

MiR-21-5p inhibition attenuates Warburg effect and stemness maintenance in osteosarcoma cells *via* inactivation of Wnt/ β -catenin signaling

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MicroRNA (miR)-21 has been found to be overexpressed in osteosarcoma (OS). The aim of the present study was to investigate the effect of miR-21-5p on the Warburg effect and stemness maintenance in OS cells and its potential molecular mechanism. Herein, miR-21-5p was overexpressed or inhibited in MG-63 cells *via* transfection with mimics or inhibitors. The effect of miR-21-5p on cell viability, apoptosis, Warburg effect and stemness maintenance were explored in OS cells. The results demonstrated that miR-21-5p inhibition suppressed MG-63 cell viability and enhanced their apoptosis. Additionally, miR-21-5p inhibition attenuated the stemness maintenance of MG-63 cells, as demonstrated by the reduced proportion of CD133-positive MG-63 cells, the decrease in tumorsphere formation capacity, and the downregulation of Sox2, Oct4, and Nanog proteins. Moreover, miR-21-5p inhibition suppressed the Warburg effect in MG-63 cells, as indicated by the decrease in glucose uptake, lactic acid production, and ATP level and the downregulation of proteins involved in the Warburg effect (GLUT1, LDHA, HK2, and PKM2). Furthermore, the results suggested that the effect of miR-21-5p suppression on stemness and the Warburg effect may be associated with the decreased activity of the Wnt/ β -catenin pathway in OS cells. Our findings suggest a novel potential biomarker for OS therapy.

Keywords: osteosarcoma; MiR-21-5p; Warburg effect; stemness; Wnt/ β -catenin pathway

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Abbreviations: miR, MicroRNA; OS, osteosarcoma; CD133, Cluster of differentiation 133; Sox2, SRY-Box Transcription Factor 2; Oct4, octamer-binding transcription factor 4; GLUT1, glucose transporter 1; LDHA, lactate dehydrogenase A; HK2, hexokinase 2; PKM2, pyruvate kinase isomer 2; NC, negative control; qRT-PCR, quantitative reverse transcription polymerase chain reaction; PC, positive control; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; AV-FITC, Annexin V-fluorescein isothiocyanate; PI, propidium iodide; ATP, Adenosine triphosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CSCs, cancer stem cells; n.s., No significance

INTRODUCTION

Osteosarcoma (OS) is the most prevalent primary bone tumor occurring in young adults and teenagers, exhibiting high aggressiveness and metastatic potential

(Li *et al.*, 2019; Otoukesh *et al.*, 2018). Epidemiological investigation indicated that three out of every million young adults and children suffer from OS, and the incidence reaches a peak at 11–15 years of age with six in every million children (Lu *et al.*, 2020). Although much effort has been dedicated to OS therapy, the five-year survival and prognosis of OS patients remain unimproved, particularly for patients with recurrence, multi-drug resistance, or lung metastasis, due to insufficient diagnosis at the early stage and ineffective therapeutic approaches (Zhang *et al.*, 2018). Therefore, it is necessary to identify novel clinical prognostic biomarkers and investigate the potential molecular mechanisms of OS to develop more effective therapeutic strategies (Tu *et al.*, 2020).

MicroRNAs (miRNAs) are endogenous, non-coding, and single-stranded small molecular RNA containing about 22 nucleotides (Liu *et al.*, 2018). They have demonstrated important functions in biological processes including cell proliferation, differentiation, and apoptosis (Najm *et al.*, 2019). MiRNAs are involved in regulating gene expression by complete or incomplete complementary pairing with the 3'-untranslated regions of the target mRNA, resulting in target mRNA degradation to silence specific genes or the inhibition of protein translation (Bartel, 2018). Numerous investigations have revealed that miRNAs are abnormally expressed in a variety of tumors, suggesting that they play an essential role in tumorigenesis and tumor progression (Iacona & Lutz, 2019; Zhang *et al.*, 2019). MiRNA-21 (miR-21) is located at 17q23.2 in the human chromosome and has exhibited proto-oncogenic activity (Li *et al.*, 2018b; Ribas *et al.*, 2012). Previous studies have shown that miR-21 was highly expressed in hepatocellular carcinoma (Chen *et al.*, 2019), hepatoblastoma (Liu *et al.*, 2019), and colorectal cancer (Xie *et al.*, 2019). MiR-21 is also associated with the regulation of tumors cell proliferation and apoptosis through its interaction with target genes (Bautista-Sanchez *et al.*, 2020). In addition, miR-21 was involved in the modulation of glycolysis and stemness maintenance in tumor cells by regulating relevant signaling pathways, thereby affecting tumor development. Zhu and others (Zhu *et al.*, 2019) found that miR-21 inhibition suppressed glycolysis in breast cancer cells by regulating the phosphatidylinositol-4,5-bisphosphate 3-kinase/Akt pathway, in turn inhibiting breast cancer tumorigenesis. Fu and others (Fu *et al.*, 2013) reported that NPV-LDE-225 inhibited the self-renewal of glioblastoma cells by downregulating the expression of Nanog, Oct4, and Sox2 through suppressing miR-21.

