Prostate cancer (PCa) is a clinically heterogeneous multifocal and highly aggressive disease. Although early stage PCa is clinically manageable, the evolution of PCa to a hormone-independent disease is invariably associated with advanced metastasis, which limits the therapeutic options. Combinations of methods are currently employed to aid in the diagnosis and prognosis of PCa, including serum prostate-specific antigen (PSA) levels and the Gleason score. However, these methods carry some limitations, such as frequent underestimation of the Gleason score and the fact that PSA levels can be elevated in conditions other than PCa. Thus, there is a crucial need to improve our understanding of the molecular mechanisms underlying PCa to assist in the development of novel diagnostic and prognostic markers and treatment strategies.

MicroRNAs (miRNAs) are small (approximately 20 nucleotides) noncoding RNAs that play important roles in cell physiology by regulating mRNA expression and stability. Many miRNAs have been shown to function as classical oncogenes or tumor suppressors and are deregulated in various cancer subtypes, including PCa. For example, miR-1 and miR-31, which are downregulated in PCa, are considered to function as tumor suppressors through regulation of Notch3 and cyclin dependent kinase 1 (CDK1) mRNAs. Understanding the roles of the downregulated miRNAs and their target genes in PCa progression could identify new options for the therapeutic use of miRNA mimics (agomirs) or inhibitors (antagomirs) in PCa. Indeed, we previously showed that inhibition of the expression of miR-450b-5p by the cytokine transforming growth factor-β1 (TGF-β1) reverses the differentiation of rhabdomyosarcoma via effects on the miRNA target gene.

In addition to rhabdomyosarcoma, TGF-β1 plays an essential role in promoting several adenocarcinomas, including PCa, colorectal cancer, pancreatic ductal adenocarcinoma, and lung adenocarcinoma. Moreover, the progression of PCa to an androgen-independent state is accompanied by changes in the effects of growth factor signaling pathways, including an increase in TGF-β1 production that promotes metastasis.

Transforming growth factor-β1 (TGF-β1) acts as a tumor promoter in advanced prostate cancer (PCa). We speculated that microRNAs (miRNAs) that are inhibited by TGF-β1 might exert anti-tumor effects. To assess this, we identified several miRNAs downregulated by TGF-β1 in PCa cell lines and selected miR-3691-3p for detailed analysis as a candidate anti-oncogene miRNA. miR-3691-3p was expressed at significantly lower levels in human PCa tissue compared with paired benign prostatic hyperplasia tissue, and its expression level correlated inversely with aggressive clinical pathological features. Overexpression of miR-3691-3p in PCa cell lines inhibited proliferation, migration, and invasion, and promoted apoptosis. The miR-3691-3p target genes E2F transcription factor 3 (E2F3) and PR domain containing 1, with ZNF domain (PRDM1) were upregulated in miR-3691-3p-overexpressing PCa cells, and silencing of E2F3 or PRDM1 suppressed PCa cell proliferation, migration, and invasion. Treatment of mice bearing PCa xenografts with a miR-3691-3p agomir inhibited tumor growth and promoted tumor cell apoptosis. Consistent with the negative regulation of E2F3 and PRDM1 by miR-3691-3p, both proteins were overexpressed in clinical PCa specimens compared with noncancerous prostate tissue. Our results indicate that TGF-β1-regulated miR-3691-3p acts as an anti-oncogene in PCa by downregulating E2F3 and PRDM1. These results provide novel insights into the mechanisms by which TGF-β1 contributes to the progression of PCa.

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Keywords: E2F transcription factor 3; miR-3691-3p; PR domain containing 1, with ZNF domain; prostate cancer; transforming growth factor-β1

INTRODUCTION

Prostate cancer (PCa) is a clinically heterogeneous multifocal and highly aggressive disease. Although early stage PCa is clinically manageable, the evolution of PCa to a hormone-independent disease is invariably associated with advanced metastasis, which limits the therapeutic options. Combinations of methods are currently employed to aid in the diagnosis and prognosis of PCa, including serum prostate-specific antigen (PSA) levels and the Gleason score. However, these methods carry some limitations, such as frequent underestimation of the Gleason score and the fact that PSA levels can be elevated in conditions other than PCa. Thus, there is a crucial need to improve our understanding of the molecular mechanisms underlying PCa to assist in the development of novel diagnostic and prognostic markers and treatment strategies.

