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Hyperglycaemia-induced miR-301a promotes cell proliferation by repressing p21 and Smad4 in prostate cancer

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ABSTRACT

Hyperglycaemia promotes the development of Prostate cancer (PCa). However, the roles of miRNAs in this disease process and the underlying mechanisms are largely unknown. In this study, we recruited 391 PCa patients in China and found that PCa patients with high level blood glucose (≥100 mg/dL) trended to have high Gleason score (GS ≥ 7). miRNA-301a levels were significantly higher in prostate cancer than that in normal prostate tissues. Hyperglycaemia or high glucose treatment induced miR-301a expression in prostate tissues or PCa cell lines. miR-301a suppressed the expression of p21 and Smad4, and subsequently promoted G1/S cell cycle transition and cell proliferation *in vitro* and xenograft growth in nude mice *in vivo*. Furthermore, knockdown of p21 and Smad4 mimicked the effects of miR-301a overexpression. Restoration of p21 and smad4 could interrupt the effects of miR-301a overexpression. Importantly, inhibition of miR-301a severely blocked high glucose-induced PCa cell growth both *in vitro* and *in vivo*. These results revealed a novel molecular link between hyperglycaemia and PCa. The miR-301a plays an important role in the hyperglycaemia-associated cancer growth, and represents a novel therapeutic target for PCa.

1. Introduction

Prostate cancer (PCa) is the most common male malignancy in the US and the second leading cause of death in men with cancer [1]. The increasing prevalence of diabetes mellitus (DM) has become a major public health problem worldwide. Hyperglycaemia is the main characteristic of DM. Epidemiological studies found a significant link between glycaemic index and the increased cancer risk [2]. Moreover, hyperglycaemia is associated with high grade, high Gleason score (GS ≥ 7) and higher risk of recurrence in PCa in western countries [3–6], implying that hyperglycaemia plays important roles in the initiation and progression of PCa.

Hyperglycaemia produces advanced glycosylated end-products with resultant oxidative stress, which leads to DNA damage [7]. Meanwhile, chronic inflammation during DM results in the release

Abbreviations: PCa, Prostate cancer; DM, diabetes mellitus; GS, Gleason score; miRNA, MicroRNA; UTR, untranslated region; mRNA, messenger RNA; STZ, streptozocin; siRNA, interfering RNAs; NC, negative control; qRT-PCR, quantitative real-time polymerase chain reaction; EdU, 5-ethynyl-2′-deoxyuridine; FACS, fluorescence-activated cell sorting; SD, Standard deviation.

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of several cytokines that promotes tumour growth [8]. Moreover, Hyperglycaemia could elevate insulin levels, activate the insulin/insulin-like growth factor pathway and then promote the growth of PCa cells [9]. However, whether the elevated glucose can directly affect intracellular signal transduction in PCa remains largely unknown.

MicroRNAs (miRNAs) belong to a class of small noncoding RNAs that repress the expression of protein-coding genes by either mRNA cleavage or translational repression *via* binding to the 3′ untranslated regions (UTRs) of their target messenger RNAs (mRNAs) [10]. It is well documented that miRNAs participate in cancer initiation, progression, and metastasis [11]. Interestingly, several miRNAs, which are associated with obesity and diabetes, have also been implicated in carcinogenesis. Loss of miR-145 expression [12] and overexpression of miR-124a and miR-30d [13] were related to the development of breast cancer complicated by type 2 DM, respectively. Oncogenic miR-221 expression positively correlated with the body mass index [14]. Elevation of miR-221 promoted tumour growth and metastasis in breast, multiple myeloma and prostate cancers [15–17]. miR-34a, which was up-regulated in obesity, was shown to promote EMT-mediated metastasis in colorectal and hepatocellular cancer [18–20]. These studies provided evidence for the involvement of miRNAs in the association between diabetes, obesity, and cancer. However, the precise mechanisms of miRNAs in linking cancer with diabetes and obesity are still largely unknown.

p21, a cyclin-dependent kinase (CDK) inhibitor, inhibits the activity of cyclin-CDK2 and cyclin-CDK4 complexes and blocks the cell cycle transition from the G1 to S phase [21]. Smad4 is the pivotal transducer of the TGF-β signalling, which could induce G1/S cell cycle arrest by transactivating a cohort of target genes, including p21 [22,23].

In this study, we identified that miR-301a was up-regulated by high glucose both *in vitro* and *in vivo*. miR-301a simultaneously inhibited p21 and Smad4 expression, thereby promoting the cell growth and regulating G1/S transition. We discovered a novel molecular link between hyperglycaemia and the increased frequency of aggressive prostate cancer.

2. Materials and methods

2.1. Patients and human specimens

The PCa patients were all histopathologically diagnosed between 2000 and 2015 in the Third Affiliated Hospital, Sun Yat-sen University. The patient characteristics including age, diagnostic PSA level, fasting blood glucose level closest to the date of diagnosis, and the Gleason sore of specimens were collected. The categories established by the American Diabetes Association (ADA) for normal (blood glucose level <100 mg/dL), impaired glucose (100–125 mg/dL) and diabetes (>125 mg/dL) were used to classify the recruited patients. Binary analyses were performed using the normal ADA glucose level as cut-off (<100 mg/dL *vs* ≥ 100 mg/dL). Informed consent was obtained from each subject at recruitment. This study was approved by the institutional research ethics committee.

2.2. Induction of hyperglycaemia in mice

Mice were intraperitoneally injected with streptozocin (STZ, 40 mg/kg body weight, dissolved in ice-cold 0.1 M citrate buffer, Sigma, St. Louis, MO, USA) for five consecutive days [24]. Age-matched controls were injected with citrate buffer only. The blood samples from tail vein were subjected to glucose level test from 48 h post STZ injection. Mice with blood glucose>250 mg/dL were selected for the experiments.

2.3. Cell culture and transfection

Human PCa cell lines PC3, DU145, C4-2 and LNCaP, and a non-malignant human prostatic epithelial cell line RWPE-1 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured according to the ATCC protocol as described previously [25]. PC3, DU145, C4-2 and LNCaP cells were authenticated by KeyGEN BioTECH Co., LTD (Nanjing, P.R.China). RWPE-1 cells were authenticated by ForeverGEN (Guangzhou, P.R.China).

