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Metformin inhibits epithelial–mesenchymal transition in prostate cancer cells: Involvement of the tumor suppressor miR30a and its target gene SOX4

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ABSTRACT

Tumor metastasis is the leading cause of mortality and morbidity of prostate cancer (PCa) patients. Epithelial–mesenchymal transition (EMT) plays a critical role in cancer progression and metastasis. Recent evidence suggested that diabetic patients treated with metformin have lower PCa risk and better prognosis. This study was aimed to investigate the effects of metformin on EMT in PCa cells and the possible microRNA (miRNA)-based mechanisms. MiRNAs have been shown to regulate various processes of cancer metastasis. We herein showed that metformin significantly inhibits proliferation of Vcap and PC-3 cells, induces G0/G1 cell cycle arrest and inhibits invasiveness and motility capacity of Vcap cells. Metformin could inhibit TGF-β-induced EMT in Vcap cells, as manifested by inhibition of the increase of N-cadherin ($p = 0.013$), Vimentin ($p = 0.002$) and the decrease of E-cadherin ($p = 0.0023$) and β-catenin ($p = 0.034$) at mRNA and protein levels. Notably, we demonstrated significant upregulation of miR30a levels by metformin ($P < 0.05$) and further experiments indicated that miR30a significantly inhibits proliferation and EMT process of Vcap cells. Interestingly, we identified that SOX4, a previously reported oncogenic transcriptional factor and modulator of EMT, is a direct target gene of miR30a. Finally, we screened the expression of miR30a and SOX4 in 84 PCa cases with radical prostatectomy. Of note, SOX4 overexpression is significantly associated with decreased levels of miR30a in PCa cases. In all, our study suggested that inhibition of EMT by metformin in PCa cells may involve upregulation of miR30a and downregulation of SOX4.

1. Introduction

Metformin (1,1-Dimethylbiguanide) is one of the most commonly used drugs for type II diabetes. It reduces glucose levels through activation of the AMP-activated protein kinase (AMPK) pathway and inhibition of hepatic gluconeogenesis [1]. Recently, multiple epidemiological studies have shown that metformin may reduce cancer risk and/or improve cancer prognosis [2]. In addition, metformin affects the progression and relapse of breast, prostate, and lung cancer mouse xenografts, when combined with suboptimal doses or standard chemotherapeutic agents [3]. So far, possible mechanisms by which metformin exerts its potential antineoplastic properties include activation of AMPK, and thereby inhibition of m-TOR signaling pathway; selective impairment of P53-deficient cells; decrease of Cyclin D1 level and downregulation of C-myc [4–6].

Prostate cancer (PCa) is a common heterogeneous disease with marked variability in progression [7]. Tumor metastasis is the leading cause of mortality in PCa patients. Epithelial–mesenchymal transition (EMT), characterized by the loss of epithelial characteristics and acquisition of a mesenchymal phenotype, plays a critical role in cancer progression and metastasis [8]. Multiple transcription factors such as Snail, Slug and ZEB, act as molecular switches, respond to the known signaling pathways and regulate the EMT program [8]. Previously, we have reported that SOX4 gene, an oncogenic transcription factor, may contribute to PCa metastasis

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by initiating a transcriptional program that enables EMT phenotype [9].

Although preliminary, several studies suggested metformin may suppress EMT in malignancy [10–12]. Vazquez-Martin et al. demonstrated metformin can prevent generation of breast cancer stem cell phenotype by downregulating some EMT regulators ("EMT status") independently of changes in EMT functioning ("EMT phenotype") [13]. Additionally, Qu et al. observed that metformin reversed EMT phenotype and decreased the invasive capacity of MCF7/5-FU and MDA-MB-231 cells [12]. However, the link between metformin and the EMT process in PCa progression remains unclear.

MicroRNAs (miRNAs) are short, stable, noncoding RNAs that target the mRNA of coding genes for degradation, thus effectively reducing the levels of the gene products [14]. Of note, a growing number of miRNAs were reported to regulate tumor invasion/metastasis through EMT-related and/or non-EMT-related mechanisms [15]. Interestingly, metformin has been shown to upregulate tumor suppressive miRNA let-7a and miRNA96 and downregulate oncogenic miRNA181a [16]. This can be the key preventing self-renewal of cancer-initiating cells arising from EMT. Given the potential ability of metformin to broadly affect multiple pathways, we hypothesized that miRNA modulation could be part of its mechanism of reversing EMT in cancer. In the current study, we investigated the effects of metformin on EMT in PCa cells and analyzed the possible microRNA (miRNA)-based mechanisms.