MicroRNAs (miRNAs) are small (approximately 20 nucleotides) noncoding RNAs that play important roles in cell physiology by regulating mRNA expression and stability. Many miRNAs have been shown to function as classical oncogenes or tumor suppressors and are deregulated in various cancer subtypes, including PCa. For example, miR-1 and miR-31, which are downregulated in PCa, are considered to function as tumor suppressors through regulation of Notch3 and cyclin dependent kinase 1 (CDK1) mRNAs. Understanding the roles of the downregulated miRNAs and their target genes in PCa progression could identify new options for the therapeutic use of miRNA mimics (agomirs) or inhibitors (antagomirs) in PCa. Indeed, we previously showed that inhibition of the expression of miR-450b-5p by the cytokine transforming growth factor-β1 (TGF-β1) reverses the differentiation of rhabdomyosarcoma via effects on the miRNA target gene.

In addition to rhabdomyosarcoma, TGF-β1 plays an essential role in promoting several adenocarcinomas, including PCa, colorectal cancer, pancreatic ductal adenocarcinoma, and lung adenocarcinoma. Moreover, the progression of PCa to an androgen-independent state is accompanied by changes in the effects of growth factor signaling pathways, including an increase in TGF-β1 production that promotes metastasis. TGF-β1 has been shown...
to modulate the expression of several miRNAs, such as miR-224, miR-15a and miR-16,21,22 in androgen-independent PCa. Therefore, we hypothesized that overexpression of TGF-β1-downregulated miRNAs might reverse the malignant phenotype in PCa, similar to the effects of overexpressing miR-450b-5p in rhabdomyosarcoma14 and miR-196a-3p in breast cancer.23 According to large amounts of literature reports, TGF-β1 plays essential role in the regulation of different adenocarcinomas, including procreate cancer,24 colorectal cancer,15 pancreatic ductal adenocarcinoma16 and lung adenocarcinoma.17

In the present study, we first screened a dataset of miRNAs differentially expressed in TGF-β1-deficient and wild-type human colorectal adenocarcinoma cells (GS53337), and we identified five miRNAs that were significantly differentially regulated by TGF-β1 (miR-4723-3p, miR-324-3p, miR-4313, miR-196a-3p, and miR-3691-3p). We then examined their expression in TGF-β1-treated human PCa cell lines and clinical specimens; identified miR-196a-3p as a TGF-β1-inhibited miRNA in PCa; and investigated the expression and function of miR-196a-3p as a potential tumor suppressor using in vitro assays, a mouse xenograft model, and human clinical PCa specimens.

MATERIALS AND METHODS

Cell lines and culture

The human prostate carcinoma cell lines PC-3 and DU145 were obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and were maintained in RPMI 1640 basic medium or Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. All cell culture reagents were purchased from Gibco (Burlington, ONT, Canada). The cell lines were maintained in a humidified 5% CO₂ incubator at 37°C.

Clinical samples

All pairs of primary PCa and benign prostatic hyperplasia (BPH) tissue samples were collected from patients seen at the Second Affiliated Hospital of Soochow University (Suzhou, China), from January, 2001 to December, 2011. The boundary between the adjacent noncancerous and cancerous tissues was at least 1.5 cm, and the identities of both tissue types were verified by pathologists. Tissues were sliced into 10-µm-thick sections using a cryostat microtome, placed in 1.5 ml microtubes (Corning, Tewksbury, MA, USA), and stored at −80°C until analysis. Signed informed consent was obtained from all patients and the study was approved by the Clinical Research Ethics Committee of the Second Affiliated Hospital of Soochow Hospital (JD-LK-2019-076-01).

