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MiR-382-5p's Function as a Suppressor in Osteosarcoma (OS) by Targeting PDPK1

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KEYWORDS Inhibitor. miR-382-5p. Osteosarcoma. PDPK1. Suppressor

ABSTRACT miR-382-5p engages in development of osteosarcoma (OS). However, the regulatory system of miR-382-5p in osteosarcoma remains to be revealed. This research studied the interplay between PDPK1 and miR-382-5p in OS. RT-PCR was used to evaluate miR-382-5p and PDPK1 expression in OS cells and normal human osteoblast cells. Dual-luciferase reporter assay validated PDPK1 as a miR-382-5p target. CCK-8 evaluated the cell viability. Flow cytometric method determined cell apoptosis rate. Transwell and Scratch assays estimated the cell metastasis. miR-382-5p was inhibited in OS cells. Further functional results showed miR-382-5p upregulation reduced cell viability, and mobility by mediating PDPK1 in OS cells.

INTRODUCTION

Osteosarcoma (OS) is frequently diagnosed in juveniles and the old (Ottaviani and Jaffe 2009; Xu et al. 2018). It is considered to be related with the bone-forming mesenchymal stem cells (Li et al. 2021). It also shows common lung metastasis during early stages of check-ups (Ritter and Bielack 2010; Yang et al. 2020). Currently, the practice of operating resection and chemical therapy are the effective functional therapy (Jones et al. 2012; Wong et al. 2013). On the other hand, close cooperation within a knowledgeable group comprising of paediatric or medical oncologists, pathologists, surgeons and radiologists, is one of the requirements for the successful therapy for patients with OS (Ritter and Bielack 2010). Though, chemotherapy is related with undesirable outcomes due to the acquired drug resistance among OS patients (Shao et al. 2019; Wang et al. 2019). Hence, spotting the predictive biomarkers and exploring new techniques for OS therapy are urgent (Fu et al. 2016).

MicroRNAs are part of a family of short non-coding RNAs that modulate gene expression via binding to the 3' untranslated area of mRNAs (Farazi et al. 2013). A rising number of researches have showed that miRNAs bear very important duties in cell functions, including the cell differentiation, invasion, migration, survival and apoptosis (Nishikawa et al. 2015; Wang et al. 2015). miRNAs could work as tumour repressors or oncogenes in cancers, which modulate tumour progression through influencing cell growth and cell invasive ability (Xu et al. 2014; Zhu et al. 2015). Upregulated miRNA-34a modulated the mobility of OS cells by inhibiting CD44 expression (Zhao et al. 2013). miR-542-5p was found to be downregulated in OS and its upregulation could inhibit the tumour progression (Zhu et al. 2020). Recent studies found that miR-382-5p is associated with the progression of breast cancer, serving as a promoter (Ho et al. 2017). Recently, miR-382-5p was also found to be linked to the OS angiogenesis (Xiao et al. 2020) and metastasis (Wang et al. 2020). However, further mechanism related in OS requires further investigation.

On the other hand, PDPK1 an associate of the AGC kinases serine group, and is a confirmed crucial regulator in cell migration, invasion, apoptosis and survival (Li et al. 2017; Zheng et al. 2015). In cancers, PDK1 is closely related to the canonical pathways PI3K/AKT (Bamodu et al. 2020; Gagliardi et al. 2018). PDK1 has an vital role in modulating cell migration as well

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(Raimondi and Falasca 2011). PDK1 plays a significant part in the progress of vascular cancers (Zheng et al. 2015). However, the association between PDPK1 and OS remains unclear. Authors hypothesized that miR-382-5p might be an inhibitor in OS through PDPK1 regulation.

Objective

To validate the functions of miR-382-5p in OS and also further explore its interplay with PDPK1 in OS in vitro.

MATERIAL AND METHODS

Cell Culture

OS cell lines including MG-63, Saos-2, HOS and U2OS were purchased from Procell in China, with the normal human osteoblast cell line NHOst as a control. Cells were cultured in DMEM (Thermo Fisher, NY, US), accompanied by ten percent FBS (Abcam, Cambridge, UK) in the cell incubator with five percent carbon dioxide at 37 °C.