2. Materials and methods

2.1. Reagents

Metformin was obtained from Sigma–Aldrich (St. Louis, MO, USA). TGF-β was purchased from RD Biosciences (San Diego, CA, USA).

2.2. Cell culture and treatment

Human PCa cell lines Vcap, DU-145, Lncap, PC-3, normal prostate epithelial cell line RWPE and Kidney HEK293T cells were from American Type Culture Collection (Rockville, MD, USA) and cultured following the manufacturer's recommendations. For experiments, PCa cells were treated with metformin or vehicle at indicated concentration for up to 24, 48 and 72 h. TGF-β (5 ng/ml for 72 h) was utilized to treat Vcap cells. Cell lysates were then collected for qRT-PCR or Western blot.

2.3. Plasmid constructs

The 3′-UTR of SOX4 gene was amplified from HEK293T and cloned into the pmirGLO dual luciferase reporter vector using Xba I and Sal I restriction sites. The primers was as followings: (F) GCTCTAGAAATGTAGACTTTTGACACTT, (R) ACGCGTCGACAGAG CAGTATTTCGTTTAAA.

2.4. Cell transfection

Cell transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. miR30a mimic, miR30a inhibitor and pre-miR negative control were designed and synthesized by Gene-Pharma (Shanghai, China). The mock group was defined as the ones supplemented with the transfection reagent only.

2.5. MTS, migration and invasion assays

Cell proliferation was measured using the MTS assay with CellTiter 96 AQueous One Solution Reagent (Promega, Mannheim, Germany) following the manufacturer's protocol. Wound healing and invasion assays were performed as previously described [9]. For quantification of invading cells, cells were counted in five randomly selected microscopic fields (200×). Three independent experiments were performed.

2.6. Cell cycle phase distribution analysis

Cells were washed twice with cold PBS and then stained with propidium iodide (PI) (0.1 mg/mL with 0.1% TritonX-100) for 30 min in the dark at room temperature. The PI staining was evaluated by flow cytometry (FACSCalibur, BD, USA). Raw data were analyzed using FlowJo Version 9.5.2 flow cytometric (FCM) analysis software (Tree Star, Ashland, OR).

2.7. qRT-PCR

RNA extraction and qRT-PCR was performed as previously described [9]. The primers for each gene in qRT-PCR were listed in Supplementary Table 1. The relative mRNA expression levels of E-cadherin, Vimentin, β-catenin, and N-cadherin were calculated using the comparative Ct method [9], with GAPDH as a reference gene.

2.8. Western blot

Western blot was performed as previously described [9]. The membrane was incubated with primary antibodies for SOX4 (Abcam, Cambridge, UK), β-catenin (Cell Signaling Technology, MA, USA), Vimentin (Cell Signaling Technology, MA, USA) and GAPDH (Santa Cruz Biotechnology, CA, USA), respectively. The signals were detected with an enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK).

2.9. Luciferase reporter assays

Cells were seeded in triplicate in 24-well plates and allowed to settle for 24 h. To experimentally verify whether the SOX4 gene is an *in vivo* target of miR30a, reporter constructs and 200nM miRNAs and their controls were transfected into 293T cells using Lipofectamine 2000. Dual-Luciferase Reporter Assay (Promega, Madison, WI, USA) was performed at 48 h after transfection. Firefly luciferase activity was normalized to Renilla luciferase expression for each sample. Each experiment was performed in triplicate.

2.10. Patients and tissue microarray construction

Our study consisted of 84 PCa patients with radical prostatectomy between 2005 and 2013 at Qilu Hospital of Shandong University (Jinan, China) and the Affiliated Hospital of Medical College Qingdao University (Qingdao, China). A tissue microarray was constructed by incorporating two representative cores of each tumor. The morphology was confirmed by a pathologist (B.H.). This study was approved by the Institutional Review Board of Shandong University. Informed written consent was obtained from the PCa patients.

2.11. Immunohistochemistry (IHC)

IHC was performed as previously described [9]. Immunohistochemical staining was done using the standardized labeled streptavidin biotin kit (DakoCytomation, Carpinteria, CA, USA). The slides

were incubated with rabbit polyclonal anti-SOX4 antibody (1:100 dilution, Abcam, Cambridge, MA, USA) and were evaluated blindly by a pathologist (B.H.) and the scoring system was utilized as previously described [9].