Oligonucleotide synthesis and transfection

For overexpression studies, miR-3691-3p mimic and a control mimic sequence were purchased from RiboBio (Guangzhou, China). PC-3 and DU145 cells were seeded into 6-well plates at 10⁴ cells per well in medium without antibiotics, incubated overnight, and transfected with mimics (100 nmol ⁻¹ final concentration) using Lipofectamine™ 2000 (Thermo Fisher Scientific, Carlsbad, CA, USA) according to the manufacturer's protocol. After 24 h or 48 h, the cells were harvested, washed, and used for experiments. For RNA interference studies, small interfering RNAs (siRNAs) specific for the miR-3691-3p target genes E2F transcription factor 3 (E2F3) and PR domain containing 1, with ZNF domain (PRDM1) were designed using BLOCK-iT RNAi Designer (Invitrogen, San Diego, CA, USA) and synthesized by RiboBio. After confirmation of the specificity and efficacy of target gene knockdown by western blot analysis, three siRNAs were selected for the experiments: E2F3 siRNA, GCACCTCGATGGTCTTTATG; and control scrambled siRNA, UUUTGATCAUTGATGAAA. PCa cells were transfected with siRNAs (30 nmol ⁻¹) using the same method as for miRNA mimics.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RNA was extracted from patient tissues samples or PCa cell lines using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Primers for amplification of miRNAs were designed and synthesized by GeneCopoeia (Guzhongzhou, China). RNA was reverse transcribed using the RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). RT-qPCR reactions were performed on an ABI PCR 7500 Real-Time System (Applied Biosystems, Foster City, CA, USA) with U6 as an internal control. For miRNA amplification, cDNA was synthesized with a miRNA-specific stem-loop primer and qPCR was performed with the following primers: U6 forward 5’-CCTTCGAACATTGCTGATCAT-3’; U6 reverse: 5’-CGCCTTCAGAATTGCTGATCAT-3’; Has-miR-3691-3p 5’-GGACCAAGCTTGTGCTCAT-3’; Has-miR-4313 5’-GAAAGGGCCCTGCGCC-3’; Has-miR-196a-3p 5’-GGAAACGGACACACAGAATCT-3’; Has-miR-324-3p 5’-GGAACTGCCCCAGGTGC-3’; and Has-miR-4723-3p 5’-GAAACCTCTCTGGGCTCCT-3’. RT-qPCR cycling conditions were 10 min at 95°C followed by 40 cycles of 95°C for 15 s and 60°C for 6 s. Relative quantification of miRNA levels was performed using the comparative cycle threshold (Ct) method.

Cell viability assay

Cell viability was measured at 1 day, 3 day, and 5 days using the 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, PCa cells were seeded into 96-well plates at 10⁴ cells per well and incubated at 37°C for the indicated times. Aliquots of 20 µl per well of 5 mg ml⁻¹ MTT (Sigma-Aldrich, St. Louis, MO, USA) were added to the cells and the plates were incubated for an additional 4 h at 37°C. The supernatant was removed, and the formazan crystals were dissolved by the addition of 150 µl per well dimethyl sulfoxide. Absorbance at 570 nm was measured using a microplate reader (Thermo Scientific, Thermo Electron Co., Carlsbad, CA, USA).

Wound-healing migration assay

DU145 and PC-3 cells were transfected for 24 h, seeded into 6-well plates, and grown to confluency. A sterile pipette tip was then used to scratch a wound across the cell monolayer, and the wells were incubated at 37°C for 48 h. The cells were photographed at 0 h, 24 h, and 48 h and the wound width was measured. The wound-healing rate was calculated using data from five high-magnification visual fields for each well.

Transwell invasion assay

DU145 and PC-3 cell lines were transfected for 24 h, washed, resuspended in serum-free medium, and added at 5 × 10⁴ cells per well to the upper chamber of Transwell chambers (catalog# 354481, Corning, Tewksbury, MA, USA). Medium containing 10% FBS was added to the lower chamber. The plates were incubated at 37°C for 24 h, and the invaded cells present on the lower side of the membrane were stained with crystal violet. Cells in five visual fields per well were counted for each condition.

