

miR-199a-3p/Sp1/LDHA axis controls aerobic glycolysis in testicular tumor cells

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Abstract. Aerobic glycolysis is one of the characteristics of tumor metabolism and contributes to the development of tumors. Studies have identified that microRNA (miRNA/miR) serves an important role in glucose metabolism of tumors. miR-199a-3p is a member of the miR-199a family that controls the outcomes of cell survival and death processes, and previous studies have indicated that the expression of miR-199a-3p is low and may be an inhibitor in several cancer types, including testicular tumors. The present study discussed the role and underlying mechanism of miR-199a-3p in aerobic glycolysis of Ntera-2 cells and identified its downstream factors. Firstly, miR-199a-3p exhibited an inhibitory effect on lactic acid production, glucose intake, and reactive oxygen species (ROS) and adenosine 5'-triphosphate (ATP) levels in Ntera-2 cells. Then, using bioinformatics, recombinant construction and a dual luciferase reporter gene system, transcription factor Specificity protein 1 (Sp1) was determined as the direct target of miR-199a-3p. Also, downregulation of Sp1 by RNA interference decreased lactic acid production, glucose intake, and ROS and ATP levels in Ntera-2 cells. Subsequently, through a functional rescue experiment, it was identified that the overexpression of Sp1 may abate the inhibition of miR-199a-3p on glucose metabolism, with the exception of ATP level, suggesting a reciprocal association between Sp1 and miR-199a-3p. Finally, it was determined that miR-199a-3p overexpression and Sp1 knockdown decreased lactate dehydrogenase A (LDHA) protein expression, which indicated that LDHA is a downstream target of the miR-199a-3p/Sp1 signaling pathway. To additionally verify the regulation of LDHA expression by 199a-3p/Sp1, a LDHA promoter reporter plasmid was generated and the high

activity of the promoter, which contained 3 potential Sp1 binding elements, was confirmed. In addition, the overexpression of Sp1 led to the increased activity of the LDHA promoter, whereas knockdown of Sp1 exhibited the opposite effect. Therefore, the results of the present study demonstrated that miR-199a-3p can inhibit LDHA expression by downregulating Sp1, and provided mechanistic evidence supporting the existence of a novel miR-199a-3p/Sp1/LDHA axis and its critical contribution to aerobic glycolysis in testicular cancer cells.

Introduction

Aerobic glycolysis is one of the characteristics of tumor metabolism (1). Normally, 90% adenosine 5'-triphosphate (ATP) is produced via mitochondrial oxidative phosphorylation in non-cancerous cells, whereas only 10% of ATP is obtained from aerobic glycolysis. By contrast, cancer cells exhibit active glucose uptake, enhancement of aerobic glycolysis and possess an abnormal metabolic phenotype in which glucose carbons are predominantly converted to lactate, even under the conditions of adequate oxygen. This unique phenomenon of metabolic alteration in cancer is referred to as the 'Warburg effect' (2). In previous years, aerobic glycolysis has become a focus of study due to its crucial role in the maintenance and development of tumor cells. Additionally, the close association between increased levels of lactate and poor patient prognosis or overall survival in different cancer types has been demonstrated by clinical studies (3). Therefore, the investigation of the molecular mechanisms resulting in this phenotype and their contributions to cancer initiation and development is of major importance for clinical treatment.

MicroRNAs (miRNAs/miRs) are endogenous non-coding RNA molecules between 21-25 nucleotides in length, and serve complicated and various roles in a number of cellular biological processes by post-transcriptionally modulating gene expression through binding to the 3'untranslated region (3'UTR) of the target mRNA (4). At present, the critical roles of miRNAs in cancer have been well-documented and miRNA-based medical treatment approaches are expected to result in a breakthrough for cancer biotherapy (5). miR-199a-3p, the well-conserved miRNA, is derived from its precursor miR-199a which exists in two different loci: miR-199a-1 is located in the intron 15 of the Dynamin 2 gene

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on chromosome 19; miR-199a-2 is located in intron 14 of Dynamin 3 gene on chromosome 1 (6,7). Generally, the functions and roles of miRNAs are defined by their expression level and relevance of their target genes. miR-199a-3p was identified to be important in multiple biological processes including cell cycle regulation, cell proliferation, apoptosis, differentiation, osteogenesis, chondrogenesis, adipogenesis and tumorigenesis through association with the modulation of different target genes, including mechanistic target of rapamycin, MET proto-oncogene, receptor tyrosine kinase, MYC proto-oncogene, BHLH transcription factor, cyclin D1, SMAD family member 1, LIF, interleukin 6 family cytokine, cyclooxygenase-2 and mitogen-activated protein kinase kinase II (7). It was also demonstrated to be dysregulated and may be an inhibitor in several cancer types including hepatocellular carcinoma (8), renal cell carcinoma (9), papillary thyroid carcinoma (10), and prostate (11) and gastric cancer (12). The present study group and Liu *et al* (13) demonstrated that the expression of miR-199a-3p is low in testicular tumors and that overexpression of miR-199a-3p *in vitro*, including in the Ntera-2 cell line, inhibits cell proliferation, migration and invasion. Chen *et al* (14) demonstrated that the deregulation of miR-199a-3p expression in testicular germ cell tumors may be due to hypermethylation of its promoter. The similar phenomenon was also investigated in ovarian cancer (15). In addition, miR-199a-3p was identified to improve cisplatin sensitivity in cholangiocarcinoma cells or breast cancer cells (16,17). Although these studies have addressed the association between miR-199a-3p and tumorigenesis using different models, little is known about its role in the Warburg effect.

Previously, our study group suggested that miR-199a-3p markedly increased the lactate production in Ntera-2 cells, and 4 significant genes [lactate dehydrogenase A (LDHA), tumor protein 53 induced glycolysis regulatory phosphatase (TIGAR), phosphoglycerate kinase 1 (PGK1) and solute carrier family 16 member 1 (MCT1)] downregulated by miR-199a-3p were identified through qPCR array technology (13). The mechanism of how miR-199a-3p downregulated these metabolic genes was unclear as the 4 selected genes lacked the potential recognition sites of miR-199a-3p, indicating that the regulation of miR-199a-3p on these 4 genes is indirect. Therefore, the present study performed bioinformatics analyses to search for potential explanations. All target genes of miR-199a-3p predicted by TargetScan databases were identified and termed the 'A' data set, and the transcription factor binding sites of the promoters of the 4 metabolic genes were predicted using the Genomatix transcription factor database and termed the 'B' data set. By integrating data sets 'A' and 'B', the transcription factor specificity protein 1 (Sp1) was demonstrated to overlap across the two datasets, suggesting that it may be associated with miR-199a-3p and the 4 metabolic genes. In the present study, this observation was discussed and the potential of the miR-199a-3p/Sp1/LDHA axis to modulate the glycolytic pathway, consequently promoting tumor development, was demonstrated.

Materials and methods

Cell culture and transient transfection. Human testicular teratoma Ntera-2 (CRL-1973 cells, American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI-1640

Table I. Sequences of microRNA and siRNA.

| miR/siRNA | Primer sequence (5'-3') |
|--------------------|--|
| miR-199a-3p mimics | F: 5'-ACAGUAGUCUGCACAUUGGUUA-3' R: 5'-ACCAAUGUGCAGACUACUGUUU-3' |
| Sp1-homo-si588 | F: 5'-CCUGGAGUGAUGCCUAAUATT-3' R: 5'-UAUUAGGCAUCACUCCAGGTT-3' |
| Sp1-homo-si721 | F: 5'-GUGCAAACCAACAGAUUAUTT-3' R: 5'-AUAUUCUGUUGGUUUGCACTT-3' |
| Sp1-homo-si1093 | F: 5'-GUGCAAACCAACAGAUUAUTT-3' R: 5'-AUAUUCUGUUGGUUUGCACTT-3' |

miR, microRNA; siRNA, short interfering RNA; F, forward; R, reverse; Sp1, specificity protein 1.

medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C with 5% CO₂ and 95% humidity. Cell transfection was performed using TurboFectTM *in vitro* Transfection reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. For the rescue experiments, Ntera-2 cells were transfected with pcDNA3.1-Sp1 or pcDNA3.1 empty plasmids (Vigene Biosciences, Ji'nan, Shandong, China) with miR-199a-3p mimics (100 nM; Table I; Shanghai GenePharma Co., Ltd., Shanghai, China) and cultured for 72 h to investigate whether Sp1 overexpression rescued the metabolic phenotype of Ntera-2 cells.