Cells were transfected with 50 nM miRNA duplex or 200 nM miRNA inhibitor using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA). Interfering RNAs (siRNAs) targeting p21 and Smad4 were transfected at concentration of 40 nM. A RNA duplex with random sequence were used as negative control (NC) for both miRNA and siRNA.

RNA transfection efficiency was 60–75%, and RNA duplex persisted in cells for at least 4 days. All RNA oligoribonucleotides were obtained from Genepharma (Shanghai, P.R. China), and the sequences were shown in Supplementary Table 1.

2.4. MiRNA array

Total RNA samples were extracted using Trizol (Invitrogen) and RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturers' instructions. The LNA array contained capture probes for 348 rat miRNAs as listed in the miRBASE version 12.0 release [26]. Scanning was performed using the Axon GenePix 4000B microarray scanner, and the raw intensity of the image was captured by GenePix pro V6.0 software. Microarray data are available in GEO with accession No. GSE77092. (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE77092).

2.5. Database

The data of miR-301a levels of prostate cancer and normal prostate tissues were obtained from TCGA (https://gdac.broadinstitute.org/).

2.6. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells and tissues using Trizol (Invitrogen). Smad4 and p21 mRNA levels were quantified using SYBR® premix Ex *Taq*II (Takara, Japan). β-actin was used as the internal control gene. miR-301a expression was quantified using the Hairpin-itTM Real-Time PCR Kit (GenePharma). U6 was used as the internal control gene. qRT-PCR was performed on the ABI Prism 7500 fast Sequence Detection System (Applied Biosystem). Sequences of the primer were shown in Supplementary Table 2.

2.7. Luciferase reporter assay

Cells were seeded in triplicate in 24-well plates and incubated for 24 h. The luciferase reporter plasmids were co-transfected with the indicated duplexes into cells using Lipofectamine 2000 reagent (Invitrogen). Forty-eight hours after transfection, the Dual-Luciferase Reporter Assay (Promega, Madison, WI, USA) was performed according to the manufacturer's instructions, as described previously [25].

2.8. Analysis of cell proliferation and cell cycle analysis

Cell proliferation was determined by CCK-8 assay, colony formation assays, cell cycle analyses, and 5-ethynyl-2′-deoxyuridine (EdU) incorporation assay, as described previously [27]. Cell cycle

analyses were performed using fluorescence-activated cell sorting (FACS) cytometry (FACSCalibur, BD Biosciences, San Jose, CA, USA).

2.9. Immunoblotting and immunohistochemical analysis

Immunoblotting and immunohistochemical analysis were performed as described previously [28]. Anti-Smad4, anti-p21, anti-cyclin D1, anti-ppRb, anti-pRb, anti-ki-67 and anti-β-actin antibodies were purchased from Cell Signalling Technology (Beverly, MA, USA). Anti-cyclin E monoclonal antibody was purchased from Millipore (Billerica, MA, USA).

2.10. Tumorigenicity assay in nude mice

Four-week-old male BALB/c nude mice (Sun Yat-sen University Experimental Animal Center) were used. A total of 5×10^6 cells suspended in 100 μl Matrigel (BD Biosciences) were injected subcutaneously into the dorsal thighs of mice. Tumour growth was monitored regularly for 5 weeks, and the tumour volume was calculated using the formula length $\times$ width$^2 \times 0.5$. Five weeks later, the mice were euthanized, and the tumour tissues were harvested and frozen in liquid nitrogen or processed immediately to isolate RNA.

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Sun Yat-Sen University.

2.11. Statistical analysis

The data are presented as the mean ± standard deviation (SD) from at least three independent experiments. Student's *t* tests were performed using SPSS software (SPSS Inc., Chicago, IL, USA) to analyze the differences between two groups. χ^2 test was used for binary analyses. P values < 0.05 were considered statistically significant.

3. Results

3.1. The tumour grade in PCa patients with high glucose levels is higher than that with normal glucose levels

To explore the association between hyperglycaemia and PCa in China, 391 PCa patients were recruited, including 251 (64.2%) patients with glucose levels<100 mg/dL and 140 (35.8%) patients with glucose levels≥100 mg/dL. As shown in Supplementary Table 3, high Gleason score (GS ≥ 7) was more common in the patients with glucose levels≥100 mg/dL (66.4%) than that with normal glucose levels (52.2%) ($P = .006$). However, the association between the diagnostic PSA and glucose levels was not found ($P = .72$). This result suggests that PCa in patients with high glucose levels are more malignant.

3.2. Expression of miR-301a is elevated by high glucose in experimental hyperglycaemia rat models and PCa cell lines

To investigate the effects of hyperglycaemia on PCa, we first established a hyperglycaemic rat model by intraperitoneal injection of STZ, which destroyed pancreas and subsequently caused hyperglycaemia. miRNA expression profiling was performed using miRNA microarray. Overall, 384 miRNAs were detected, of which 4 miRNAs were upregulated and 2 miRNAs were downregulated in the prostate tissues of hyperglycaemic rat compared with that in the control group (Fig. 1A). Among them, miR-301a was chosen for further analysis because the expression of miRNA-301a in prostate cancer tissues was significantly higher than that in normal prostate tissues (Fig. 1B), and it could promote malignant transformation and cancer progression by negatively regulating tumour suppressor genes such as FOXF2 and NKRF [29,30]. As expected, qPCR analysis confirmed the increase of miR-301a level in the hyperglycaemic rat prostates (Fig. 1C). Furthermore, compared with normal concentration glucose (NG, 5.5 mM), culture with high concentration glucose (HG, 25 mM) resulted in dramatic elevation of miR-301a in the androgen refractory PCa cells (PC3, DU145 and C4-2, Fig. 1D–F), and slight but statistically significant elevation in the non-malignant human prostatic epithelial cell line RWPE-1 (Fig. 1D and G). However, high glucose had no effect on the miR-301a levels in LNCaP, an androgen-dependent PCa cells (Fig. 1D). These data suggest that hyperglycaemia or high glucose induces miR-301 expression in PCa cells.

