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Cancer Research

miR-99 Family of MicroRNAs Suppresses the Expression of Prostate-Specific Antigen and Prostate Cancer Cell Proliferation

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Abstract

MicroRNAs (miRNA) have been globally profiled in cancers but there tends to be poor agreement between studies including in the same cancers. In addition, few putative miRNA targets have been validated. To overcome the lack of reproducibility, we profiled miRNAs by next generation sequencing and locked nucleic acid miRNA microarrays and verified concordant changes by quantitative RT-PCR. Notably, miR-125b and the miR-99 family members miR-99a, -99b, and -100 were downregulated in all assays in advanced prostate cancer cell lines relative to the parental cell lines from which they were derived. All four miRNAs were also downregulated in human prostate tumor tissue compared with normal prostate. Transfection of miR-99a, -99b, or -100 inhibited the growth of prostate cancer cells and decreased the expression of prostate-specific antigen (PSA), suggesting potential roles as tumor suppressors in this setting. To identify targets of these miRNAs, we combined computational prediction of potential targets with experimental validation by microarray and polyribosomal loading analysis. Three direct targets of the miR-99 family that were validated in this manner were the chromatin-remodeling factors SMARCA5 and SMARCD1 and the growth regulatory kinase mTOR. We determined that PSA is posttranscriptionally regulated by the miR-99 family members, at least partially, by repression of SMARCA5. Together, our findings suggest key functions and targets of miR-99 family members in prostate cancer suppression and prognosis. *Cancer Res; 71(4); 1313–24. ©2011 AACR.*

Introduction

A major advance in biology in the last decade is the discovery of small regulatory noncoding RNAs including microRNA (miRNA), siRNA, tiny noncoding RNAs (tncRNAs), and Piwi-interacting RNA (piRNA; ref. 1). miRNAs typically bind to the 3′-untranslated region (3′-UTR) of a target mRNA and posttranscriptionally regulate its expression by degrading the mRNA and repressing translation. Many studies have shown that miRNAs play critical roles in cancer (2). Each type and/or stage of cancer exhibits a characteristic miRNA expression profile, which therefore has the potential to serve as a useful tool in cancer diagnosis and prognosis (3, 4).

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Prostate cancer is the most frequently diagnosed cancer and the second leading cause of cancer-related deaths in the male population of the United States. Initially, prostate cancers depend on androgens for their growth. Therefore, the primary treatment of metastatic prostate cancer is androgen deprivation therapy, achieved by orchiectomy or antiandrogens. However, prostate cancer often progresses into a castration-resistant metastatic stage (5, 6). Therefore, understanding the mechanism of progression to androgen refractoriness may allow the treatment of advanced prostate cancer.

Several lines of evidence have noted the role of miRNAs in prostate cancer. Comparison of an aggressive PC3 line to LNCaP and 22Rv1 cell lines showed that upregulation of miR-221 and miR-222 promoted cell proliferation through targeting a cell-cycle regulator p27 (7). Advanced cancer cell lines PC3 and C4-2B have a reduced level of miR-146a, whose ectopic overexpression suppresses their growth (8). Ectopic expression of miR-126* was also shown to suppress the proliferation and invasion of androgen-dependent LNCaP cells (9). miR-125b is differentially expressed between the androgen-dependent cell line LNCaP and castration-resistant cell lines Cds1 and Cds2, and in benign and malignant prostate tumor tissues (10). A recent review points out that there is extensive disagreement between the published profiles of miRNAs in prostate cancer (11). The differences could arise from limitations of the hybridization method for detecting the

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small RNAs and from the wide variability of malignant tissue content in clinical samples.

All published expression profiles of miRNAs in prostate cancer depended on microarray hybridization and none utilized the recently available profiling method of next generation sequencing of miRNAs. In this article, we systematically examined the expression profile of miRNAs in an androgen-dependent human prostate cancer cell line, LNCaP, and its derivative castration-resistant more advanced cell line C4-2, using both Roche 454 sequencing and miRCURY LNA microarray platform. We confirmed the changes that were concordant by the two methods by miRNA-specific quantitative RT-PCR (qRT-PCR) to identify 4 miRNAs, miR-125b and members of miR-99 family (miR-99 a, -99b, and -100), that were downregulated in C4-2 relative to LNCaP. These miRNAs were decreased in human prostate tumor tissue compared with normal prostate tissue as well, indicating their importance in the development of prostate cancer. One of the bottlenecks in the study of miRNAs is the identification of relevant miRNA targets. Computational methods make too many predictions with an unacceptable level of false-positive rates. After it became apparent that miRNAs often lead to the degradation of target mRNAs, we and others used microarray analysis to find downregulated mRNAs to improve target prediction. In this report, we have added a third approach, the transfer of mRNAs bound to miRNAs from polyribosomes to monoribosomes, to identify 3 *bona fide* targets of the miR-99 family. We propose that the miR-99 family regulates the growth and prostate-specific antigen (PSA) production of prostate epithelial cells, at least partially, through repressing these 3 targets, SMARCA5, SMARCD1, and mTOR.

