microRNA-199a-3p functions as tumor suppressor by regulating glucose metabolism in testicular germ cell tumors

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Abstract. microRNA (miR)-199a-3p serves critical roles in cancer development and progression. In order to improve knowledge of the functional mechanism of miR-199a-3p in testicular tumors, the present study characterized the regulation of aerobic glycolysis by miR-199a-3p and its impact on metabolism. Using 3-4,5-dimethylthiazol-2-yl-2,5 diphenyl tetrazolium bromide, wound healing and flow cytometry assays, it was determined that overexpression of miR-199a-3p in Ntera-2 cells caused suppression of cell growth and migration. Further biochemical methods and high-throughput quantitative polymerase chain reaction array of metabolic genes showed that inhibition of miR-199a-3p markedly elevated lactate production and 12 differentially expressed genes, including 2 upregulated and 10 downregulated genes, were identified following treatment with miR-199a-3p in Ntera-2 cells. In clinical samples, four selected genes, *lactate dehydrogenase A, monocarboxylate transporter 1, phosphoglycerate kinase 1* and *TP53-inducible glycolysis and apoptosis regulator*, were significantly overexpressed in malignant testicular germ cell tumor, and their expression inversely correlated with the expression of miR-199a-3p, suggesting that these four genes may be affected by miR-199a-3p. Using bioinformatics analysis, the transcription factor Sp1 binding site was identified in the promoter region of the four selected genes. In addition, miR-199a-3p was predicted to bind to conservative target sequences in the 3'-untranslated region of Sp1 mRNA, suggesting that miR-199a-3p may downregulate these four metabolic genes through Sp1. It was demonstrated the dysregulated expression and activation of miR-199a-3p may serve important roles in aerobic glycolysis and tumorigenesis in patients with testicular cancer. Therefore, miR-199a-3p may be a potential biomarker in the prognosis and treatment of testicular tumors.

Introduction

micro (mi)RNAs are endogenous, small and non-coding RNAs, which negatively regulate protein expression by mRNA cleavage or translation repression (1). Since the first miRNA was reported in *Caenorhabditis elegans* by Lee *et al* (2), an large quantity of miRNAs have been identified and subsequent studies indicated that they serve crucial roles in various biological process and regulate the expression of up to 30% of human genes (3,4). Previous studies suggested that some specific miRNAs have an important role in the pathogenesis of tumors by functioning as oncogenes or tumor suppressor genes (5-7). In addition, expression profiling of miRNAs can also be used for differentiation of the major histological subtypes of tumors (8,9). As a result of their high sensitivity and the relatively easy method of detection, miRNAs have been shown to exhibit great potential as novel biomarkers for diagnosis, prognosis and therapy in cancer (9).

Testicular germ cell tumors (TGCTs) arise from carcinoma *in situ* cells, which resemble malignant primordial germ cells of fetal origin. Histologically, TGCTs are divided into seminomas and non-seminomas. For the clinical management of TGCTs, blood-based markers, including lactate dehydrogenase, α-fetoprotein and human chorionic gonadotropin are used for diagnosis, risk assessment and determining patient prognosis (9). However, these biomarkers are useless for ~40% of patients with TGCTs, suggesting that it is necessary to discover novel candidate biomarkers. Recently, certain miRNAs, including miR-372, -373, -449, -383 and -199a have been identified to be dysregulated in TGCTs and they contribute to the development of TGCTs (9-11). Among them, miR-199a-3p, one of the miR-199a family, is of particular interest since it was reported to be specifically upregulated by transforming growth factor (TGF)-β1 signaling in mouse GC-1 spg germ

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cells and may be associated with germ cell development in our previous study (12). In testicular cancer, miR-199a-3p has been reported to suppress cell growth, migration, invasion and metastasis by regulating TGF-β1 signaling through the regulation of Smad4 (13). Chen *et al* (11) recently found that miR-199a-3p negatively regulated DNA methylation, partly through targeting DNA (cytosine-5)-methyltransferase 3A, in TGCTs (11). Although these interesting functional studies have addressed the role of miR-199a-3p in cancer development and progression, the regulatory role and underlying mechanisms of miR-199a-3p in metabolism of TGCTs remains unknown.

The present study characterized the regulation of aerobic glycolysis by miR-199a-3p and its impact on testicular cancer metabolism. It was revealed that miR-199a-3p suppressed cell growth and migration, and inhibition of miR-199a-3p increased the production of lactate in Ntera-2 cells and affected testicular tumor metabolism by downregulating metabolic genes, including *lactate dehydrogenase A (LDHA), monocarboxylate transporter 1 (MCT1), phosphoglycerate kinase 1 (PGK1)* and *TP53-inducible glycolysis and apoptosis regulator (TIGAR)*, suggesting that miR-199a-3p functions as a tumor suppressor in TGCTs by regulating the metabolism signaling pathway.

Materials and methods

Clinical tissue specimens. Formalin fixed paraffin embedded (FFPE) human testicular tumors tissue specimens, including adjacent non-tumor tissues, were collected at the Department of Pathology, Xiangya Hospital of Central South University (Hunan, China). Clinical tissue specimens included five normal testes and five TGCTs tissues. The present study was approved by the Independent Ethical Committee of Xiangya Hospital of Central South University.

Cell culture and transient transfections. The human Ntera-2 testicular tumor cell line [CRL-1973; American Type Culture Collection (ATCC), Rockville, MD, USA] and the human embryonic kidney cell line HEK293 (CRL-1573; ATCC) were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 units/ml penicillin and 100 µg/ml streptomycin. The cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2 in air. The cells were seeded into a 6-well plate and cultured for 24 h prior to transfection. Then either miR-199a-3p mimics, inhibitors or the negative control (NC) were transfected into the cells at a final concentration of 50 or 100 nM using TurboFect™ *in vitro* Transfection reagent (Fermentas, Burlington, Canada), according to the manufacturer's protocol. The culture medium was discarded following transfection for 12 h and was replaced with the fresh medium. The miR-199a-3p mimics, inhibitors and the NC were all synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China).