Apoptosis assay

DU145 and PC-3 cells were transfected for 24 h and 48 h, respectively, and washed. Samples of 5 × 10⁴ to 5 × 10⁶ cells were stained with propidium iodide (PI) and annexin V (Annexin V-FITC-P1 kit; Beyotime, Shanghai, China) according to the manufacturer's protocol.
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RESULTS

TGF-β1-regulated expression of miR-3691-3p in PCa cell lines and tissues

To identify TGF-β1-regulated miRNAs in adenocarcinomas, we first screened a dataset of miRNAs in a TGF-β1-deficient human colorectal adenocarcinoma cell line compared with the parental cell line (GSE53337) and identified five significantly differentially expressed miRNAs: miR-4723-3p, miR-324-3p, miR-4313, miR-196a-3p, and miR-3691-3p. To determine whether the same miRNAs were regulated by TGF-β1 in human PCa cells, we performed RT-qPCR analysis of DU145 and PC-3 human PCa cell lines incubated with or without TGF-β1 (10 ng ml⁻¹) for 24 h. As shown in Figure 1a, four of the miRNAs, the exception being miR-4313, were downregulated in TGF-β1-treated PC-3 cells compared with the untreated controls (fold change [FC] = 0.60; P < 0.05 for all four), whereas in TGF-β1-treated DU145 cells, miR-4313 (FC = 0.12; P < 0.01) and miR-3691-3p (FC = 0.15; P < 0.01) were markedly decreased by TGF-β1, miR-324-3p and miR-4723-3p were slightly decreased (both FC = 0.55; P < 0.05), and miR-196a-3p was marginally and insignificantly increased (FC = 1.27). BPH is a known risk factor for PCa,[4–26] and the majority of PCa patients have a history of chronic prostatitis. Therefore, we also examined expression of the five miRNAs in five paired samples

**Figure 1:** Expression levels of miR-3691-3p in PCa cell lines and tissues. (a) RT-qPCR analysis of five miRNAs in PC-3 and DU145 cells incubated with or without 5 ng ml⁻¹ TGF-β1 for 24 h. (b) RT-qPCR analysis of five TGF-β1-regulated miRNAs in five paired samples of human PCa and BPH tissues. (c) RT-qPCR analysis of miR-3691-3p expression in 100 paired samples of PCa and normal prostate tissues. Data are presented as the mean ± s.d. of three independent experiments. (d) PCa patients were dichotomized into high and low miR-3691-3p expression groups using the median expression level as the cut-off value. Kaplan–Meier curves show BCR-free survival. Data are presented as the mean ± s.d. of one experiment, representative of three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001. RT-qPCR: real-time quantitative polymerase chain reaction; TGF-β1: transforming growth factor beta 1; PCa: prostate cancer; BPH: benign prostatic hyperplasia; BCR: biochemical recurrence; s.d.: standard deviation.
of PCa and BPH specimens. As shown in Figure 1b, miR-3691-3p expression was markedly lower in the primary PCa samples compared with BPH samples (FC = 0.05; \(P < 0.01\)), miR-196a-3p, miR-324-3p, and miR-4723-3p were slightly lower in PCa (FC = 0.82), and excepted miR-4313 (FC = 0.04; \(P < 0.01\)). We also analyzed 100 pairs of PCa and adjacent noncancerous tissues and again observed that miR-3691-3p was significantly decreased in PCa tissues compared with control tissues (\(P < 0.001\); Figure 1c). These data prompted us to select miR-3691-3p for further investigation. Whether the decrease in miR-3691-3p expression in PCa tissues is related to disease progression requires further analysis.

**Correlation between miR-3691-3p expression and prognosis**

To assess the relationship between miR-3691-3p expression level and clinicopathological and prognostic features in PCa, we assigned the 100 patients to groups of low and high miR-3691-3p expression using the median relative expression level of miR-3691-3p in PCa tissues as the cut-off. We found that lower miR-3691-3p expression was significantly associated with advanced pathological stage (\(P = 0.0251\)), positive lymph node metastasis (\(P = 0.003\)), high preoperative PSA level (\(P = 0.0019\)), and positive angiolymphatic invasion (\(P = 0.0017\)). However, there was no statistically significant association between miR-3691-3p expression and other features, including Gleason score and age (Table 1).

