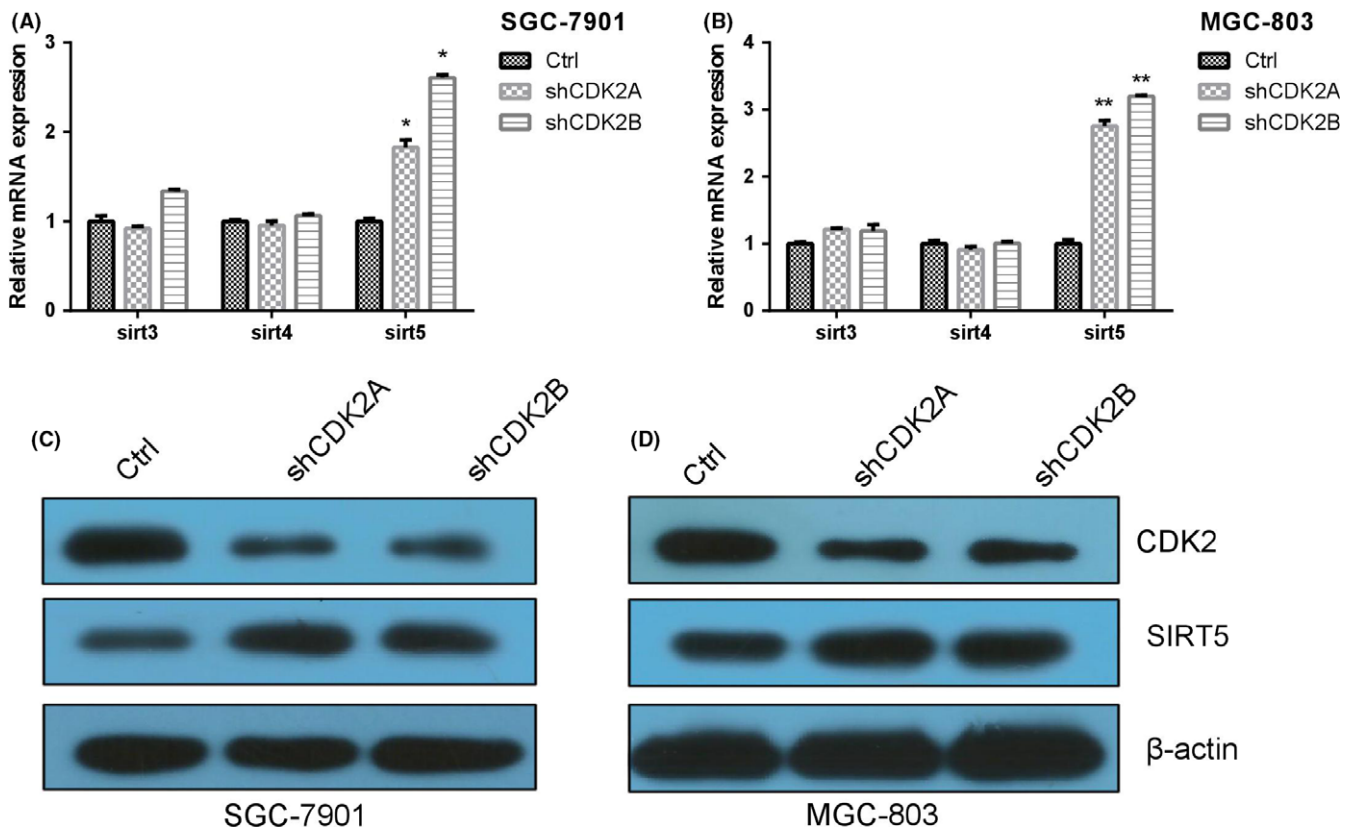
FIGURE 2 CDK2 negatively regulated SIRT5 expression in gastric cancer cells. A,B, To explore the molecular mechanism underlying CDK2 in glycolysis regulation, we examined the impact of CDK2 on expression status of mitochondrial sirtuin family members, and our results indicated that CDK2 silencing increased SIRT5 expression, but had only slight impact on SIRT3 and SIRT4 in SGC-7901 and MGC-803 cells, suggesting that SIRT5 might be a CDK2 target. C,D, Western blot analysis confirmed the impact of CDK2 silencing on SIRT5 expression, and SIRT5 protein levels increased in CDK2 knockdown of SGC-7901 and MGC-803 cells. $*P < 0.05$; $**P < 0.01$

