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Epigenetic deregulation of miR-29a and miR-1256 by isoflavone contributes to the inhibition of prostate cancer cell growth and invasion

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The epigenetic regulation of genes has long been recognized as one of the causes of prostate cancer (PCa) development and progression. Recent studies have shown that a number of microRNAs (miRNAs) are also epigenetically regulated in different types of cancers including PCa. In this study, we found that the DNA sequence of the promoters of miR-29a and miR-1256 are partly methylated in PCa cells, which leads to their lower expression both in PCa cells and in human tumor tissues compared with normal epithelial cells and normal human prostate tissues. By real-time PCR, western blot analysis and miRNA mimic and 3′-UTR-Luc transfection, we found that TRIM68 is a direct target of miR-29a and miR-1256 and that the downregulation of miR-29a and miR-1256 in PCa cells leads to increased expression of TRIM68 and PGK-1 in PCa cells and in human tumor tissue specimens. Interestingly, we found that a natural agent, isoflavone, could demethylate the methylation sites in the promoter sequence of miR-29a and miR-1256, leading to the upregulation of miR-29a and miR-1256 expression. The increased levels of miR-29a and miR-1256 by isoflavone treatment resulted in decreased expression of TRIM68 and PGK-1, which is mechanistically linked with inhibition of PCa cell growth and invasion. The selective demethylation activity of isoflavone on miR-29a and miR-1256 leading to the suppression of TRIM68 and PGK-1 expression is an important biological effect of isoflavone, suggesting that isoflavone could be a useful non-toxic demethylating agent for the prevention of PCa development and progression.

Introduction

Prostate cancer (PCa) is the most common cancer in men in the US.[1] Epigenetic regulation of genes has long been recognized as one of the many causes of cancer development and progression in human tumor system including PCa.[2] The epigenetic regulation of specific genes in human cancer cells include DNA methylation and histone modifications, leading to the changes in the expression of genes without altering the DNA sequences. DNA methylation has been well studied for understanding the epigenetic mechanisms of gene regulation. The methylation of promoter sequences in the DNA causes epigenetic gene silencing through the obstruction of transcriptional activators in or near the promoter. Importantly, recent studies have shown that a number of microRNAs (miRNAs) are also epigenetically regulated in different types of cancers including PCa,[3-5] which is directly responsible for the regulation of coding mRNAs. The methylation of DNA in the promoter sequence of miRNAs causes decreased expression of miRNA, leading to increased expression of their target mRNAs and proteins. This mechanism leads to aberrant expression of oncogenes, which is in part responsible for the development and progression of cancers including PCa.

In the past five years, miRNAs have received increased attention in cancer research. These small, non-coding RNAs could inhibit target gene expression by binding to the 3' untranslated region (3'UTR) of target mRNA, resulting in either mRNA degradation or inhibition of translation. It is becoming well known that miRNAs participate in many normal biological processes; however, miRNAs also play critical roles in the pathophysiological processes including cancer development and progression. It has been found that aberrant miRNA expression is correlated with the occurrence, progression, and treatment outcome of cancers.[6,7] The miRNAs could be oncogenic or tumor suppressive dependent on their regulatory effects on cancers. It is commonly accepted that some miRNAs, such as let-7, miR-29, miR-34, miR-122, miR-152 and miR-330, function as tumor suppressor.[8] The methylation in the DNA sequence of the promoter of these tumor suppressive miRNAs could result in the low expression of tumor suppressive miRNAs, leading to the development and progression of cancers. Therefore, targeting the methylation of DNA sequences in the promoter of miRNAs could be a novel approach to inhibit the development and progression of cancer including PCa.

Interestingly, recent studies have shown that several natural agents including isoflavone could demethylate DNA sequences

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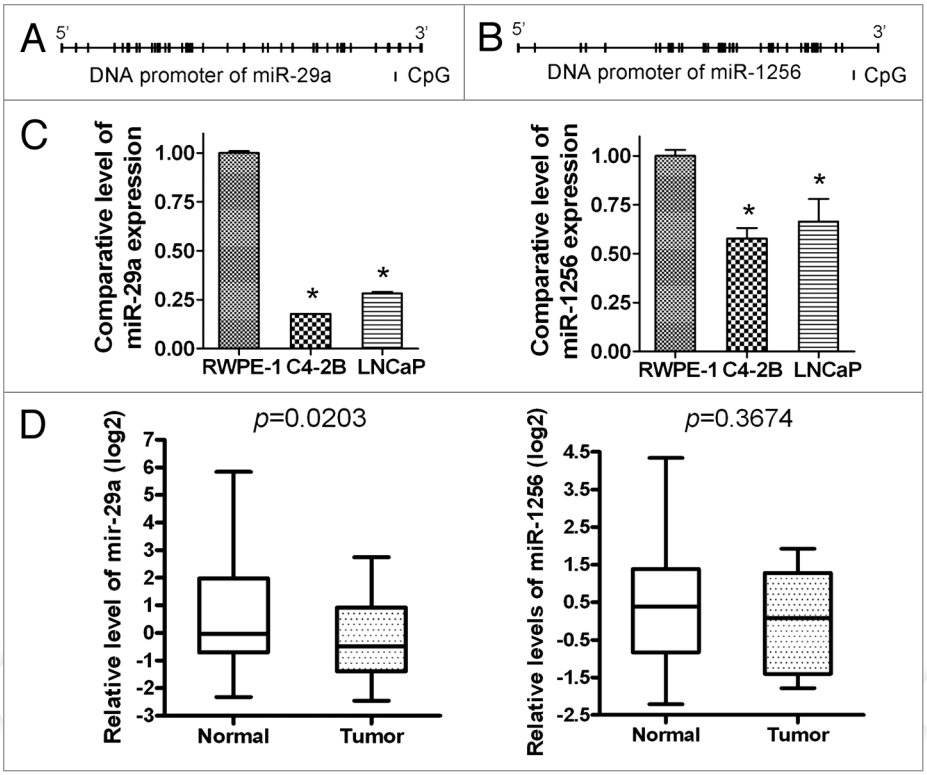