Bioinformatics analysis. The expression abundance of miR-199a-3p in different human tissues was analyzed by GEO Datasets (http://www.ncbi.nlm.nih.gov/gds/). Using both 'miR-199a-3p' and 'Expression profiling by array' as key words, three data sets, including GSE65626, GSE53437 and GSE14985, were selected and categorized using statistical analysis. The data of GSE65626 was derived from glioblastoma tissue and matched adjacent normal tissue. The data of GSE53437 was derived from heart failure tissue and matched adjacent normal tissue. The data from GSE14985 was from multiple cancer tissue and matched adjacent normal tissue. The potential target genes of miR-199a-3p were searched for using TargetScan 6.2 (http://www.targetscan.org/). An RNAhybrid tool (bibiserv.techfak.uni-bielefeld.de/rnahybrid) was subsequently used to analyze the minimum free energy hybridization of the miR-199a-3p and target mRNAs. RNAFold WebServer (rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) was used to predict the secondary structure of single-stranded target mRNAs. Bioinformatics analysis of promoter sequence and potential transcription factor binding sites within the 5'-regulatory region of the selected metabolic genes was performed using the EPD database (http://epd.vital-it.ch/) and MatInspector (http://www.genomatix.de/online_help/help_matinspector/matinspector_help.html).

RNA isolation of FFPE testicular tissues and Ntera-2 cells. The total RNA from FFPE tissue specimens, including testicular tumors and normal testes, were isolated using RecoverAll™ Total Nucleic Acid Isolation kit (Ambion; Thermo Fisher Scientific), according to the manufacturer's protocol. For total RNA isolation of Ntera-2 cells, RNAiso (Takara Bio., Inc., Otsu, Japan) was used.

cDNA synthesis and quantitative polymerase chain reaction (qPCR) for miRNA and mRNA detection. PrimeScript miRNA qPCR Starter kit Ver. 2.0 (Takara Bio., Inc.) was used to detect the expression of miRNA. Briefly, 3 µg total RNA was digested with DNase I (Fermentas) and used for miRNA reverse transcription by PrimeScript miRNA qPCR Starter kit Ver. 2.0 (Takara Bio., Inc.) or for mRNA reverse transcription by PrimeScript RT reagent kit with gDNA Eraser (Takara Bio., Inc.). Following this, real-time qPCR was performed using a SYBR Premix Ex Taq II kit and the MX3000 instrument (Stratagene, La Jolla, CA, USA). The thermocycling conditions were as follows: Initial denaturation, 95°C for 10 min; 40 cycles of 95°C for 10 sec, 60°C for 30 sec and 72°C for 32 sec. For miRNA, has-miR-15a was used as an internal control and for mRNA, GAPDH was used as an internal control. Primer sequences were as follows: GAPDH, forward: 5'-GACCCCTTCATTGACCTCAA-3' and reverse: 5'-GCATGGACTGTGGTCATGAGT-3'. The results were quantified using the $2^{-\Delta\Delta Cq}$ method (14).

Cell viability analysis by 3-4,5-dimethylthiazol-2-yl-2,5 diphenyl tetrazolium bromide (MTT) assay. Ntera-2 cells at a density of $5x10^3$ cells/well were seeded into a 96-well plate with 100 µl medium for 24 h. When the cells reached 70% confluence, they were trasfected with NC, miR-199a-3p mimics or inhibitor as above. MTT reagent (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added to each well for 0, 24, 72 h. The cells were subsequently incubated for 4 h at 37°C and 150 µl dimethyl sulfoxide was added to each well following the removal of the medium to dissolve the formazan products. The absorbance at a wavelength of 570 nm in each well was determined using an ELISA reader.

Cell migration analysis by wound healing assay. Ntera-2 cells at a density of $1x10^5$ cells/well were seeded into a 6-well-plate
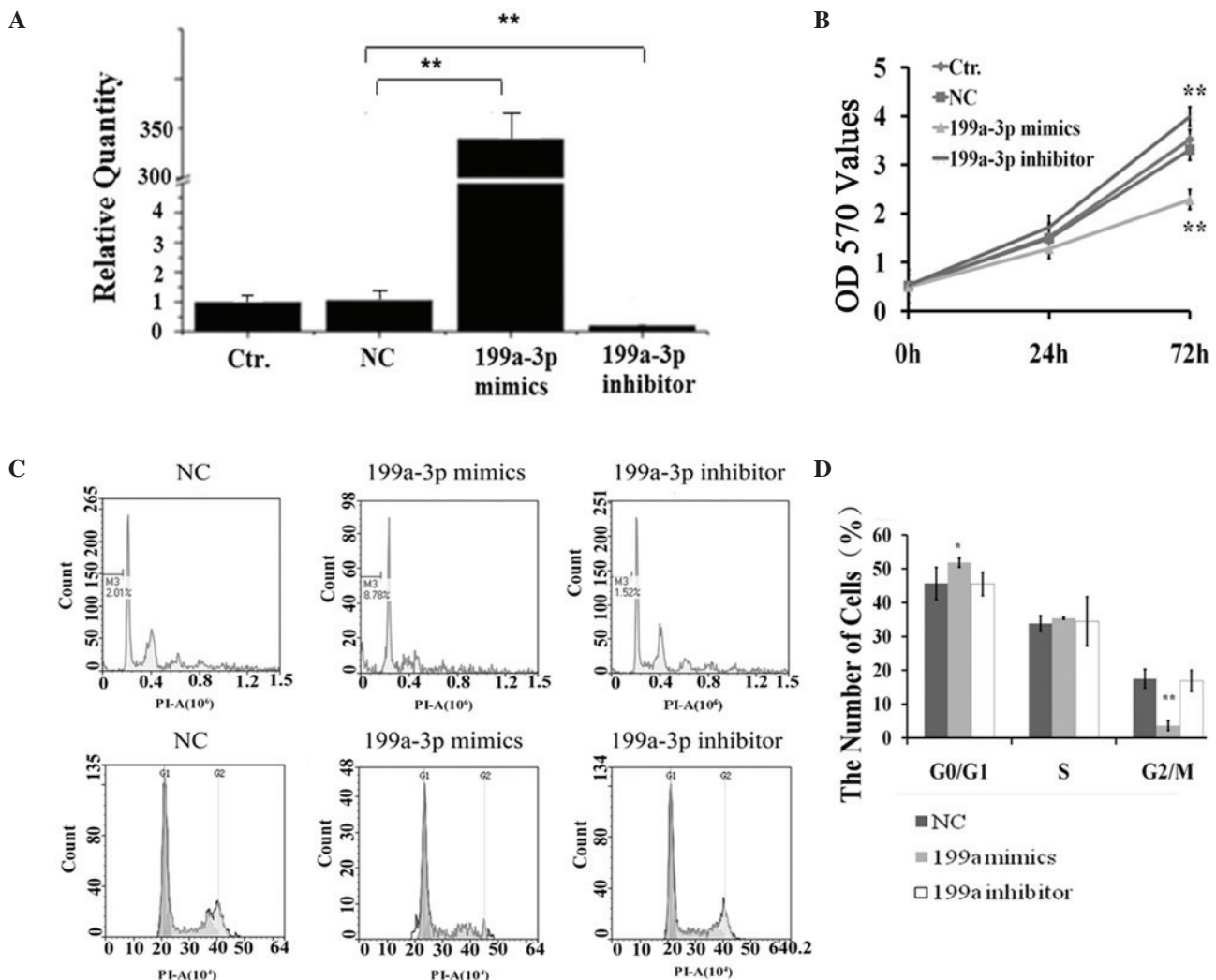
Figure 1. miR-199a-3p inhibits the proliferation of Ntera-2 cells. (A) The transfection efficiency of miR-199a-3p mimics or inhibitor after 48 h was assessed using reverse transcription-quantitative polymerase chain reaction. The values were normalized against that of the NC group. (B) The effects of miR-199a-3p mimics or inhibitor at 0, 24 or 72 h on the viability of Ntera-2 cells were assessed using an 3-4,5-dimethylthiazol-2-yl-2,5 diphenyl tetrazolium bromide assay. (C and D) The effects of miR-199a-3p mimics or inhibitor on the cell cycle and apoptosis of Ntera-2 cells were analyzed by flow cytometry following staining with propidium iodide. (C) Representative cell apoptosis (above) and cell cycle distribution plots (below) and (D) the numbers of cells in each phase of the cell cycle are shown (total 1x10^5). The data are expressed as the mean ± standard deviation (n=3; **P<0.01 vs. NC). miR, microRNA; OD, optical density; NC, cells transfected with scramble sequence.