For the RNA interference (RNAi)-mediated inhibition of Sp1 expression, Ntera-2 cells were transfected with 3 kinds of Sp1 small interfering RNA (siRNA) oligonucleotides or control siRNA oligonucleotides (Table I; Shanghai GenePharma Co., Ltd.) and cultured for 72 h before subsequent experimentation.

Measurement of glucose consumption and lactate production. Ntera-2 cells were transfected with either miR-199a-3p mimics or negative control miRNA mimics (NC mimics) as above. Following transfection, cells were cultured in complete medium as above for 48 h at 37°C, the supernatants were harvested by centrifugation (1,000 x g for 5 min at room temperature) and detected for the measurement of intracellular glucose utilization and lactate concentration using an Automatic Biochemical Analyzer (7170A; Hitachi, Ltd., Tokyo, Japan).

Measurement of reactive oxygen species (ROS) and ATP. For the measurement of ROS, 10⁵ Ntera-2 cells were plated into blank 96-well plates. Following transfection as above and culture for 48 h, the cells were washed with Hanks' Balanced Salt Solution and then incubated with 10 μM dichlorofluorescein diacetate (DCF-DA) in RPMI-1640 for 1 h at 37°C. The formation of the fluorescent-oxidized derivative of DCF-DA was monitored at an emission wavelength of 525 nm and excitation wavelength of 500 nm in a fluorescence multi-detection reader.

For the measurement of ATP, 10⁵ Ntera-2 cells were lysed with ATP detection kit lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) in 6-well plates and moved to

Table II. Primers for reverse transcription quantitative polymerase chain reaction analysis.

| Gene | Primer sequence (5'-3') | Length, bp |
|----------------|---|------------|
| GAPDH | F: GTCTCCTCTGACTTCAACAGCG R: ACCACCCTGTTGCTGTAGCCAA | 243 |
| Sp1 | F: CTCCAGACCATTAAACCTCAGTG R: ATCATGTATTCCATCACCACCAG | 147 |
| TIGAR | F: ACTCAAGACTTCGGGAAAGGA R: CACGCATTTTCACCTGGTCC | 144 |
| LDHA | F: TTGACCTACGTGGCTTGGAAG R: GGTAACGGAATCGGGCTGAAT | 90 |
| MCT1 | F: GGTGGAGGTCCTATCAGCAGT R: CAGAAAGAAGCTGCAATCAAGC | 107 |
| PGK1 | F: TGGACGTTAAAGGGAAGCGG R: GCTCATAAGGACTACCGACTTGG | 152 |
| Sp1 3' UTR wt | F: <u>CCGCTCGAGGCACTCCTTCCCTAACTCAA</u> (Xho I site is underlined) R: <u>ATTTGCGGCCGCGTCCAAATACTCCACTAACTCCTG</u> (Not I site is underlined) | 445 |
| Sp1 3' UTR mut | F1: <u>CCGCTCGAGGCACTCCTTCCCTAACTCAA</u> (Xho I) R1: TGCCTGCG TTATTGGTAT F2: ATGCCAATAACGCAGGCA R2: <u>ATTTGCGGCCGCGTCCAAATACTCCACTAACTCCTG</u> (Not I) | 207 256 |
| pLDHA1257 | F: CGGGGTACCGGCAATGGAATCAGCAAGAATACAG (KpnI) R: CCCAAGCTTCGTGGCAATGAGATCCGGAAT (HindIII) | 1,257 |

F, forward; R, reverse; Sp1, specificity protein 1; LDHA, lactate dehydrogenase A; TIGAR, tumor protein 53 induced glycolysis regulatory phosphatase; PGK1, phosphoglycerate kinase 1; MCT1, solute carrier family 16 member 1; UTR, untranslated region.

1.5 ml tubes, and then centrifuged at 13,200 x g for 5 min at 4°C. Subsequently, the supernatant was treated with an ATP detection kit (Beyotime Institute of Biotechnology), according to the manufacturer's protocol. Finally, the fluorescence intensities of the samples were measured with Glomax 20/20 (Promega Corporation, Madison, WI, USA) instrument to calculate the concentration of ATP.

Polymerase chain reaction (PCR) amplification and recombinant plasmid construction. The human genomic DNA was extracted from Ntera-2 cells with a genomic DNA kit (Tiangen Biotech, Co., Ltd., Beijing, China). For the construction of Sp1-3' UTR reporter plasmid and its mutant, the wild-type 3'UTR (3'UTR-wt) of Sp1 containing the miR-199a-3p binding sites was obtained by PCR. DNA was extracted from Ntera-2 cells using 2X TSINGKE Master Mix (TSINGKE, Beijing, China) and primer sequences are given in Table II. The reaction conditions: 95°C for 5 min, 30 cycles of 95°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec, 72°C for 5 min. The mutant Sp1 3'UTR (3'UTR-mut), in which the mutations occurred in the conserved binding sites for miR-199a-3p, were generated by using overlapping extension PCR: Sp1 3'UTR-mut primers F1 and R1, F2 and R2 (Table II) were respectively used to amplify 2 fragments by PCR first (95°C for 5 min, 30 cycles of 95°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec, 72°C for 5 min). There is an overlap region in these 2 fragments. Then primers

F1, R2 and 2 fragments were used to do overlapping PCR (95°C for 5 min, 30 cycles of 95°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec, 72°C for 5 min). The fragment of Sp1 3'UTR-wt (1 µg) and the mutant 3'UTR fragment (1 µg) were inserted into XhoI/NotI-digested pmiR-RB-Reporter™ Vector (Guangzhou RiboBio Co., Ltd., Guangzhou, China) containing both firefly and *Renilla* luciferase reporter genes, respectively. Recombinant plasmid (1 µg) were characterized by sequencing analysis (TSINGKE) and enzyme digestion (Fastdigest XhoI FD0694 and Fastdigest NotI FD0593, incubated at 37°C for 2 h; Thermo Fisher Scientific, Inc.).

For the construction of the LDHA promoter reporter plasmid, a 1,257 bp fragment containing LDHA 5'UTR sequences from -1,040 to +217 bp (Table III) relative to the transcription initiation site, was amplified by PCR using human genomic DNA obtained from Ntera-2 cells (DNA polymerase; TSINGKE) as template and then subcloned into the pGL3-basic plasmid. The primer sequences are given in Table II and the reaction conditions of PCR were: 95°C for 5 min, 30 cycles of 95°C for 15 sec, 55°C for 30 sec and 72°C for 90 sec. Based on the length of the promoter fragments, the recombinant was labelled pLDHA1257. Recombinant plasmid (1 µg) were characterized by sequencing analysis (TSINGKE) and enzyme digestion (Fastdigest KpnI FD0524 and Fastdigest HindIII FD0504, incubated at 37°C for 2 h, Thermo Fisher Scientific, Inc.). All the primers used are summarized in Table II.