by ECAR examination (Figure 4A,B). OCR measurement results showed that SIRT5 overexpression increased mitochondrial respiration capacity (Figure 4C,D). We also measured the impact of SIRT5 on the expression status of glycolysis genes, including GLUT1, HK2, LDHA and PDK1. Real-time PCR results showed that the introduction of SIRT5 into SGC-7901 and MGC-803 cells decreased the expression of these glycolysis genes (Figure 4E,F).

3.5 | SIRT5 inhibited the tumor formation capacity of gastric cancer cells

As observed above, SIRT5 inhibited cell proliferation and aerobic glycolysis in vitro in gastric cancer cells. However, little is known about its role in tumor formation. Thus, we assessed the impact of SIRT5 on the tumor formation capacity of SGC-7901 cells. We s.c. injected SIRT5-overexpressing SGC-7901 cells into nude mice. Growth curve measurements indicated that SIRT5 overexpression inhibited the tumor formation capacity of SGC-7901 cells (Figure 5A). Furthermore, SIRT5 overexpression inhibited tumor volume and tumor weight of SGC-7901 tumors (Figure 5B,C).

We demonstrated a novel function of the cell cycle regulator CDK2 in aerobic glycolysis in gastric cancer cells. Mechanism explorations indicated that the mitochondrially localized tumor suppressor

SIRT5 might be the downstream effector of CDK2 in aerobic glycolysis regulation. Furthermore, SIRT5 played negative roles in cell proliferation and glycolysis. Collectively, our results uncovered a novel axis of CDK2/SIRT5 in gastric cancer, a finding that might help the development of novel strategies for the treatment of gastric cancer (Figure 6).

4 | DISCUSSION

In the present study, we showed that the cell cycle regulator CDK2 played negative roles in regulating aerobic glycolysis in gastric cancer. Mechanism studies demonstrated that CDK2 regulated aerobic glycolysis by suppressing expression of SIRT5, a tumor suppressor and negative regulator of metabolism in cancer.