with RPMI-1640 medium were grown to 80% confluence and transiently transfected with 100 nM NC, miR-199a-3p mimics or inhibitors as above. The 100% confluent monolayer Ntera-2 cells were subsequently scraped with a sterile 100 µl pipette tip and the cell debris was washed with D-hanks solution. The cells were incubated at 37°C with 5% CO_2 overnight. Images were captured under bright field light microscopy with a Nikon Eclipse E600 microscope and an RS Photometrics CoolSNAP camera at 0 and 48 h after scraping.

Flow cytometry analysis. For apoptosis or cell cycle analysis, 1-5x10^5 Ntera-2 cells from each sample were digested with 0.25% trypsin and fixed in 70% cold ethanol overnight after resuspending in D-hanks three times. The cells were subsequently collected and were mixed with propidium iodide staining solution at a final concentration of 50 µg/ml for 30 min at room temperature in the dark. All samples were analyzed using flow cytometry, according to the manufacturer's protocols.

Glucose metabolic assay. Ntera-2 cells were transfected with either miR-199a-3p mimics or inhibitor, using NC as a control. The supernatants were collected after 24 and 48 h, and detected using an Automatic Biochemical Analyzer (7170A; Hitachi, Tokyo, Japan).

qPCR array screening for metabolic genes. Briefly, the total RNA was isolated from Ntera-2 cells, which were treated with either NC, miR-199a-3p mimics or inhibitor for 48 h. The RNA was subsequently converted into cDNA. qPCR of 148 metabolic genes was performed using a MX3000 instrument (Stratagene) by mixing equal quantities of cDNA, SYBRGreen mix (CWBio, Beijing, China) and specific primers. Each sample was analyzed in triplicate. All real-time data were normalized against GAPDH and analyzed using the RT^2 profiler PCR Array Data Analysis software version 3.5 (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). The primers used in qPCR screening are as described previously (15).
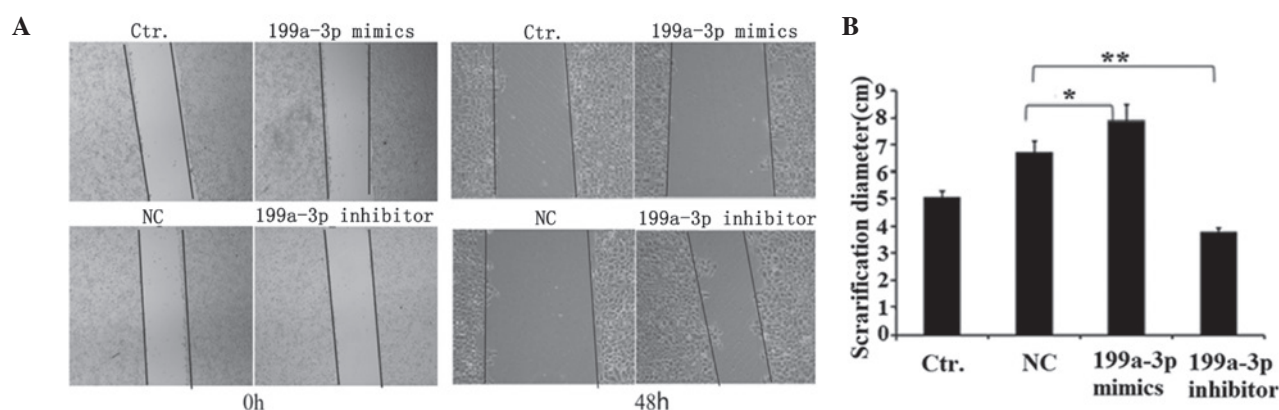
Figure 2. miR-199a-3p inhibits migration of Ntera-2 cells. (A) Ntera-2 cells were transfected with either miR-199a-3p mimics or inhibitor, using NC as control. Then cell migration was measured by wound healing assay. Images were captured at 0 and 48 h after Ntera-2 cells were scraped. (B) The scarification diameter of the wound healing assay results (*P<0.05, **P<0.01 compared with the NC). Ctr., control untransfected; NC, cells transfected with scramble sequencer; miR, microRNA.
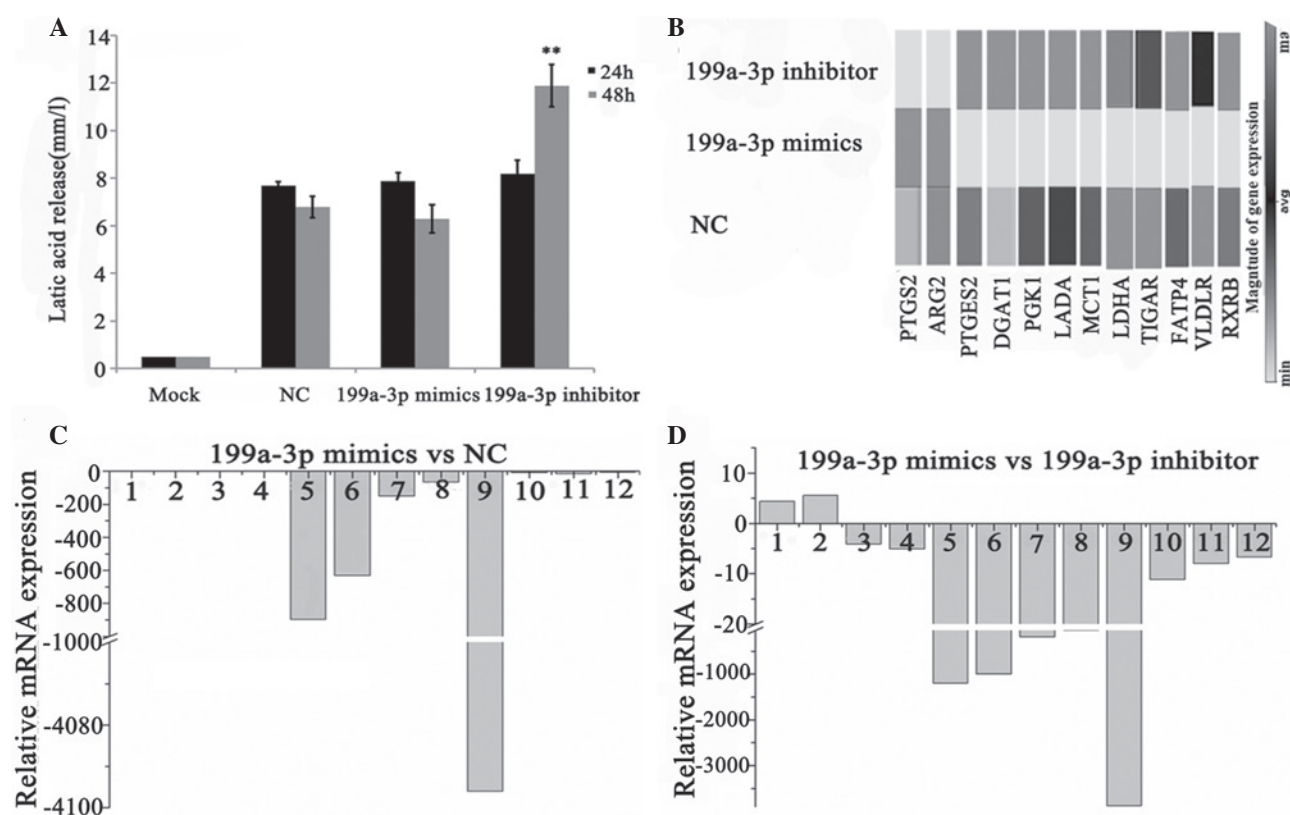

