

## Original Article

# Metformin inhibits cell growth by upregulating microRNA-26a in renal cancer cells

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**Abstract:** Accumulating evidence suggests that metformin, a biguanide class of anti-diabetic drugs, possesses anti-cancer properties and may reduce cancer risk and improve prognosis. However, the mechanism by which metformin affects various cancers, including renal cancer still unknown. MiR-26a induces cell growth, cell cycle and cell apoptosis progression via direct targeting of Bcl-2, cyclin D1 and PTEN in cancer cells. In the present study, we used 786-O human renal cancer cell lines to study the effects and mechanisms of metformin. Metformin treatment inhibited RCC cells proliferation by increasing expression of miR-26a in 786-O cells ( $P < 0.05$ ). As a result, protein abundance of Bcl-2 and cyclin D1 was decreased and PTEN was increased in cells exposed to metformin. Also over-expression of miR-26a can inhibited cell proliferation by down-regulating Bcl-2, cyclin D1 and up-regulating PTEN expression. Therefore, these data for the first time provide novel evidence for a mechanism that the anticancer activities of metformin are due to upregulation of miR-26a and affect its downstream target gene.

**Keywords:** Metformin, renal cancer, proliferation, miR-26a

## Introduction

Renal cell carcinoma (RCC) is the third most prevalent urologic malignancy, and the sixth leading cause of cancer deaths in the United States. Each year, around 200,000 patients are diagnosed with this malignancy resulting in approximately 100,000 deaths, and its incidence is increasing steadily in recent years [1]. Its incidence has gradually increased during the last decades [2]. Surgical intervention is the primary treatment for RCC, which includes radical nephrectomy and nephron sparing surgery (NSS). However, 30% of patients develop metastatic disease after surgery, and the median survival for those patients is only 13 months [3]. Therefore, novel therapeutic strategy as well as prophylactic regimen is urgently required.

Metformin (1,1-dimethylbiguanide hydrochloride) is an oral hypoglycemic drug with a remarkable record of safety that has been prescribed world-wide for treatment of Type II diabetes. It

reduces glucose levels through activation of the AMP-activated protein kinase (AMPK) pathway and inhibition of hepatic gluconeogenesis [4]. A large number of epidemiologic data have revealed that the oral use of metformin in patients with diabetes mellitus elicits a protective effect by decreasing incidence of different tumors and improving prognosis of patients with cancers [5, 6]. Recent evidence indicates that metformin can inhibit the proliferation of several cancer cell types, such as colon, lung and pancreatic carcinoma [7-9]. However, the mechanism underlying the suppression of RCC growth by metformin remains unknown.

miRNAs are small non-coding RNAs which regulate coding RNAs at the post-transcriptional level [10]. Several recent reports implicate miRNAs in the growth and metastasis of various cancers [11, 12]. Down-regulation of miR-26 has been detected in a number of cancers which suggesting that this miRNA could serve as a biomarker for these cancers [13]. Several studies have implicated miR-26a in oncogene-

sis. For example, Yu found that the expression of miR-26 in oral squamous cell carcinoma in Syrian hamsters was decreased [14]. Visone reported that miR-26a was significantly decreased in anaplastic carcinomas (ATC) in comparison to normal thyroid tissue. The overexpression of miR-26 in 2 human ATC-derived cell lines significantly decreased thyroid carcinogenesis, suggesting a crucial role for miR-26a down-regulation in thyroid carcinogenesis. Here, we have shown that metformin inhibits the growth of renal cancer by upregulation of miR-26a. At the molecular level, miR-26a targets Bcl-2, cyclin D1 and PTEN expression to affect cell proliferation progression [15, 16]. Therefore, downregulation of miR-26a is considered as a promising biomarker for cancers.

### Materials and methods

#### *Reagents and antibodies*

Metformin was purchased from Sigma Chemicals (St Louis, MO, USA). Antibodies against Bcl-2, cyclin D1 and PTEN were purchased from Cell Signaling (Beverly, MA, USA). RPMI1640 medium and fetal bovine serum were obtained from Gibco (New York, NY, USA). AMPK siRNAs were purchased from Shanghai GenePharma (Shanghai, China). Lipofectamine 2000 was bought from Invitrogen (Carlsbad, CA, USA).