Cancerous cells harbor deficiencies in the cell cycle regulatory machine, leading to uncontrolled proliferation of cancer cells.[17] Cells must shift their metabolism pattern to produce sufficient energy and building blocks for the synthesis of proteins, nucleic acids, and lipids. The impact of cell cycle regulators on cancer cell metabolism has received increasing attention. For example, somatic mutations of the p53 gene have been implicated as driving events in the formation of a large number of common human cancers.[18,19] Several lines of
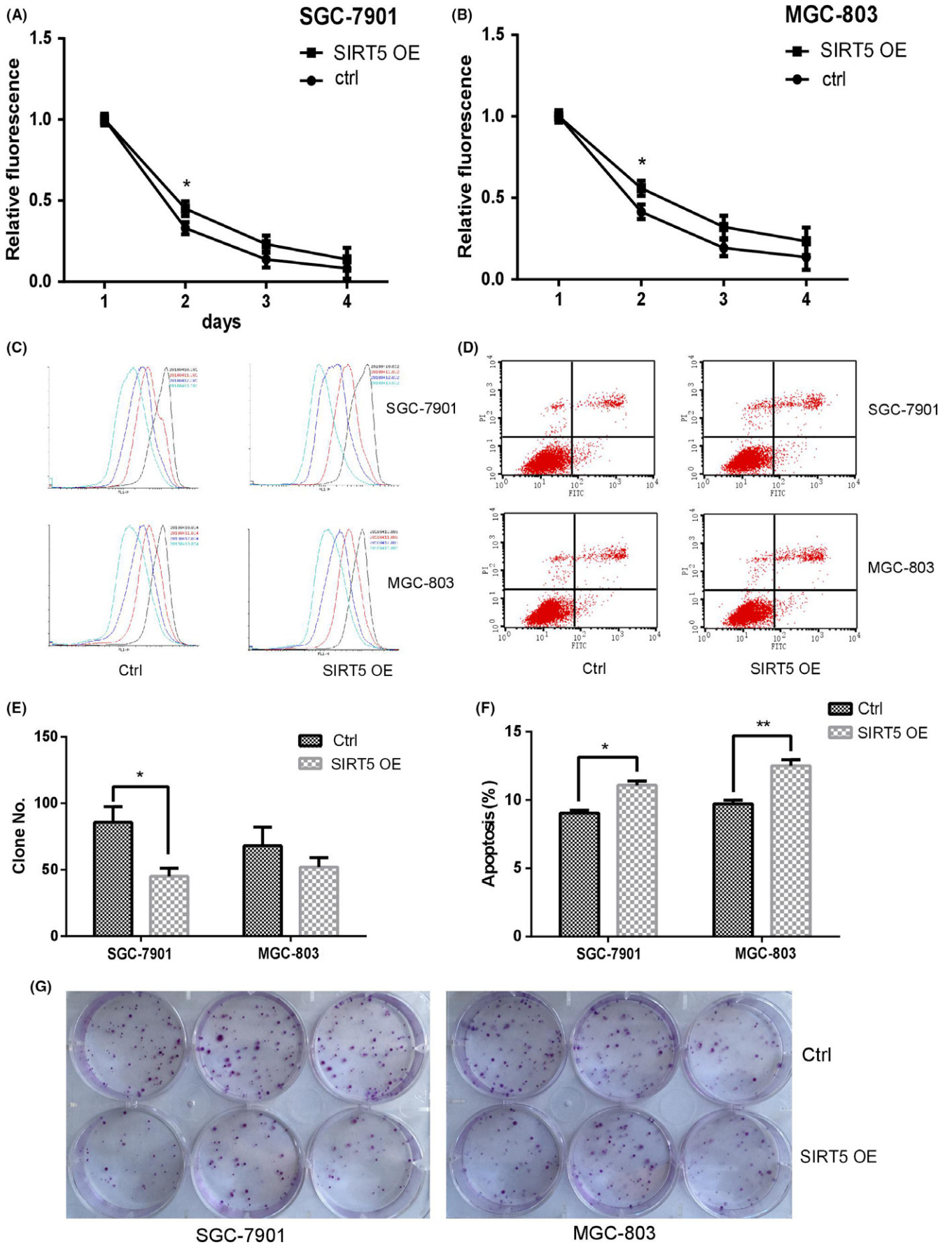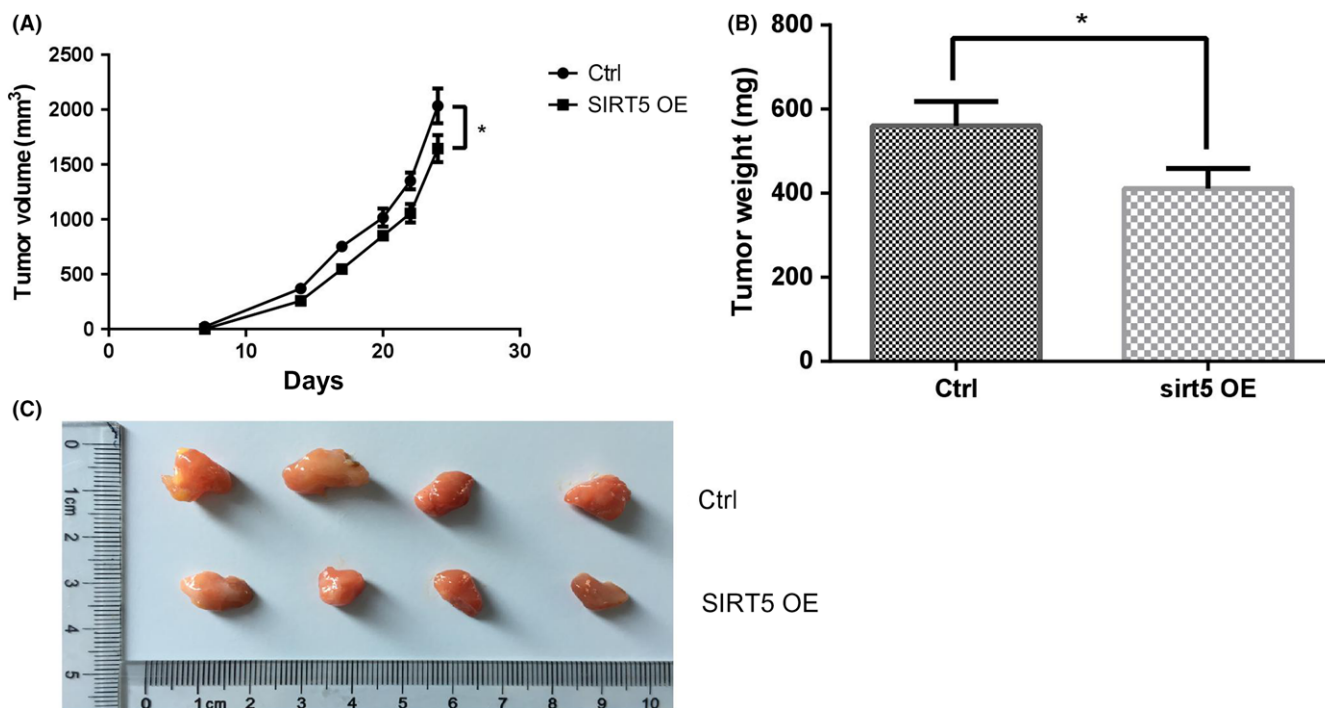
FIGURE 3 SIRT5 inhibited gastric cancer cell proliferation. To confirm the impact of SIRT5 on gastric cancer cell proliferation, we overexpressed SIRT5 in SGC-7901 and MGC-803 cells. A-C, 5(6)-Carboxyfluorescein diacetate *N*-succinimidyl ester assay results demonstrated that overexpression of SIRT5 in gastric cancer cells inhibited cancer cell proliferation. E,G, SIRT5 introduction into SGC-7901 and MGC-803 cells inhibited colony formation capacity of these 2 cell lines. D,F, SIRT5 overexpression resulted in increased cell apoptosis in SGC-7901 and MGC-803 cells. *P < 0.05; **P < 0.01