2.12. miRNA isolation and real-time quantitation of miRNA expression levels using RT-qPCR

miRNA extraction from tissues was performed with a miRNeasy FFPE kit (Qiagen, Valencia, CA, USA). miRNA expression levels were quantitated using a MicroRNA Assay Kit (Applied Biosystem, Foster City, CA, USA) according to the manufacturer's instructions. All reactions were run in duplicate. The universal small nuclear RNA U6 was used as an endogenous control for miRNAs. The primer sequences were as follows: miR30a mimics: AAGGAGCUCACAGUC UAUUGAG; miR30a inhibitor: CUCAAUAGACUGUGAGCUCCUU.

2.13. Statistical analysis

Statistical analyses were carried out using the Statistical Package for Social Sciences, version 19.0 (SPSS). Data were presented as median ± S.D. Differences were analyzed with Student's *t*-test between two groups or with one-way analysis of variance among multiple groups. For continuous data, the Mann–Whitney U-test was used for unpaired data and the Wilcoxon matched pairs test for paired data. The correlation between miR30a level and SOX4 protein expression in PCas was calculated by Spearman's correlation. A *p*-value <0.05 was considered statistically significant.

3. Results

3.1. Metformin inhibits cellular proliferation, migration and invasion of PCa cell lines

Human PCa cell lines Vcap and PC-3 as well as normal prostate epithelial cell line RWPE were utilized to determine metformin regulation of cell proliferation. Consistent with previous findings [4], metformin was growth inhibitory for both Vcap and PC-3 cells through time- and dose-dependent manner. Metformin at 5 mM significantly inhibited the proliferation of Vcap and PC-3 cells at 48 and 72 h, whereas there was no effect on RWPE cells, accordingly (Fig. 1A).

Wound healing assay indicated that Vcap cells treated with 5 mM metformin for 48 h displays a significant decrease in cell migratory ability compared to negative controls (Fig. 1B). Invasion assay further showed that 5 mM metformin can significantly inhibit the invasive capacity of Vcap cells. Compared with the control