Table III. Sequence of LDHA 5'UTR from -1,040 to +160 bp.

| bp | Sequence (5'-3') |
|--------|---|
| -1,040 | GGCAATGGAA TCAGCAAGAA TACAGGCCCA GAGGTAAGTA TGATAAGAAAACAAAAAATT |
| -980 | GAGCTGGGCA CGGTGGCTCA CGCCTGTAAT CCCAGCTCTT TGTGAGGCCG AGGCGGCCAG |
| -920 | ATCACTTGAG GCCAGGAGTT TGAGATCAGC CTGACCAACA TGGTGAAACC CCGTCTCTAC |
| -860 | TAAAAATTAC AAAAATTAGC CGGGCGTGGT GGCGCCTGGC TGTAATCCCA TCTTCTCAGG |
| -800 | AGGCTGAGGCAGGAGATTCG CTTGAACCCGGGAGGCGGAGGTTGCAGTGAGCCGAGATCA |
| -740 | GCCCACTGCA CTCCAGCCTG GGCGACAGAG CGACTCCGTT TCAAAAAAAAAAAAAATTAAT |
| -680 | AATTAAAAAAA AAAATCGCAG GGCAAGTGGG CGCGCTTGTA GTCCAGTCA CTTCGGGGAG |
| -620 | CCGAGGTGGGAGGATCGCTT GAGCCAGAAG GTCGAGGCTG CAGGAAGCCATGATCACGCC |
| -560 | ACTGCCCTCC AGCCTGGGTG ACAGAGTGAG ACCCTGTCTC GAAAAATACC AAAAAACAAA |
| -500 | TAAACAAACAAAAAACCAAAACCAAAAAAAAA CAAGCCACTG ACAGTTCTTG GGTATGGTTG |
| -440 | AGACTCGAGA TGAGATGCCA GTGGGGTGGG CAGTAGAAAG TGCAGAATAA AATGTACATT |
| -380 | TGAACTGAGT CACCCTGCAAGGCCTGAGAG GCCAAGGCTT CACTGTGAGT GGGAGCTGGT |
| -320 | AGGCTTAGCA GCAGAGGGAAAAGCAGCGTC GAGTTTTGGA GGTCACCTCGACTTAGGTAAG |
| -260 | AACAGACTGA CTGACTGCTA GGCATTTTCT TCCTTTCGTT CAACAAATAT TTGTGGAGTG |
| -200 | CCTATTACGT GCCAGAAGCT GTTCTGGACA CTGAGAAACA GGGATGAAGA AGAAACAGAT |
| -140 | CCAAGCCTTC CTGAGAGTAA CCTCCCCAGG TTTCATGGAT GAGGAACTG AAGGTCGTCC |
| -80 | TGACTCAGGC TCATGGCTCC GACCCCGGCT TCTGTGGTTG GAGGGCAGCA CCTTACTTAG |
| -20 | ACTCCAGCG CACGTGGAGC AGTCTGCCGG TCGGTTGTCT GGCTGCGCGC GCCACCCGGG |
| 40 | CCTCTCCAGT GCCCCGCCTG GCTCGGCATC CACCCCCAGC CCGACTCACA CGTGGGTTCC |
| 100 | CGCACGTCCG CCGGCCCCCC CCGCTGACGT CAGCATAGCT GTTCCACTTA AGGCCCTCC |
| 160 | CGCGCCCAGC TCAGAGTGCT GCAGCCGCTG CCGCCGATTC CGGATCTCAT TGCCACG |

LDHA, lactate dehydrogenase A; UTR, untranslated region; bp, base pair.

Dual luciferase reporter assay. For the reporter system analysis, Ntera-2 cells (10^5) were seeded in each well of 6- or 24-well plates 24 h prior to transfection. Each of the luciferase-containing plasmids (Vigene Biosciences), with *Renilla* (Promega Corporation) as an internal control, were co-transfected into Ntera-2 cells and cultured for 48 h.

For the measurement of Spl-3'UTR reporter activity, Ntera-2 cells were transiently co-transfected for 48 h with reporter plasmids and miR-199a-3p mimics or NC mimics (as aforementioned), then were harvested in reporter lysis buffer (Promega Corporation). Firefly luciferase and *Renilla* luciferase activities were measured using the Dual Luciferase assay kit (Promega Corporation), according to manufacturer's protocol. The luciferase activity normalized against the protein concentration was expressed as a ratio of firefly luciferase to *Renilla* luciferase units.

For the measurement of LDHA promoter activity, a luciferase assay was performed using luciferase assay reagent (Promega Corporation) according to the manufacturer's protocol (as aforementioned). The luciferase activity driven by the LDHA promoter was measured using Modulus™ (Turner BioSystems; Thermo Fisher Scientific, Inc.) instrument in Ntera-2 cells following transfection for 48 h with the indicated LDHA promoter reporters in the absence (Spl siRNA treatment) or presence (overexpression of pcDNA3.1-Spl constructed by our group) of Spl. LDHA promoter-luciferase activities in transfected cells were normalized to *Renilla* activity. All results were representative of at least three independent experiments performed in triplicate.

cDNA synthesis and reverse transcription-quantitative PCR (RT-qPCR) for miRNA and mRNA detection. Total cellular RNA was extracted using TRIzol® reagent (Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's protocol. Then, the PrimeScript miRNA qPCR Starter kit version 2.0 (Takara Biotechnology Co., Ltd.) was used for miRNA RT, and PrimeScript RT reagent kit with gDNA Eraser (Takara Biotechnology Co., Ltd.) was used for mRNA RT, according to the protocol of the manufacturer. Finally, qPCR was performed using 2X SYBR Green qPCR Master Mix (Low Rox) kit (Bimake, Houston, TX, USA) and MX3000 (Stratagene; Agilent Technologies, Inc., Santa Clara, CA, USA) instrument. The reaction conditions of PCR were: 95°C for 5 min, 40 cycles of 95°C for 15 sec, 58°C for 30 sec and 72°C for 30 sec. The relative expression was analyzed by the $2^{-\Delta\Delta C_q}$ method. For miRNA, U6 was used as an internal control. For mRNA, GAPDH was used as an internal control and primer sequences are summarized in Table II.

Western blot analysis. Protein samples were isolated from whole cell lysates (RIPA lysis buffer, Beyotime) and the concentration of total protein was measured using BCA Protein Assay kit (Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China). Amounts of samples (50 µg/lane) were separated by 10% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane. Subsequent to blocking in 5% skimmed milk dissolved in TBST at 4°C for 2 h, the membrane was incubated with specific primary antibodies. The