Materials and Methods

Cells and tissues

Human prostate cancer cells LNCaP, C4-2, and WPE1-NB26 and immortalized human prostate epithelial cell RWPE1 were obtained from ATCC. LNCaP and C4-2 were maintained in RPMI 1640 medium, supplemented with 10% FBS, RWPE1, and WPE1-NB26. For experiments on androgen responsiveness, cells were cultured in phenol red–free RPMI 1640 medium, supplemented with charcoal:dextran-stripped FBS (Hyclone) for 48 hours before the addition of the androgen analogue R1881 (Perkin-Elmer). RWPE-1 and WPE1-NB26 were maintained in keratinocyte serum-free medium, supplemented with bovine pituitary extract and human recombinant epidermal growth factor. De-identified mid-Gleason-grade prostate cancer and normal prostate were obtained from the University of Virginia mid-Atlantic CHTN (Cooperative Human Tissue Network). A pathologist screened sections so that at least 70% of the cells in a cancer section were malignant.

mRNA microarray

mRNA microarray analysis was carried out with Affymatrix HG-U133 Plus 2.0 array. The data set can be found in GEO with accession number of GSE26332.

Transfection of siRNA and miRNA duplex

Transfection of siRNA/miRNA duplex or 2′-*O*-methyl antisense oligonucleotide was done with Lipofectamine RNAiMax reagent (Invitrogen) as described (12).

Western blotting

The antibodies used were as follows: anti-SMARCD1 (BD Bioscience), anti-SMARCA5 (Santa Cruz), anti-mTOR (BD Bioscience), anti-PPFIA3 (ProteinTech Group), anti-AR (BD Bioscience), and anti–β-actin (Sigma). The Western blot image was captured by G:Box iChemi XT gel documentation and analysis system. Signal intensity of Western blots was quantified with GeneTools from SynGene.

RNA isolation and quantification of miRNA

Total RNA was extracted using TRIzol (Invitrogen). One microgram of total RNA was reverse transcribed using NCode miRNA First-Strand cDNA Synthesis kit (Invitrogen). The expression level of miRNAs was measured by qPCR with NCode SYBR GreenER miRNA qPCR kit (Invotrogen) in triplicate. U6 small nuclear RNA (snU6) was used to normalize the expression data of miRNAs. The primer sequence of snU6 is 5′-CTGCGCAAGGATGACACGCA-3′. miRNA microarray profiling was carried out using Exiqon miRCURY LNA array system (v.9.2).

Cloning of small RNAs and Roche 454 deep sequencing

Small RNA cloning was carried out as described in Lau's article with minor modifications (13). Small RNA with a size of 17 to 26 nucleotides (nt) was gel purified from 500 μg of total RNA. Purified small RNA was ligated with a modified 3′-adaptor, followed by a 5′-adaptor ligation, PCR amplification, and concatamerization. Concatamerized DNAs with a size of 200 to 250 nt were subjected to Roche 454 deep sequencing (VBI Core Lab at Virginia Bioinformatics Institute in Virginia Tech).

Luciferase reporter assay

The 3′-UTR fragments of SMARCD1, SMARCA5, mTOR, and PPFIA3 containing miR-99 family binding sites were cloned into a modified vector pRL-CMV (12). The mutations were made to the miR-99 family binding sites in the 3′-UTR-MUT clones. The primers used in 3′-UTR or 3′-UTR-MUT cloning are described in Supplementary Table S4. The luciferase reporter assay was carried out as previously described (12).

Polyribosome fractionation and qRT-PCR

Forty-eight hours after miRNA duplex or si-GL2 transfection in C4-2 cells, polysome fractionation assay was carried out as described (14). The total RNAs from monoribosome and polyribosome fractionations were extracted separately and subjected to qRT-PCR analysis for individual mRNAs.