MiR-21 has been identified as a biomarker of poor prognosis in OS patients, as it was shown to be overexpressed in OS and associated with poor clinicopathological characteristics (Ren *et al.*, 2016). However, the effect of miR-21 on glycolysis and stemness maintenance in OS cells remains to be elucidated. Moreover, evidence suggests that miR-21 was involved in the regulation of Wnt/ β -catenin signaling (Du *et al.*, 2019), which is associated with glycolysis and stemness maintenance in tumor cells (Manna *et al.*, 2014; Udoh *et al.*, 2019). Whether miR-21 has an effect on the activity of the Wnt/ β -catenin pathway in OS cells remains unknown. On this basis, the current study was performed to explore the effect of miR-21-5p on glycolysis and stemness maintenance in OS cells and clarify the underlying molecular mechanism involved therein.

MATERIALS AND METHODS

Cell culture and transfection

MG-63 human osteosarcoma cells were purchased from the Shanghai Institutes for Biological Sciences, Chinese Academy of Science and maintained in minimum essential medium supplemented with 10% fetal bovine serum (Gibco, MD, USA) at 37°C with 5% CO₂. After the cell confluence reached 70%, the cells were transfected with commercially generated miR-21-5p mimic or inhibitor, or their corresponding negative control (NC) (40 pmol/ml, Ribo Biotechnology Co. LTD, Guangzhou, China) for 24 h using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction to overexpress or silence miR-21-5p expression. The transfection efficiency was then evaluated using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Doxorubicin (200 nM; Kamata *et al.*, 2017, Aladdin, Shanghai, China), a prevalent antineoplastic drug (Qin *et al.*, 2018), served as positive control (PC).

qRT-PCR

Total RNA was extracted using Trizol (Ambion, Texas, USA) and reverse-transcribed into cDNA. The collected cDNA was then amplified using the SYBR Green PCR kit (KAPA Biosystems, USA) according to the manufacturer's protocol. The primer sequences are as follows: miR-21-5p forward: 5'-GGGTAGCTTATCAGACT-3', reverse: 5'-AACTGGTGTCTGGAGTCCGGC-3'; U6 forward: 5'-CTCGCTTCGGCAGCACACA-3', reverse: 5'-AACGCTTCACGAATTTGCCGT-3'. U6 served as an endogenous control for miR-21-5p. The data were analyzed using the 2^{- $\Delta\Delta C_t$} method (Livak and Schmittgen, 2001).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

MTT was performed to evaluate the effect of miR-21-5p on MG-63 cell proliferation. Harvested cells were seeded into a 96-well plate (100 μ l per well) at 3 \times 10³ cells/well and cultured overnight at 37°C with 5% CO₂. 10 μ l of MTT (5 mg/ml, Solarbio, Beijing, China) was then added to the cells and maintained for 4 h at 37°C with 5% CO₂. After the supernatant was removed, the cells were incubated in 150 μ l of dimethylsulfoxide for 10 min and measured using a microplate reader (Allsheng, Hangzhou, China) at 490 nm.