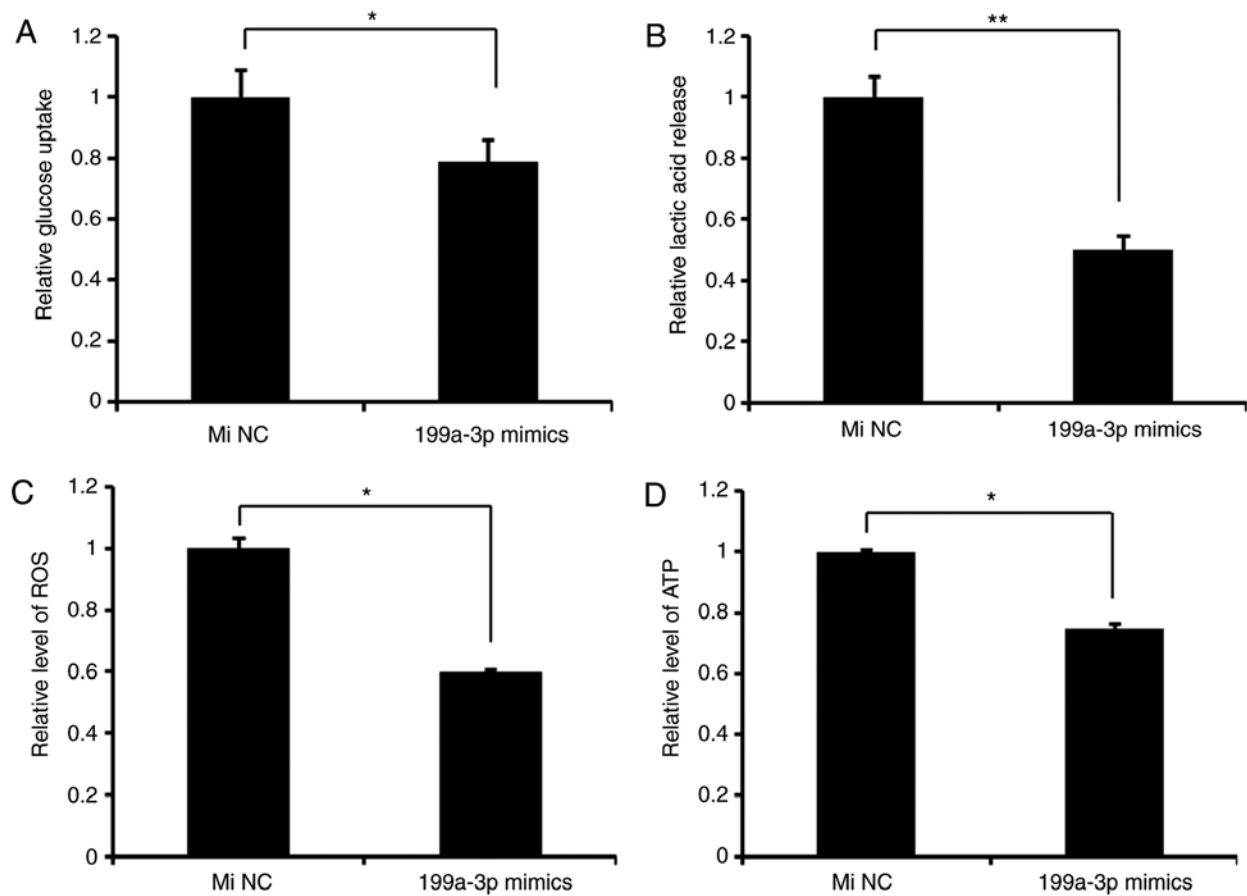


Figure 1. miR-199a-3p inhibits glycolysis in Ntera-2 cells. The effects of miR-199a-3p on (A) uptake of glucose, (B) production of lactate, (C) ROS level and (D) ATP level in Ntera-2 cells were analyzed. * $P \leq 0.05$ and ** $P \leq 0.01$ vs. MiNC group. miR, microRNA; MiNC, miRNA negative control mimics; ROS, reactive oxygen species; ATP, adenosine 5'-triphosphate.

following primary antibodies were incubated at 4°C for 12 h: Sp1 (1:500; cat. no., CSB-PA050124; Cusabio Technology LLC, Wuhan, China), LDHA (1:2,000; cat. no. 19987-1-AP; Wuhan Sanying Biotechnology, Wuhan, China), TIGAR (1:1,000, cat. no., CSB-PA162542, Cusabio Technology LLC), MCT1 (1:1,000; cat. no., sc-365501; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), PGK1 (1:1,000; cat. no., CSB-PA00035A0Rb; Cusabio Technology LLC), actin (1:10,000; cat. no., AC026; ABclonal, Woburn, MA, USA) and GAPDH (1:5,000; cat. no., AP0063; Bioworld Technology, Inc., St. Louis, MN, USA). Goat anti-mouse (1:2,000; cat. no., A0216) and anti-rabbit (1:2,000; cat. no., A0208) IgG antibodies conjugated to horseradish peroxidase were used as the secondary antibodies (both Beyotime Institute of Biotechnology). Secondary antibody were incubated with the membrane at 4°C for 2 h. The blotted proteins were then determined by using enhanced chemiluminescence reagents (SuperSignal™ West Pico PLUS Chemiluminescent Substrate, Thermo Fisher Scientific, Inc.). Image Lab version 5.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to analyze the protein expression.

Bioinformatics prediction. TargetScan (www.targetscan.org) and Genomatix (www.genomatix.de) were applied to predict the putative target genes of miR-199a-3p and the binding sites of Sp1 on LDHA promoter, according to the given protocols.

Statistical analysis. All experiments were performed in triplicate, and the results are presented as the mean \pm standard deviation. All statistical analyses were performed using SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA). All data were analyzed with analysis of variance and Student-Newman-Keuls test for continuous variables. $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

miR-199a-3p inhibits glycolysis in Ntera-2 cells. To determine the effect of miR-199a-3p on glucose metabolism *in vitro*, miR-199a-3p mimics or miNC were transfected in Ntera-2 cells, and the results indicated that compared with miNC groups, miR-199a-3p decreased the rates of lactic acid production and glucose intake, and ROS and ATP levels, indicating that miR-199a-3p inhibits glycolysis in Ntera-2 cells (Fig. 1).

Sp1 is a target of miR-199a-3p. TargetScan predicted that miR-199a-3p binds to the target sequences (location 886-893) in the 3'UTR of Sp1 mRNA (Fig. 2A-a) (18). To confirm the reliability of the putative recognition site of miR-199a-3p, the expression of Sp1 in Ntera-2 cells treated with miR-199a-3p mimics was first detected. As demonstrated in Fig. 2B, miR-199a-3p markedly suppressed the expression of Sp1 at mRNA and protein levels. Then,