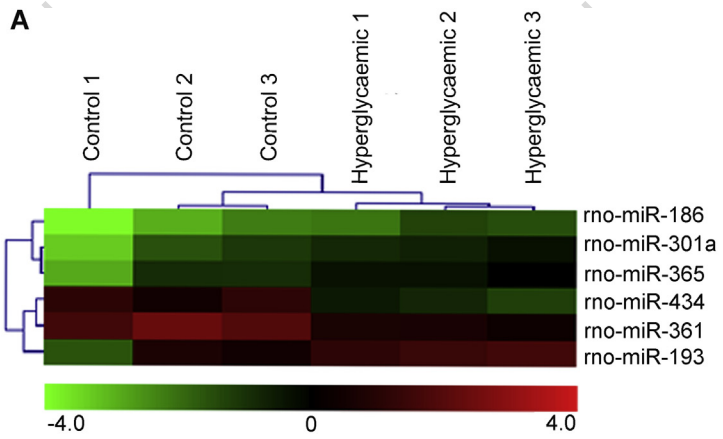
3.3. MiR-301a mediates high glucose-induced cell growth and G1/S transition in PCa cells

To evaluate the effect of high glucose-induced miR-301a on PCa cells, cells were cultured with normal and high glucose medium respectively. The CCK-8 assay revealed that high glucose promoted the growth of PC3, DU145, RWPE-1 and LNCaP cells, comparing with normal glucose (Fig. 2A and Fig. S1). Furthermore, cells cultured with normal glucose medium were transfected with miR-301a mimics or miR-control duplex. Interestingly, ectopic miR-301a promoted cell growth in PC3 and DU145 cells (Fig. 2A), but had marginal effect in RWPE-1 and LNCaP cells (Fig. S1). Consistently, inhibition of miR-301 by transfection with antimiR-301a oligonucleotides largely blocked the growth-promoting effect of high glucose in PC3 and DU145 cells (Fig. 2A). Similarly, the colony-formation assay demonstrated that high glucose culture induced more colonies in PC3 and DU145 cells (Fig. 2B). Ectopic expression of miR-301a mimicked the effects of high glucose, whereas silencing miR-301a significantly compromised high glucose-induced colony formation in both cell lines (Fig. 2B).

To dissect the biological events responsible for the growth-promoting effect of miR-301a, cells were synchronized with serum deprivation and then released by serum re-addition, and FACS was applied to analyze the distribution of cell cycle phases. PC3, DU145 and C4-2 cells displayed significantly decreased proportion of G1 phase and elevated percentage of S phase upon high glucose culture or miR-301a transfection (Fig. 2C and Fig. S2). Consistently, miR-301a antagonist hampered the G1/S transition induced by high glucose treatment (Fig. 2C). However, ectopic miR-301a had no effects on G1/S transition in RWPE-1 and LNCaP cells (Fig. S3). Furthermore, immunofluorescent staining for EdU incorporation revealed the increased DNA synthesis in both PC3 and DU145 cells upon high glucose treatment or miR-301a transfection (Fig. 2D). Importantly, the high glucose-induced DNA synthesis was significantly blocked by miR-301a antagonist (Fig. 2D). These data suggest that high glucose-induced G1/S transition and cell growth were mediated at least partially by the elevated miR-301a in PCa cells.

3.4. MiR-301a targets Smad4 and p21

To elucidate how miR-301a promotes G1/S transition and cell growth, we used TargetScan, a bioinformatic tool for microRNA target prediction. We identified p21 and Smad4, two central cell cycle genes among the list of predicted targets of miR-301a. p21 is a cyclin-dependent kinase (CDK) inhibitor, which could inhibit the activity of cyclin-CDK2 and cyclin-CDK4 complexes, and subsequently blocks G1/S transition and DNA replication [21]. Recent data showed that Smad4 was a putative suppressor of prostate tumour progression [22,23]. We further experimentally validated whether p21 and Smad4 were the direct targets of miR-301a. Obviously, miR-301a inhibited the activity of luciferase with the



miRNAs	Fold change (H/C)	P-value (H/C)
Up-regulated		
miR-186	2.405	0.0460
miR-301a	2.202	0.0462
miR-365	2.093	0.0345
miR-193	2.317	0.0464
Down-regulated		
miR-434	0.298	0.0082
miR-361	0.386	0.0151