Figure 1. The methylation of miR-29a and miR-1256 promoters leads to decreased expression of miR-29a and miR-1256. Computerized analysis showed that there are many CpG sites in the promoters of miR-29a (A) and miR-1256 (B). Real-time RT-PCR showed that the expressions of miR-29a and miR-1256 were significantly downregulated in PCa cells [(C) *p < 0.05; n = 3] and in human prostate tumor tissues [(D) n = 46] compared with normal prostate epithelial cells and normal human prostate tissues.

in the promoters of genes or miRNAs, leading to increased expression of genes or miRNAs.[9,10] We have previously reported that isoflavone could alter the miRNA expression profiles, leading to the inhibition of cancer cell growth, induction of apoptosis, reversal of epithelial-to-mesenchymal transition (EMT), and enhancement of efficacy of conventional cancer therapeutics.[11,12] In this study, we investigated whether isoflavone could demethylate DNA sequences in the promoter of tumor suppressive miR-29a and other miRNAs and thus affect the expression of their target genes. We found that isoflavone could demethylate the DNA sequences of the methylated promoter of miR-29a and miR-1256, which led to the upregulation of miR-29a and miR-1256. Increased expression of miR-29a and miR-1256 induced by isoflavone resulted in the downregulation in the expression of their target genes TRIM68 and PGK-1, which led to the inhibition of PCa cell growth and invasion.

Results

The DNA sequences in the promoters of miR-29a and miR-1256 were methylated in PCa cells, leading to lower expression of miR-29a and miR-1256. It is well known that the promoter activity of a specific miRNA regulates the expression level of the miRNA. In the DNA sequences of the promoter of miR-29a and miR-1256, there is a lot of CpG site that could be methylated, leading to the downregulation of miR-29a and miR-1256 expression (**Fig. 1A and B**). By Methylation450 assay, we found that the methylation index of miR-29a and miR-1256 promoters in PCa cell group was much higher than that in prostate epithelial cell group (**Table 1**), suggesting more methylation events happened in the promoters of miR-29a and miR-1256 in PCa cells compared with non-cancer cells (normal cells). To confirm whether the methylation could downregulate the expression of miR-29a and miR-1256, we conducted specific miRNA assays. We found that the expression of miR-29a and miR-1256 was significantly lower in C4–2B and LNCaP PCa cells compared with RWPE-1 prostate epithelial cells (**Fig. 1C**). Moreover, we also found that lower expression of miR-29a and miR-1256 in the human prostate tumor tissues from patients with PCa compared with their adjunct normal prostate tissues (**Fig. 1D**). These results suggest that increased methylation of miR-29a and miR-1256 promoters in PCa could lead to decreased expression of miR-29a and miR-1256.

Isoflavone increased the expression of miR-29a and miR-1256 by demethylating DNA sequences in the promoters of miR-29a and miR-1256. To explore the effects of isoflavone on methylation, we obtained the methylation profiles of C4–2B and LNCaP PCa cells treated with isoflavone and Aza-dC by conducting Methylation450 array assay. We found that both Aza-dC and isoflavone decreased the methylation level the DNA sequences in the promoters of miR-29a and miR-1256 (**Table 2**). We also conducted methylation specific PCR to confirm the data from Methylation450 array data. The results showed that Aza-dC and isoflavone partly inhibited the methylation of miR-29a and miR-1256 promoters (**Fig. 2A**). By miRNA expression assay, we found that both Aza-dC and isoflavone significantly upregulated the expression of miR-29a and miR-1256 (**Fig. 2B and C**), suggesting that both isoflavone and Aza-dC treatment leads to increased expression of miR-29a and miR-1256, which is mediated by demethylation of the DNA sequence in the promoters of miR-29a and miR-1256. However, we also observed differential effects of isoflavone and Aza-dC on the expression of miR-155 and miR-421, which are known as oncogenic miRNAs. The expression of miR-155 and miR-421 was increased by Aza-dC treatment and decreased by isoflavone treatment (**Fig. 2D**), suggesting the inhibitory effects of isoflavone on the oncogenic miRNAs through non-demethylation related regulation.

TRIM68 is a target of miR-29a and miR-1256 and was upregulated in PCa. By comparing computerized prediction of miRNA targets with mRNA microarray analysis, we found that TRIM68 could be a target of both miR-29a and miR-1256. miR-29a and miR-1256 could bind to 3'UTR of TRIM68 with high binding score (**Fig. 3A**). To confirm whether miR-29a and miR-1256 could biologically bind to the 3'UTR of TRIM68, we co-transfected TRIM68 3'UTR-Luc reporter vector with miR-29a, miR-1256 or the negative control miRNA into LNCaP and C4–2B cells. We found that the luciferase activity was inhibited by the introduction of miR-29a or miR-1256 in C4–2B and LNCaP cells (**Fig. 3B**), suggesting that miR-29a or miR-1256 did bind to the 3'UTR of TRIM68. Furthermore, using real-time RT-PCR and western blot analysis, we observed that miR-29a or miR-1256 transfection inhibited the expression of TRIM68 mRNA (**Fig. 3C**) and protein (**Fig. 3D**), suggesting that TRIM68 is a target of both miR-29a and miR-1256. In addition, we also observed that miR-1256 transfection could inhibit the expression of PGK-1 (**Fig. 3C and D**), which could be another target of miR-1256 that was also found through the computerized prediction of miRNA target analysis.

Since we found decreased expression of miR-29a and miR-1256 in PCa cells and in human prostate tumor tissues compared with normal prostate epithelial cells and tissues, we expected that PCa cells should have high expression of TRIM68 and PGK-1. Indeed, we found that the mRNA (**Fig. 4A and B**) and protein (**Fig. 4C**) expression of TRIM68 and PGK-1 were higher in PCa cells and human tumor tissues compared with normal cells or normal human prostate tissues. Our data was consistent with other investigators' reports showing higher expression of TRIM68 and PGK-1 in PCa, which can be found in ONCOMINE database (www.oncomine.org).