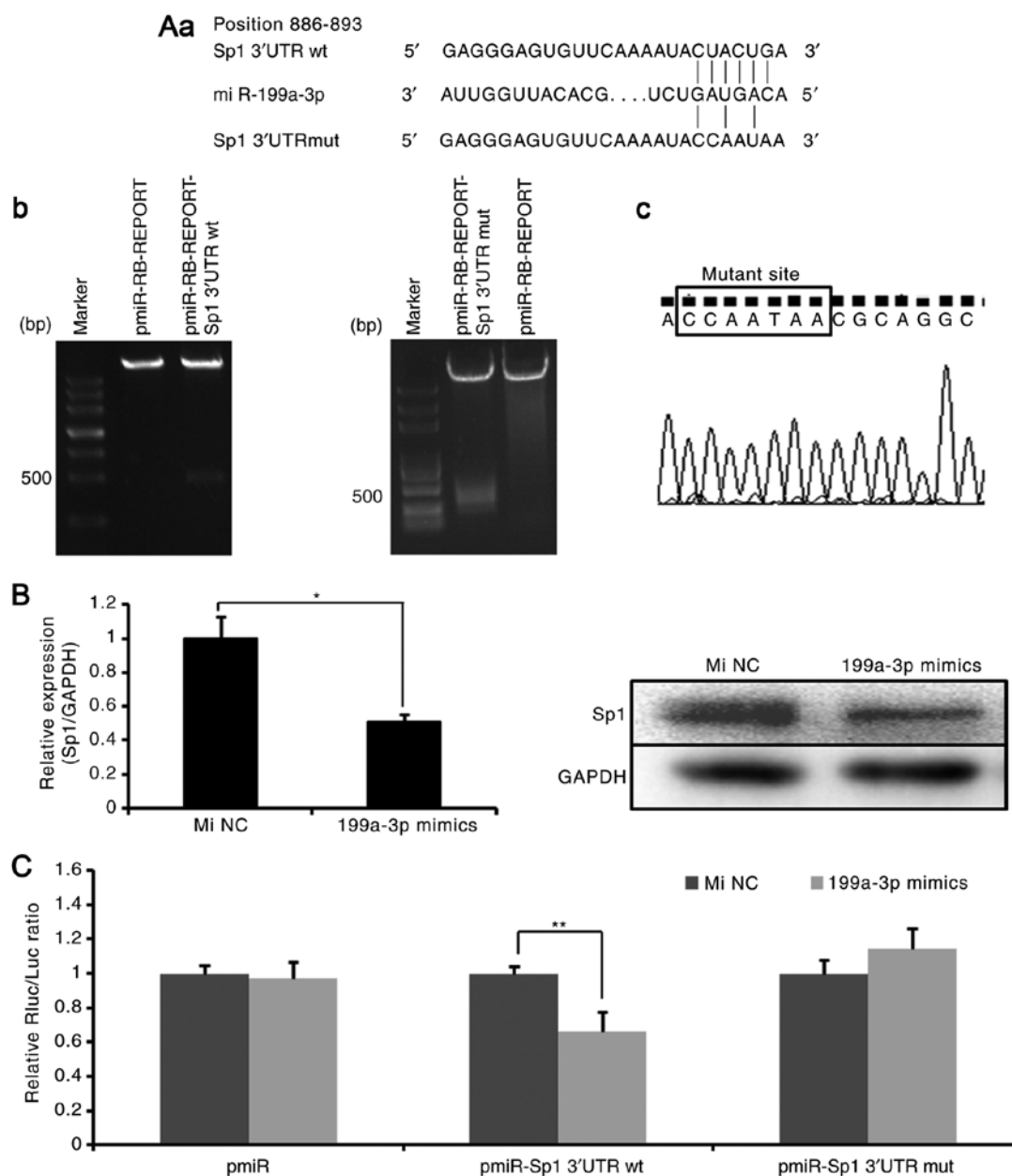


Figure 2. Sp1 is a target of miR-199a-3p in Ntera-2 cells. (A-a) miR-199a-3p binds to the target sequences (location 886-893) in the 3'UTR of Sp1 mRNA; (b) Enzyme digestion analysis of pmiR-RB-REPORT Sp1-3'UTRwt and pmiR-RB-REPORTmut, and (c) sequence analysis of pmiR-RB-REPORT Sp1-3'UTRmut. (B) The mRNA and protein expression of Sp1 in miR-199a-3p mimics-treated Ntera-2 cells were detected by reverse transcription quantitative polymerase chain reaction and western blot analysis, respectively. (C) Analysis of target recognition of miR-199a-3p and Sp1-3'UTR by a dual luciferase reporter system. * $P \leq 0.05$ and ** $P \leq 0.01$ vs. MiNC group. miR, microRNA; MiNC, miRNA negative control mimics; Sp1, specificity protein 1; UTR, untranslated region; wt, wild-type; mut, mutant.

wild-type recombinant (pmiR-RB-REPORT Sp1-3'UTRwt) and point mutation recombinant (pmiR-RB-REPORT Sp1-3'UTRmut) plasmids were generated. The recombinant plasmids were confirmed by double-enzyme digestion (Fig. 2A-b) and sequencing (Fig. 2A-c). Next, the plasmids were transfected into Ntera-2 cells combined with miR-199a-3p mimics or miRNA NC mimics. As indicated in Fig. 2C, in the Sp1-3'UTRwt group, the dual luciferase reporter system demonstrated a significant decrease in relative luciferase activity following miR-199a-3p mimics transfection compared with miRNA NC mimics transfection. However, in the Sp1-3'UTRmut group, no difference between miR-199a-3p mimics treatment and miRNA NC

mimics treatment was identified. These observations indicated that miR-199a-3p may directly target the 3'UTR of Sp1 mRNA and decrease Sp1 expression.

RNAi-mediated silence of Sp1 phenocopies the effect of miR-199a-3p on glucose metabolism in Ntera-2 cells. To understand the functional association of Sp1 and miR-199a-3p in the regulation of glucose metabolism, knockdown of Sp1 by RNAi was performed to detect the effect on glycolysis in Ntera-2 cells. As indicated in Fig. 3A, high endogenous Sp1 expression was silenced by all 3 siRNA oligonucleotides, and Si588 was selected for subsequent analyses. It was identified that the knockdown of Sp1 by Si588 markedly decreased

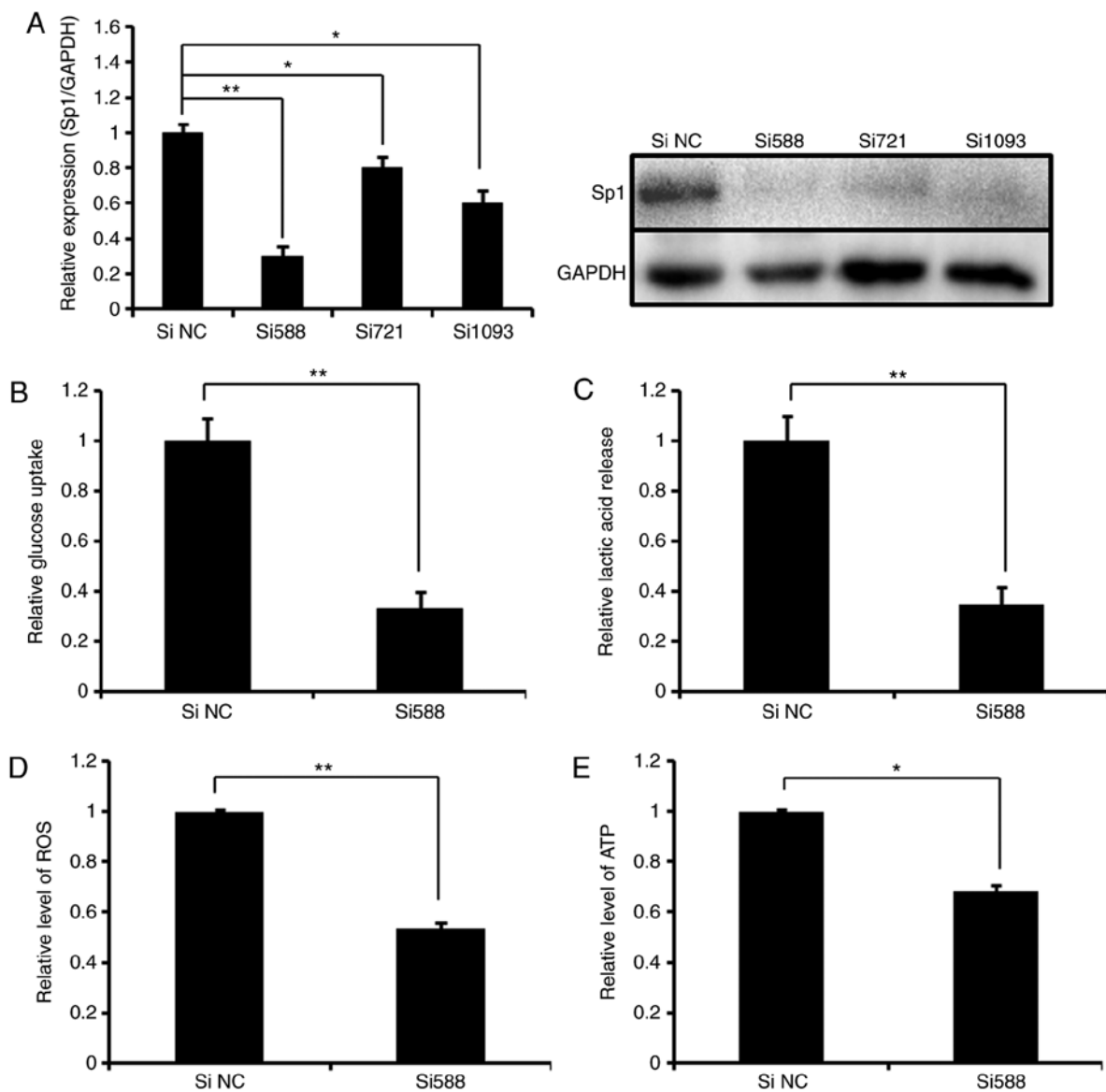


Figure 3. Sp1 siRNA decreases glycolysis in Ntera-2 cells. (A) The mRNA and protein expression of Sp1 in siRNA-treated Ntera-2 cells were detected by reverse transcription quantitative polymerase chain reaction and western blot, respectively. The effects of Sp1 siRNA (Si588) on (B) uptake of glucose, (C) production of lactate, (D) ROS level and (E) ATP level in Ntera-2 cells were then analyzed. * $P \leq 0.05$ and ** $P \leq 0.01$ vs. SiNC group. Sp1, specificity protein 1; siRNA, small interfering RNA; SiNC, siRNA negative control; ROS, reactive oxygen species; ATP, adenosine 5'-triphosphate.