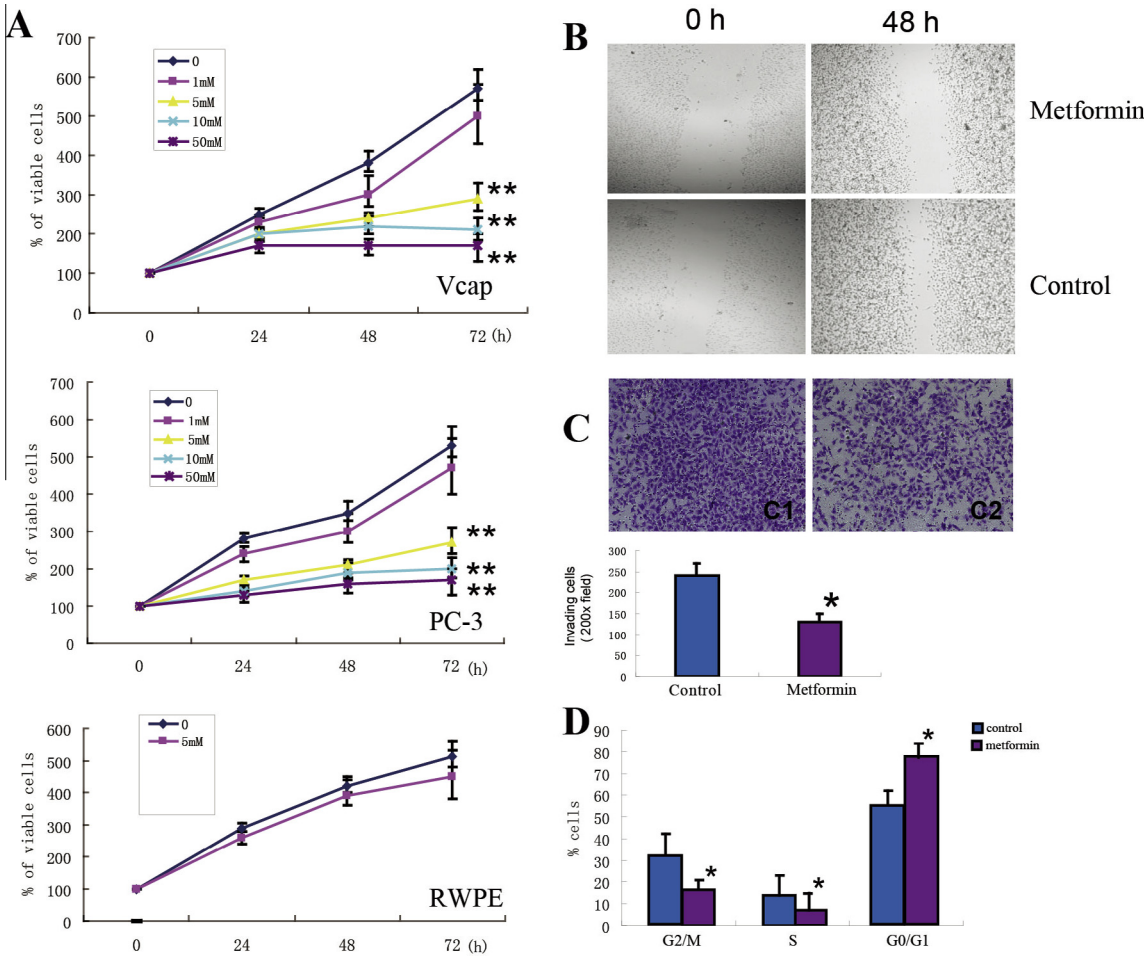

Fig. 1. Biological roles of metformin on PCa cells. (A) Cell viability as assessed by MTS assay at different time points ranging from 0 to 72 h in Vcap and PC-3 cells, respectively. (B) Vcap cells were treated with 5 mM metformin for 48 h or left untreated. Effects of metformin on Vcap cell migration was measured by wound healing assay. Original magnification, ×100; (C) cellular invasive capacity of Vcap cells (C1–C2) as assessed by invasion chambers; (C1) Control, PBS; (C2) 5 mM metformin. (D) Cell cycle distribution was monitored by FCM using a PI staining assay. The data shown and each bar represent the mean ± SD of three independent samples. $^{*}p < 0.05$, $^{**}p < 0.01$ when compared with negative control.

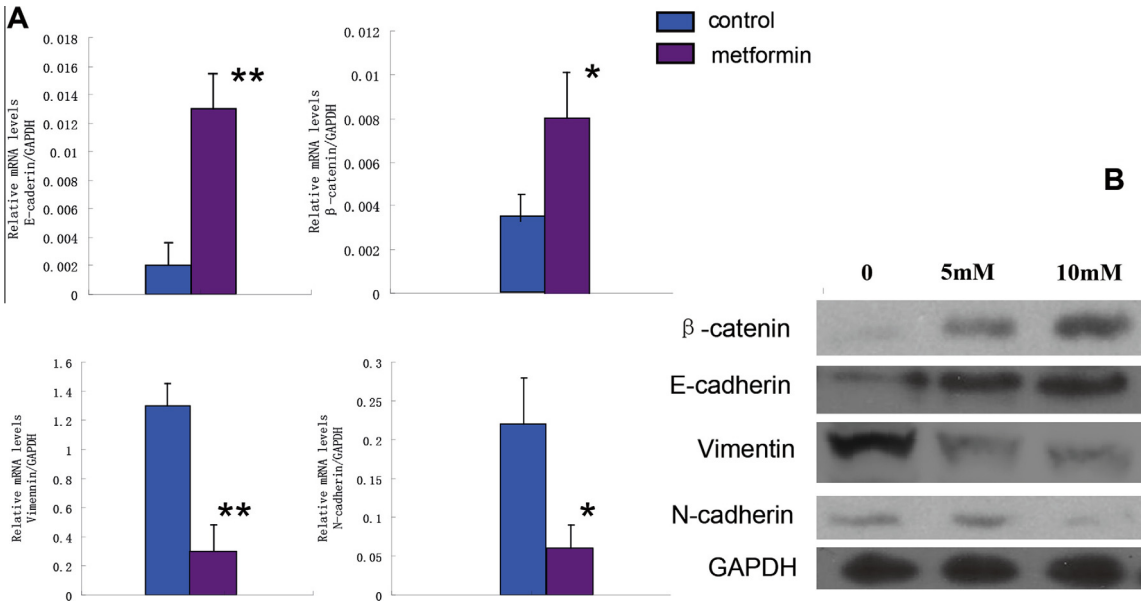

Fig. 2. Metformin inhibits TGF-β-induced EMT in PCa cells. (A) mRNA expression of mesenchymal markers (Vimentin, N-cadherin) and the epithelial markers (E-cadherin, β-catenin) was shown by qRT-PCR after metformin treatment or left untreated in TGF-β-induced Vcap cells; (B) The protein expression levels of Vimentin, N-cadherin, E-cadherin and β-catenin were presented by Western blot after metformin treatment (5 mM & 10 mM) in TGF-β-induced Vcap cells. Representative results from triplicate experiments are shown as mean ± SD. ($^{*}p < 0.05$, $^{**}p < 0.01$). All of relative expression of target genes was normalized to GAPDH.