Flow cytometry

Flow cytometry was performed to detect the apoptosis and proportion of CD133-positive cells. For apoptosis: After treatment, 1 \times 10⁶ cells were collected and centrifuged at 4°C at 400 \times g for 5 min, followed by resuspension in 1 ml of phosphate-buffered saline (PBS) and centrifugation at 4°C at 400 \times g for 5 min. Then, the cells were resuspended in 200 μ l of PBS and incubated with 10 μ l of Annexin V-fluorescein isothiocyanate (FITC) (BD, Shanghai, China) and 10 μ l of propidium iodide (PI) in the dark at 4°C for 30 min. After 300 μ l of PBS was added, the cells were evaluated by flow cytometry (ACEA Biosciences, San Diego, California, USA).

For the detection of CD133-positive cells: Cells (1 \times 10⁶) were resuspended in 100 μ l of PBS, followed by the addition of 2 μ l of phycoerythrin-conjugated CD133 antibodies (eBioscience, CA, USA). The cells were cultured in the dark at 4°C for 30 min. Then, the cells were centrifuged at 4°C at 400 \times g for 5 min, resuspended in the dark in 400 μ l of PBS, and evaluated by flow cytometry (ACEA Biosciences).

Tumorsphere formation

Cells were resuspended in Dulbecco's modified eagle medium containing 2% B27 (Gibco), 20 ng/ml epidermal growth factor (PeproTech, New Jersey, USA), 10 μ g/ml basic fibroblast growth factor (PeproTech), 5 μ g/ml insulin (Solarbio), and 10 μ g/ml transferrin (Solarbio). The cells were then incubated in ultra-low adherent 6-well dishes (Corning, New York, USA) at 2 \times 10⁶ cells/ml (2 ml per well) at 37°C with 5% CO₂ for 7 days. Thereafter, tumorspheres were observed under a DMIL LED microscope (Leica, Wetzlar, Germany).

Biochemical detection

Adenosine triphosphate (ATP) content (A095-1-1) and lactic acid levels (A019-2-1) in MG-63 cells were detected using corresponding commercial kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) following the manufacturer's instruction. Glucose uptake (361510) was evaluated using a corresponding commercial kit purchased from Shanghai Rongsheng Biotechnology Co., LTD following the manufacturer's instruction.

Western blot

Total proteins were extracted using radioimmunoprecipitation assay lysis buffer (Solarbio) and quantified using a bicinchoninic acid assay kit (Solarbio). 20 μ g of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Solarbio) and transferred onto polyvinylidene fluoride membranes (Millipore, Massachusetts, USA). After blocking with 5% skim milk, the membranes were incubated for 1 h at room temperature with primary antibodies against sex determining region Y-box 2 (Sox2), octamer-binding transcription factor 4 (Oct4), Nanog, glucose transporter 1 (GLUT1), lactate dehydrogenase A (LDHA), hexokinase 2 (HK2), pyruvate kinase isomer 2 (PKM2), Wnt1, β -catenin, and GAPDH (all purchased from Myhalic Biotechnology Co., Ltd., Bioswamp, Wuhan, China). The membranes were then incubated for 1 h at room temperature with goat anti-rabbit IgG secondary antibodies (Myhalic Biotechnology Co., Ltd). GAPDH acted as the internal reference.