glucose uptake, lactic acid production, and ROS and ATP levels in Ntera-2 cells (Fig. 3B-E), which were similar with the data obtained from the cells treated with miR-199a-3p mimics (Fig. 1). The results suggest that in the process of Ntera-2 metabolism, Sp1 and miR-199a-3p have a reciprocal functional association, indicating that RNAi-mediated silence of Sp1 phenocopies the effect of miR-199a-3p on glycolysis.

Overexpression of Sp1 rescues the effects of miR-199a-3p on glucose metabolism in Ntera-2 cells. A pcDNA3.1-Sp1 plasmid, which lacked the Sp1 3'UTR, was constructed to induce ectopic expression of Sp1. Then, four co-transfection groups (miRNA NC mimics+pcDNA3.1, miRNA NC mimics+pcDNA3.1-Sp1, miR-199a-3p mimics+pcDNA3.1 and miR-199a-3p mimics+pcDNA3.1-Sp1) were used to detect and confirm whether miR-199a-3p exerts its effects on glycolysis

by targeting Sp1. As demonstrated in Fig. 4, with the exception of ATP level, pcDNA3.1-Sp1 was able to abate the inhibition of miR-199a-3p on glucose consumption, lactate production and ROS level in cultured cells. These results suggested that the restoration of Sp1 may, to a large extent, rescue the effects of miR-199a-3p on the metabolic phenotypes of Ntera-2 cells.

LDHA is identified as the downstream target of miR-199a-3p/Sp1 signaling pathway in controlling aerobic glycolysis. In order to determine how miR-199a-3p/Sp1 affected glucose metabolism, using data from our previous study (13), the expression levels of LDHA, MCT1, PGK1 and TIGAR in Ntera-2 cells following transfection of Sp1 siRNA or miR-199a-3p mimics were detected. The results indicated that the mRNA level of LDHA and MCT1 were significantly decreased following Sp1 knockdown or miR-199a-3p treatment, whereas PGK1 expression was downregulated only

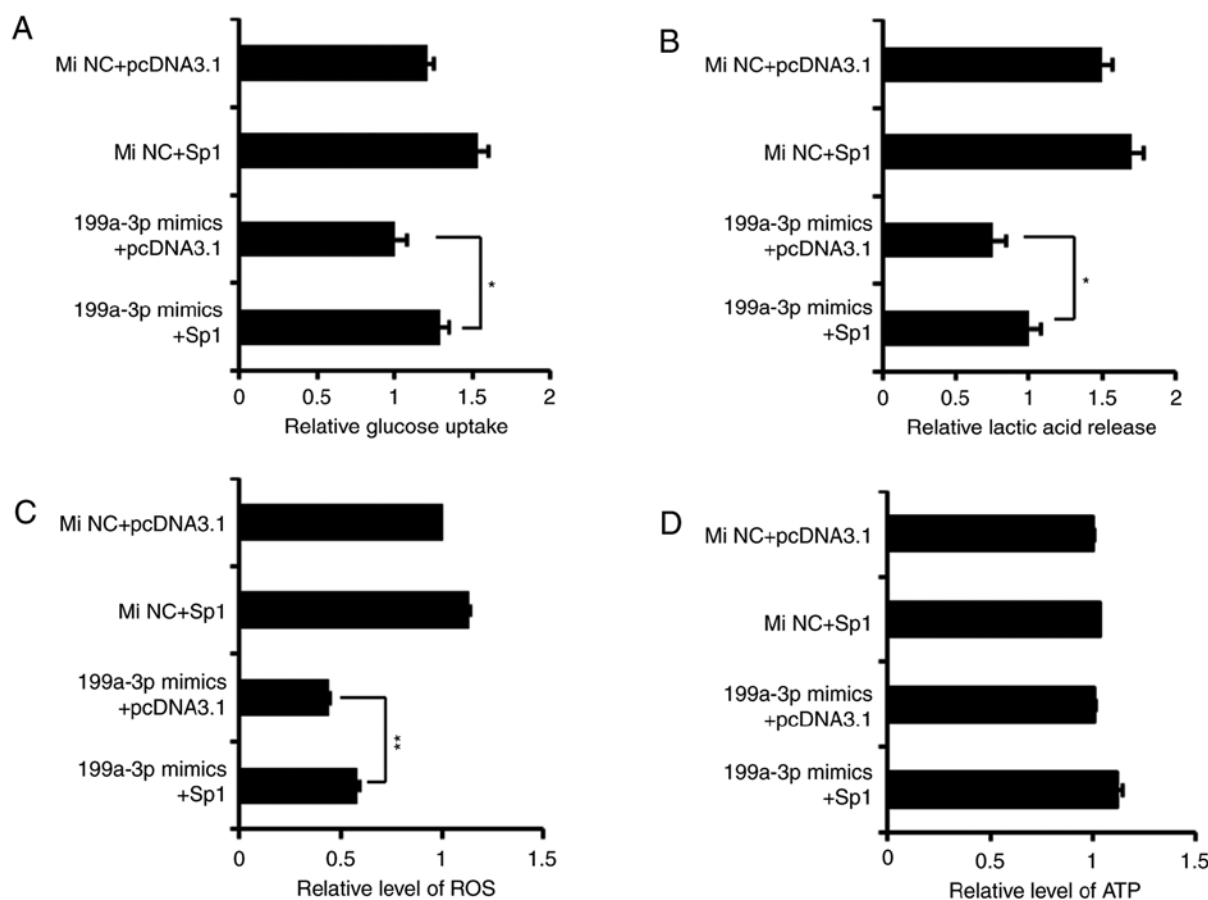


Figure 4. Overexpression of Sp1 rescues the effects of miR-199a-3p on glucose metabolism in Ntera-2 cells. The (A) uptake of glucose, (B) production of lactate, (C) ROS level and (D) ATP level were detected in Ntera-2 cells which were respectively treated by four different groups (MiNC+pcDNA3.1, MiNC+pcDNA3.1-Sp1, miR-199a-3p mimics+pcDNA3.1, miR-199a-3p mimics+pcDNA3.1-Sp1). * $P \leq 0.05$ and ** $P \leq 0.01$ vs. pcDNA3.1 group. miRNA, microRNA; MiNC, miRNA negative control mimics; Sp1, specificity protein 1; ROS, reactive oxygen species; ATP, adenosine 5'-triphosphate.

by Sp1 si588 and there was no significant difference in the expression of TIGAR (Fig. 5A). At the protein level, the results of western blot analysis confirmed the suppression of LDHA, MCT1 and PGK1, but not TIGAR, by miR-199a-3p mimics (Fig. 5B). Concurrently, only LDHA and PGK1 expression were visibly downregulated following Sp1 si588 transfection. These data indicated that either the overexpression of miR-199a-3p or knockdown of Sp1 may decrease LDHA protein level, and that the suppression of LDHA was the most marked among the 4 genes. Therefore, we hypothesized that LDHA was the most important downstream target of miR-199a-3p/Sp1.