#### *Cell culture*

The human RCC cell lines 786-O were purchased from American Type Culture Collection (ATCC). The cell lines were maintained in RPMI-1640 with 10% fetal bovine serum (FBS) and 1% antibiotic (100 U/mL penicillin and 100 mg/L streptomycin). Cells were maintained in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C.

#### *Cell viability assay*

Cells were seeded at 2×10<sup>3</sup> cells per well in 96-well plates and incubated in medium containing 10% FBS. 24 hours after seeding, cells were treated with metformin (0, 1, 5, 10, 20, 40 mM). At the indicated intervals, MTT was added to each well and incubated for 4 hours at 37°C. Finally, the medium was discarded carefully, and 150 µL DMSO was added to each well to dissolve MTT. The absorbance at 570 nm was

measured using the Universal Microplate Reader (Bio-Tek Instruments, Winooski, VT). The percentages of surviving cells from each group relative to controls were calculated. The experiment was independently repeated three times.

#### *Analysis of miRNA expression using TaqMan RT-PCR*

Total RNA from cell lines was harvested using a miRNA isolation kit. Expression of mature miRNAs was assayed using Taqman Metformin Assay specific for miR-26a. Briefly, 10 ng total RNA were reverse transcribed to cDNA with specific stem-loop RT primers. Real-time PCR was performed by using an Applied Biosystems 7500 Real-time PCR System and a TaqMan Universal PCR Master Mix. All the primers were obtained from the TaqMan miRNA Assays. Small nuclear U6 snRNA was used as an internal control.

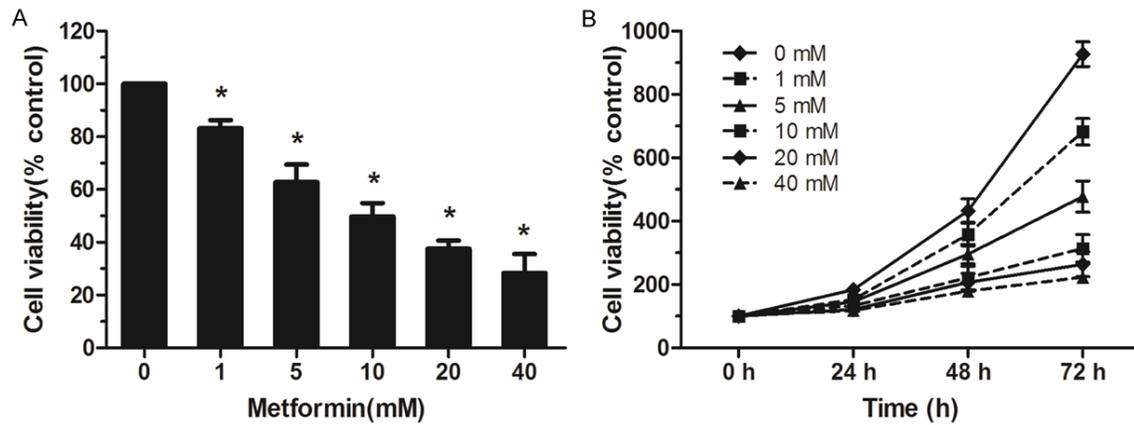
#### *mRNA isolation and real-time PCR*

Total RNA was isolated from cells using TRIzol according to the manufacturer's protocol (Invitrogen). The concentration and quality of the extracted total RNA were determined by measuring OD<sub>260</sub> and the OD<sub>260</sub>:OD<sub>280</sub> ratio. The first strand cDNA was synthesized using SuperScript II RNase H Reverse Transcriptase and Oligo (DT) primer from 2 µg of total RNA, according to the manufacturer's instructions (Invitrogen). The PCR amplification were performed for 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, on a Applied Biosystems 7900HT (Applied Biosystems) with 1.0 µl of cDNA and SYBR Green real-time PCR Master Mix (TaKaRa). Data was collected and analyzed by SDS 2.3 Software (Applied Biosystems). The expression level of each candidate gene was internally normalized against that of the GAPDH. The relative quantitative value was expressed by the 2<sup>-ΔΔCt</sup> method, representing the amount of the candidate gene expression with the same calibrators. Each experiment was performed in triplicates and repeated three times.