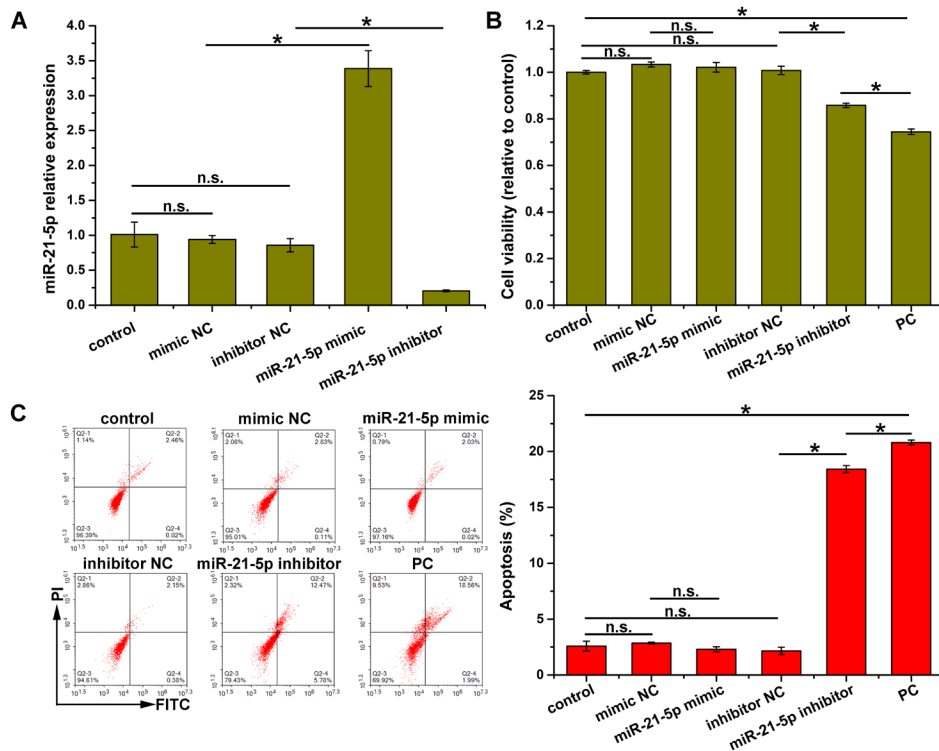


Figure 1. MiR-21-5p inhibition attenuates the viability and promotes the apoptosis of MG-63 cells.

(A) The expression of miR-21-5p in MG-63 cells was detected by qRT-PCR after miR-21-2p mimic and inhibitor transfection. (B) Detection of MG-63 cell viability using MTT assay. (C) Detection of MG-63 cell apoptosis by flow cytometry. Data represent the mean \pm S.D. (n=3), * P <0.05.

Statistical analysis

Data are shown as the mean \pm standard deviation (S.D.). Differences among groups were analyzed using one-way analysis of variance followed by Tukey test. P <0.05 was considered to be statistically significant.

RESULTS

miR-21-5p inhibition attenuates proliferation while promoting apoptosis of MG-63 cells

As shown in Fig. 1A, the expression of miR-21-5p in MG-63 cells was increased and decreased by miR-21-5p mimic and inhibitor transfection, respectively. The corresponding NC showed no effect. Compared to the control or NC, the viability of MG-63 cells (Fig. 1B, P <0.05) was decreased by miR-21-5p inhibition, while apoptosis (Fig. 1C, P <0.05) was enhanced. The effect of miR-21-5p inhibition on cell viability and apoptosis was similar to that of doxorubicin. These results demonstrated that miR-21-5p inhibition attenuates the proliferation while promoting the apoptosis of MG-63 cells.

miR-21-5p inhibition attenuates stemness maintenance in MG-63 cells

Tumorspheres are spherical and solid structures originating from cancer stem cells. Figure 2A indicates that miR-21-5p inhibition suppressed tumorsphere formation of MG-63 cells. Flow cytometry demonstrated that miR-21-5p inhibition reduced the proportion of MG-63 cells with positive expression of CD133 (Fig. 2B), a specific marker of cancer stem cells (Bi *et al.*,

2016). In addition, the expression of stem cell-related proteins was evaluated. miR-21-5p inhibition attenuated the protein expression of Sox2, Oct4, and Nanog in MG-63 cells, while miR-21-5p overexpression showed an opposite effect (Fig. 2C). The effect of miR-21-5p inhibition on the stemness of MG-63 cells was similar to that of doxorubicin. These results indicated that miR-21-5p inhibition attenuates stemness maintenance in MG-63 cells

miR-21-5p inhibition attenuates the Warburg effect in MG-63 cells

Compared to the control and NC, glucose uptake (Fig. 3A), lactic acid generation (Fig. 3B), ATP content (Fig. 3C), and the expression of metabolism-related enzymes associated with the Warburg effect (LDHA, GLUT1, PKM2, and HK2) (Fig. 3D and E) were decreased by miR-21-5p inhibition (P <0.05), but increased by miR-21-5p mimics (P <0.05). The effect of miR-21-5p inhibition on MG-63 cells was similar to that of doxorubicin. These results indicated that miR-21-5p inhibition attenuates the Warburg effect in MG-63 cells.

miR-21-5p inhibition attenuates the activity of Wnt/ β -catenin signaling

As shown in Fig. 4, the expression of Wnt and β -catenin was decreased by miR-21-5p inhibitors compared to that of the control or inhibitor NC groups (P <0.05), while it was increased by miR-21-5p mimics compared to that of the control or mimic NC groups (P <0.05). These results demonstrated that miR-21-5p inhibition attenuates the activity of Wnt/ β -catenin signaling.