To additionally verify the regulation of LDHA expression by miR-199a-3p/Sp1, a LDHA promoter reporter plasmid (pLDHA 1257) that included 3 potential Sp1 binding sites was generated (Fig. 6A) and characterized by sequencing analysis (data not shown) and enzyme digestion (Fig. 6B). Compared with the pGL3-basic plasmid, the activation of luciferase activity driven by pLDHA1257 was significantly increased in Ntera-2 cells (Fig. 6C), indicating the presence of promoter activity. Additionally, co-transfection of Ntera-2 cells with pLDHA and pcDNA3.1-Sp1 markedly increased the LDHA promoter activity (Fig. 6D), whereas knockdown of Sp1 expression with Si588 decreased this activity in the cells (Fig. 6E). These data suggested that the Sp1 binding sites, as positive regulatory elements in the LDHA promoter,

were involved in upregulating LDHA gene transcription of Ntera-2 cells.

Discussion

Testicular cancer is a relatively rare tumor type, accounting for ~1% of all types of cancer in men (19). However, testicular cancer is the most commonly diagnosed malignancy for young males aged 15-40 years (20). Furthermore, ~95% of all malignant tumors of the testis are germ cell tumors (TGCTs) (21). TGCTs are classified as seminoma or non-seminoma, according to their origin, clinical behavior and chromosomal constitution (21). Non-seminoma tumors include several subtypes of cancers, including embryonal cell carcinoma, choriocarcinoma, yolk sac tumors and teratoma (teratocarcinoma) (22-24). The Ntera-2 cell line examined in the present study was derived from non-seminomatous tumors. For the clinical management of TGCTs, lactate dehydrogenase (LDH) has been regarded as one of critical serum tumor markers, suggesting the existence of aerobic glycolysis and metabolic reprogramming in TGCTs.

The high glycolytic flux in tumor cells is induced by a number of enzymes including LDH, glucose transporter types 1/3, hexokinases and pyruvate kinase type M (25,26). LDHA, which catalyzes the final step of glycolysis, is a major subunit of LDH and abnormally upregulated in cancer (27). Experimental knockdown or downregulation of LDHA

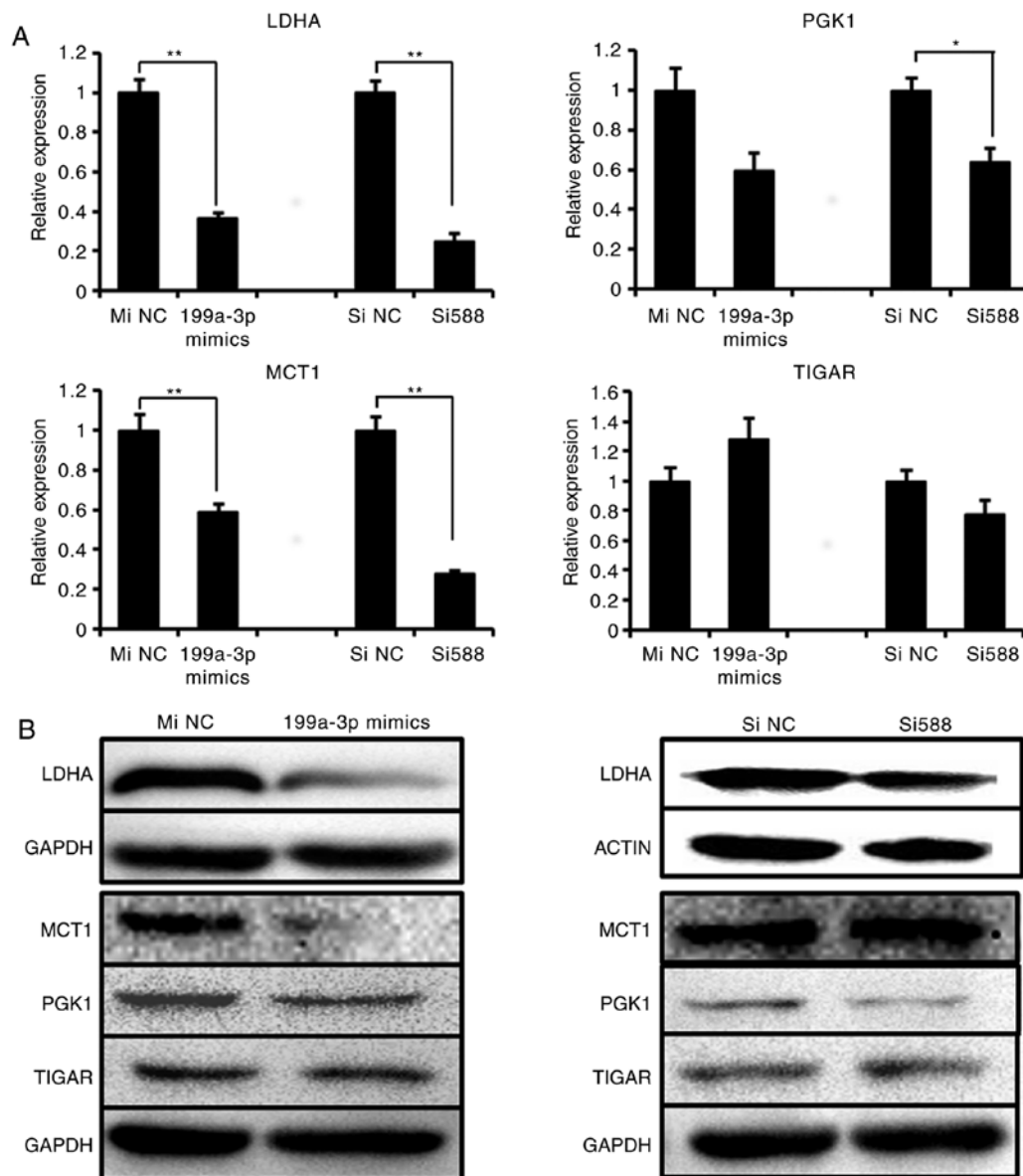


Figure 5. Sp1 Si588 and miR-199a-3p suppress the LDHA expression. (A) The mRNA expression and (B) protein expression of 4 metabolic genes (LDHA, PGK1, MCT1 and TIGAR) were detected in Ntera-2 cells following treatment with miR-199a-3p mimics or Sp1 Si588, respectively. * $P \leq 0.05$ and ** $P \leq 0.01$ vs. NC group. miRNA, microRNA; MiNC, miRNA negative control mimics; SiNC, siRNA negative control; Sp1, specificity protein 1; siRNA, small interfering RNA; LDHA, lactate dehydrogenase A; TIGAR, tumor protein 53 induced glycolysis regulatory phosphatase; PGK1, phosphoglycerate kinase 1; MCT1, solute carrier family 16 member 1.

in diverse malignant cell types has been demonstrated to decrease glycolytic activity and attenuate lactate, thereby controlling cell proliferation, migration and invasion (28,29). In addition, a number of studies have suggested that LDHA mRNA may be degraded at an epigenetic regulation level by miRNAs: miR-34a, -34c, -369-3p, -374a and -4524a/b (30,31). In testicular tumor cells, certain miRNAs, including miR-372, -373, -449, -383 and -199a have been identified to be dysregulated and indicated to contribute to the development of TGCTs (22). In the present study, miR-199a-3p as a suppressor in TGCTs, was demonstrated to repress LDHA expression and glucose metabolism by inhibiting lactic acid production, glucose intake, and ROS and ATP levels. As LDHA lacks the recognition site for miR-199a-3p, future studies will aim to identify the bridge molecule in this metabolic pathway.