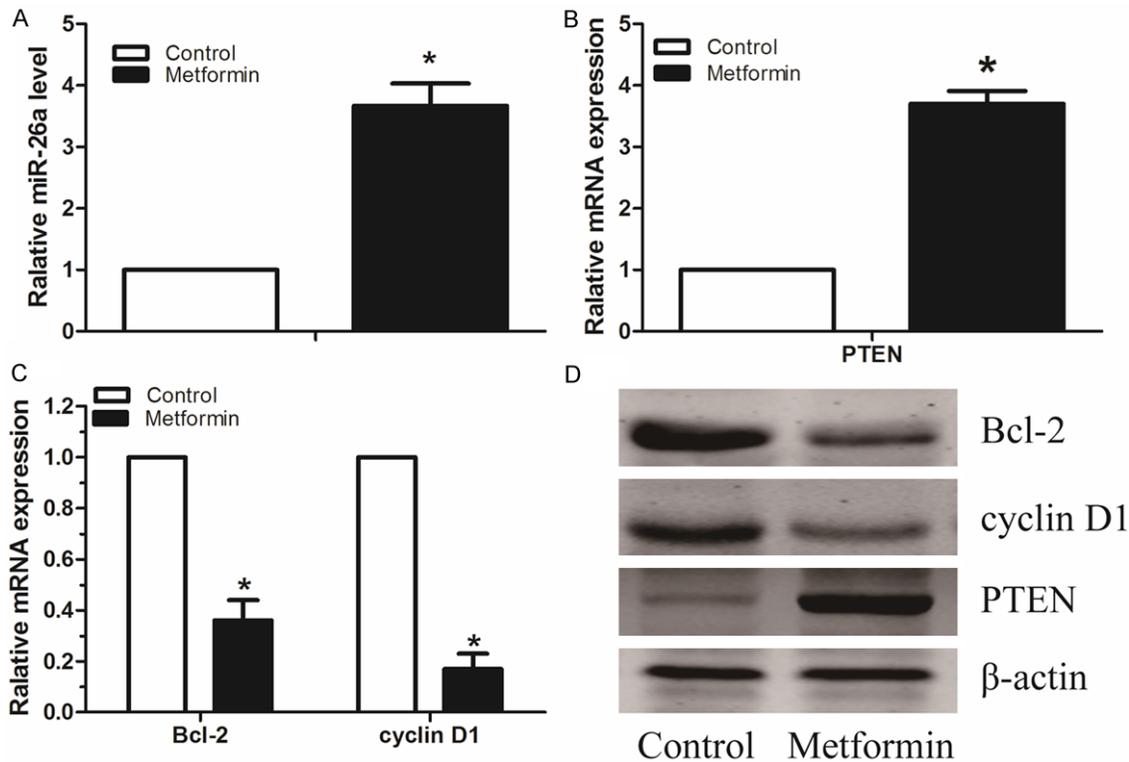
#### *miRNA and transfection*

To modify miR-26a expression levels in RCC cell lines, we obtained recombinant lentivirus vectors from Genechem (Genechem, Shanghai, China) that included genes such as pre-miR-26a, the negative control precursor miRNA.

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**Figure 1.** Metformin inhibits RCC 786O cells proliferation. A. 786-O cells were treated with metformin (0, 1, 5, 10, 20 and 40 mM) for 48 hours, and cell viability was measured by MTT assay. The results were expressed as percent of cell viability compared with control. B. 786-O cells were treated with metformin at different concentrations for 24, 48 and 72 hours. Cell proliferation was measured by MTT assay. The results are the mean  $\pm$  SD of three independent experiments, \* $P < 0.05$ .