Isoflavone inhibited the expression of TRIM68 and PGK-1 mediated through upregulation of miR-29a and miR-1256 expression. Interestingly, we found that isoflavone and Aza-dC significantly inhibited the expression of TRIM68 and PGK-1 at mRNA (**Fig. 5A and B; Table 3**) and proteins (**Fig. 5C and D**) levels in C4–2B and LNCaP cells. These observations are consistent with our findings showing that isoflavone and Aza-dC significantly upregulated the expression of miR-29a and miR-1256 in C4–2B and LNCaP cells (**Fig. 2B and C**). These results suggest that downregulation of TRIM68 and PGK-1 by isoflavone could be mediated by the upregulation of miR-29a and miR-1256, which directly target TRIM68 and PGK-1 though binding to the 3'UTR of TRIM68 and PGK-1.

Isoflavone inhibited cell growth and invasion mediated through the upregulation of miR-29a and miR-1256, and downregulation of TRIM68 and PGK-1 expression. Transfection of C4–2B and LNCaP cells with miR-29a and miR-1256 mimic significantly inhibited the growth of C4–2B and LNCaP PCa cells (**Fig. 6A**). Likewise, isoflavone treatment also showed similar growth inhibition of C4–2B and LNCaP cells (**Fig. 6A**). More importantly, we found that isoflavone treatment and the transfection of miR-29a or miR-1256 mimic into PCa cells significantly suppressed the invasive activity of C4–2B and LNCaP cells (**Fig. 6B**). These results clearly suggest that isoflavone could inhibit the growth of PCa cells as well as inhibit their invasive capacity, which is in part mediated through the upregulation of miR-29a and miR-1256 expression, and consequent downregulation of TRIM68 and PGK-1 expression in PCa cells. By Ingenuity Pathway analysis, we found that isoflavone treatment regulated the signal transduction in the TRIM68/AR and PGK-1 (**Fig. 6C**) signaling networks, which appears to be responsible for the inhibition of cancer cell growth and invasion.

Table 1. The methylation index of miR-29a and miR-1256 promoters in prostate cancer group and normal cells group measured by Methylation450 chip assay

	Cancer cell group	Normal cell group
miR-29a	0.8853	0.6551
miR-1256	0.7674	0.5557

Table 2. The methylation index of miR-29a and miR-1256 promoters altered by isoflavone (G2535) and Aza-dC in prostate cancer cells measured by Methylation450 chip assay

miRNA	C4–2B			LNCaP		
	Control	Aza-dC	G2535	Control	Aza-dC	G2535
miR-29a	0.9240	0.7274	0.7228	0.9171	0.8321	0.6948
miR-1256	0.8823	0.6642	0.6418	0.8382	0.5874	0.7983

Discussion

The methylation of DNA sequences in the promoter of several tumor suppressive genes has been found to play critical roles in PCa development and progression. In recent years, emerging evidence has shown that the promoter DNA sequence of tumor suppressive miRNAs could be methylated in PCa cells, leading to the loss of expression of specific miRNAs. In this study, we found that there are many CpG sites in the DNA sequence of the promoters of miR-29a and miR-1256 and that higher level of methylation was present in the promoter region of miR-29a and miR-1256 in PCa cells compared with normal prostate epithelial cells. The methylation of miR-29a has been previously found in lymphoma.[13] The methylation of miR-29a resulted in lower expression of miR-29a, and consequently results in higher expression of its target gene Mcl-1, which leads to apoptosis blockade and tumor cell survival in lymphoma.[13] It was also found that the treatment of cells with demethylating agent Aza-dC led to the induction in the expression of miR-29a in pancreatic cancer cells, suggesting that the methylation of miR-29a in pancreatic cancer cells is responsible for the loss of miR-29a expression.[14] Our results are consistent with these reports, demonstrating that the DNA sequences in the promoter of miR-29a are partly methylated in PCa cells, resulting in lower expression of miR-29a compared with normal prostate epithelial cells.

The role of miR-29a has been investigated in various cancers showing that miR-29a functions as a tumor suppressor. Downregulation of miR-29a expression has been found in colorectal, gastric, and lung cancers, acute myeloid leukemia, and lymphoma.[13,15-18] Furthermore, a significant downregulation