Figure 3. Impact of miR-199a-3p on the metabolism of Ntera-2 cells. (A) The production of lactate of Ntera-2 cells was detected following treatment with NC, miR-199a-3p mimics or inhibitor for 24 or 48 h. (B) A heat-map of 12 selected metabolic genes in Ntera-2 cells, which were regulated by miR-199a-3p. The expression levels are depicted by a scale from black (high expression) to gray (low expression). (C) The relative mRNA expression of 12 selected metabolic genes in the miR-199a-3p mimics group compared with the NC group. (D) The relative mRNA expression of 12 selected metabolic genes in miR-199a-3p mimics group compared with miR-199a-3p inhibitor group. (**P<0.01). 1-12 refers to *PTGS2, ARG2, PTGES, DGAT1, PGK1, LADA, MCT1, TIGAR, LDHA, FATP4, VLDLR* and *RXRB,* respectively. NC, cells transfected with scramble sequence; PTGS2, prostaglandin-endoperoxide synthase 2; ARG2, arginase 2; PTGES, prostaglandin E synthase; DGAT1, diacylglycerol O-acyltransferase 1; PGK1, phosphoglycerate kinase 1; LADA, latent autoimmune diabetes of adults; MCT1, monocarboxylate transporter 1; TIGAR, TP53-inducible glycolysis and apoptosis regulator; LDHA, lactate dehydrogenase A; FATP4, fatty acid transport protein 4; VLDLR, very-low-density-lipoprotein receptor; RXRB, retinoid X receptor β.

Statistical analysis. The data are expressed as the mean ± standard deviation (n=3). Statistical analysis was performed using the Student's t-test. P≤0.05 was considered to indicate a statistically significant difference. For the qPCR array assay, the fold changes of ≥4 or ≤0.5 (P≤0.05) were analyzed using t-test and P-value.