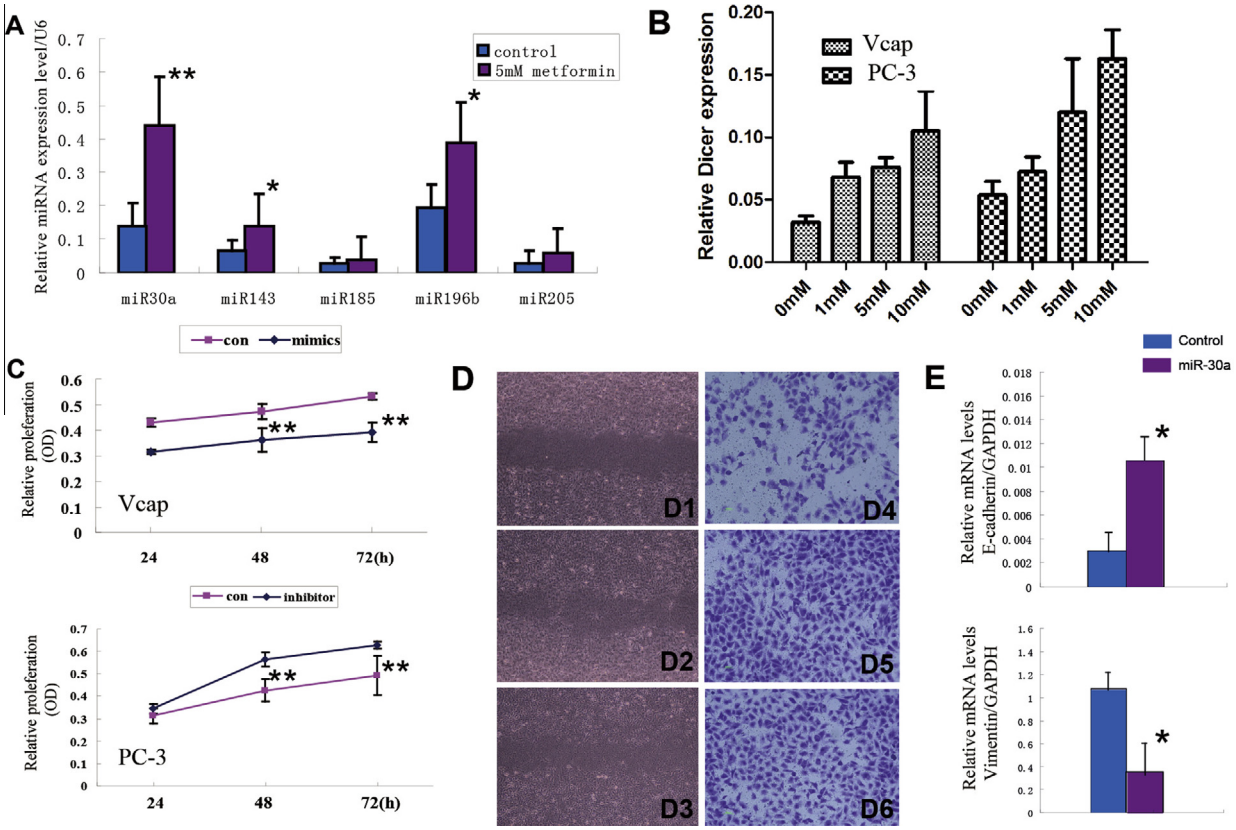

Fig. 3. Upregulation of miR30a expression levels by metformin and effect of miR30a on the proliferation and EMT of Vcap cells. (A) Modulation of selected miRNA levels by 5 mM metformin in Vcap cells. (B) qPCR. mRNA levels of Dicer from Vcap and PC-3 cells treated with metformin at the indicated concentrations for 24 h. (C) Cell viability as assessed by MTS assay at different time points ranging from 0 to 72 h in Vcap and PC-3 cells treated with miR30a mimics and miR30a inhibitor, respectively. Each bar represents the mean ± SD of three independent samples. (D) miR30a inhibits migration and invasive capacity of Vcap cells. Vcap cells were treated with miR30a mimics or miR30a inhibitor for 48 h or left untreated. Original magnification, ×100; (D1, D4) miR30a mimics; (D2, D5) miR30a inhibitor; (D3, D6) Mock. (E) mRNA expressions of Vimentin and E-cadherin were shown by qRT-PCR after miR30a mimics treatment or left untreated in TGF-β-induced Vcap cells ($^{*}p < 0.05$, $^{**}p < 0.01$).

group, the invasive capacity of Vcap cells was reduced to 55% by 5 mM metformin treatment for 24 h (Fig. 1C).