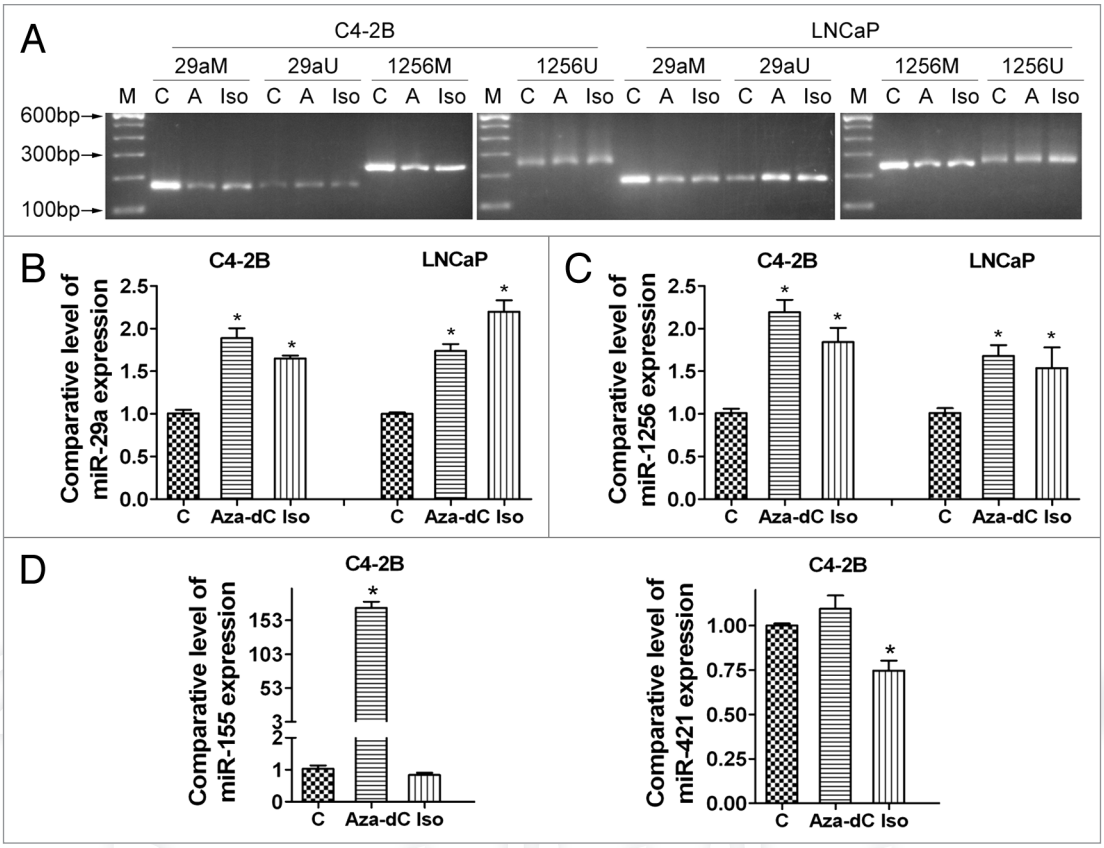

Figure 2. The effects of isoflavone on miR-29a and miR-1256 expression. Methylation specific PCR showed that isoflavone or Aza-dC treatments could partly demethylate the promoters of miR-29a and miR-1256 (A). Real-time RT-PCR showed increased expression of miR-29a (B) and miR-1256 (C) after isoflavone or Aza-dC treatment. However, isoflavone and Aza-dC had differential effects on the expression of miR-155 and miR-421 (D). (C, control; A, Aza-dC; Iso, isoflavone; M, methylation; U, unmethylation)

in the expression of miR-29a has been found to be correlated with short survival, more invasive phenotype, and early recurrence.[16-18] Therefore, miR-29a is believed to play important roles in the inhibition of cancer development and progression and the loss of its expression is in part responsible for tumor development and progression. However, there is no report on the expression level of miR-29a in PCa cells, normal prostate epithelial cells, human PCa specimens or normal human prostate tissues. In this study, we found that miR-29a expression was significantly downregulated in PCa cell lines and in human prostate tumor tissues compared with normal prostate epithelial cells and tissues, suggesting that miR-29a also function as a tumor suppressor in PCa development and progression. The biological function of miR-1256 is unknown. We found that the expression level of miR-1256 was also lower in PCa cells and human prostate tumor tissues compared with normal cells and normal human prostate tissues, suggesting that miR-1256 may also function as a tumor suppressor.

Recently, the studies on tripartite motif (TRIM) proteins have shown that some members of the TRIM family function as important regulators of carcinogenesis.[19] The tripartite motif containing 68 (TRIM68) has been found to be significantly upregulated in prostate cancer tissues compared with normal prostate tissues.[20] TRIM68 could functionally interact with co-activators of androgen receptor (AR) and promote the transactivation of AR.[20] Knockdown of TRIM68 could decrease the secretion of PSA and inhibit PCa cell growth and colony-forming ability.[20] Consistent with other investigators' report, we also found that the mRNA and protein expression of TRIM68 was significantly upregulated in PCa cells and human prostate tumor tissues, suggesting the oncogenic function of TRIM68 in prostate carcinogenesis. Importantly, we found that both miR-29a and miR-1256 directly targeted TRIM68 by binding to the 3'UTR of TRIM68, leading to the downregulation of TRIM68 expression.

Phosphoglycerate kinase 1 (PGK-1) is an important molecule involved in the process of glycolysis. Studies have found upregulation of PGK-1 in gastric, pancreatic and metastatic prostate cancers.[21-23] Interestingly, the high expression of PGK-1 is significantly correlated with tumor progression, metastasis and multidrug resistance in various cancers.[21,22,24-26] It has also been reported that the expression of PGK-1 in PCa cells could induce bone formation[27] and that cancer-associated fibroblasts, which showed strong expression of PGK-1, could promote PCa growth,[28] suggesting that PGK-1 plays important roles in PCa development, progression and bone metastasis. In addition, both PGK-1 and AR genes are located within Xqll-Xql3 region and PGK-1 short tandem repeat polymorphism linkage to AR