evidence suggest that wild-type p53 plays negative roles in regulating cell proliferation. When mutated, p53 loses its ability to induce cell cycle arrest, thereby accelerating the progression of cancer cells.[20] To meet the sustained demands of cell proliferation, cancer cells shift their metabolic pattern. For example, in lung cancer cells, wild-type p53 could repress hypoxia-mediated glycolysis. p53 was shown to transcriptionally repress the expression of GLUT1 and GLUT4 to decrease glucose uptake in cancer cells.[21] Furthermore, p53 induced the expression of TIGAR (TP53-induced glycolysis and apoptosis regulator), which decreases the intracellular concentrations of fructose-2,6-bisphosphate, and thus reduces glycolysis and diverts glucose catabolism to the pentose phosphate pathway (PPP).[22-24] However,
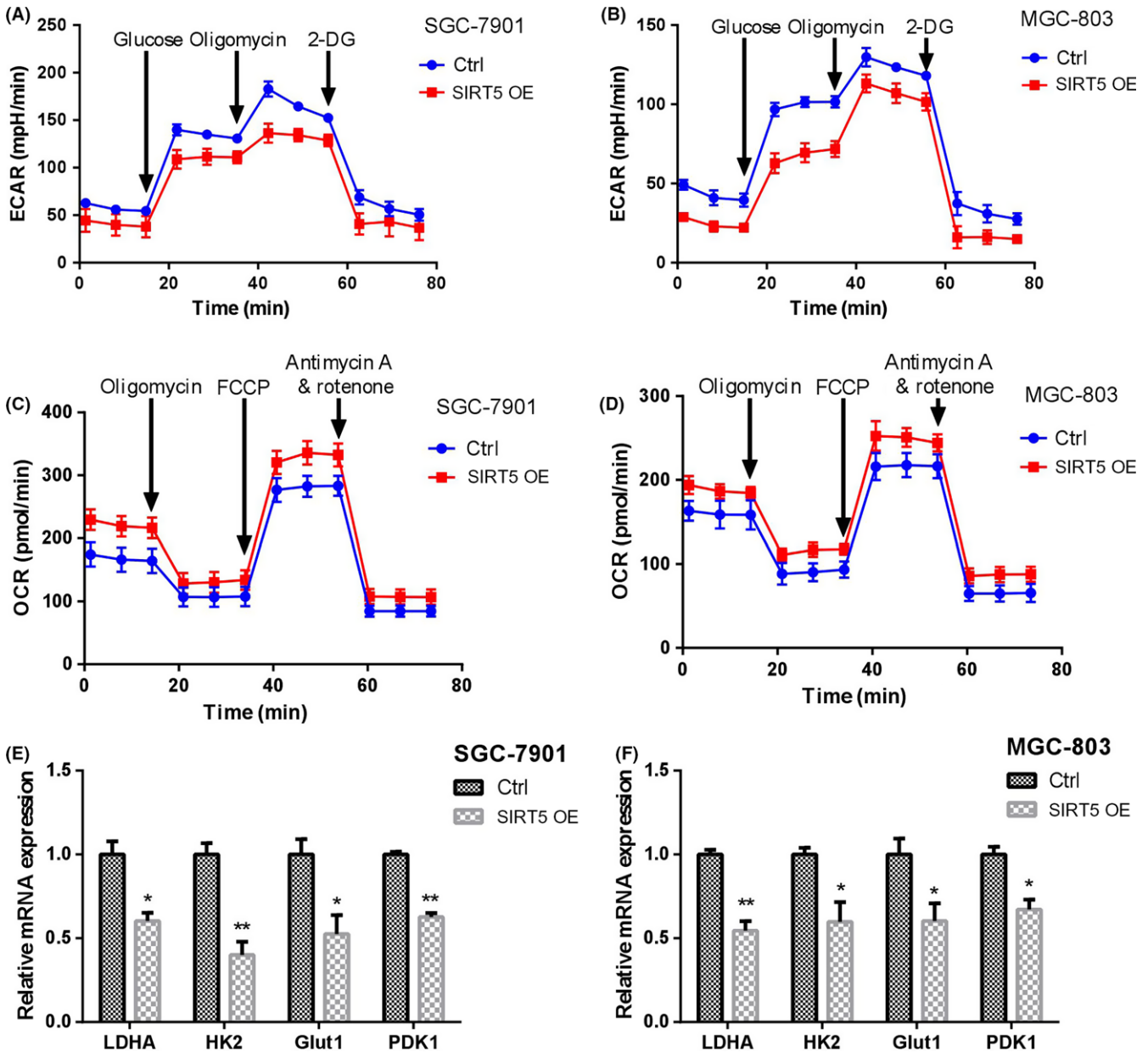
FIGURE 4 SIRT5 inhibited aerobic glycolysis in gastric cancer cells. A,B, Overexpression of SIRT5 in SGC-7901 and MGC-803 cells inhibited extracellular acidification rate (ECAR) values, suggesting that SIRT5 is a negative regulator of glycolysis. C,D, SIRT5 overexpression increased oxygen consumption rate (OCR) values, indicating that SIRT5 has a positive role in mitochondrial respiration. E,F, SIRT5 introduction decreased the expressional status of glucose transporter 1 (GLUT1), hexokinase 2 (HK2), lactate dehydrogenase A (LDHA) and pyruvate dehydrogenase kinase 1 (PDK1), which further supports the negative role of SIRT5 on glycolysis. *P < 0.05; **P < 0.01