Transfection Assays

Transfection methods were conducted to differentiate the RNA expression in cell groups so as to further validate the impacts of miR-382-5p or PDPK1 in cell functions. MiR-382-5p mimics were purchased from Beyotime with the miR-NC as a negative control. PDPK1 siRNA (si-PDPK1-1,2,3) and negative control siRNA (si-control) were purchased from Tianjin Future Current Biotech (Tianjin, China). Cells were planted in 6-well plates. Cell transfection was conducted with Lipofectamine 2000 (Thermo Fisher). It was completed in accordance with the producer's recommendations. After 48 hours, the transfected cells were collected for functional assays.

RT-PCR

Cells after transfection were collected. Total RNAs was extracted utilising TRIzol (Thermo Fisher, Cambridge, UK) in accordance with the producer's directions (Tang et al. 2019). First-strand cDNA synthesis was completed using the TaqMan MicroRNA kit for miR-382-5p while the BeyoRT™ cDNA was for PDPK1 (Beyotime, Shanghai, China). RT-PCR was operated using TaqMan Master Mix II on an ABI 7500 (Thermo Fisher). Thermocycler conditions were described as follows, that is, 95°C used for 5 minutes, followed by 40 cycles on 95°C intended for 30 seconds, 55°C aimed at 40 seconds and 72°C for 30 seconds. The primers for miR-382-5p and PDPK1 were purchased from GenePharma (Suzhou, China). MiR-382-5p expression was standardised to that of U6 and PDPK1 to GAPDH. The $2^{-\Delta\Delta CT}$ method was adopted to calculate the RNA relative expression.

CCK-8

Cells were planted in 96-well plates at 2×10^4 per well and incubated for 24 hours, 48 hours and 72 hours. Ten ul CCK-8 (Beyotime) was dripped into each well at 24, 48 and 72 hours, and after another 2 hours the cell viability was observed at 450 nm utilising a lab microplate tool. Each group was performed in triplicate at different time points.

Transwell Assays

The BD Matrigel invasion chambers and migration chambers (Thermo Fisher, CA, US) were utilised to determine mobility of OS cells. Subsequently, cells were put in upper chambers with no serum. FBS was added in lower chambers. After 48 hours, the invaded or migrated cells were collected for fixation using four percent paraformaldehyde. The cells were dyed with 0.10 percent crystal violet and observed under the lab Olympus light microscope (Nikon, Japan) and images were taken from five random fields from each group. Cells were counted in each field and average and standardised deviation (sd) values were reached.

Flow Cytometry (FCM) Assay for Apoptosis

For cell apoptosis analysis, over 10,000 cells were planted in six-well plates. Cells were stained using Annexin V for 15 minutes. Then 50 μg/ml PI was used to stain the cells for 1 hour at 37°C. The lab flow cytometry machine was applied to measure the apoptosis changes in all groups (BD Biosciences, CA, USA).

Dual-luciferase Reporter Gene Experiment

The target scan software (targetscan.org/vert_71/) identified PDPK1 as a possible target. The pGL3-basic vector PDPK1 fragment, predicted as the target sequences of miR-382-5p, was inserted into the pGL3-basic vector to establish the wild type plasmid, pGL3-PDPK1-Wt. The mutated sequence was positioned into the vector to establish the mutant type plasmid, pGL3-PDPK1-Mut. The cells were transfected with miR-NC or miR-382-5p mimic and the indicated plasmids. Luciferase activity was evaluated employing the Dual-Luciferase Reporter System (Abcam Cambridge UK).

Statistical Analysis

Analysis of statistical results was performed on GraphPad (GraphPad 8, CA, USA). Data was expressed as means and standard deviation(sd). Student's t-test was applied within two groups and One-Way ANOVA was adopted for multiple comparisons among groups. P< 0.05 was regarded significant.