3.2. The influence of metformin on Vcap cell cycle distribution

Using FCM analysis, we demonstrated that Vcap cells treated with 5 mM metformin for 48 h clearly shows G1 phase arrest (77.2% vs. 54.1%) and significant decrease in the percentage of cells in S (6.7% vs. 13.8%) and G2/M (16.1% vs. 32.1%) phase (Fig. 1D). These results suggested that metformin causes cell cycle arrest of Vcap cells.

3.3. Metformin inhibits TGF-β-induced EMT in Vcap cells

TGF-β is known to play a major role in EMT induction. As shown in Fig. 2A, 5 mM metformin treatment for 24 h suppressed the TGF-β-induced EMT, as shown by inhibition of the increase of N-cadherin ($p = 0.013$), Vimentin ($p = 0.002$) and the decrease of E-cadherin ($p = 0.0023$) and β-catenin ($p = 0.034$) at mRNA levels. In addition, in Vcap cells treated by 5 mM and 10 mM metformin for 48 h, Western blot showed that the expression of E-cadherin and β-catenin at protein levels were significantly elevated, while the expressions of Vimentin and N-cadherin protein levels were significantly decreased (Fig. 2B).

3.4. miRNAs modulated by metformin

To investigate the possible miRNA-based mechanisms, we next selected a series of miRNAs that have been reported to play a role in PCa invasion and metastasis, and evaluated the expression of these miRNAs after metformin treatment. Compared with the control group, 5 mM metformin treatment was found to notably upregulate the expressions of miR30a, miR143 and miR196b, but has no effect on miR185 and miR205. Remarkably, miR30a showed the highest alteration level (~fourfold increase) after metformin treatment (Fig. 3A).

3.5. Metformin modulates the levels of DICER

To understand how metformin could modulate the levels of the intracellular miRNAs, we evaluated the effect of metformin on the RNASE III endonuclease DICER, one of the key enzymes of miRNA biogenesis. As shown in Fig. 3B, metformin treatment for 24 h led to a significant increase in DICER mRNA levels in PCa cells.

3.6. Functional characterization of miR30a in PCa cells

Among the metformin-regulated miRNAs, we focused on the miR30a owing to its robust modulation by metformin. Interest-