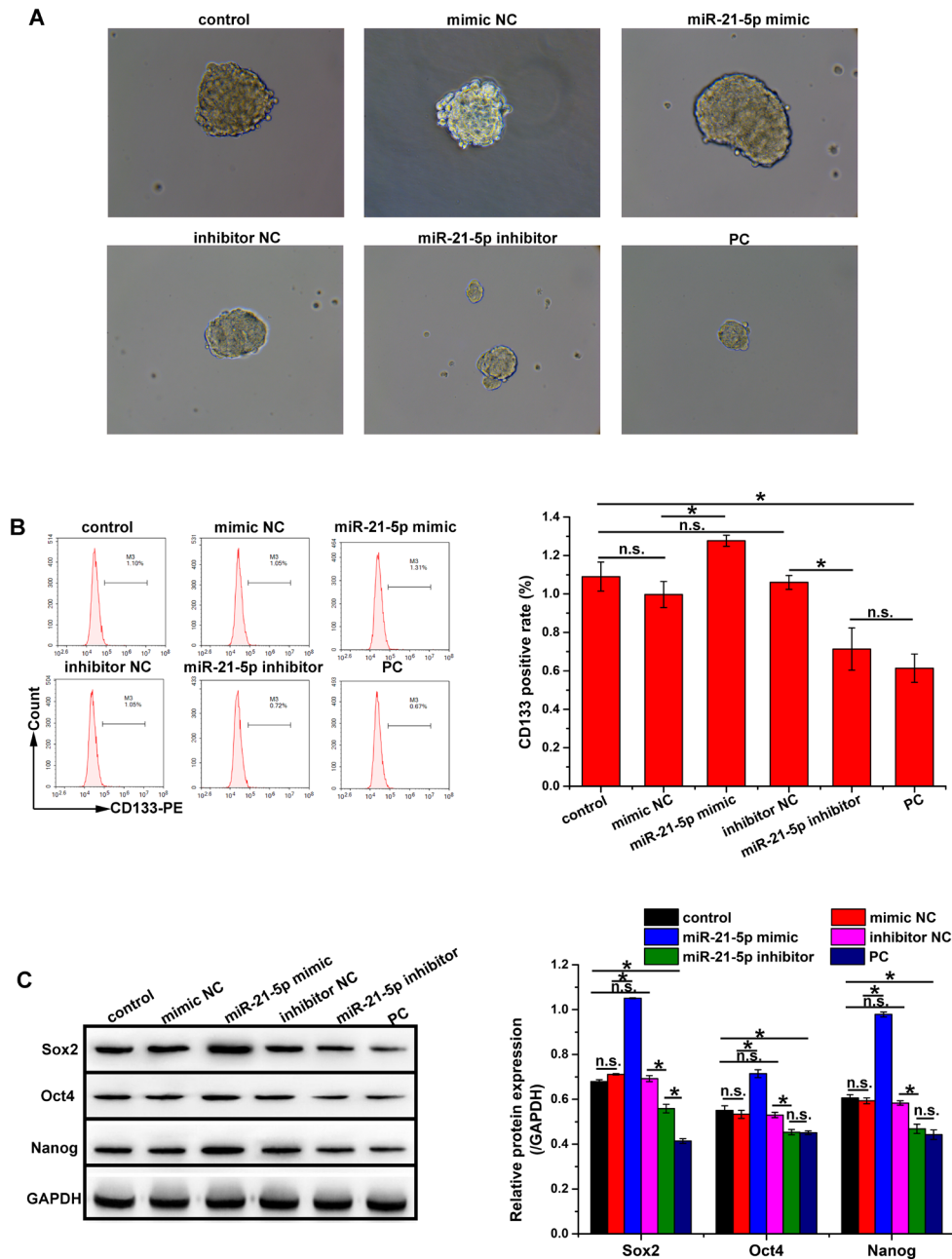


Figure 2. MiR-21-5p inhibition attenuates the stemness maintenance of MG-63 cells.