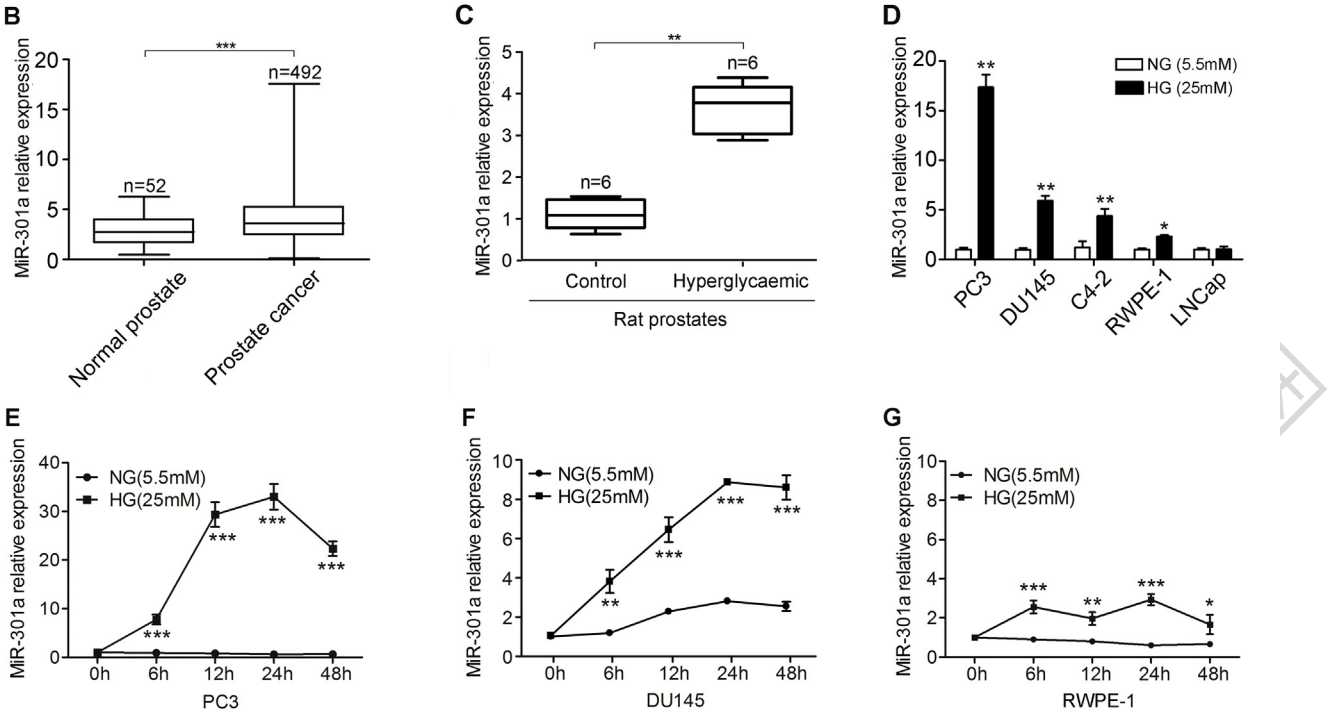


Fig. 1. Expression of miR-301a in prostate is upregulated by high glucose. (A) Hierarchical clustering heat map of significantly differentially expressed miRNAs. Each row represents an individual miRNA and each column represents an individual tissue sample. Different colours depict different relative expression values, as indicated by the colour scale bar at the bottom of the heat map, with the lowest and highest values in green and red, respectively. (B) miR-301a was elevated in the prostate cancer tissues. The miRNA-301a levels of normal prostate (n = 52) and prostate cancer (n = 492) were obtained from TCGA. (C) miR-301a was elevated in the hyperglycaemic rat prostate tissues. (D) High glucose treatment induced the expression of miR-301a in prostate cells. (E–G) The time course of miR-301a level upon high glucose treatment in PC3 (E), DU145 (F) and RWPE-1 (G) cells. For (C–G), the level of miR-301a was examined by qPCR and normalized to that of U6. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

wild-type but not mutant 3′-UTR of p21 and Smad4 (Fig. 3A and B). Furthermore, both gain-of-function and loss-of-function analyses disclosed the suppressive effect of miR-301a on the expression of p21 mRNA and protein (Fig. 3C), and the suppressive effect on the Smad4 protein level but not mRNA level (Fig. 3D). Moreover, consistent with the miR-301a levels (Fig. 1D), high glucose treatment significantly decreased p21 and Smad4 protein levels, and the suppressive effects were reversed by miR-301a inhibitor in both PC3 and DU145 cells (Fig. 3C and D).

Furthermore, we examined whether knockdown of Smad4 and p21 mimicked the effect of miR-301a overexpression. Transfection with siRNA duplexes targeting either Smad4 or p21 resulted in significant decrease in the levels of mRNA in both PC3 and DU145 cells (Fig. 4A). As expected, silencing of either target gene led to substantial upregulation of cyclin D1 and cyclin E, and subsequently induced a prominent increase of phosphorylated Rb protein (ppRb) (Fig. 4B and C). Consistently, FACS analysis displayed decreased proportion of G1 phase and increased percentage of S phase upon p21 (Fig. 4D) or Smad4 (Fig. 4E) silencing in both PC3 and DU145 cells, which phenocopied the outcome of miR-301a overexpression. Moreover, overexpression of p21 and smad4 (Fig. S4A) could interrupt miR-301a-induced G1/S transition (Fig. S4B). These data suggest that high glucose-induced miR-301a promotes cell proliferation by suppressing Smad4 and p21, and subsequently inducing G1/S transition.