RESULTS

miR-382-5p Expression is Low While PDPK1 Expression is High in OS Cell Lines

The miR-382-5p expression in OS cells was determined through RT-PCR in comparison with normal cells (NHOst) as a control. miR-382-5p was silenced in OS cells compared to NHOst cells, and most downregulated in the Saos-2 and HOS cells while PDPK1 was upregulated in OS cells in comparison with the NHOst cells (Figs. 1A and 1B).

miR-382-5p Targets PDPK1 in OS Cells

The target scan online database predicted a possible target of miR-382-5p, PDPK1 in Homo sapiens (Fig. 2A). Luciferase results showed that

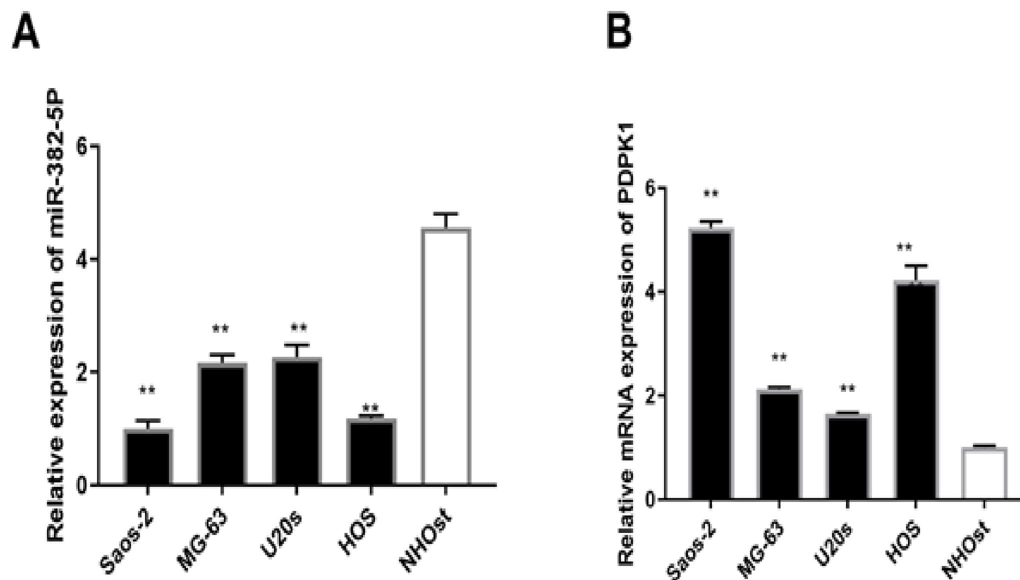

Fig. 1. miR-382-5p is down-regulated in OS cell lines
A-B. OS cell lines HOS, U2OS, MG-63 and Saos-2 and the normal human osteoblast cell line NHOst were collected for RT-PCR test to examine the relative expression of miR-382-5p. **P<0.05

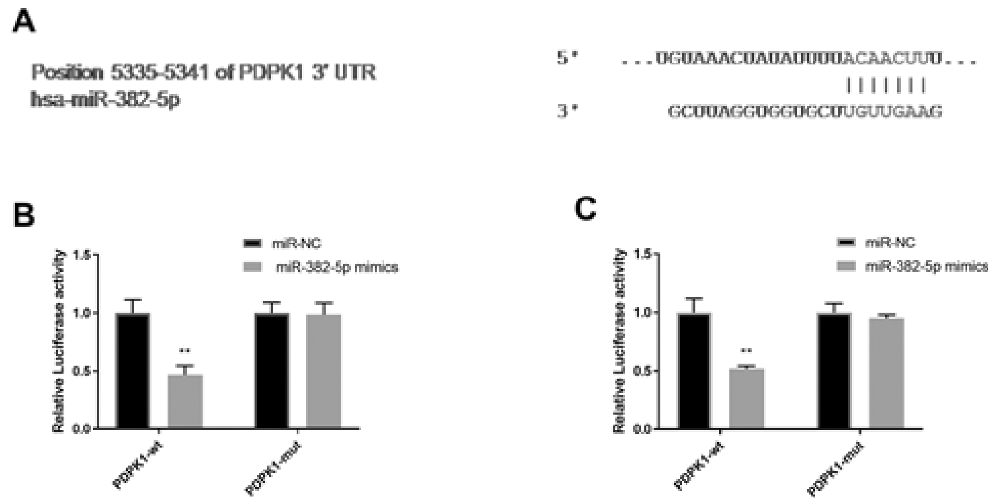

Fig. 2. miR-382-5p targets PDPK1 in OS cells TargetScan was used for target prediction (A)while the luciferase reporter assays(B) were performed to validate this in both OS cell lines.P<0.05**

luciferase activity was inhibited in the cell group transfected with pGL3-PDPK1-Wt and miR-382-5p mimic (Figs. 2B and 2C).