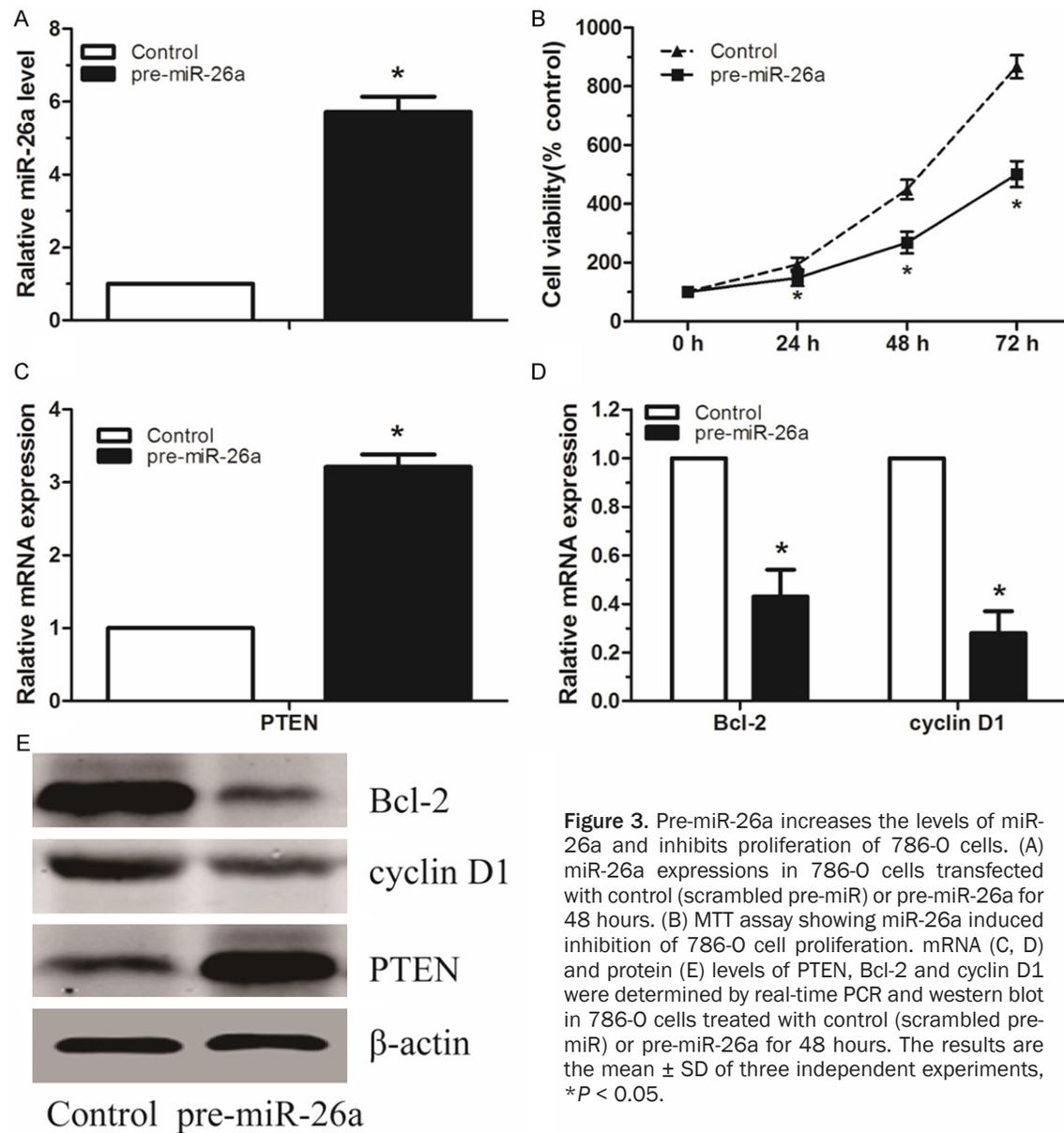
**Figure 2.** Metformin regulates expression of miR-26a and its target genes. (A) miR-26a expressions in 786-O cells treated with control (PBS) or metformin (10 mM) for 48 hours. mRNA (B, C) and protein (D) levels of PTEN, Bcl-2 and cyclin D1 were determined by real-time PCR and western blot in 786-O cells treated with control (PBS) or metformin (10 mM) for 48 hours. The results are the mean  $\pm$  SD of three independent experiments, \* $P < 0.05$ .