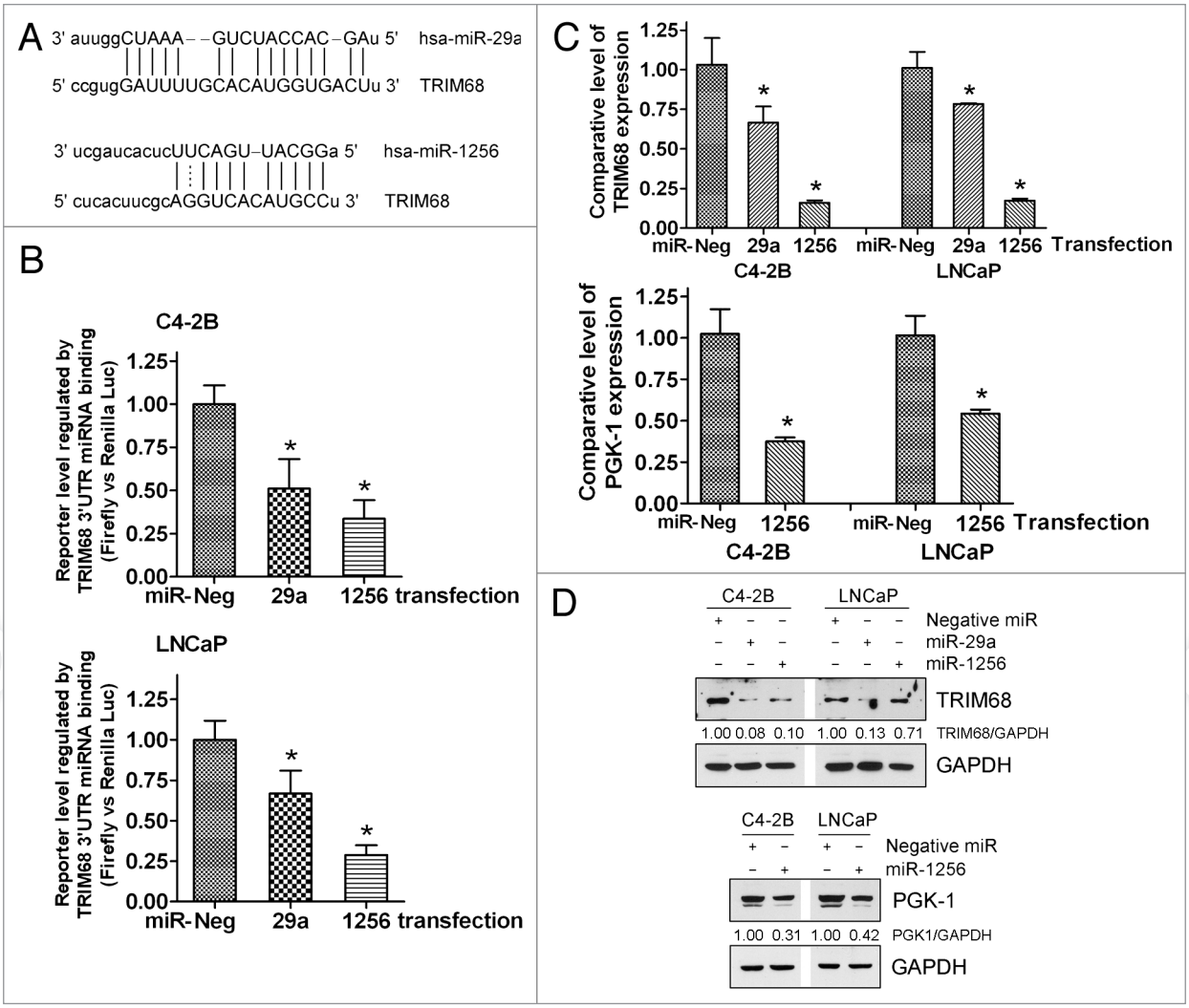

Figure 3. Targeting TRIM68 by miR-29a and miR-1256. Computerized analysis showed miR-29a and miR-1256 sequence alignment to the sequence of TRIM68 3′UTR (A). Co-transfection of TRIM68 3′UTR-Luc and miR-29a, miR-1256 mimic, or negative control miRNA showed that miR-29a and miR-1256 directly bond to the 3′UTR of TRIM68 (B). Transfection of miR-29a, miR-1256 mimic, or negative control miRNA into PCa cells caused downregulation of TRIM68 and PGK-1 at mRNA (C) and protein (D) levels. (Neg, negative control miRNA; 29a, miR-29a; 1256, miR-1256; *$p < 0.05$, $n = 3$)

was found in PCa.[29,30] In our study, we found that the expression of PGK-1 was upregulated and the expression of miR-1256 was downregulated in PCa cells and in human prostate tumor tissues. Transfection of miR-1256 mimic into PCa cells inhibited the expression of PGK-1, suggesting that PGK-1 could be a target of miR-1256. Therefore, the strategies for upregulation of miR-29a and miR-1256 could lead to the inhibition of PCa development and progression, which in part could be due to downregulation of TRIM68/AR/PGK-1 signaling network. Since transfection strategies are not practically applied for human studies, we sought to investigate whether natural nontoxic agents could be useful for the upregulation of miRNAs and thereby inactivate their targets for the inhibition of PCa cell growth.

To that end, we investigated the role of isoflavone. Isoflavone has shown its inhibitory effects on PCa cells through the regulation of multiple signaling pathways.[31] Isoflavone has also shown to reverse the methylation status of several genes including *BTG3*, *GSTP1*, *RASSF1A*, *EPH2*, *BRCA1*, *p16*, *RAR*β and *MGMT*.[32-35] Studies have also shown the regulatory effects of isoflavone genistein on the methylation of miR-221/222 and miR-145 in PCa cells.[36,37] These findings suggest the demethylating function of isoflavone. In our study, we found that isoflavone could demethylate the methylated promoter of miR-29a and miR-1256 and, in turn, increased the expression of miR-29a and miR-1256. The upregulation of miR-29a and miR-1256 by isoflavone treatment inhibited the expression of their target genes, TRIM68 and PGK-1. These results demonstrate the epigenetic regulatory effect of isoflavone. It is important to note that isoflavone is not just a pan-demethylating agent like Aza-dC. Aza-dC treatment caused the upregulation of miR-155 and miR-421 through the demethylation effects; however, isoflavone treatment downregulated the expression of miR-155 and miR-421 which are oncogenic miRNAs.[38,39] Therefore, isoflavone with its specific targeting effect on

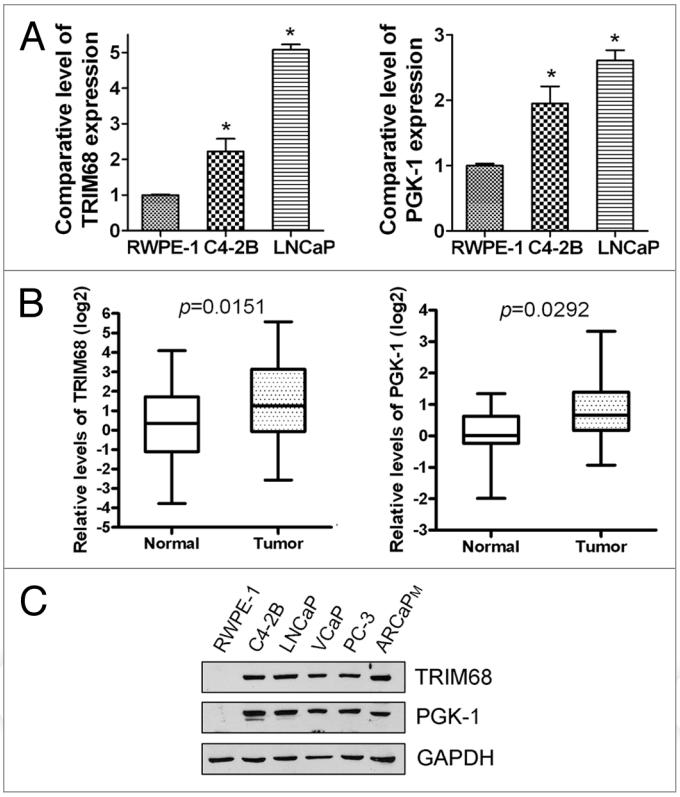

Figure 4. The expression of TRIM68 and PGK-1. A significantly higher mRNA expression of TRIM68 and PGK-1 was observed in PCa cells [(A) *: $p < 0.05$; n = 3] and in human prostate tumor tissues [(B) n = 46] compared with normal prostate epithelial cells and normal human prostate tissues measured by real-time RT-PCR. A significantly higher expression of TRIM68 and PGK-1 proteins were also observed in PCa cells compared with normal prostate epithelial cells as assessed by western blot analysis (C).

miR-29a and miR-1256 methylation could be a promising agent for the inhibition of PCa development and progression mediated through epigenetic regulation.