Results

miR-199a-3p inhibits the proliferation of Ntera-2 cells. Ntera-2 cells were transfected with either miR-199a-3p mimics or inhibitor, and the results of miRNA PCR confirmed that the transfection was highly efficient, stable and useful for the
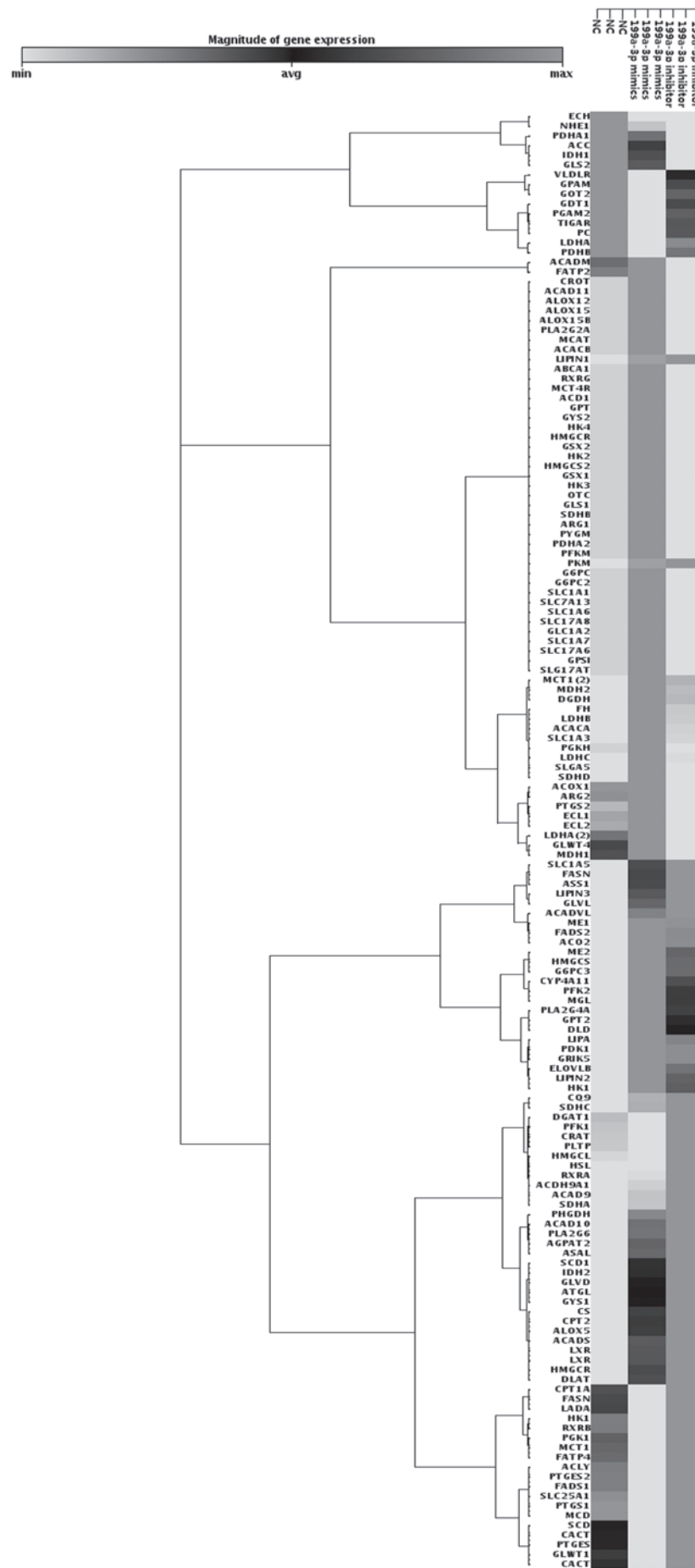
Figure 4. Impact of miR-199a-3p on the metabolism of Ntera-2 cells. A heat-map for metabolic gene profile of the Ntera-2 cells induced by miR-199a-3p was performed by quantitative polymerase chain reaction array screening. miR, microRNA; NC, cells transfected with scramble sequence.
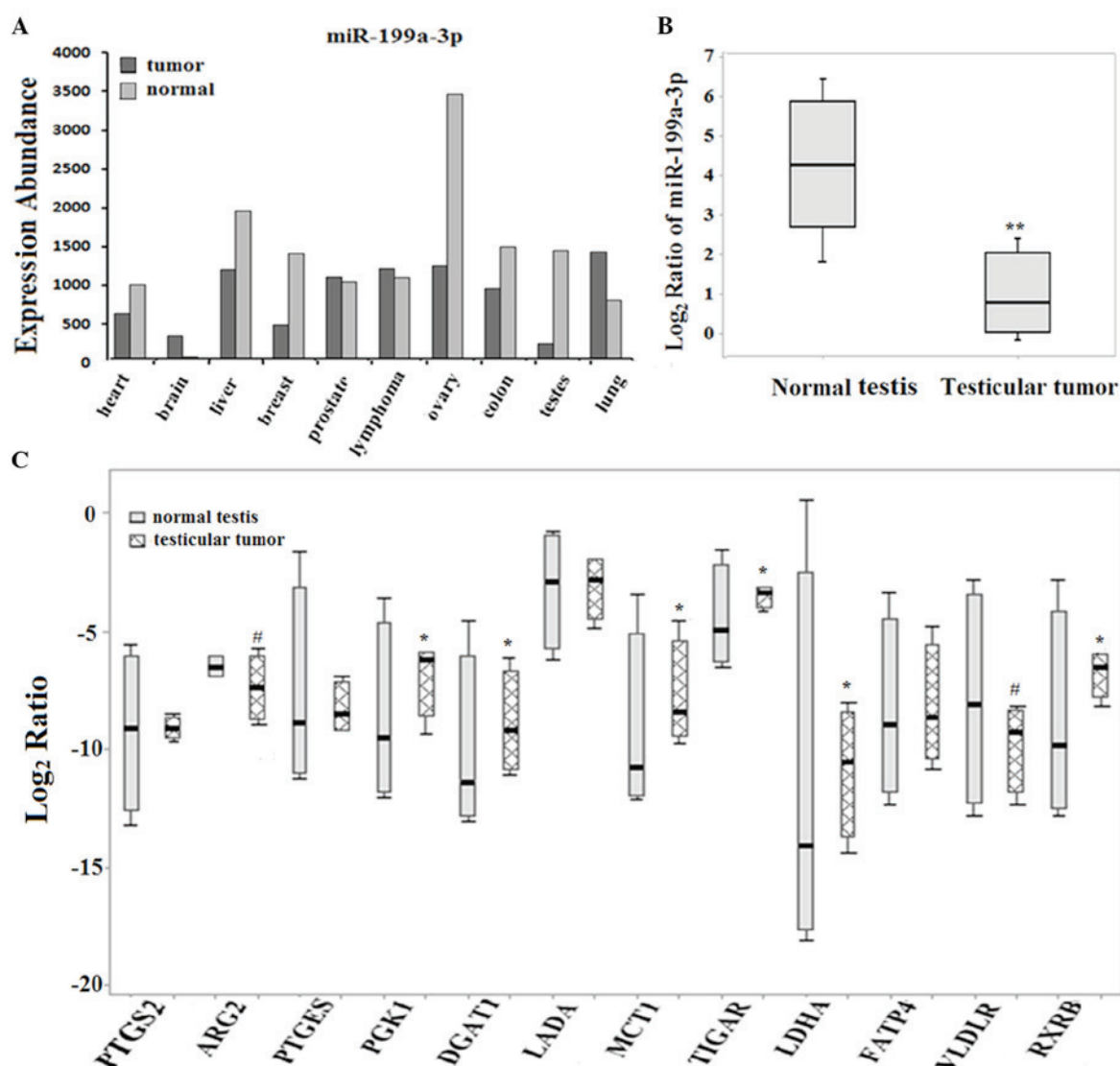
Figure 5. Expression levels of 12 selected metabolic genes in clinical testicular samples. (A) The expression profile of miR-199a-3p in various tissues was analyzed by bioinformatics. (B) The expression of miR-199a-3p in both normal testes and testicular tumors. (**P<0.01) (C) The expression of 12 selected genes in normal testes and testicular tumors. For clinical tissue specimens, the mRNA expressions were represented with the box plots by calculating the Log$_2$ ratio, which was normalized against *glyceraldehyde 3-phosphate dehyrogenase* (*P<0.05 for upregulation, #P<0.05 for downregulation). PTGS2, prostaglandin-endoperoxide synthase 2; ARG2, arginase 2; PTGES, prostaglandin E synthase; DGAT1, diacylglycerol O-acyltransferase 1; PGK1, phosphoglycerate kinase 1; LADA, latent autoimmune diabetes of adults; MCT1, monocarboxylate transporter 1; TIGAR, TP53-inducible glycolysis and apoptosis regulator; LDHA, lactate dehydrogenase A; FATP4, fatty acid transport protein 4; VLDLR, very-low-density-lipoprotein receptor; RXRB, retinoid X receptor β.