FIGURE 5 A-C, SIRT5 inhibited tumor-formation capacity of gastric cancer cells. To further confirm the negative roles of SIRT5 on gastric cancer cell proliferation in vivo, we carried out s.c. injection of SIRT5 overexpressing SGC-7901 cells into nude mice. As shown, SIRT5 overexpression decreased tumor formation capacity of SGC-7901 cells in vivo. $P < 0.05$
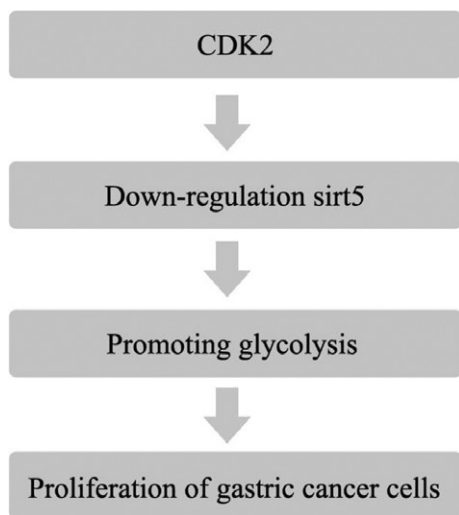
FIGURE 6 Working model of the present study. In gastric cancer cells, CDK2 positively regulated aerobic glycolysis by suppressing expression of SIRT5, a tumor suppressor and negative regulator of aerobic glycolysis