The prognostic impact of miR-3691-3p in PCa was analyzed by constructing Kaplan–Meier survival curves. Patients with lower miR-3691-3p expression level had a significantly shorter biochemical recurrence (BCR) - free survival after radical prostatectomy than did patients with higher miR-3691-3p levels (\(P = 0.0113\), log-rank test; Figure 1d).

Taken together, these data demonstrate that the decreased miR-3691-3p expression level in PCa adversely affects prognosis.

**Effect of miR-3691-3p overexpression on the behavior of PCa cell lines**

To investigate how reduced miR-3691-3p expression affects PCa behavior, we transiently transfected a miR-3691-3p mimic or control...
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sequence into PC-3 and DU145 cells, verified that miR-3691-3p was overexpressed by qPCR (Supplementary Figure 1), and then analyzed the effects of miR-3691-3p upregulation on the proliferation, migration, invasion, and apoptosis of PCa cells in vitro. The MTT proliferation assay revealed a reduction in viability on day 5 in cells transfected with the miR-3691-3p mimic compared with the control sequence for both DU145 cells (P < 0.001) and PC-3 cells (P < 0.05; Figure 2b). Moreover, transfection with the miR-3691-3p mimic increased the apoptotic rate of PCa cells, as detected by annexin V-FITC/PI staining and flow cytometry (Figure 2b). Thus, the mean percentage of miR-3691-3p-overexpressing DU145 cells in early apoptosis (annexin V-positive/PI-negative) and late apoptosis (annexin V-negative/PI-positive) was 18.3% and 10.1%, respectively, compared with 4.5% and 1.2%, respectively, for control DU145 cells (Figure 2b). Similarly, the mean percentage of miR-3691-3p-overexpressing PC-3 cells in early and late apoptosis was 7.7% and 11.3%, respectively, compared with 3.8% and 4.5%, respectively for the control cells (Figure 2b). We next analyzed the effect of miR-3691-3p overexpression on PCa cell migration and invasion using wound-healing and Transwell assays, respectively. Notably, DU145 and PC-3 cells transfected with the miR-3691-3p mimic displayed markedly and significantly inhibited migration (P < 0.001; Figure 2c) and invasion (P < 0.001; Figure 2d) compared with cells transfected with the control sequence.

Prediction and expression of candidate miR-3691-3p target genes in PCa cells

We used the DIANA-MICROT (diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index), Target Scan (http://www.targetscan.org/vert_72/), and MIRDB databases (http://mirdb.org/) to predict potential target genes of miR-3691-3p. Among the 200 target genes identified, 9 were commonly identified by all three algorithms. Using miR-Oncology database screening, we selected three target genes known to be associated with the occurrence and development of cancer; namely, Cyclin Dependent Kinase 17 (CDK17), PRDM1, and E2F3 (Figure 3a). To validate these potential miR-3691-3p target genes, we performed western blot analysis of PC-3 cells transfected with the control mimic or miR-3691-3p-mimic. As shown in Figure 3b, E2F3 protein was present at significantly lower levels in miR-3691-3p-mimic-expressing cells than in control DU145 and PC-3 cells. In contrast, PRDM1 expression was significantly decreased by the miR-3691-3p mimic only in DU145 cells, while CDK17 was not significantly affected by the miR-3691-3p mimic in either cell line (Figure 3b). We next confirmed these findings by RT-qPCR analysis and found that transfection with the miR-3691-3p mimic significantly decreased E2F3 mRNA levels in both DU145 cells (P < 0.001) and PC-3 cells (P < 0.01); significantly decreased PRDM1 mRNA levels in DU145 cells but not PC-3 cells (P < 0.01); and did not significantly affect CDK17 mRNA levels in either cell line (Figure 3c), which was consistent with the results of the western blot analyses. Based on these results, E2F3 and PRDM1 were considered to be likely target genes of miR-3691-3p in PCa cells. Finally, these results were further substantiated by demonstrating concentration-dependent decreases in E2F3 and PRDM1 expression levels in both cell lines transfected with 10 nmol l⁻¹–100 nmol l⁻¹ miR-3691-3p mimic (Supplementary Figure 2).