3.5. Hyperglycaemia-induced miR-301a promotes the proliferation of PCa cells in the nude mice model

To verify the effect of miR-301a *in vivo*, BALB/c nude mice were

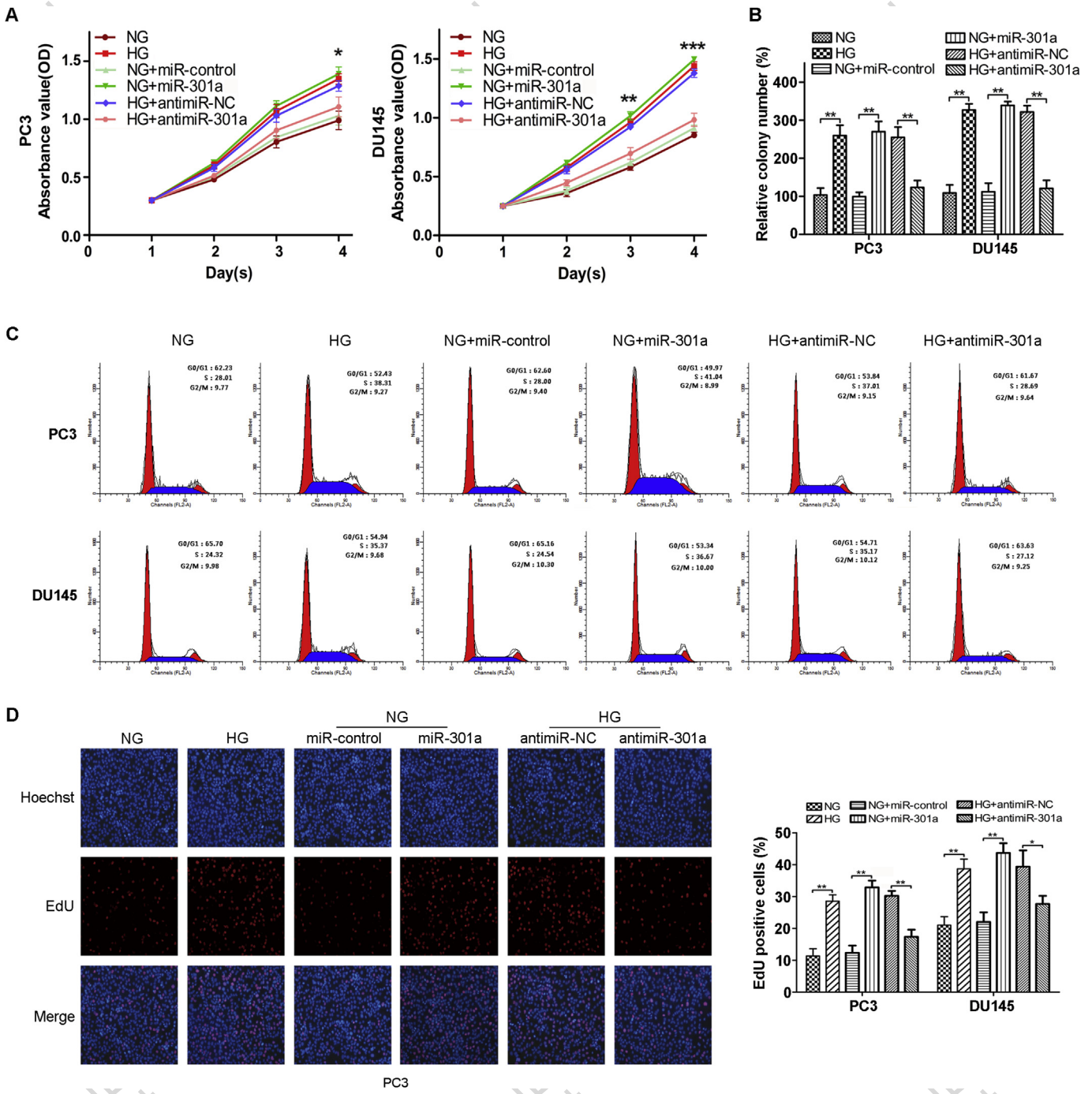


Fig. 2. MiR-301a mediates high glucose-induced PCa cell growth and G1/S transition. (A) CCK-8 assays revealed the cell growth curves of the indicated cells. (B) Relative quantification of colony formation of the indicated cells. For (A and B), PC3 and DU145 cells were transfected with the indicated oligonucleotides, then cultured in normal (NG) or high glucose (HG) condition. (C) FACS analyzed cell cycle of the indicated cells. Cells transfected with the indicated oligonucletides were cultured in the normal (NG) or high glucose (HG) condition. Cells were synchronized with serum deprivation for 24 h, and then stimulated to enter S-phase by serum re-addition. Cells were harvested for FACS analysis at 12 h post release. (D) Representative micrographs (left) and quantification (right) of EdU incorporation assay. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$.

first subjected to intraperitoneal injection of STZ, which led to hyperglycaemia. 5×10^6 PC3 cells were injected subcutaneously into either control or hyperglycaemic mice. Accompanying the elevation of miR-301a level (Fig. 5A), PCa tumors grew much faster and larger in the hyperglycaemic mice, comparing with that in the control mice (Fig. 5B and C). To manipulate the miR-301a levels, PC3 cells were infected by the lentivirus expressing miR-301a or the lentivirus expressing inhibitory sequence to repress the endogenous miR-301a levels before inoculation (Fig. 5A). As expected, overexpression of miR-301a significantly accelerated tumour growth *in vivo* (Fig. 5B and C). Consistently, inhibition of miR-301a severely impaired hyperglycaemia-induced tumour growth in hyperglycaemic mice (Fig. 5B and C). Furthermore, immunohistochemical staining confirmed the tumour cell proliferation by Ki-67 staining, and revealed the reverse correlation between the miR-301a levels and the expression of Smad4 and p21 protein and cell proliferation (Fig. 5D). Together, these data indicate that the hyperglycaemia-induced miR-301a promotes cell proliferation by