as direct regulators of the cell cycle, the impact of CDK on cancer cell metabolic reprogramming remains unreported. Previous studies suggested that CDK2 was a positive regulator of the gastric cancer cell cycle, which can be abnormally activated by increasing degrees of malignancy and the invasion of cancer cells.[25] Thus, it is perceivable that CDK2 could regulate aerobic glycolysis. Consistent with this hypothesis, we did observe a positive role of CDK2 in regulating glycolysis. Our mechanism studies indicated that CDK2 could regulate

metabolism by suppressing SIRT5. There are other possible mechanisms. For example, CDK2 could phosphorylate Myc at Ser-62, which inhibits cellular senescence.[26] The Myc protein functions as a key regulator of metabolism and participates in metabolic reprogramming such as glucose and glutamine metabolic reprogramming and in serine generation, which collectively meet the demands for the proliferation of cancer cells.[27]

Sirtuin family members are key regulators of aging, mitochondria-related processes and reactive oxygen species (ROS) generation.[28] Among them, SIRT3, SIRT4 and SIRT5 localize to the mitochondria and are reported to play negative roles in tumor formation and progression.[29] For example, the deletion or decreased expression of SIRT3 has been observed in breast cancer cells. Decreased SIRT3 expression increased ROS generation, leading to HIF1α stabilization and enhanced glycolysis.[30] SIRT4 has been reported to regulate glutamine and glucose metabolism in cancer cells.[31] Furthermore, SIRT4 has been reported to regulate cancer cell metabolism in response to genotoxic stress.[32] SIRT5 has been reported to be a negative regulator of metabolism, but its impact on cancer, especially gastric cancer, has seldom been reported. In our study, we indicated that SIRT5 plays a negative role in gastric cancer cell metabolism and proliferation. The underlying molecular mechanism requires further investigation. Previous studies showed that SIRT5 could bind, desuccinylate and activate Cu/Zn superoxide dismutase (SOD1) to scavenge ROS generation.[33] As discussed above, decreased ROS could inhibit glycolysis. Thus, it is necessary to examine the impact of SIRT5 on ROS generation in gastric cancer cells. Moreover, the underlying molecular mechanism might reside in the impact of SIRT5 on the activity of glycolysis genes. For

example, tumor cells tend to express higher levels of pyruvate kinase M2 (PKM2). PKM2 could be activated by succinylation at lysine 498 (K498). SIRT5 could bind and desuccinylate PKM2, inhibiting its activity.[34,35] Thus, further investigations are needed to examine the desuccinylating roles of SIRT5 in gastric cancer.

In conclusion, our present study uncovered novel functions of CDK2 and SIRT5 in gastric cancer and may shed light on novel therapeutic and prognostic markers, with the aim of improving the overall survival of gastric cancer patients.

CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

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