Effect of E2F3 and PRDM1 knockdown on the behavior of PCa cell lines

E2F3 and PRDM1 are both transcriptional regulators that influence biological processes such as the cell cycle and apoptosis. To determine whether E2F3 and PRDM1 mediate the effects of miR-3691-3p in PCa cells, we transfected cells with E2F3- or PRDM1-specific siRNAs and examined the effects on cell proliferation, invasion, and migration. We first confirmed effective inhibition of E2F3 and PRDM1 expression at both the mRNA level (P < 0.001; Supplementary Figure 3a) and protein level (Supplementary Figure 3b) by RT-qPCR and western blot analysis, respectively. Analysis of proliferation using the MTT assay showed that E2F3 and PRDM1 silencing suppressed the proliferation of both PCa cell lines (Figure 3d), consistent with the effects of the miR-3691-3p mimic. The wound-healing migration assay showed that PC-3 cells expressing E2F3- or PRDM1-targeting siRNAs migrated shorter distances than control cells, while in DU145 cells, only PRDM1 silencing reduced cell migration (Figure 3e). Last, the invasion capability of the two lines was significantly reduced by transfection with either E2F3 or PRDM1 siRNA compared with the control siRNA (P < 0.001; Figure 3f). These data suggest that downregulation of miR-3691-3p and concomitant upregulation of E2F3 and PRDM1 may play a key role in the progression of PCa.

Effect of miR-3691-3p agomir administration on PCa growth in a mouse xenograft model

To verify that our in vitro results with cell lines translated to the in vivo situation, we established a PCa xenograft mouse model. For this, PC-3

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**Table 1: Correlation between miR-3691-3p expression and clinicopathological features in prostate cancer patients**

| Clinicopathological feature | Cases (n) | miR-3691 expression status | P     |
|----------------------------|-----------|----------------------------|-------|
| Age (year)                 |           | Low, n (%)                 | High, n (%) |       |
| <70                        | 57        | 35 (62.5)                  | 22 (37.5) | NS    |
| ≥70                        | 43        | 23 (53.5)                  | 20 (46.5) |       |
| Preoperative PSA           |           |                            |       |
| <10 ng ml⁻¹                | 32        | 10 (31.2)                  | 22 (68.8) | 0.0019** |
| >10 ng ml⁻¹                | 68        | 48 (70.6)                  | 20 (29.4) |       |
| Gleason score              |           |                            |       |
| 4–6                        | 55        | 30 (54.5)                  | 25 (45.5) | NS    |
| 7                          | 26        | 18 (69.2)                  | 8 (30.8)  |       |
| 8–10                       | 19        | 10 (52.6)                  | 9 (47.4)  |       |
| Pathological stage         |           |                            |       |
| T1                         | 54        | 17 (31.5)                  | 37 (68.5) | 0.0251* |
| T2/T3                      | 46        | 41 (89.1)                  | 5 (10.9)  |       |
| Lymph node metastasis      |           |                            |       |
| Negative                   | 78        | 40 (51.3)                  | 38 (48.7) | 0.003** |
| Positive                   | 22        | 18 (81.8)                  | 4 (18.2)  |       |
| Angiolymphatic invasion    |           |                            |       |
| Negative                   | 84        | 45 (53.6)                  | 39 (46.4) | 0.0017** |
| Positive                   | 16        | 13 (81.3)                  | 3 (18.8)  |       |

*P<0.05; **P<0.01. PSA: prostate-specific antigen; NS: not significant

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**Table 2: Expression of E2F3 and PRDM1 in prostate cancer and normal prostate tissues**

| Tumor (n=168) | E2F3 expression | PRDM1 expression | P     |
|--------------|-----------------|-----------------|-------|
| Low, n (%)   | High, n (%)     | Low, n (%)      | High, n (%) |       |
| Tumor (n=168) | 116 (69.0)      | 52 (31.0)       | 0.031* |
| BPH (n=65)   | 58 (89.2)       | 7 (10.8)        |       |