These vectors, with their packaging vectors were transfected into 293T cells using Lipofectamine 2000 (Invitrogen). 786-O cells were then transfected with virus following the manufacturer's instruction.

### siRNA and transfection

The siRNAs to AMPK (5'-ACCAAGGGCAGCCA-UACCCUU-3') or control siRNA (5'-CAUACGCU-UAAUACUACGUCCA-3') were all purchased from

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**Figure 3.** Pre-miR-26a increases the levels of miR-26a and inhibits proliferation of 786-O cells. (A) miR-26a expressions in 786-O cells transfected with control (scrambled pre-miR) or pre-miR-26a for 48 hours. (B) MTT assay showing miR-26a induced inhibition of 786-O cell proliferation. mRNA (C, D) and protein (E) levels of PTEN, Bcl-2 and cyclin D1 were determined by real-time PCR and western blot in 786-O cells treated with control (scrambled pre-miR) or pre-miR-26a for 48 hours. The results are the mean  $\pm$  SD of three independent experiments, \* $P < 0.05$ .

Shanghai GenePharma. Cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were incubated for 48 hours before further treatment.

### Western blot assay

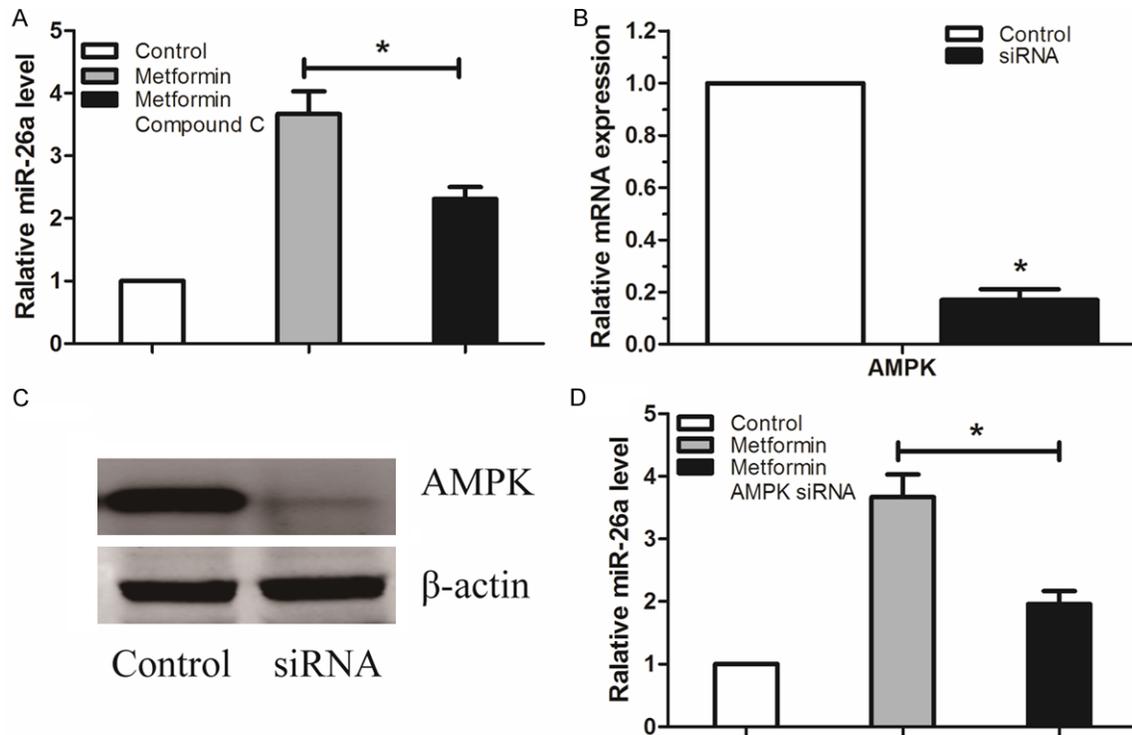
Cells were lysed in lysis buffer containing protease inhibitor. Protein concentration was determined using a Bio-Rad protein assay system (Bio-Rad). Equivalent amounts of proteins were separated by SDS-PAGE, and then transferred to polyvinylidene difluoride membranes (Bio-