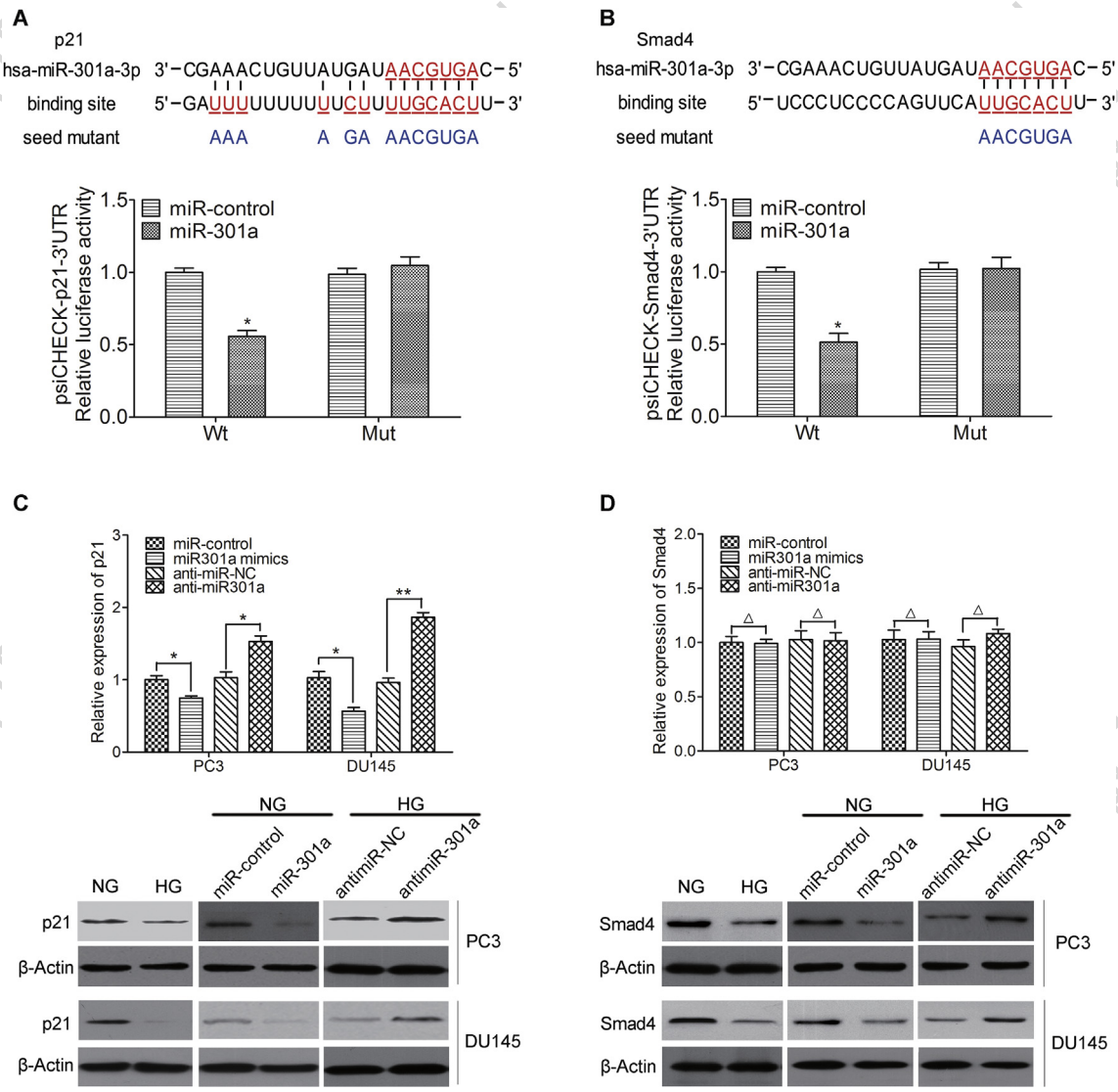


Fig. 3. miR-301a targets p21 and Smad4 in PCa cells. (A–B) miR-301a inhibited the activity of the reporter with the 3′UTR of *p21* (A) and *Smad4* (B). For (A and B), the predicted binding sites of miR-301a were underlined. Mutations were generated as indicated (up). 293 T cells were co-transfected with luciferase reporter plasmid containing either wild-type or mutant 3′ UTR for *p21* (A) or *Smad4* (B) and either miR-301a or miR-control duplexes (down). (C) miR-301a suppressed the expression of both mRNA and protein of p21. (D) miR-301a inhibited the expression of Smad4 protein. For (C and D), cells were transfected with the indicated oligonucleotides and cultured under normal or high glucose condition. Cells were subjected to both qRT-PCR and immunoblotting analysis, and β-actin were used as the internal control. $^{*}P < .05$; $^{**}P < .01$; △ not significant.

repressing Smad4 and p21 *in vivo*.

4. Discussion

Diabetes continues to be a major health problem globally. Various cancers are related to diabetes. Understanding of the molecular mechanism underlying the involvement of diabetes in cancer is important for better cancer treatment. Studies in prostate, breast and colon cancer revealed that higher blood glucose levels were associated with greater risk of disease progression [6,31,32]. In this study, we found that PCa patients in China with high Gleason score (GS ≥ 7) were associated with high blood glucose. Our data, together with the results from western countries [3–6], emphasized the effects of hyperglycaemia on PCa development and promoted us to investigate their molecular tumour biology.

Using STZ-treated hyperglycaemic rat model and several PCa cell lines, we demonstrated that hyperglycaemia or high glucose treatment induced significant miR-301a overexpression in prostate cells both *in vivo* and in *vitro*. It was reported that high glucose could affect the signal transduction of multiple pathways, including insulin/IGF-1, transforming growth factor-β and peroxisome proliferator-activated receptor pathways, and the activities of transcription factors including Runx2, CREB and C/EBPs *via* O-GlcNAcylation of protein [33]. However, the mechanism underlying the transactivation of miR-301a by high glucose is needed to be further study. Interestingly, miR-301a was dramatically elevated in androgen refractory PCa cells (PC-3 and DU145), moderately upregulated in androgen receptor positive androgen refractory PCa C4-2 cells, while slightly increased in RWPE-1 cells, and was invariable in LNCaP cells upon high glucose treatment. This discrimination might be attributed to the different background of androgen receptor in prostate cell lines we used. Androgen receptor is expressed in C4-2, RWPE-1 and LNCaP cells, while silenced in PC3 and DU145 cells due to hypermethylation of its promoter [34]. As a transcription factor, androgen receptor could either activate or repress the expression of target genes involved in glucose metabolism and cell proliferation by recruiting different coregulators [35]. Therefore, we speculated that androgen receptor may severely