By upregulating miR-29a and miR-1256 expression, isoflavone significantly suppressed the expression of TRIM68 and PGK-1, leading to the inhibition of PCa cell growth and invasion. Other investigators have reported that downregulation of TRIM68 could inhibit the secretion of PSA and the growth of PCa cells by suppression of AR signaling.[20] We have previously reported that isoflavone could also inhibit AR signaling.[40] Therefore, the epigenetic regulation of miR-29a and miR-1256 by isoflavone could be one of the molecular mechanisms by which isoflavone regulates AR signaling and inhibits PCa growth. In addition, upregulated expression of PGK-1 in tumors has been correlated with metastatic phenotype of tumor.[22,23,25] Thus, downregulation of PGK-1 through epigenetic regulation by isoflavone could be another molecular mechanism by which isoflavone would be able to inhibit PCa invasion. However, more mechanistic studies are warranted. In conclusion, the epigenetic regulation of genes and miRNAs by isoflavone would make it a promising agent for the prevention of prostate cancer development and progression.

Materials and Methods

Cell lines, reagents and antibodies. LNCaP (ATCC), VCaP (ATCC), PC-3 (ATCC), C4–2B and $ARCaP_M$ (Novicure) prostate cancer (PCa) cells were maintained in RPMI 1640 (Invitrogen) or MCaP (for $ARCaP_M$, Novicure) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2 atmosphere at 37°C. RWPE-1 (ATCC) and CRL2221 (ATCC) prostate epithelial cells were cultured in keratinocyte serum-free medium supplemented with 5 ng/ml of epidermal growth factor (EGF) and 50 μg/ml of bovine pituitary extract (Invitrogen). The cell lines have been tested and authenticated through the core facility Applied Genomics Technology Center at Wayne State University. The method used for testing was short tandem repeat (STR) profiling using the PowerPlex 16 System from Promega. Isoflavone mixture G2535 (70.5% genistein, 26.3% daidzein and 0.31% glycetein manufactured by Organic Technologies and obtained from NIH) was dissolved in DMSO to make a stock solution containing 50 mM genistein. The concentrations of isoflavone we described in this article all refer to the concentration of genistein in isoflavone mixture. 5-aza-2'-deoxycytidine (Aza-dC, Sigma) was dissolved in DMSO to make a stock solution of 10 mM. Anti-TRIM68 (Santa Cruz), anti-PGK-1 (Santa Cruz), anti-β-actin (Sigma) and anti-GAPDH (Sigma) primary antibodies were used for Western Blot analysis.

Methylation450K chip analysis. PCa cells were treated with 5 μM Aza-dC or 20 μM G2535. Control cells received 0.05% DMSO (solvent control). Fresh Aza-dC, G2535 and DMSO were administered everyday along with a change of culture medium. After 5 d of treatment, genomic DNA was isolated and purified by using the Wizard Genomic DNA Purification Kit (Promega). The purified DNA from each sample was preceded to bisulfate conversion using the EZ DNA Methylation-Gold Kit (Zymo Research). The converted DNA was then applied to the HumanMethylation450 BeadChip (Illumina). The Illumina Infinium 450K methylation files obtained from the Illumina iScan scanner were uploaded to GenomeStudio (V2011.1, Illumina) using the Methylation module. Data was normalized using the Controls Normalization method. Differentially methylated probes were identified using the Illumina Custom Error Model with Benjamini-Hochberg False Discovery Rate correction. A p value was associated with every probe (detection p value) and probes were discarded if this Detection p value was more than 0.05. All data analysis and statistical evaluation was assisted by Dr Dyson.

Methylation-specific PCR. Cell treatment and DNA extraction were described above. After DNA extraction, 500 ng of genomic DNA was treated with bisulfite using the EZ DNA Methylation-Gold Kit (Zymo Research). The modified DNA was eluted with a final volume of 12 μl. Conventional PCR was performed using 100 ng modified DNA and ZymoTaq DNA Polymerase Kit (Zymo Research) in a thermocycle (Eppendorf) with initial 95°C 10 min and 40 cycles of 95°C 30 sec, 55°C 40 sec, and 72°C 60 sec. The PCR product was visualized in 2% agarose gel with ethidium bromide staining. The primers

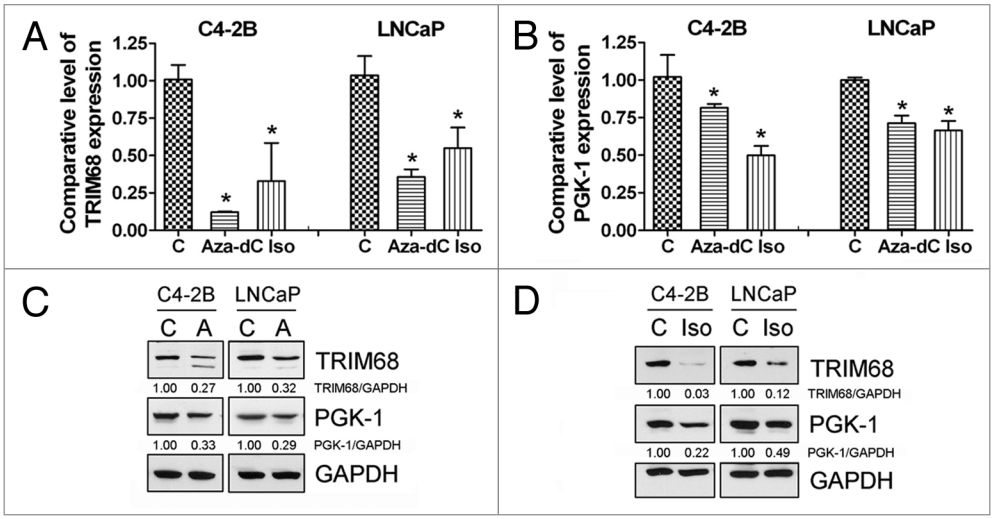

Figure 5. TRIM68 and PGK-1 expression was altered by isoflavone and Aza-dC. (A and B) Real-time RT-PCR showed that the expression of TRIM68 (A) and PGK-1 (B) mRNA were significantly inhibited by isoflavone and Aza-dC treatment. (C and D) western blot analysis showed that the expression of TRIM68 (C) and PGK-1 (D) proteins were inhibited by isoflavone (D) and Aza-dC (C). (C, control; Iso, isoflavone; A, Aza-dC; *p < 0.05, n = 3)