following experiments (Fig. 1A). An MTT assay revealed that the viability and growth of Ntera-2 cells were significantly inhibited by miR-199a-3p mimics compared with the negative control. Conversely the proliferation of cells was notably induced following treatments with the miR-199a-3p inhibitor (Fig. 1B). Using flow cytometry analysis, cell apoptosis and cell cycle distribution of Ntera-2 cells treated with NC, miR-199a-3p mimics or inhibitor were also assessed. As shown in Fig. 1C and D, compared with the cell population of the NC group (G0/G1, 45.66±1.79%; S, 33.83±2.07%; G2/M, 17.15±2.29%) and the miR-199a-3p inhibitor group (G0/G1, 45.53±1.46%; S, 34.44±3.76%; G2/M, 16.92±2.54%), the miR-199a-3p mimics group exhibited an increased G0/G1 phase cell population (51.83±3.25%) and decreased G2/M phase cell population (3.61±3.15%), but no obvious effect on the percentage of S

phase cells (35.4±1.34%). In addition, the apoptotic cell population (pre-G1 peak) was notably increased in the miR-199a-3p mimics group (8.78%), but not in the other groups. Therefore, these data suggested that miR-199a-3p has an anti-proliferation effect on Ntera-2 cells and it may induce Ntera-2 cells apoptosis.

miR-199a-3p inhibits the migration of Ntera-2 cells. Cell migration was measured using a wound healing assay in Ntera-2 cells treated with NC, miR-199a-3p mimics or inhibitor. The cells treated with miR-199a-3p mimics were less migratory compared with the NC control or untreated cells at 48 h after scratching (Fig. 2A and B). By contrast, inhibition of miR-199a-3p significantly promoted the migration of Ntera-2 cells (P<0.01; Fig. 2B). This result revealed that miR-199a-3p may inhibit the migration of Ntera-2 cells.
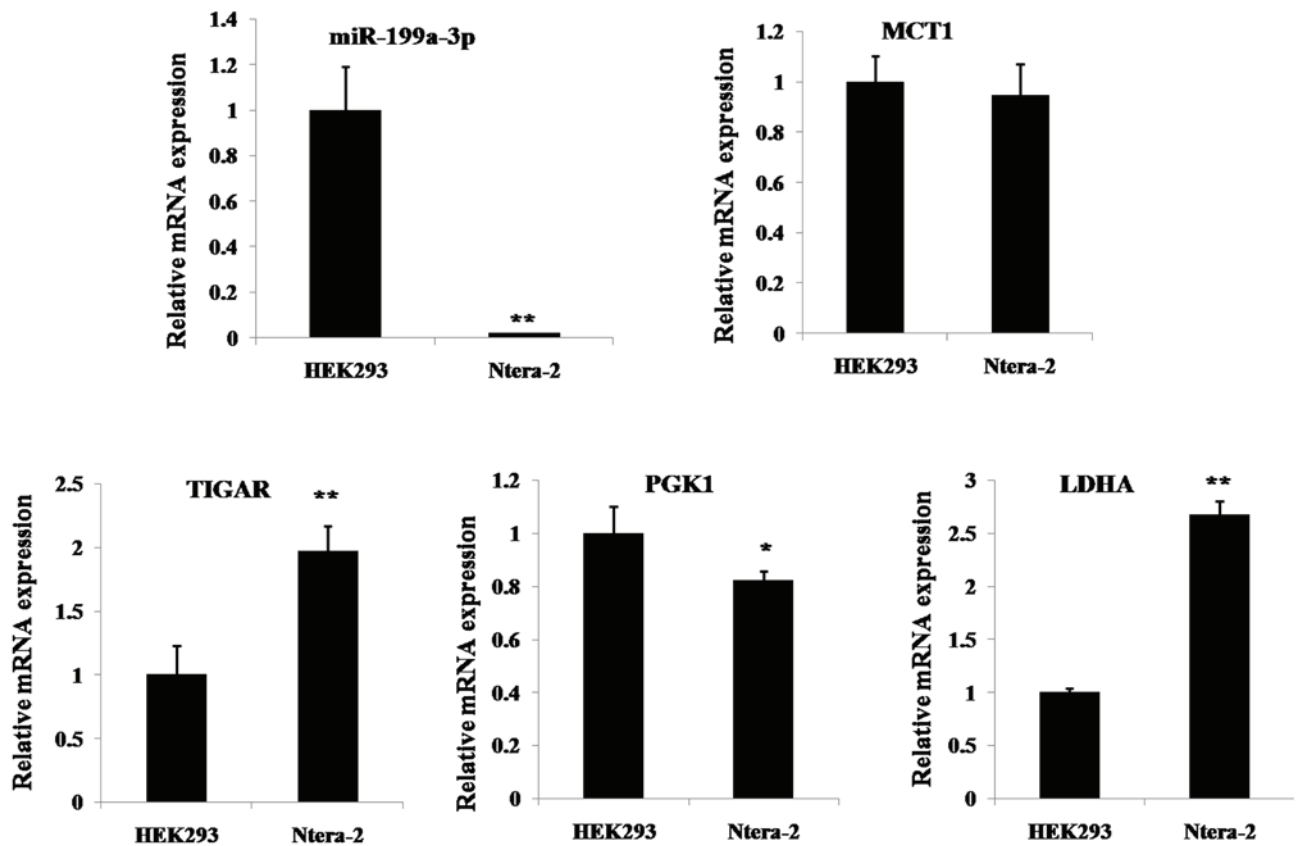
Figure 6. mRNA expression levels of miR-199a-3p and four selected metabolic genes in Ntera-2 cells and HEK293 cells. The mRNA expression levels of the selected genes were determined by reverse transcription-quantiitative polymerase chain reaction. $^*P<0.05$, $^{**}P<0.01$ vs. the HEK293 cells. MCT1, monocarboxylate transporter 1; TIGAR, TP53-inducible glycolysis and apoptosis regulator; LDHA, lactate dehydrogenase A; PGK1, phosphoglycerate kinase 1.
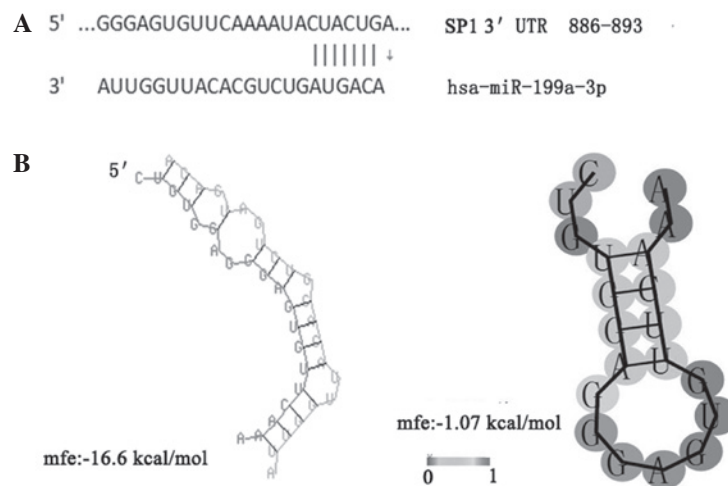