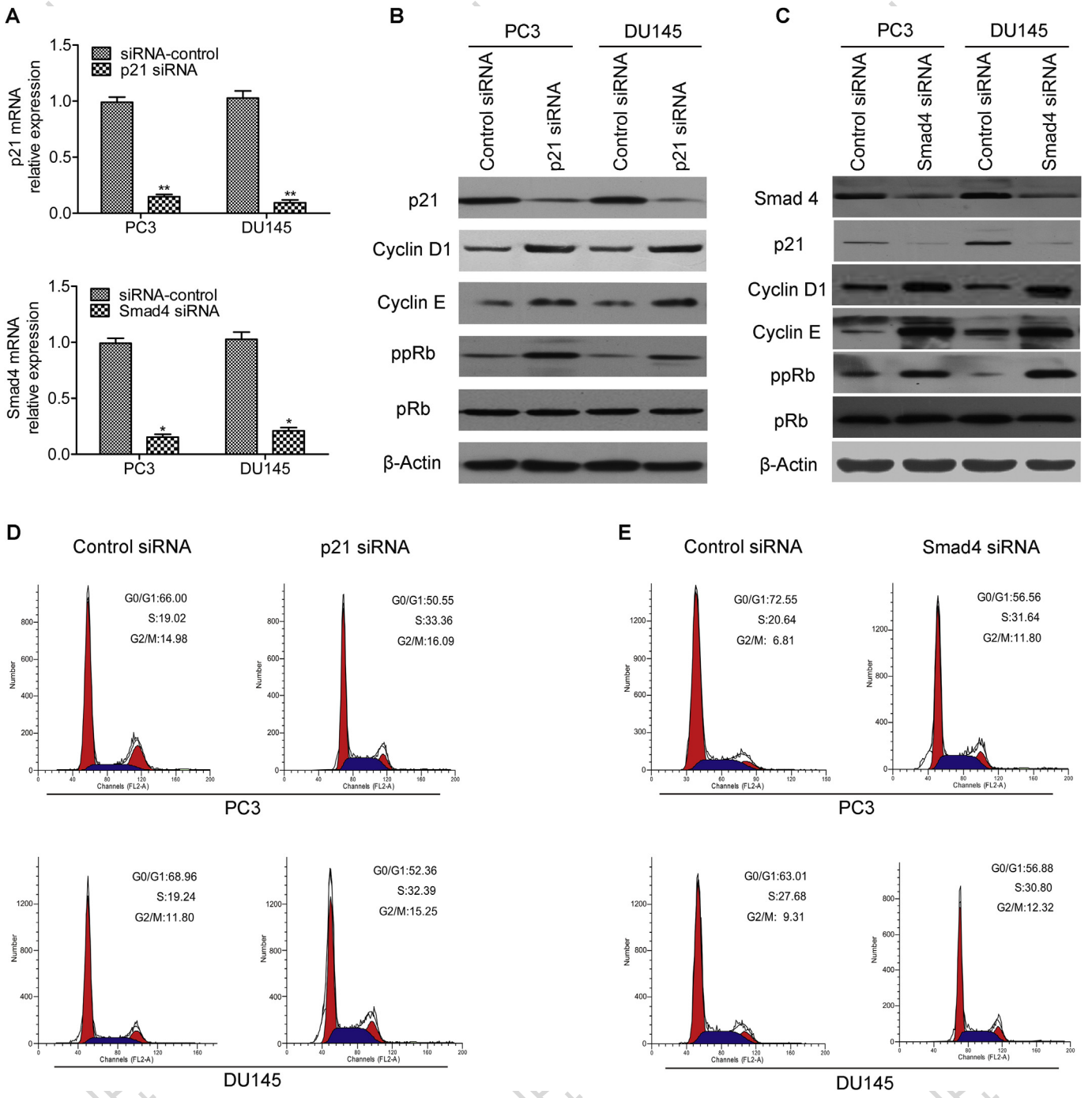


Fig. 4. p21 and Smad4 are involved in the regulation of G1/S transition in PCa cells. (A) siRNA inhibited the expression of p21 and Smad4 mRNA. (B–C) Knockdown of p21 (B) or Smad4 (C) altered the expression of the essential G1/S transition-related proteins. For (A–C), PC3 and DU145 cells were transfected with the indicated duplexes and subjected to qRT-PCR analysis (A) and immunoblotting (B and C). β-actin were used as the internal control for qRT-PCR and immunoblotting. (D–E) Knockdown of p21 (D) or Smad4 (E) promoted G1/S transition in PCa cells. PC3 and DU145 cells transfected with the indicated oligonucletides were synchronized with serum deprivation for 24 h, stimulated to enter S-phase by serum re-addition for 12 h, and then subjected to FACS analysis. $^{*}P < .05$; $^{**}P < .01$.

hamper the glucose pathway-activated expression of miR-301a, while absence of androgen receptor could release glucose pathway to induce the expression of miR-301a in androgen refractory cells. However, the precious mechanism for the regulation of miR-301a expression, especially under the castration-resistant pathologic condition is worth further study.

miR-301a has been reported to function as an oncogene in multiple human cancers [29,30,36,37]. In prostate cancer, high levels of miR-301a are associated with an increased risk of recurrence and/or metastasis [38]. miR-301a could promote colony-forming ability, EMT and motility of prostate cancer cells [38–40]. Furthermore, hypoxia-induced miR-301a decreases autophagy and promotes radioresistance of prostate cancer cells [41]. However, its role in malignant combined with diabetes remains largely unclear. We demonstrated that high glucose-induced miR-301a promoted G1/S transition and the proliferation of PCa cells by inhibiting the expression of p21 and Smad4. miRNA can repress gene expression by either mRNA cleavage or translational