Sp1 was the focus of the present study as it is bound directly to the promoter regions of not only the LDHA gene, but also the TIGAR, PGK1 and MCT1 genes (32). Among these 4 selected metabolic genes from our previous study (13), Sp1 demonstrated the most marked effect on LDHA gene transcription and translation, suggesting that LDHA is the key component of miR-199a-3p/Sp1 pathway in glycolytic metabolism of Ntera-2 cells. Using a dual luciferase reporter system, the present study identified that the overexpression of Sp1 led to increased activity of the LDHA promoter, whereas knockdown of Sp1 had the opposite effect, which verified the positive regulation of Sp1 on LDHA transcription. On the other hand, it was also confirmed that miR-199a-3p directly targets the 3'-UTR of Sp1 mRNA. Sp1 is one of the most well characterized transcription factor that serves an important role in controlling

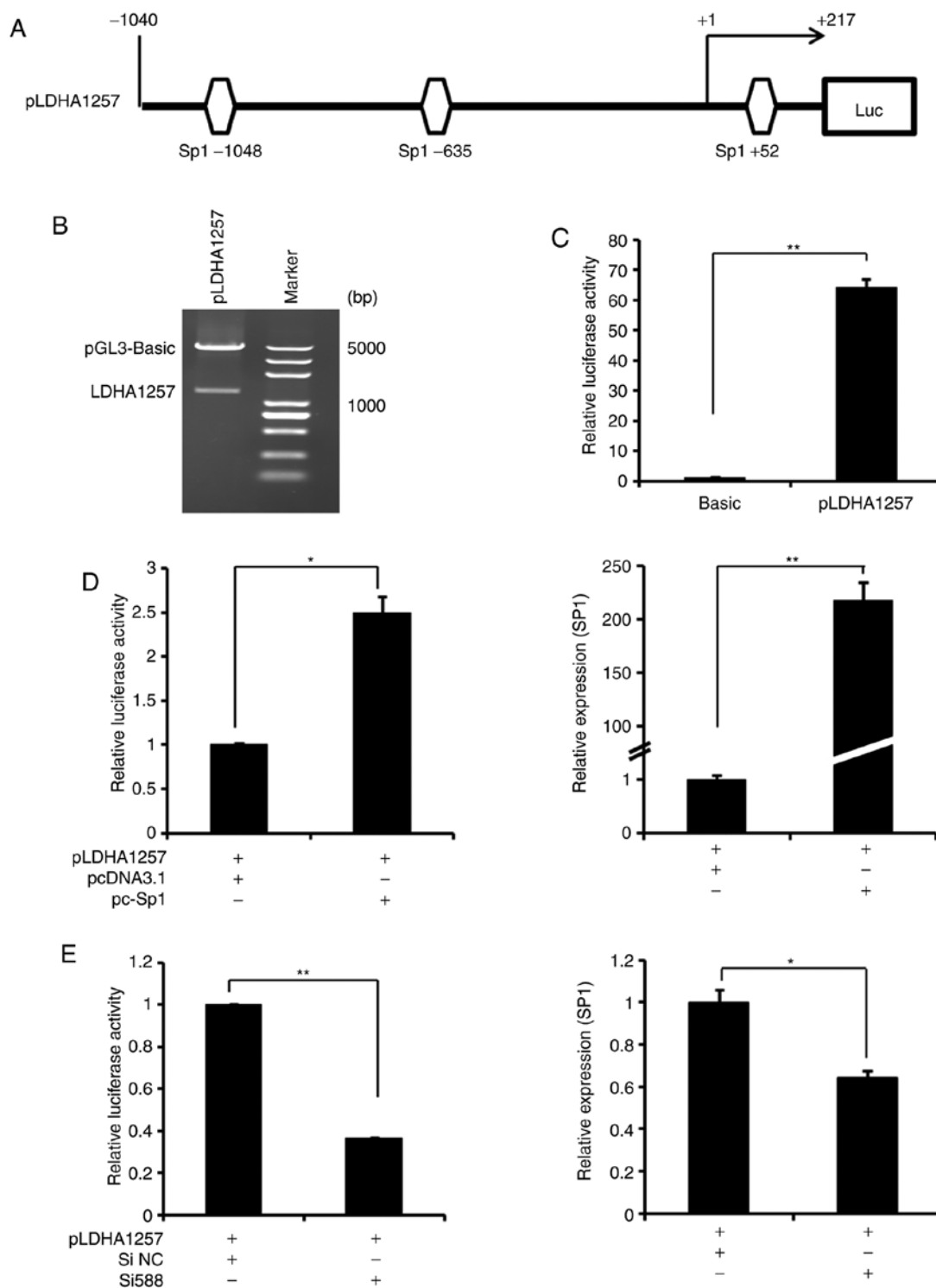


Figure 6. Transcriptional action of LDHA expression by Sp1 in Ntera-2 cells. (A) A schematic representation of the LDHA promoter, which contains 3 potential Sp1 binding sites. (B) Enzyme digestion analysis of the recombinant LDHA promoter (pLDHA1257). (C) Analysis of transcriptional activity of pLDHA1257 promoter in Ntera-2 cells using a reporter system. ** $P \leq 0.01$ vs. pGL3-basic group. (D) Analysis of transcriptional activity of pLDHA1257 promoter in Ntera-2 cells following with pcDNA3.1-Sp1 overexpression using a reporter system (left). Concurrently, Sp1 mRNA expression was detected following transfection (right). (E) Analysis of transcriptional activity of pLDHA1257 promoter in Ntera-2 cells following transfection with Sp1 siRNA (Si588) using a reporter system (left). Concurrently, Sp1 mRNA expression was detected subsequent to transfection (right). In each reporter system experiment, *Renilla* was used as an internal control. * $P \leq 0.05$ and ** $P \leq 0.01$ vs. control group. pc-Sp1, pcDNA3.1-Sp1. Sp1, specificity protein 1; LDHA, lactate dehydrogenase A; Luc, luciferase; si, small interfering; NC, negative control.

the transcription of numerous genes that contain GC boxes (consensus sequence: 5'-(G/T) GGGCGG (G/A) (G/A) (C/T)-3') in their promoters, particularly of those genes associated with the metabolism of nucleic acids and biosyn-

thesis (33). Abnormal activation of Sp1 occurs in a wide variety of human tumor types and high levels of Sp1 protein are considered to be a negative prognostic factor (34-36). The data of the present study suggested that the silencing of

Sp1 by RNAi may decrease aerobic glycolysis in testicular cancer cells. More importantly, in miR-199a-3p mimics-treated cells, the restoration of Sp1 expression markedly promoted glucose intake, lactate concentrations and ROS levels, implying that the overexpression of Sp1 rescues the effects of miR-199a-3p on glucose metabolism in Ntera-2 cells. Therefore, the data supported the hypothesis that Sp1 may serve as bridge molecule between miR-199a-3p and LDHA to regulate abnormal metabolism of testicular cancer cells.

In conclusion, the present study demonstrated that miR-199a-3p downregulated LDHA and consequently inhibited the Warburg effect through targeting Sp1, and suggested that the miR-199a-3p/Sp1/LDHA metabolic pathway is an underlying pathway in the pathogenesis of TGCTs.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

DL designed the research and wrote the manuscript. SZ and ZM performed the research and wrote the manuscript. KS, SQ, JZ, HD, HL, XL and ZG performed the research. All authors analyzed the data and were involved in writing the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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