(A) Tumorsphere formation of MG-63 cells after miR-21-2p mimic and inhibitor transfection. (B) Proportion of CD133+ cells in MG-63 cells after miR-21-2p mimic and inhibitor transfection, detected by flow cytometry. (C) Relative protein expression of Oct4, Sox2, and Nanog in MG-63 cells after miR-21-2p mimic and inhibitor transfection, detected by western blot. Data represent the mean \pm S.D. (n=3), * P <0.05.

DISCUSSION

The phenomenon wherein cancer cells preferentially utilize glucose for energy via glycolysis, even under aerobic conditions, was identified as the “Warburg effect” (Spencer & Stanton, 2019). It is associated with oncogenic expression, abnormal expression of glycometabolic enzymes, the tumor microenvironment, and tumor development. Thus, the Warburg effect has been widely characterized as a hallmark of tumor, and anti-cancer therapeutic strategies targeting the Warburg ef-

fect are being development (Li *et al.*, 2018a). Compared to non-tumorigenic cells, tumorigenic cells exhibit dramatically increased glucose uptake, followed by the rapid conversion of pyruvate into lactic acid even under aerobic conditions, during which the glycolysis rate is upregulated and ATP is produced (Lebelo *et al.*, 2019; Lu *et al.*, 2015). Evidence has suggested that the glycolytic process is associated with GLUTs, LDHA and rate-limiting enzymes including HK2, and PKM2 (Ruzzo *et al.*, 2020; Wiese & Hitosugi, 2018). GLUTs are involved in the transport of glucose across the plasma