Figure 7. miR-199a-3p and its predicted target gene Sp1. (A) The predicted miR199a-3p binding sites in 3'UTR of Sp1 mRNA sequence. (B) The analysis of minimum free energy hybridization. The figure on the left shows the minimum free energy hybridization of miR199a-3p and Sp1 mRNA was predicted by RNAhybrid; the figure on the right shows the secondary structure of single-stranded Sp1 mRNAs was predicted by the RNAFold WebServer. UTR, untranslated region; miR, microRNA.

Inhibition of miR-199a-3p significantly increases the production of lactate in Ntera-2 cells. The production of lactate is an important index of glycolysis. By means of detecting lactate release of Ntera-2 cells after miR-199a-3p treatment, it was revealed that no statistically significant changes occurred in the miR-199a-3p

mimics group for both 24 and 48 h treatment compared with the NC group (Fig. 3A). Additionally, the miR-199a-3p inhibitor group notably increased the production of lactate at 48 h (P<0.01). These results indicated that inhibition of miR-199a-3p significantly enhanced the metabolism of glucose.

Table I. Expression of miR-199a-3p and 12 selected genes in Ntera-2 cells transfected with the miR-199a-3p mimics or inhibitor and in testicular tumors (n=5) vs. normal clinical samples (n=5).

Gene	miR-199a-3p mimics vs. inhibitor in Ntera-2 cells	Testicular tumors vs. normal testes
miR-199a-3p	↑	↓
PTGS2	↑	No change
ARG2	↑	↓
PTGES	↓	No change
PGK1	↓	↑
DGAT1	↓	↑
LADA	↓	No change
MCT1	↓	↑
TIGAR	↓	↑
LDHA	↓	↑
FATP4	↓	No change
VLDLR	↓	↓
RXRB	↓	↑

↑, upregulation; ↓, downregulation; miR, microRNA; PTGS2, prostaglandin-endoperoxide synthase 2; ARG2, arginase 2; PTGES, prostaglandin E synthase; DGAT1, diacylglycerol O-acyltransferase 1; PGK1, phosphoglycerate kinase 1; LADA, latent autoimmune diabetes of adults; MCT1, monocarboxylate transporter 1; TIGAR, TP53-inducible glycolysis and apoptosis regulator; LDHA, lactate dehydrogenase A; FATP4, fatty acid transport protein 4; VLDLR, very-low-density-lipoprotein receptor; RXRB, retinoid X receptor β.

Regulation of miR-199a-3p on the metabolic gene expression profile of Ntera-2 cells. qPCR array screening was applied to investigate the changes at the mRNA level in Ntera-2 cells following miR-199a-3p transfection by comparison with the control group (Figs. 3 and 4). Global gene expression levels induced by miR-199a-3p in Ntera-2 cells from the qPCR array analysis are presented in Fig. 3B and Fig. 4. The differentially expressed genes with fold changes of ≥4 or ≤0.5 (P≤0.05) were analyzed using t-test and P-value. As shown in Fig. 3D and E, the expression of 12/148 genes were altered following treatment with the miR-199a-3p mimics compared with the inhibitor treatment. Of these, two genes, *prostaglandin-endoperoxide synthase 2* (*PTGS2*) and *arginase 2* (*ARG2*) were upregulated. The other 10 genes, *prostaglandin E synthase* (*PTGES*), *diacylglycerol O-acyltransferase 1* (*DGAT1*), *PGK1*, *latent autoimmune diabetes of adults* (*LADA*), *MCT1*, *TIGAR*, *LDHA*, *fatty acid transport protein 4* (*FATP4*), *very-low-density-lipoprotein receptor* (*VLDLR*) and *retinoid X receptor β* (*RXRB*) were downregulated specifically. Whereas, eight downregulated genes (*PGK1, LADA, MCT1, TIGAR, LDHA, FATP4, VLDLR, RXRB*) were observed in miR-199a-3p mimics group compared with the NC group.

Expression abundance of miR-199a-3p and selected metabolic genes in both TGCT tissues and cell lines. The clinical relevance of aberrant metabolism caused by dysregulated

miR-199a-3p signaling was determined using TGCTs and normal testis tissue specimens. In accordance with the bioinformatics analysis (Fig. 5A), the miR-199a-3p expression in testicular tumors was obviously decreased compared with that in normal testes. For the selected genes, *PTGS2, PTGES, LADA* and *FATP4* revealed no significant change between normal testes and testicular tumors, while *ARG2* and *VLDLR* were downregulated in the clinical samples (Fig. 5C; Table I). Notably, the other four genes, including *LDHA, MCT1, PGK1* and *TIGAR*, were markedly upregulated in testicular tumors, which were similar to the results in Ntera-2 cells. In order to further understand the association between miR-199a-3p and the four selected metabolic genes, *LDHA, MCT1, PGK1* and *TIGAR*, the expression levels in cell lines, including HEK293 and Ntera-2 cells were detected. Fig. 6 demonstrated that miR-199a-3p is expressed at a higher level in HEK293 cells compared with the levels in Ntera-2 cells. For the four selected genes, the mRNA level of *MCT1* was similar in both HEK293 and Ntera-2 cells, while the mRNA expression of *PGK1* was slightly higher in HEK293 cells compared with in Ntera-2 cells. By contrast, a significantly lower expression of both *TIGAR* and *LDHA* was present in HEK293 cells compared with the Ntera-2 cells, which is the opposite expression signature compared with miR-199a-3p.

miR-199a-3p may downregulate the expression of four metabolic genes via the Sp1 binding site. Using TargetScan analysis, it was demonstrated that none of the four selected genes (*LDHA, MCT1, PGK1* and *TIGAR*) have the potential recognition site of miR-199a-3p, which suggests that the regulation is not direct. In order to understand how miR-199a-3p downregulates the expression of these genes, the promoter sequence of the four genes was analyzed and revealed that the binding site of Sp1, a common transcription factor, was in the promotor region of all four selected genes (data not shown). Notably, it was predicted using TargetScan analysis that miR-199a-3p binds to the target sequences in the 3'-untranslated region (UTR) of Sp1 mRNA. In addition, further bioinformatics analysis revealed that the minimum free energy hybridization of miR-199a-3p binding with the target gene Sp1 3'UTR was markedly lower compared with that of the secondary structure of single-stranded Sp1 mRNA, indicating that Sp1 and miR-199a-3p have a higher possibility for binding (Fig. 7). Therefore, the present study hypothesized that miR-199a-3p may downregulate the four metabolic genes via Sp1.