Rad). After being blocked in Tris buffered saline (TBS) containing 5% non-fat milk, the membranes were incubated with specific primary antibodies (Cell Signaling, Beverly, MA) at 4°C for 12 hours and then with horseradish peroxidase-conjugated second antibody for 2 hours at room temperature. ECL detection reagent (Amersham LifeScience, Piscataway, NJ) was used to demonstrate the results.

### Statistical analysis

Statistical analysis was performed with SPSS version 17.0 (SPSS Inc., Chicago, IL). Values are

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**Figure 4.** Roles of AMPK signaling in the regulation of miR-26a by metformin. miR-26a expressions in 786-O cells (A) treated with vehicle control (PBS) or metformin (10 mM). Cells were pretreated with AMPK inhibitor (Compound C) for 4 hours. (B, C) Real-time PCR and western blot analysis of AMPK in 786-O cells treated with siRNA targeting AMPK or scramble control siRNA oligos. (D) miR-26a expression in 786-O cells treated with vehicle control (PBS) or metformin (10 mM). Cells were pretreated with siRNA oligos for 24 hours. The results are the mean  $\pm$  SD of three independent experiments, \* $P < 0.05$ .

expressed as the mean  $\pm$  SD. The difference between groups was analyzed using a Student t test when comparing only two groups or one-way analysis of variance when comparing more than two groups.  $P < 0.05$  was considered statistically significant.

### Results

#### *Metformin inhibits proliferation of 786-O cell lines*

In order to determine whether metformin affected the proliferation of human renal cancer cells, we investigated the effect of metformin on growth of human renal cancer cell lines 786-O. Cells were grown in 10% FBS and treated with metformin at different concentrations for 48 hours. Cell viability was then examined by MTT. As shown in **Figure 1A**, the MTT viability assay demonstrated that metformin led to a dose-dependent inhibition of cell proliferation in renal cancer cell lines 786-O. At the concen-

tration of 10 mM, metformin decreased the cell viability of 786-O cells by 51%. Therefore, 10 mM metformin was selected for the further analysis of genes expression in 786-O cell lines. To discern the direct relationship between the decrease in cell viability and the inhibition of cell proliferation, we followed the course of proliferation over three days after the addition of metformin. MTT assay showed that metformin decreased cell proliferation in a dose- and time-dependent manner in 786-O cells (**Figure 1B**). These results demonstrate that metformin inhibits the proliferation of renal cancer cells.

#### *Expression of miR-26a, Bcl-2, cyclin D1 and PTEN protein in metformin-treated cells*

Metformin can affect tumor cell proliferation by regulation of some genes [17]. Here, we found that miR-26a expression was significantly increased in 786-O cells exposed by metformin (**Figure 2A**). Next, we analyzed the expression contents of Bcl-2, cyclin D1 and PTEN, which

are also known as key molecules involved in cell proliferation. The expression levels of Bcl-2, cyclin D1 were decreased and PTEN was significantly increased in 786-O cell lines treated with metformin (**Figure 2B-D**).