Upregulated miR-382-5p Inhibits Cell Viability, Mobility and Enhances Apoptosis Level of OS Cells

miR-382-5p was upregulated in the miR-382-5p mimic group (Fig. 3A). CCK-8 was carried out to measure the cell viability of the transfected OS cells and the results highlighted that cell viability was curbed in the miR-382-5p mimic group (Figs. 3B and 3C). To evaluate the apoptosis rate, flow cytometric method was used. Apoptosis rates in miR-382-5p mimics group was enhanced compared to the miR-NC group (Figs. 3D and 3E). Also, overexpressed miR-382-5p inhibited the number of migrated and invaded OS cells (Figs. 4A to 4D).

PDPK1 Downregulation Inhibits the Cell Viability and Mobility

In OS cells, the PDPK1 mRNA expression was inhibited by the miR-382-5p upregulation (Fig. 5A). Furthermore, the researchers transfected the cells with the siRNAs of PDPK1 and RT-PCR verified that the si-PDPK1-2 had the best knockdown effect (Figs. 5B and 5C). Thereafter, it was found that the cell viability was decreased by PDPK1 downregulation in OS cells (Figs. 3D and 3E). Apoptosis was detected to be enhanced by the PDPK-1 downregulation (Fig. 5F). Transwell methods revealed that the inhibition of PDPK1 inhibited the mobility in OS cells (Figs. 5G to 5J).

DISCUSSION

At present, various researches have pointed out the importance that miRNAs could serve as diagnostic and therapeutic biomarkers in cancers and diseases. microRNAs target specific oncogenes or tumour suppressor genes (Galka-Marciniak et al. 2021; Hayes et al. 2014; Jansson and Lund 2012). miR-382-5p was discovered to be inhibited in glioma and upregulation of miR-382-5p could inhibit the invasion, migration and proliferation of glioma cells (He et al. 2019). In cervical cancer, miR-382-5p was reported as an inhibitor in vitro targeting VEGFA (Guo et al. 2021). Similarly, in ovarian cancer, miR-382-5p was downregulated in cancer cells and tissues and its upregulation could suppress the cell proliferation in vitro (Xu et al. 2020). In breast can-