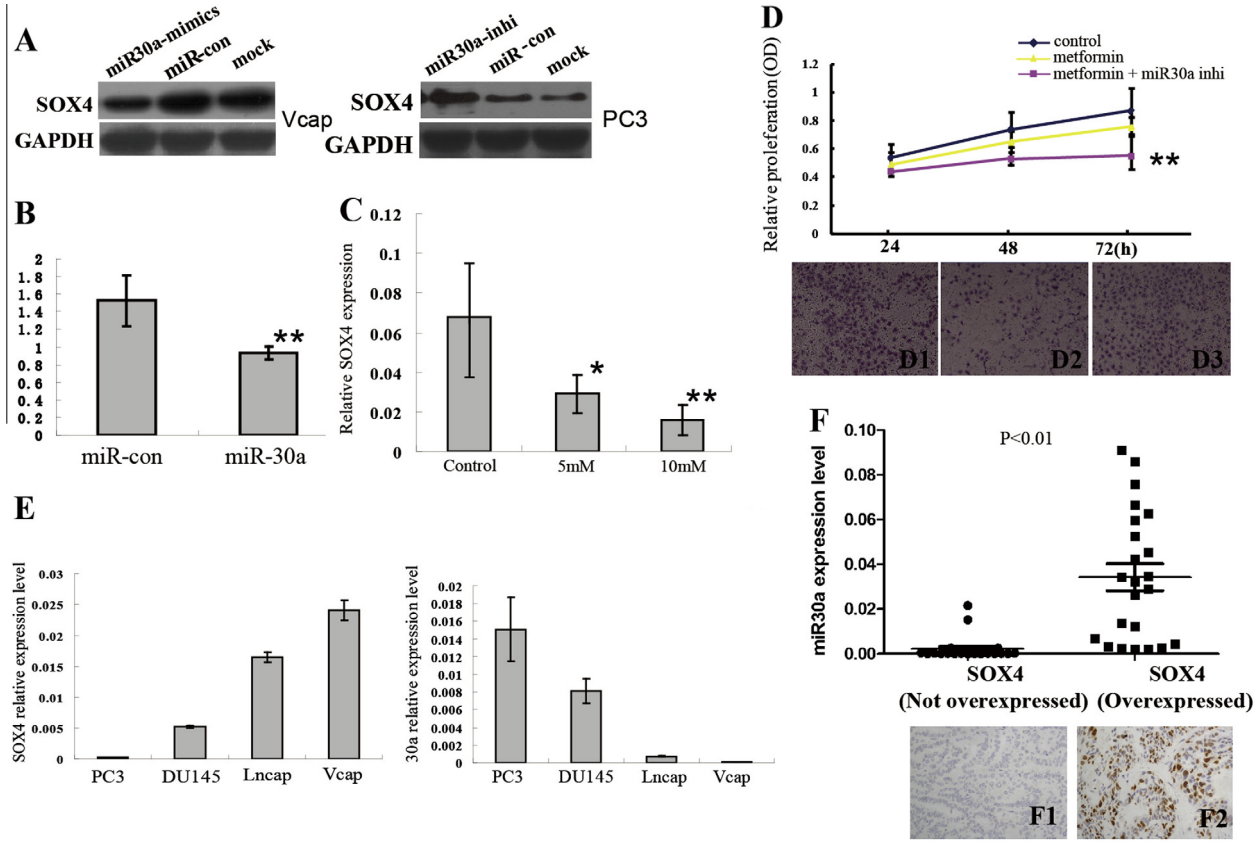

Fig. 4. miRNA30a targets SOX4 and inverse correlation of SOX4 overexpression with decreased level of miR30a in PCa. (A) Vcap cells were transfected with miR30a mimic, and the PC-3 cells were transfected with miR30a inhibitor. Western blot was performed to examine the effects of miR30a on SOX4 protein levels. GAPDH served as a reference control. (B) The SOX4 3′UTR containing a putative binding site for miR30a was cloned into a firefly luciferase in a luciferase vector and co-transfected into H293T cells. Luciferase measurements were done 24 h post-transfection. Renilla luciferase was used as control for transfection efficiency. (C) mRNA expression of SOX4 was shown by qRT-PCR after metformin treatment (5 mM & 10 mM) for 24 h. (D) Cellular proliferation as assessed by MTS assay with 5 mM metformin treatment alone for 24 h or treatment with 5 mM metformin plus miR30a inhibitor transfection for 24 h in PC-3 cells (Upper); Invasive capacity as assessed by invasion assay with 5 mM metformin treatment alone for 24 h or treatment with 5 mM metformin plus miR30a inhibitor transfection for 24 h in PC-3 cells (Lower); (D1) Control, PBS; (D2) 5 mM metformin; (D3) 5 mM metformin + miR30a inhibitor. (E) mRNA expression of SOX4 and miR30a in PCa cells lines by qRT-PCR analysis. (F) MiR30a expression was validated in 40 of 84 tumors of PCa cases and found to be downregulated in patients with SOX4 overexpression by IHC. ($p < 0.01$) Representative PCa cases with or without SOX4 overexpression were shown in (F1) and (F2), respectively.

ingly, miR30a is a recently described tumor suppressor in lung cancer and breast cancer [17,18]. We firstly transfected Vcap (low miR30a level) cells with miR30a mimics, and found that miR30a efficiently inhibits the proliferation, cell migration and invasion ability (reduced ~60% $P < 0.01$) of Vcap cells. (Fig. 3C and D). By contrast, miR30a inhibitor significantly increased proliferation of PC-3 (high miR30a level) cells (Fig. 3C).

Further, using qRT-PCR analysis, we showed that miR30a mimics significantly upregulates expression of E-cadherin, but downregulates expression of Vimentin at the mRNA level, compared with the miR30a-negative control (Fig. 3E). These results suggested that miR30a inhibits EMT process of Vcap cells.

3.7. miR30a directly targets SOX4 in Vcap cells

To further characterize by which miR30a suppresses EMT in PCa cells, we used three algorithms (Targetscan, Pictar and Miranda) to identify potential miR30a targets in human PCas. Among a series of target genes predicted to be regulated by miR30a, SOX4 gene was selected. Previously, SOX4 overexpression has been reported to be a poor prognostic factor in PCa patients by our group [9]. As shown in Fig. 4A, miR30a mimics significantly decreased SOX4 protein expression level in Vcap cells. By contrast, miR30a inhibitor increased expression level of SOX4 in PC-3 cells. These data suggested that miR30a regulates SOX4 expression at protein level. Next, we showed miR30a regulates the luciferase activity of the wild-type SOX4 3′UTR reporter. As shown in Fig. 4B, miR30a significantly reduced the activity of the luciferase reporter gene fused to the SOX4 3′-UTR by 39.5 ± 11.4% in Vcap cells, compared with the miR-negative control ($P < 0.05$). In addition, we confirmed downregulation of SOX4 by metformin treatment at mRNA level (Fig. 4C). Collectively, these data suggests that SOX4 was a direct target gene of miR30a.