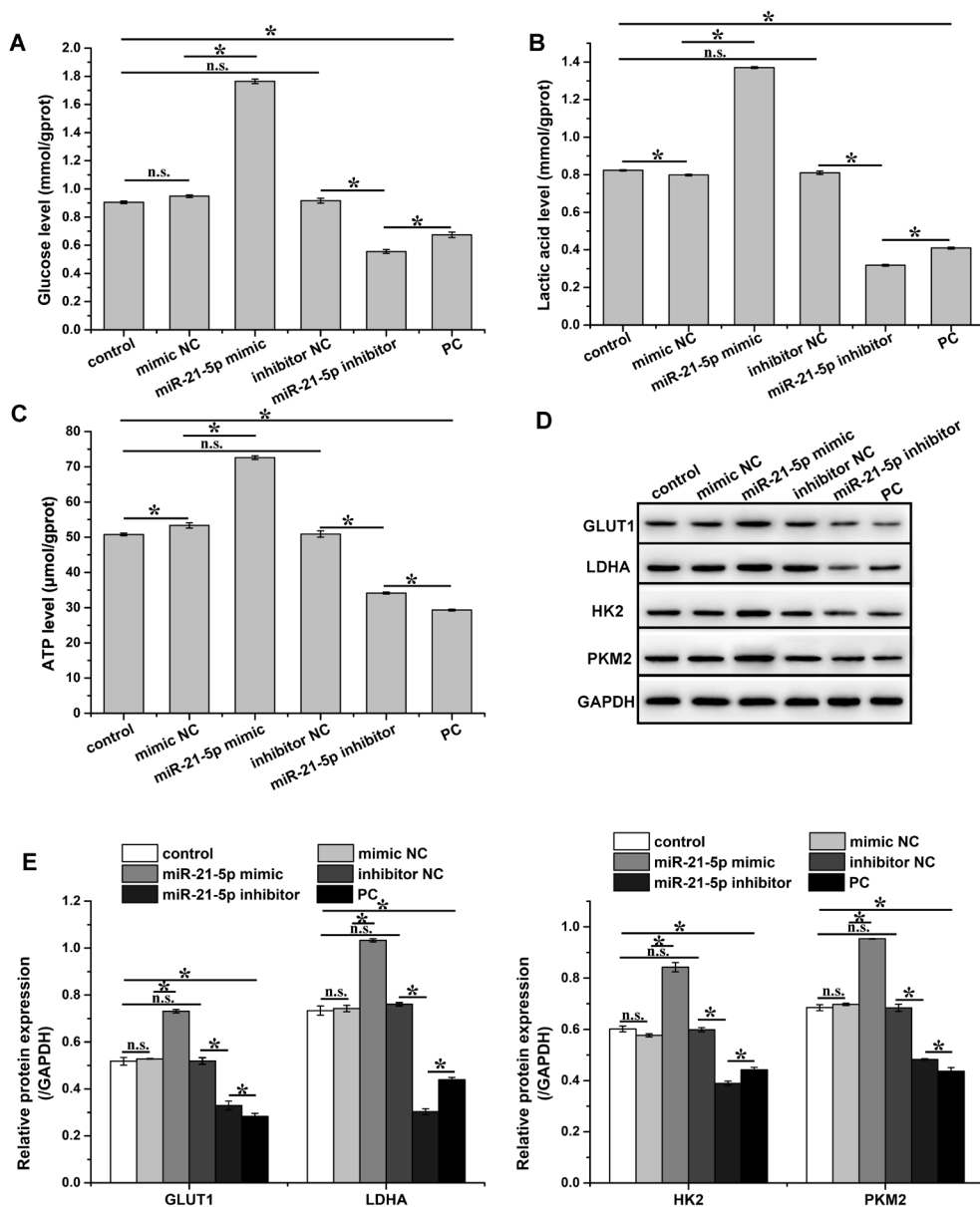


Figure 3 MiR-21-5p inhibition attenuates the Warburg effect in MG-63 cells.

(A) Glucose uptake, (B) lactic acid level, (C) and ATP level in MG-63 cells after miR-21-2p mimic and inhibitor transfection, detected using corresponding kits. (D) Relative protein expression of GLUT1, LDHA, HK2, and PKM2 in MG-63 cells after miR-21-2p mimic and inhibitor transfection, detected by western blot. (E) Quantification of (D). Data represent the mean \pm S.D. (n=3), * P <0.05.

membrane, thereby mediating glucose uptake (Barron *et al.*, 2016). GLUT1 is upregulated in numerous types of cancer and can act as a target for anti-cancer treatment because of its effect on glucose uptake (Lebelo *et al.*, 2019). HK2 facilitates glucose phosphorylation to generate glucose-6-phosphate, which is the first irreversible step in the glucose metabolism pathway, thereby enhancing aerobic glycolysis (Xu *et al.*, 2019). Thus, it is identified as a vital player in the Warburg effect, and like GLUT1, it has been regarded as a therapeutic target for tumors (Patra *et al.*, 2013). Previous studies have found that HK2 deficiency exhibited remarkable reduced LDHA expression, a crucial functional enzyme in glycolysis that promotes pyruvate conversion into lactic acid (Pathria *et al.*, 2018; Xu *et al.*, 2019). PKM2 is another important component of the Warburg effect that promotes rapid energy generation during glycolysis (Palsson-McDermott & O'Neill, 2013). Overexpression

of HK2, PKM2, LDHA, and GLUT1 contributed to the Warburg effect in cancer cells (Yan *et al.*, 2019; Yu *et al.*, 2019). The present work showed that miR-21-5p inhibition suppressed the expression of HK2, PKM2, LDHA, and GLUT1, thereby inhibiting glucose uptake and the levels of lactic acid and ATP in OS cells, indicating that miR-21-5p inhibition attenuates the Warburg effect in OS cells. As for HK2, PKM2, LDHA, and GLUT1 proteins detection, GAPDH, another glycolytic enzyme (Liao *et al.*, 2019), was used as the loading control. The present study showed no effect of miR-21-5p on GAPDH expression, as indicated by no statistical difference of gray value in different group, which was in consistency with previous studies that miR-21-5p inhibition or overexpression did not affect GAPDH expression (Huang *et al.*, 2021; Zhang *et al.*, 2021)

As an important characteristic of metabolic reprogramming in tumor cells, the Warburg effect contrib-

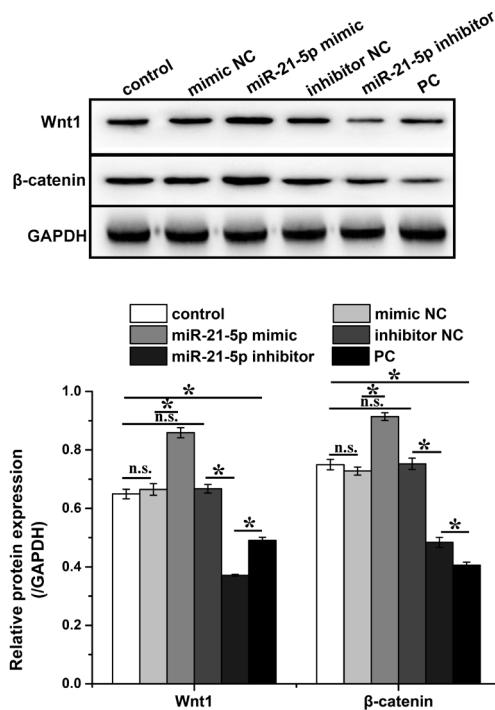


Figure 4. MiR-21-5p inhibition attenuates the activity of Wnt/ β -catenin signaling pathway.