were designed by using Methylation Primer Express (Applied Biosystems). The sequences of primers used were: 29aM-2-F: TTA AGA ATT ATA TTA TGA CGG C; 29aM-2-R: ATA TCG CTT CCG AAA TAA; 29aU-2-F: ATA TTA AGA ATT ATA TTA TGA TGG T; 29aU-2-R: CTA ATA TCA CTT CCA AAA TAA; 1256M-2-F: GGC GCG ATT TTA GTT TATC; 1256M-2-R: TTT AAT TAC CAA CCG AAT ACG; 1256U-2-F: AGT GGT GTG ATT TTA GTT TAT T; 1256U-2-R: TTT AAT TAC CAA CCA AAT ACA ATA.

Microarray analysis of isoflavone and Aza-dC treated PCa cells. C4–2B and LNCaP cells were treated with 20 μM G2535 or 5 μM Aza-dC for 5 d as described earlier. Total RNA from each sample was isolated by using RNeasy Mini Kit and RNase-free DNase Set (QIAGEN) according to the manufacture's protocols. RNA purity and integrity were evaluated by microfluidics analysis using Agilent 2100 bioanalyzer (Agilent Technologies). The samples were processed for cDNA synthesis, transcriptional cRNA labeling, and hybridization to HumanHT-12 v4 BeadChip (Illumina) following standard protocol from Illumina. The HumanHT-12 v4 BeadChip provides coverage for more then 47,000 transcripts and known splice variants across the human transcriptome. The raw data of gene expression from scanning was normalized and compared by using GenomeStudio (Illumina) with gene expression module. The fold changes were computed between the different groups. The gene expression data was also integrated with methylation data to compare the methylation status and gene expression status using GenomeStudio.

Total RNA extraction, miRNA and mRNA detection. Total RNA was extracted by using the miRNeasy Mini Kit and RNase-free DNase Set (QIAGEN) following the protocol provided by the manufacturer. The expression level of miR-29a, miR-1256, miR-155, and miR-421 in isoflavone and Aza-dC treated C4–2B and LNCaP cells was analyzed by using TaqMan MicroRNA Assay Kit (Applied Biosystems) following manufacturer's protocol. Briefly, total RNA from each sample was subjected to reverse transcription with a specific miRNA primer (Applied Biosystems). Real-time PCR reactions were then performed in StepOnePlus (Applied Biosystems). The total RNA extracted from paraffin-embedded tissues obtained from PCa patients was also subjected to miR-29a and miR-1256 assay using Universal cDNA Synthesis Kit (Exiqon), specific LNA™ PCR primer set (Exiqon), and SYBR Green RT-PCR Reagents (Applied biosystems). The PCR program was initiated by 10 min at 95°C before 40 thermal cycles, each of 15 sec at 95°C and 1 min at 60°C. Data were analyzed according to the comparative Ct method and were normalized by RNU48 and RNU44 expression in each sample. The expression level of miRNA was statistically evaluated by Student's t-test using GraphPad StatMate software (GraphPad Software Inc.).

Table 3. TRIM68 and PGK-1 mRNA expression altered by isoflavone (G2535) and Aza-dC measured by microarray

Gene		Average expression level		
Symbol	Access #	Control	Aza-dC	G2535
C4–2B				
TRIM68	NM_018073.5	436.1949	366.7657	143.9768
PGK1	NM_000291.2	5948.721	4354.008	1749.751
LNCaP				
TRIM68	NM_018073.5	613.4414	402.655	231.0056
PGK1	NM_000291.2	4011.298	3675.979	1584.556

The expression level of TRIM68 and PGK-1 in isoflavone and Aza-dC treated C4–2B and LNCaP cells, and in the human tissues obtained from PCa patients was analyzed by real-time RT-PCR using High Capacity RNA-to-cDNA Kit and SYBR Green Master Mixture from Applied Biosystems. The sequences of primers used were: TRIM68-F: GGA GCA AAT CTT GGA GCT TG; TRIM68-R: TCC TCA GAC ACG ATG AGA CG; PGK1-F: CTG TGG GGG TAT TTG AAT GG; and PGK1-R: CTT CCA GGA GCT CCA AAC TG. The PCR was initiated