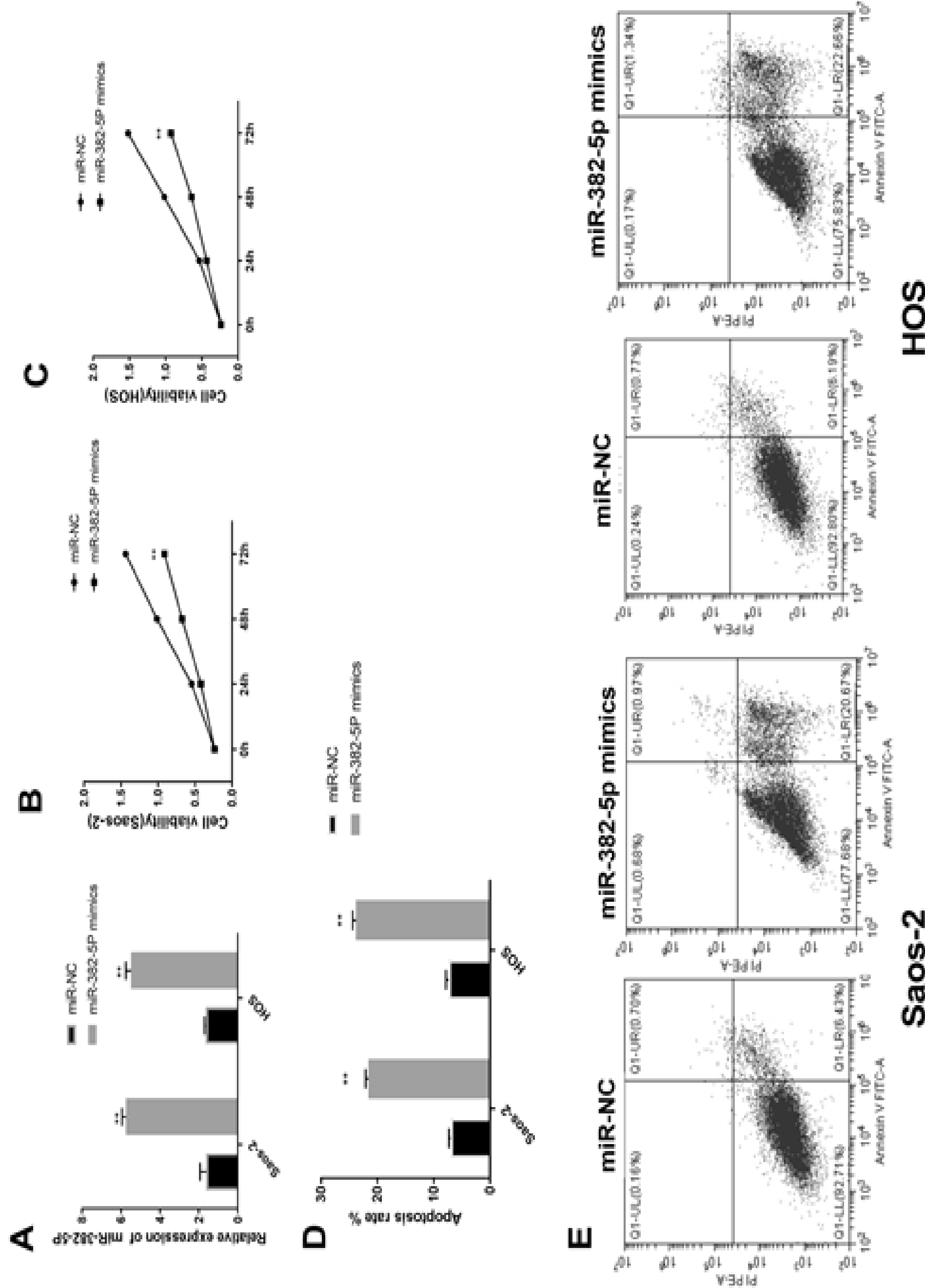

Fig. 3. Upregulated miR-382-5p inhibits cell viability, migration and invasion and enhances apoptosis level of OS cells
Cells were transfected with miR-382-5p mimics and miR-NC
A. RT-PCR examined the miR-382-5p expression after transfection
B-C. CCK8 assays measured the changes of viability
D-E. Apoptosis rates were measured by Flow cytometry methods.P<0.05**