*P<0.05; **P<0.01. E2F3: E2F transcription factor 3; PRDM1: PR domain containing 1, with ZNF domain; BPH: benign prostatic hyperplasia
or DU145 cells were injected into the mammary pads of female nude mice, and tumors were allowed to develop. The mice were then injected intratumorally with a control sequence or a miR-3691-3p-agomir three times a week for 2 weeks. At the end of the experiment, the tumors were excised for analysis. As shown in Figure 4a–4c, the volume and weight of PCa tumors injected with the miR-3691-3p agomir were smaller than the tumors injected with the control sequence, indicating that elevation of miR-3691-3p concentrations locally at the tumor site suppressed tumor cell growth. We also performed IHC staining of tumor sections to investigate the expression levels of E2F3, PRDM1, and two key proteins involved in cell proliferation (Ki67) and apoptosis (cleaved caspase-3). These analyses showed that miR-3691-3p agomir injection inhibited tumor expression of E2F3 and PRDM1 (Figure 4d) and the proliferation marker Ki67 and increased the expression of the
apoptosis effector cleaved caspase-3 (Figure 4e), consistent with the results of the in vitro analyses. These results were observed in tumors formed by both DU145 and PC-3 cell lines. Collectively, these data indicate that miR-3691-3p suppresses the growth of PCa cell lines both in vitro and in vivo.

Expression of E2F3 and PRDM1 in PCa clinical specimens

Finally, we examined the expression levels of E2F3 and PRDM1 by IHC staining of 168 PCa tumors and 65 BPH tissues. The results showed that E2F3 and PRDM1 were mainly expressed in the nucleus of prostatic epithelial cells. The two proteins were both strongly expressed in cancer tissues but were present at lower levels in BPH tissues (Supplementary Figure 4). Quantification of protein expression showed strong E2F3 staining in a higher proportion of PCa tissues (31.0% vs 52/168) than BPH tissues (10.8%, 7/65; P < 0.05; Table 2), and similarly, significantly more PCa tissues than normal tissues showed positive staining for PRDM1 (28.6% [48/168] vs 16.9% [11/65]; P < 0.01; Table 2). Thus, the observed elevated expression of E2F3 and PRDM1 target genes was consistent with the reduced expression of miR-3691-3p detected in PCa compared with BPH clinical specimens.

DISCUSSION

Previous studies have shown that aberrant expression of several miRNAs play a role in the occurrence, development, and progression of PCa, which has implications for the development of novel diagnostic, prognostic, and therapeutic tools. TGF-β1 is well established to be involved in the growth and progression of a variety of cancers, including those of the breast, colorectum, and prostate, and serum TGF-β1 protein and mRNA levels are significantly increased in these cancers. TGF-β1, a member of the TGF-β superfamily, plays a dual role in cancer in that it is generally tumor suppressive but promotes the progression of late-stage cancers. The TGF-β1 signaling pathway can promote small mother against decapentaplegic (SMAD) protein binding to miRNA promoter genes, thereby altering the expression of numerous miRNAs.

We previously showed that TGF-β1-mediated inhibition of miR-450b-5p reversed the differentiation of rhabdomyosarcoma. In the present study, we identified miR-3691-3p as the most significantly downregulated miRNA among those tested in primary PCa compared with BPH samples. Moreover, low miR-3691-3p expression correlated positively with advanced pathological stage, lymph node metastasis, and shorter BCR-free survival after radical prostatectomy, thereby supporting the potential application of miR-3691-3p as a prognostic indicator and possible therapeutic target in PCa.

Our data suggest that miR-3691-3p is an anti-oncogene. Overexpression of miR-3691-3p inhibited the proliferation of PCa cells in vitro and in vivo and reduced metastatic behavior in vitro. In addition, we predicted and validated E2F3 and PRDM1 as miR-3691-3p target genes and demonstrated that knockdown of endogenous E2F3 and PRDM1 in PCa cell lines attenuated malignant behaviors such as proliferation and metastasis.