### *Inhibitory effected of miR-26a on proliferation of 786-O cells*

To further explore the biological significance of miR-26a in RCC, we transfected a pre-miR-26a expression vector into human RCC 786-O cell lines. Expression of miR-26a was verified by TaqMan Universal PCR (**Figure 3A**). Up-regulation of miR-26a in 786-O resulted in significant suppression of cell proliferation (**Figure 3B**). Further we examined a number of the main miR-26a target genes, including Bcl-2, cyclin D1 and PTEN. Expression of Bcl-2, cyclin D1 were significantly decreased and PTEN was increased in 786-O cells which were transfected with pre-miR-26a vector (**Figure 3C-E**).

### *Inhibition of AMPK pathway reverses the roles of metformin*

We tested whether the inhibition effect of metformin on miR-26a expression is mediated by AMPK in renal cancer cells. As shown in **Figure 4A**, pretreatment with the AMPK inhibitor (Compound C) could reverse the inhibitory effect of metformin on miR-26a. To rule out possible nonspecific effects of Compound C, siRNA oligo-mediated knockdown of AMPK performed (**Figure 4B, 4C**). As a result, we also observed that the inhibitory roles of miR-26a were also blocked by AMPK depletion (**Figure 4D**). Interestingly, both compound C and AMPK siRNA followed by PBS treatment seem to elevate miR-26a expression a little bit comparing to DMSO and control siRNA, respectively, suggesting that AMPK signaling might repress miR-26a expression at the basal condition. Together, our results suggested that the regulation of miR-26a expression by metformin in renal cancer was relied on AMPK signaling.

## Discussion

In our study, we used human renal cancer cell lines 786-O to investigate the effects and mechanisms of metformin. Our result showed that metformin treatment could significantly upregulate the expression of miR-26a in renal cancer cells. As a result, protein abundance of

Bcl-2, and cyclin D1 were decreased but protein abundance PTEN was increased in cells exposed to metformin. Transfected pre-miR-26a vector in 786-O up-regulated miR-26a expression and resulted in significant suppression of Bcl-2 and cyclin D1 but enhance PTEN expression compared with the negative control. Therefore, these results provide a novel evidence for the mechanism that may contribute to the anticancer effects of metformin suggested by recent population studies and justify further work to explore potential roles for it in renal cancer treatment. Diabetic patients treated with metformin have a reduced incidence of cancer and cancer-related mortality. Recent studies showed that metformin affects engraftment and growth of bladder cancer tumour in mice, and this correlates with the induction of metabolic changes compatible with clear anti-neoplastic effects [18].

Recent studies showed that metformin modulation underlies the antineoplastic metabolic actions of metformin. For example, Blandino found that metformin increases DICER mRNA and protein expression and its effects are no longer able to affect tumor engraftment in knock-down DICER cells. Conversely, ectopic expression of DICER recapitulates the effects of metformin [19]. Wang found that metformin up-regulated p27, p57 and PTEN expression through modulation of metformins in lung cancer [8]. In colon cancer, Nangia-Makker showed that metformin treatment decreased miRNA21 and increased miR-145 expression [20]. In pancreatic cancer cells, Bao found that metformin treatment increased the relative expressions of let-7a, let-7b, miR-26a, miR-101, miR-200b and miR-200c in a dose-dependent manner [21]. Indeed, forced expression of miR-26a significantly inhibited cell proliferation, invasion, migration and increased cell apoptosis, whereas knockdown of miR-26a obtained the opposite effect [21]. In addition, the miRNA expression was also markedly altered with the treatment of metformin in esophageal and breast cancer cells. Therefore, it will be interesting to further investigate the regulation of metformin expression by metformin in other tumor cells in the future.

In conclusion, our results showed that metformin was able to inhibit RCC growth by increasing expression of miR-26a in 786-O human renal cancer cell lines. As a result, protein

abundance of Bcl-2 and cyclin D1 was decreased and PTEN was increased in cells exposed to metformin. Also over-expression of miR-26a in 786-O cells can inhibited cell proliferation by down-regulating Bcl-2, cyclin D1 expression and up-regulating PTEN expression. These data provide novel evidence for a mechanism that may contribute to the antineoplastic effects of metformin and justify further work to explore potential roles for it in renal cancer treatment.

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### Disclosure of conflict of interest

None.

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