Discussion

The metabolic shift to aerobic glycolysis is a common hallmark of cancer. Normally, non-cancerous cells catabolize glucose by oxidative phosphorylation in the mitochondria to produce adenosine triphosphate (ATP). However, in proliferating cancer cells, glucose carbons were predominantly converted to lactate, even with supply of adequate oxygen. This phenomenon of metabolic alteration in cancer is termed the 'Warburg effect' (16). Advances in cancer metabolism research over the last decade have demonstrated that metabolic alterations can enhance a cancer cells capability for proliferation, migration and invasion. In addition, clinical studies have shown a close correlation between elevated levels of lactate and poor patient prognosis or

overall survival in different cancer types (17). Therefore, certain biological molecules involved in metabolic process are currently being considered as therapeutic targets for cancer.

Since it was discovered that miRNAs are abnormally expressed in cancer, increasing data has revealed that miRNAs serve crucial roles in tumor growth by regulating their target genes and a large number of miRNAs have been identified to regulate cancer metabolism (18). Previous studies showed that the alterations of miR-199a-3p, a member of miR-199a family that controls the fate of cell survival and death, were associated with the pathogenesis and progression of cancer, and it can behave either as an oncogene or as tumor suppressor in different cancer types (19-21). In the present study, bioinformatics analysis (Fig. 5A) revealed that the expression of miR-199a-3p was upregulated in tumors, including brain and lung cancer, and downregulated in tumors, including liver, breast, ovary, colon and testicular cancer, suggesting that patterns of miR-199a-3p expression tend to vary among tumor types and its functions are complicated in different tissues. The present study also investigated that overexpression of miR-199a-3p caused the inhibition of Ntera-2 cell proliferation and apoptosis, G1 and G2 phase arrest and suppressed cell migration (Figs. 1 and 2), which is consistent with a previous study and suggested miR-199a-3p was a tumor suppressor in testicular tumor cells.

To improve the understanding of the functional mechanism of miR-199a-3p as a tumor suppressor in testicular cancer, the present study performed a biochemical method and high-throughput qPCR array screening analysis to detect the effect of miR-199a-3p on global glycolysis metabolism pattern in Netra-2 cells. As shown in Figs. 3 and 4, the inhibition of miR-199a-3p increased the production of lactate and a list of metabolic genes regulated by miR-199a-3p was identified. Of these, two genes (*PTGS2, ARG2*) were upregulated and 10 genes (*PTGES, DGAT1, PGK1, LADA, MCT1, TIGAR, LDHA, FATP4, VLDLR, RXRB*) were downregulated specifically. In clinical samples, the expression of miR-199a-3p was clearly reduced in testicular germ cell tumors compared with normal testicular tissue. The selected metabolic genes, including *LDHA, MCT1, PGK1* and *TIGAR*, downregulated by miR-199a-3p in Netera-2 cells, were significantly overexpressed in malignant testicular tumors, and the expression of these genes was inversely correlated with the expression of miR-199a-3p (Fig. 5; Table I). Among these four genes, a significantly lower expression of both *TIGAR* and *LDHA* was present in human embryonic kidney cells (HEK293) compared with testicular tumor cells (Ntera-2), which is the opposite expression signature compared with miR-199a-3p (Fig. 6). These results suggested that miR-199a-3p may serve important roles in aerobic glycolysis and tumorigenesis.

During aerobic glycolysis, the majority of the pyruvate is converted into lactate in the cytoplasm by enzyme lactate dehydrogenase (LDH). Lactate is subsequently secreted outside of the cells by MCT1. Overexpression of MCT1 and LDHA (one of the predominant LDH isoforms) has been reported in a variety of solid tumor types, including colorectal, melanoma, breast and pancreatic cancer (22-25). In order to determine what may influence the expression levels of MCT1 or LDHA, several previous studies have focused on examining certain factors, including hypoxia, p53 status and chemotherapeutics (26,27). Previously, a few studies have focused on the association between glucose metabolism and miRNA regulation in tumors. For instance, miR-124 is a direct regulator of MCT1 in medulloblastoma cells and miR-375 is involved in regulation of lactate dehydrogenase B (LDHB). The present study indicated that both *LDHA* and *MCT1* may be negatively regulated by miR-199a-3p in testicular germ cell tumors.

The other selected gene in this metabolic qPCR array screening is *PGK1*, one of the major glycolytic enzymes, which catalyzes the conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate. PGK1 has been previously demonstrated to be associated with the HER-2/neu status in breast cancer (28) and a poor outcome for patients who have lung adenocarcinoma and who are multi-drug resistant (29,30), suggesting its potential as a biomarker for cancer. In lung cancer cell lines, PGK1 was revealed to be regulated by miR-29a and the present study has linked its expression to miR199a-3p in tumor biology.

However, these four selected genes (*LDHA, MCT1, PGK1* and *TIGAR*) lack the potential recognition sites of miR-199a-3p, meaning the regulation of miR-199a-3p on four genes is not direct. Notably, the present study noticed that the potential binding site of Sp1 was present in the promoter regions of all four selected genes. In addition, it was predicted that miR-199a-3p can bind to target sequences in the 3'-UTR of Sp1 mRNA by using TargetScan analysis (Fig. 7). Sp1, a member of Sp/Kruppel super family, is a ubiquitous transcription factor that serves an important role in controlling the transcription of numerous genes that contain GC boxes in their promoters, particularly to those genes associated with the metabolism of nucleic acids and biosynthesis (31,32). Abnormal activation of Sp1 occurs in a wide variety of human tumor types and high levels of Sp1 protein are considered a negative prognostic factor (33,34). The present study speculated that miR-199a-3p may downregulate the four metabolic genes through Sp1.

In conclusion, the present study characterized the role of miR-199a-3p in regulating the metabolism of Ntera-2 cells. These results suggested that miR-199a-3p is a major metabolic regulator in tumor suppression and miR-199a-3p may downregulate metabolic genes (*LDHA, PGK1, MCT1, TIGAR*) through the transcription factor Sp1. This led to the speculation that downregulation of miR-199a-3p may be a potential cause of overexpression of these metabolic genes and targeting miR-199a-3p may be an innovative strategy in cancer treatment.

Acknowledgements

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