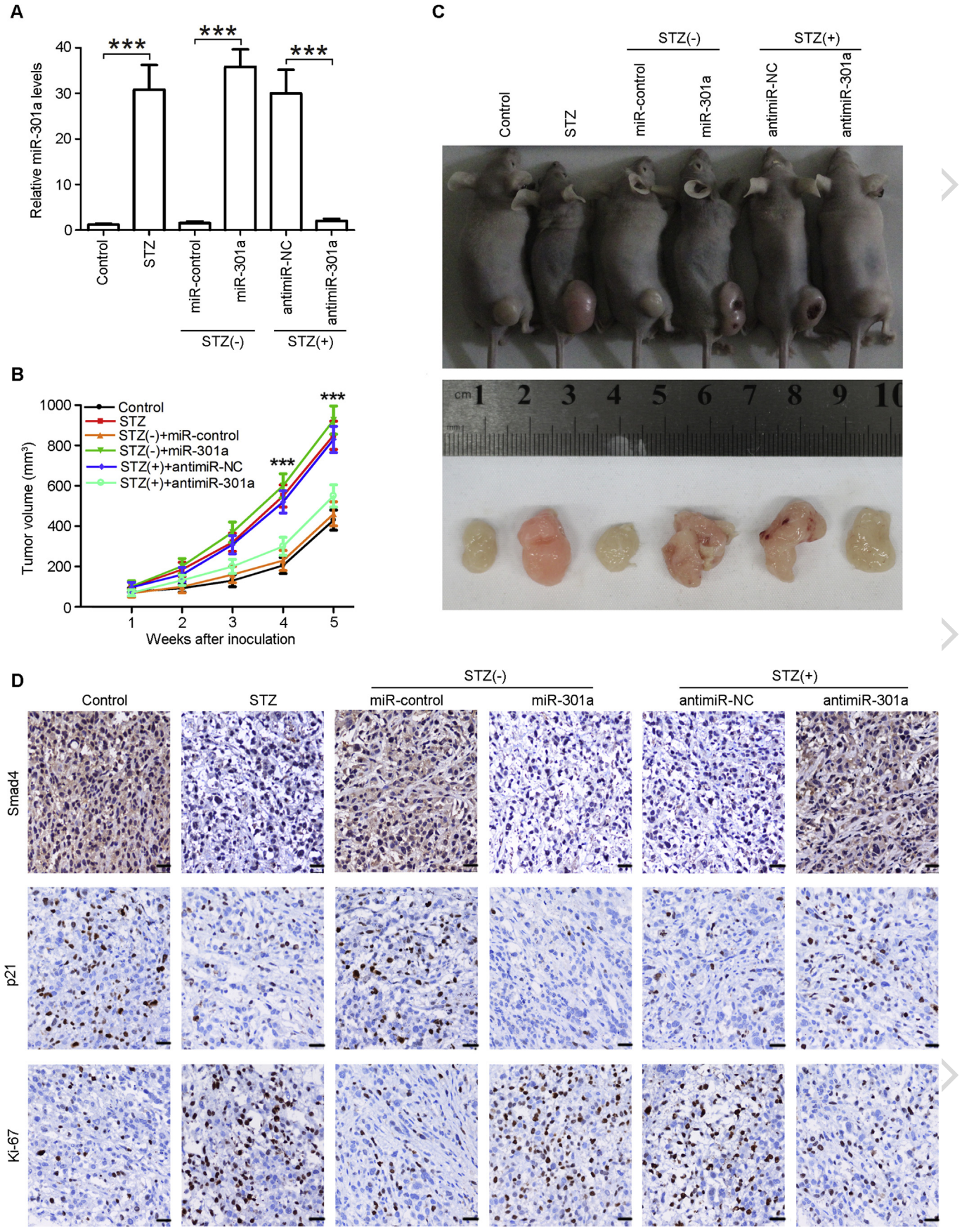


Fig. 5. Hyperglycaemia-induced miR-301a promotes xenograft growth *in invo*. (A) qRT-PCR assay analyzed the miR-301a levels in the xenografts of PC3 cells. (B) The tumour growth curves of PC3 cells in the nude mice. (C) Representative photograph of PC3 tumour formation in the nude mice. (D) Immunohistochemical analysis of Ki-67, p21 and Smad4 in the xenografts of PC3 cells. The levels of miR-301a were manipulated by lentivirus transduction as indicated, and then injected subcutaneously into hyperglycaemic (STZ+) or normoglycaemic (STZ-) mice. The tumors were monitored regularly for 5 weeks (B) and excised at the end of experiment for qRT-PCR assay (A) and Immunohistochemical staining (D). Each data represents the mean ± S.D. of six independent xenografts. Scale bar: 100 μm $^{***}P < .001$.

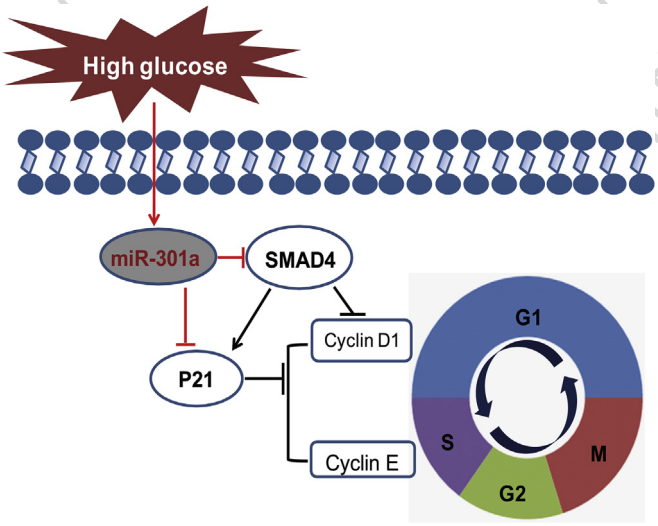


Fig. 6. Schematic overview of the mechanism for the regulation of G1/S transition by hyperglycaemia-induced miR-301a. High glucose induces the expression of miR-301a, which inhibits the expression of Smad4 and p21, and subsequently increases the levels of the essential G1/S transition proteins, such as cyclin D1 and cyclin E, thereby promotes cell cycle transition and proliferation of prostate cancer cells.

repression. The choice of posttranscriptional mechanisms is not determined by miRNA but is determined by the identity of the target [10]. According to our results, miR-301a might reduce smad4 expression by translational repression and repress p21 expression by mRNA cleavage. p21 is a potent cyclin-dependent kinase inhibitor, which causes cell cycle arrest at G1 phase. Smad4 could induce G1/S cell cycle arrest by transactivating a cohort of target genes, including p21. miR-301a simultaneously inhibited both upstream and downstream components of the cell cycle pathway, strengthening its modulation of G1/S transition. Interestingly, although high glucose promoted the proliferation of all prostate cells we tested, including PC3, DU145, C4-2, RWPE-1 and LNCaP cells, miR-301a could promote proliferation only in the androgen refractory PCa cells (PC3, DU145 and C4-2). Consistently, inhibition of miR-301a severely blocked the high glucose-induced cell growth in the androgen refractory PCa cells. Both our and previous study indicated that miR-301a overexpression led to increased proliferation in PC3 cells [38]. However, ectopic miR-301a promoted growth of LNCaP cells in the previous study [38], but had marginal effect in our study. The discrepancy might be due to the different cell culture condition. In the previous study, LNCaP cells were cultured using the common RPMI-1640 medium with 11 mM glucose. Whereas, in our study we used culture medium with 5.5 mM glucose. It was speculated that miR-301a alone could not promote proliferation of the less malignant cells, such as LNCaP cells under the low nutrient status. Furthermore, it was probably that with the surveillance of androgen signalling, suppression of p21 and Smad4 was not enough to induce cell growth. However, with activation of several bypass pathways, such as PI3K/Akt pathway in the androgen refractory PCa cells [42], inhibition of p21 and Smad4 by the elevated miR-301a were sufficient to promote cell proliferation. This explanation was supported by the recent finding that comparing with PTEN inactivation alone, concomitant PTEN and Smad4 inactivation results in a robust increase of Cyclin D1, thereby enhancing proliferation in PCa cells [22]. More importantly, the blockage of high glucose-induced cell proliferation by miR-301a inhibition argues that miR-301a is the vital mediator in the hyperglycaemia-promoted cell proliferation, at least in the androgen refractory PCa cells.

There were some limitations in our study. We adopted a single glucose measurement on admission to classify PCa patients, which may not reflect the individual's on-going glucose status. Although haemoglobin A1C level is considered as more precise index for DM diagnosis and glucose control, the HbA1C data are just obtained from DM suspected cases. In contrast, fasting blood glucose data are much more available for analyses. In this study, there was no clinical data to support miR-301a's role between NG and HG patients, which was mainly due to the lack of the clinical tissues, and further study is ongoing in our new research project.

In summary, we revealed a novel molecular link between hyperglycaemia and PCa (Fig. 6). Hyperglycaemia elevated the miR-301a levels, which repressed the expression of p21 and Smad4, and subsequently promoted G1/S transition and cell growth. Inhibition of miR-301a could severely block the hyperglycaemia-induced cell growth, suggesting that miR-301a plays an important role in the hyperglycaemia-associated cancer growth, and represents a novel therapeutic target for PCa.

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Conflicts of interest

The authors declare that they have no potential conflicts of interests.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.canlet.2018.01.031.

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