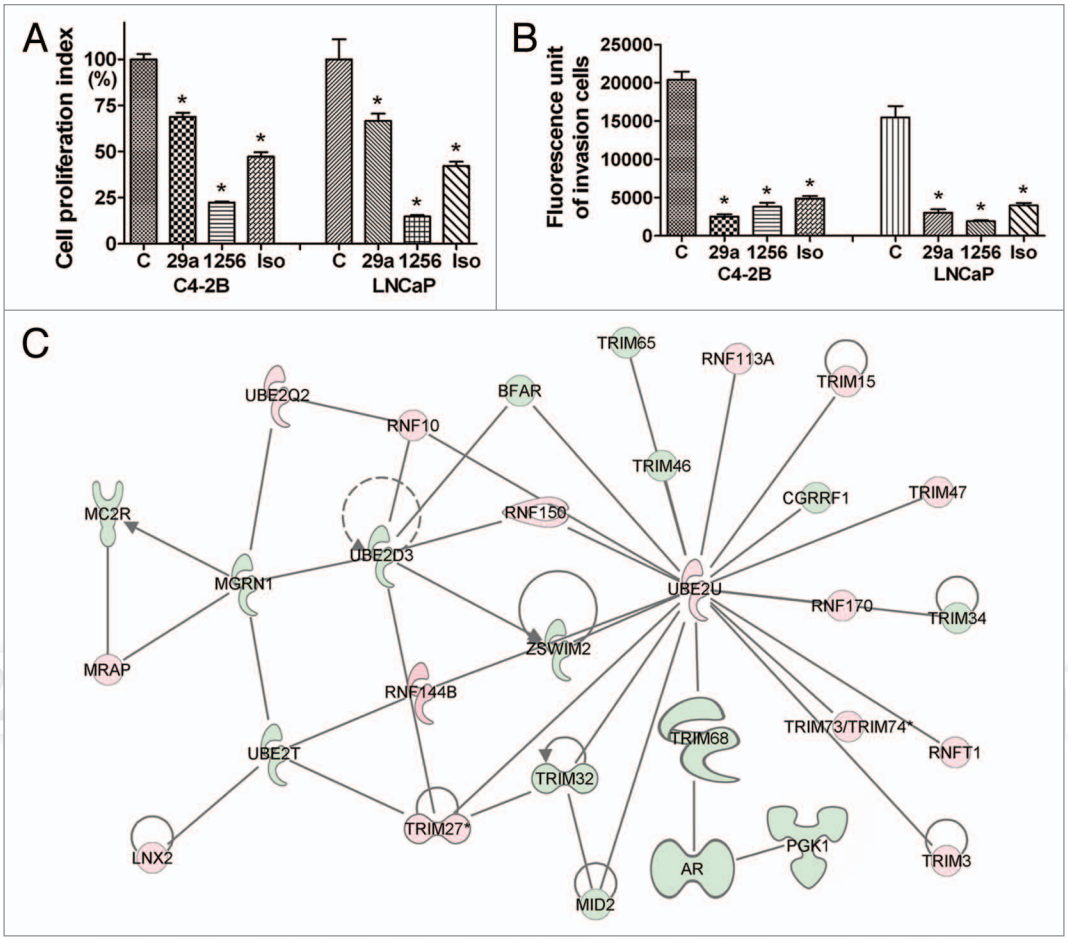

Figure 6. Inhibition of cell growth and invasion by miR-29a, miR-1256 and isoflavone through TRIM68/AR and PGK-1 signaling. Transfection of miR-29a and miR-1256 mimic into PCa cells, and isoflavone treatment significantly inhibited cell growth (A) and invasion (B) of PCa cells (*: $p < 0.05$; $n = 3$). Microarray and ingenuity pathway analysis showed that isoflavone downregulated TRIM68/AR and PGK-1 signaling (C). Green, downregulated; red, upregulated.

by 10 min at 95°C before 40 thermal cycles, each of 15 sec at 95°C and 1 min at 60°C. Data were analyzed according to the comparative Ct method and were normalized by GAPDH expression in each sample.

Western blot analysis. Whole cell lysates from isoflavone and Aza-dC treated C4–2N and LNCaP cells were subjected to standard Western Blot analysis as described previously.[41] The signal was then detected using the chemiluminescent detection system (PIERCE). The signal of western blot was scanned and quantified by using AlphaEaseFC software (Alpha Innotech). The signal ratio of each protein over loading control was calculated by standardizing the ratios of each control to the unit value.

Re-expression of miR-29a and miR-1246 in PCa cells. C4–2B and LNCaP cells were seeded in 6 well plates and transfected with miR-29a mimic, miR-1256 mimic, or miRNA negative control (Ambion) at a final concentration of 20 nM using DharmaFact Transfection Reagent (Dharmacon). After 3 d of transfection, the cells were passaged and transfected repeatedly with the miRNAs for three more days. Total RNA from each sample was then extracted and the RNA was subjected to RT-PCR as described earlier. Total proteins from each sample were also extracted and subject to Western Blot analysis as described earlier. The C4–2B and LNCaP cells transfected with miR-29a and miR-1256 were also subjected to cell growth and invasion assays.

Cell growth assay by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). C4-2B and LNCaP cells transfected with miR-29a, miR-1256, or miRNA negative control were seeded in 96-well plates. After 48 h of culture, the cells were subjected to MTT assay as described previously.[41] The growth inhibition of C4-2B and LNCaP cells after miRNA transfection was statistically evaluated by Student's t-test using GraphPad StatMate software (GraphPad Software, Inc.).

Invasion assay. The invasive capacity of C4-2B and LNCaP cells with miR-29a, miR-1256, or miRNA negative control transfection was assessed by using BD BioCoat Tumor Invasion Assay System (BD Biosciences) according to the manufacturer's protocol with modification. Briefly, the miRNA transfected C4-2B and LNCaP cells with serum free medium were seeded into the upper chamber of the system. Bottom wells in the system were filled with complete medium. After 18 h of incubation, the cells in the upper chamber were removed, and the cells, which invaded through matrigel matrix membrane, were stained with

4 μg/ml Calcein AM in Hanks buffered saline at 37°C for one hour. Then, the solution was removed and trypsin was used to collect the invaded cells. After incubation for 5 min, the fluorescence of invaded cells in bottom chamber was read in an ULTRA Multifunctional Microplate Reader (TECAN) at excitation/emission wavelengths of 530/590 nm. The significance of difference between samples was statistically evaluated by Student's t-test using GraphPad StatMate software (GraphPad Software Inc.).

Luciferase activity assay for confirming miRNA binding to target 3'UTR. C4-2B and LNCaP cells were seeded in 96-well plates and incubated for 24 h. The cells were co-transfected with TRIM68 3'UTR luciferase vector (GeneCopoeia) and miR-29a, miR-1256, or miRNA negative control using DharmaFECT Duo Transfection Reagent (Dharmacon). The vector includes TRIM68 3' UTR target sequence fused downstream to a firefly luciferase. The vector also contains renilla luciferase as internal control for signal normalization. After 48 h of transfection, firefly and renilla luciferase activities were assayed using Luc-Pair miR Luciferase Assay (GeneCopoeia) according to the manufacturer's protocol. The renilla luciferase activity was used as a control when calculation for the miRNA 3'UTR binding was assessed. The significance of difference between samples was statistically evaluated by Student's t-test using GraphPad StatMate software (GraphPad Software Inc.).

Patients and prostate tissue specimen collection. After obtaining institutional review board approval, retrospective archival pre-treatment PCa tissues and matched adjacent normal tissues were obtained from the Biospecimen Core of Karmanos Cancer Institute (KCI) collected from patients who underwent radical prostatectomy from 2004–2010 at KCI. Total RNA was extracted following our previously published procedures.[42]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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