E2F3 is a member of the E2F transcription factor family and is essential for cell proliferation. Many reports have demonstrated the importance of intricate networks between E2F3 and miRNAs in regulating the balance of proliferation, apoptosis, and metastasis in various cancers. Deregulated E2F3 transcriptional activity is present in the vast majority of human cancers and has been clearly implicated in the dysregulation of cell cycle control, proliferation, and apoptosis. E2F3 is also a key transcription factor in tumor-associated macrophages and influences both the tumor microenvironment and tumor cell metastasis. Here, we demonstrated that E2F3 regulates the malignant behavior of PCa cell lines in vitro, and showed that its expression is downregulated by miR-3691-3p agomir treatment of PCa xenografts in a mouse model. However, the exact mechanisms by which miR-3691-3p modulates E2F3 expression and the progression of PCa requires further study.

The second miR-3691-3p target gene identified in this study was PRDM1 (also known as B lymphocyte-induced maturation protein [Blimp-1]), which is known to play critical roles in the development
of many cell types in the mouse and other model organisms. 42, 43 PRDM1 is implicated in malignancy through its interactions with the p53 tumor-suppressor pathway, suggesting that PRDM1 itself may have a role in tumor suppression. 44 Another study showed that PRDM1 inhibits SW620 colon cancer cell proliferation via inhibition of c-Myc. 45 In the present study, we demonstrated that PRDM1 was significantly overexpressed in PCa compared with BPH clinical tissues, and that PRDM1 silencing in PCa cell lines inhibited their proliferation, migration, and invasion ability. Thus, our results suggest that PRDM1 may act as an oncogene in the progression of PCa.

**AUTHOR CONTRIBUTIONS**

YMH and LXL performed experiments, analyzed, and interpreted data. BZL, LS and SW analyzed data and provided samples and clinical data. LW, XZ, QZ and MMS performed mouse experiments. KT and YSZ provided tissue samples and analyzed immunohistology data. YSZ and SLW designed experiment and wrote the paper. All authors read and approved the final manuscript.

**COMPETING INTERESTS**

All authors declare no competing interests.

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Supplementary Information is linked to the online version of the paper on the Asian Journal of Andrology website.

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**Supplementary Figure 1:** Validation of miR-143-3p expression. Validation of miR-3691-3p expression by real-time PCR after transfection with miR-3691-3p mimics in PC-3 and DU145 cell lines. Each assay was conducted at least three times independently. Error bars indicate s.d.; **P < 0.01. s.d.: standard deviation; PCR: polymerase chain reaction.**

**Supplementary Figure 2:** Validation of PRDM1 and E2F3 expression. PCa cell lines (DU145 and PC-3) were transfected with different concentrations of miR-3691-3p mimic (10 nmol ml⁻¹, 50 nmol ml⁻¹, 100 nmol ml⁻¹). PRDM1 and E2F3 expression was measured at the gene level by RT-qPCR (a) and on the protein level by Western blot (b). *P < 0.05, **P < 0.01, ***P < 0.001. PRDM1: PR domain containing 1, with ZNF domain; E2F3: E2F transcription factor 3; PCa: prostate cancer; RT-PCR: reverse transcription-quantitative polymerase chain reaction.

**Supplementary Figure 3:** Synthetic siRNA reduced the expression level of candidate target genes E2F3 and PRDM1 in PCa cell lines (DU145 and PC-3). The expression of these target genes was explored by RT-qPCR at the gene level (a) and by western blot at the protein expression level (b). *P < 0.05, **P < 0.01, ***P < 0.001. E2F3: E2F transcription factor 3; PRDM1: PR domain containing 1, with ZNF domain; PCa: prostate cancer; RT-PCR: reverse transcription-quantitative polymerase chain reaction.

**Supplementary Figure 4:** Immunohistochemical staining of E2F3 and PRDM1 in PCa clinical specimens. Representative immunohistochemical staining of (up) E2F3 and (bottom) PRDM1 in clinical specimens of PCa and BPH tissues. Scale bar: 100 μm. PRDM1: PR domain containing 1, with ZNF domain; E2F3: E2F transcription factor 3; PCa: prostate cancer; BPH: benign prostatic hyperplasia.