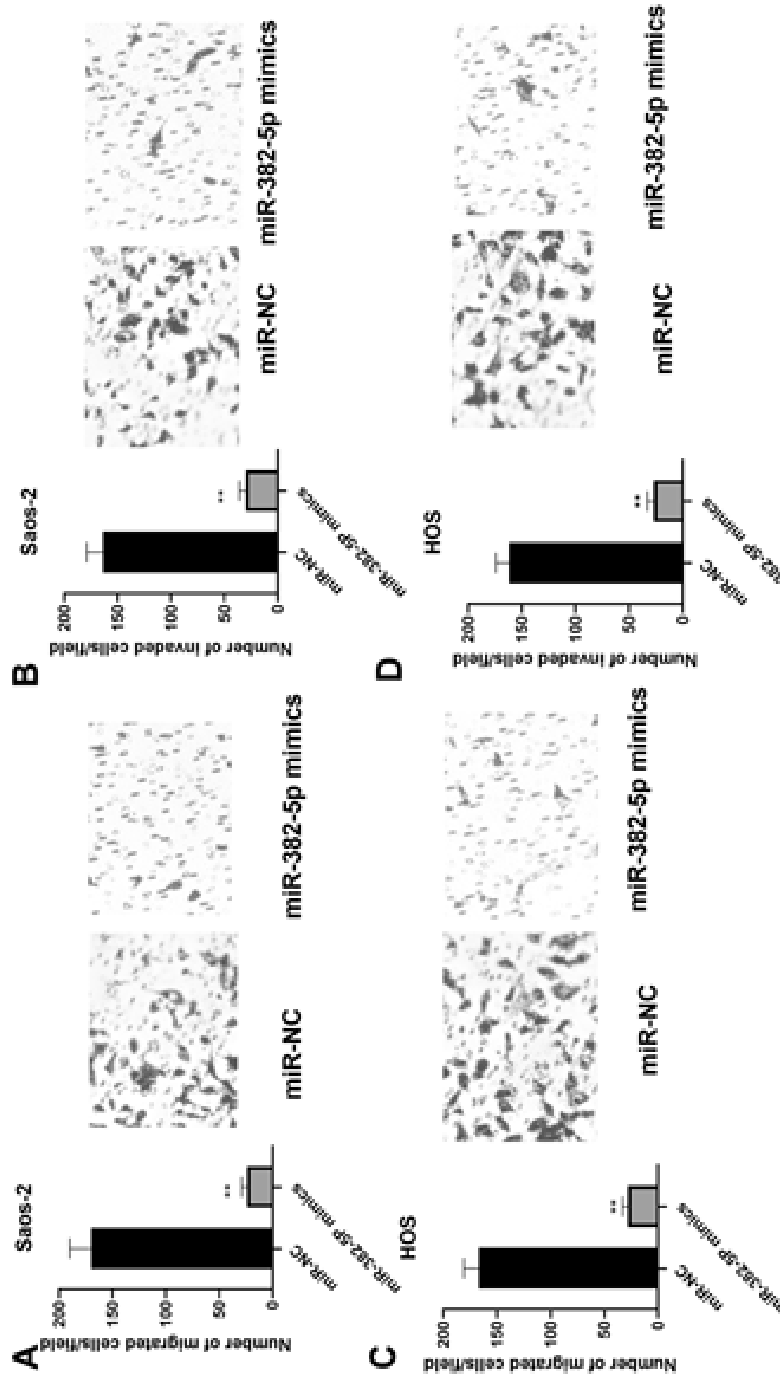

Fig. 4. miR-382-5p upregulation inhibits the invasion and migration of OS cells
A-D. Transwell assays for migration and invasion were performed to examine the effect of miR-382-5p regulation in OS cells.
****P<0.05.**