The next experiment was dedicated to assessing whether miR30a accounts for the anti-neoplastic effect of metformin in PCa cells. As shown in Fig. 4D, miR30a inhibitor can significantly rescued the suppressive effect of 5 mM metformin on the proliferation and invasion of PC-3 cells. These data suggested that miR30a is a major factor that associates with the effect of metformin in PCa cells.

3.8. SOX4 overexpression is associated with decreased level of miR30a in PCa cases

Notably, an inverse correlation was identified between miR30a level and SOX4 expression level ($P < 0.01$) both in PCa cell lines (Fig. 4E) and clinically localized PCa cases (Fig. 4F). SOX4 protein tends to be overexpressed in samples with low miR30a level. These data further corroborate that SOX4 is the direct target gene of miR30a in human PCa cases.

4. Discussion

Although earlier epidemiological studies strongly suggest a role for metformin in reducing PCa risk, the mechanism by which metformin inhibits PCa carcinogenesis and progression remains unclear. Previous studies suggested two possible mechanisms underlying the anti-proliferative of metformin on neoplastic cells in vitro and *in vivo*: reduction of systemic insulin levels and a direct action involving AMPK activation within neoplastic cells [19]. Other possible mechanisms involving p53 and cyclin D1 leading to G0/G1 or S phase arrest have also been presented [4,6]. In this study, we confirmed the significantly inhibitory effect of metformin on PCa cells and minimal inhibition on normal prostate epithelial cells, which were in line with the previous findings [2,4,5].

PCa is a heterogeneous disease with a variable natural history. It is estimated that only a small fraction of patients suffers from tumor metastasis that requires aggressive treatment [7]. Currently, although there is still a large gap between the concept and its clinical development, EMT has been reported to play a critical role in cancer progression and metastasis [8]. In the current study, our data clearly demonstrated that metformin inhibits TGFβ-induced EMT in PCa cells and markedly reduced cell migration and invasion. Most recently, several reports have suggested that metformin suppresses EMT both in breast cancer and PCa in vitro [11–13,20]. Vazquez-Martin et al. showed that metformin can regulate key drivers of the EMT machinery in breast cancer [13]. They illustrated for the first time that metformin suppresses the EMT status in CD44+ cells. This was done by transcriptional repression of key EMT drivers including ZEB1, TWIST1, SLUG and thus eliminating the breast cancer stem cell phenotypes in cell populations. In PCa, Wang et al. demonstrated that metformin inhibits EMT in DU-145 cells by down-regulating FoxM1 expression [11].

Multiple signaling pathways and EMT-related transcription factors are involved in EMT process in cancer. Of note, there are many miRNAs and/or long-chain non-coding RNA closely related to EMT and cancer metastasis. In an attempt of analyzing the possible miRNA-based mechanisms, we identified that miR30a was significantly upregulated by metformin. In several large scale miRNA expression arrays, miR30 family members have been frequently found to be downregulated in various malignancies and an elegant study by Baffa et al. has identified that miR30a is among the highly downregulated miRNAs in metastatic cancers, implying a putative function of miR30a in cancer and potentially metastasis [21]. In the present study, our data suggested that miR30a inhibits EMT process of Vcap cells. These data were in consistent with the previous findings that miR30 may play an important role in regulating TGF-beta induced EMT in lung cancer [17]. Additionally, we demonstrated that metformin induces DICER expression which implies that modulation of the miRNA by metformin in PCa cells may be associated with this process.

SOX4 is an oncogenic transcription factor and previous studies have demonstrated that SOX4 is upregulated in diverse human malignancies and its high expression indicates poor prognosis [22,23]. We have reported that SOX4 promotes the TGF-β-induced EMT, and alterations of SOX4 expression could also disturb EMT phenotype in PCa cells [9]. More importantly, SOX4 might be a metastasis contributor in cancer progression, and cooperates with other important oncogenes to promote PCa metastasis [9]. Most recently, we demonstrated a link of SOX4 and ERG gene in the development of a subset of PCa patients, and SOX4 may serve as a prognostic marker for Chinese PCa patients [24]. In the current study, for the first time, we showed that SOX4 is a target gene of miR30a in PCa cells. A significant correlation of SOX4 overexpression and miR30a lost was also identified in clinical samples. Importantly, our findings suggested reversal of EMT by metformin in PCa involves up-regulation of miR30a and down-regulation of SOX4. Therefore, it would be interesting to investigate whether or not metformin might be effective on a subset of PCa patients (SOX4+, miR30a−).

In conclusion, we demonstrated that metformin could inhibit EMT in PCa cells possibly by upregulation of miR30a and downregulation of SOX4. These data suggested a potential clinical use of metformin in treatment of a subset of PCa patients.

Conflict of interest

None.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.154.

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