Relative protein expression of Wnt1 and β -catenin in MG-63 cells after miR-21-2p mimic and inhibitor transfection, detected by western blot. Data represent the mean \pm S.D. (n=3), * P <0.05.

utes to growth, survival, and proliferation of cancer stem cells (CSCs) as well as enables the maintenance of the CSCs state (Vaupel *et al.*, 2019). Tumorigenic cells with stem cell-like characteristics are regarded as CSCs, which have strong multi-directional differentiation potential and self-renewal ability (Ayob & Ramasamy, 2018; Hajizadeh *et al.*, 2019). Emerging evidence has demonstrated that the capacity of tumor reproduction and development depends on the stem-like characteristics of cancer cells, which allow cells to remain in a dormant state and retain the ability to evade therapeutic drugs and metastasis (Adorno-Cruz *et al.*, 2015). Therefore, inhibiting the maintenance of the CSC state or the proportion of CSCs in cancer cells is an effective strategy for cancer treatment (Yoshida & Saya, 2016). The current study revealed that miR-21-5p inhibition suppressed tumorsphere formation (initiated from CSCs) of OS cells and reduced the proportion of MG-63 cells with positive expression of CD133, a specific marker of CSCs (Bi *et al.*, 2016). In addition, miR-21-5p inhibition attenuated the protein expression of Sox2, Oct4, and Nanog, which are key transcription factors that keep the self-renewal of CSC, in turn regulating cancer recurrence, progression, and drug resistance (Hattermann *et al.*, 2016). Taken together, these results indicate that miR-21-5p inhibition suppressed the stemness maintenance in OS cells, as demonstrated by the decrease in the proportion of CD133-positive OS cells and the downregulation of Sox2, Oct4, and Nanog. Whether this is associated with the suppression of the Warburg effect induced by miR-21-5p inhibition needs to be further elucidated.

The current results also demonstrated that miR-21-5p inhibition attenuates the activity of the Wnt/ β -catenin signal pathway, which has been shown to be

associated with glycolysis and stemness maintenance in tumor cells (Manna *et al.*, 2014; Udoh *et al.*, 2019). The activation of Wnt/ β -catenin signaling promoted the Warburg effect in tumor cells by increasing the level of acetyl-coenzyme A and ATP level produced by the oxidation of fatty acids (Manna *et al.*, 2014). Additionally, it was previously reported that Wnt/ β -catenin pathway inactivation may assist in eradicating OS stem cells by regulating CD133, Sox2, Oct4, and Nanog expression (Yi *et al.*, 2015). In agreement with previous research, the current study indicates that miR-21-5p inhibition suppresses the Warburg effect and stemness maintenance in OS cells, possibly by Wnt/ β -catenin signal pathway.

In conclusion, the current study provides insight suggesting that miR-21-5p inhibition suppresses the Warburg effect in OS cells by regulating the expression of proteins involved in the Warburg effect (HK2, PKM2, LDHA, and GLUT1), thereby inhibiting glucose uptake and lactic acid and ATP level in OS cells. In addition, miR-21-5p inhibition suppresses stemness maintenance in OS cells by regulating the expression of transcription factors that promote the self-renewal of CSCs (Sox2, Oct4, and Nanog). The underlying mechanism might be associated with the Wnt/ β -catenin signaling pathway.

Acknowledgement

Not applicable.

Conflict of interest

The authors declare that no conflict of interest is associated with this work.

Ethics approval and consent to participate

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

Study design: Ziyang Wu, Yi Yu. Experiment production: Ziyang Wu, Zhong Zhou, Weiguo Zhang. Data collection and molecular analysis: Ziyang Wu, Zhong Zhou. Manuscript writing and editing: Ziyang Wu, Zhong Zhou, Yi Yu

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