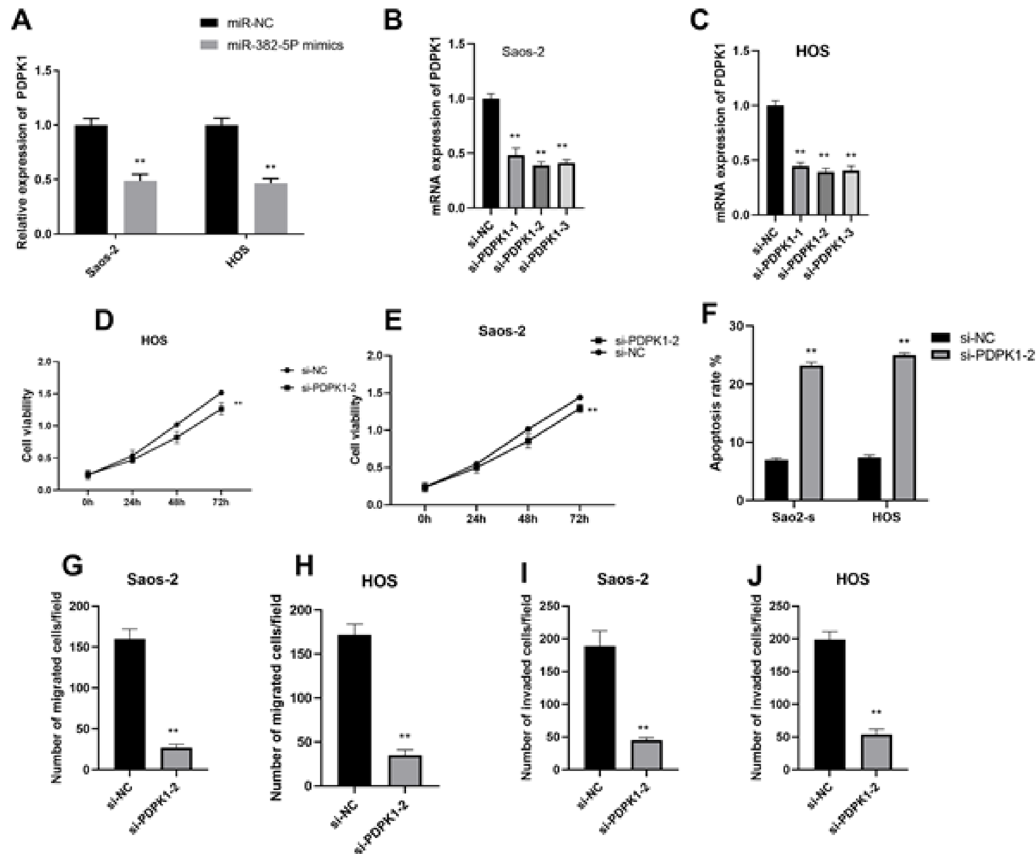

Fig. 5. PDPK1 downregulation inhibits the cell viability, invasion and migration
A. RT-PCR examined the expression of PDPK1 after miR-382-5p manipulation in OS cells.
B-C. PDPK1 was knocked down in OS cells through transfection and RT-PCR verified the knockdown efficiency.
D-E. Cell viability
F. Apoptosis
G-J. Invasion and migration assays. **P<0.05

cer, miR-382 was also reported as a suppressor in vitro (Zheng et al. 2019). Recently, it was also discovered that in OS cells, miR-382-5p targeted YB-1 and inhibited the proliferation, invasion and migration (Wang et al. 2020). In this study, researchers validated its function in two OS cell lines. Furthermore, a new regulatory route of miR-382-5p, was revealed in this study, which is through PDPK1. Based on the bioinformatic prediction, the further in vitro verification was performed by luciferase reporter assays, which proved that in OS cells, the PDPK1 was targeted by miR-382-5p. RT-PCR assays also unveiled that miR-382-5p upregulation could decrease the PDPK1 expression in OS cells.

PDPK1 was reported previously as a biomarker of overall survival rate in OS patients (Wang et al. 2020; Zhang and Yang 2018). PDPK1 is an underestimated gene, engaged in cancers and diseases (Gagliardi et al. 2018). PDPK1 was discovered to modulate the autophagy through interaction with PIK3C3 (Hu et al. 2020), and in cervical cancer, it was enhanced and targeted by miR-125b-5p, which finally facilitated the can-

cer development (Hu et al. 2020). It was also discovered through in vitro study that PDPK1 was engaged in the AKT pathway, which is a key regulator of cell functions including proliferation, apoptosis, migration and invasion (Wang et al. 2018). More specifically, PDPK1 inhibitors inhibited the survival of prostate cancer cells through inactivating the serum/glucocorticoid-regulated Kinase 3 pathway (Nalairndran et al. 2020). Although there is much research on the PDPK1 in cancers and diseases, little experimental reporting has been found in OS. Therefore, the findings in this study filled the gap. It was validated that the knockdown of PDPK1 in OS cells inhibited the viability, invasion and migration and enhanced the apoptosis.

CONCLUSION

In this study, researchers discovered that miR-382-5p might exert its inhibitory effects on cell viability, invasion and migration in OS through PDPK1 inhibition.

RECOMMENDATIONS

This study validated the inhibitory role of miR-382-5p in OS in vitro and further discovered that miR-382-5p targeted and inhibited PDPK1 in OS cells, and PDPK1 inhibition could decrease the cell survival, invasion and migration in OS.

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