Bromodeoxyuridine incorporation and PSA ELISA assay

Bromodeoxyuridine (BrdUrd) incorporation was measured as previously described (15) and was normalized to cell density measured by the MTT assay (Promega). PSA ELISA

Table 1. Ratios of miRNAs in C4-2 relative to LNCaP

	miRNA	Microarray	Sequencing	qPCR
miRNAs significantly decreased in C4-2 relative to LNCaP	hsa-miR-100	0.38	NA	0.17
	hsa-miR-125b	0.34	0.31	0.21
	hsa-miR-19b	0.48	0.43	0.23
	hsa-miR-99a	0.39	0.21	0.26
miRNAs modestly decreased in C4-2 relative to LNCaP	hsa-miR-99b	0.66	0.62	0.43
	hsa-miR-106a	0.49	0.97	0.41
	hsa-miR-21	0.71	0.7	0.5
	hsa-miR-16	0.86	0.61	0.59
miRNAs with no significant change	hsa-miR-222	1.01	2.19	0.92
	hsa-miR-29a	1.35	2.3	1.12
	hsa-miR-15b	1.07	0.84	
	hsa-let-7b	1.02	1.51	
	hsa-miR-200b	1.09	1.07	0.79
	hsa-miR-22	1.12	2.17	0.81
miRNAs significantly increased in C4-2 relative to LNCaP	hsa-miR-196b	1.39	5.32	2.48
	hsa-miR-557	1.97	NA	3.28
	hsa-miR-9	2.76	NA	5.86

NOTE: NA, not available.

assay was carried out using culture supernatant 72 hours after siRNA/miRNA duplex transfection, using Human PSA ELISA Kit (Abazyme), according to the manufacturer's instructions and normalized to the MTT assay.

Results

Screening for differential expression of miRNAs in prostate cancer cells

Small RNAs of 17 to 26 nt were cloned from 2 prostate cancer cell lines, LNCaP and C4-2, and subjected to 454 deep sequencing. A flowchart for the analysis of 454 deep sequencing data is outlined in Supplementary Figure S1. The sequencing reads, approximately 190,000 from each sample, were compared with one another to yield unique sequences and their cloning frequencies. In further analysis, we included only unique sequences cloned 5 times or more, which add up to more than a thousand. Among them, a few hundred sequences were cloned more than 50 times (Supplementary Table S1).

miRNAs were identified by BLASTing the unique sequences against the miRNA database (miRbase release 10.0; ref. 16; Supplementary Fig. S1). As expected, miRNAs are the most abundant class of small RNAs in the cloned sequences; 37% of sequencing reads were mapped to miRNAs in the database (Supplementary Table S1). A report on the analysis of the non-miRNA short RNAs has been published (17). Altogether, 293 miRNAs were cloned from the two cell lines. The most frequently cloned miRNAs were *let-7* family members, miR-125b, -99a, -200c, -17, and -21, suggesting that these miRNAs are abundant in these cell lines (Supplementary Table S2).

Significant change in the relative cloning frequency of several miRNAs between LNCaP and C4-2 (Table 1) suggested that these miRNAs are differentially expressed between the two cell lines. To confirm the changes in these miRNAs, we obtained miRNA expression profile from LNA (Locked Nucleic Acid) microarray and compared the array data to the 454 sequencing data. On the basis of these two data sets, the expression of several miRNAs was measured by qPCR (Fig. 1A). The data are summarized in Table 1. In general, the qRT-PCR results are more consistent with the microarray data than with 454 sequencing data, probably because both qRT-PCR and microarray are hybridization-based methods.

miR-100, -125b, -19b, and miR-99a were the most downregulated miRNAs in C4-2 relative to LNCaP. miR-20a, -106a, -99b, -21, and miR-16 were modestly decreased in C4-2. In contrast, miR-9, -557, and -196b were the most upregulated in C4-2 relative to LNCaP (with more than 2-fold changes confirmed by at least two methods). The differential expression of these miRNAs between LNCaP and the more advanced C4-2 lead us to hypothesize that the change of these miRNAs may be important in the progression of prostate cancer.

Confirmation of miRNA changes in other prostate cell line model and cancer tissue

To further test whether the changes in the expression level of the miRNAs seen in C4-2 and LNCaP are correlated with the progression of prostate cancer, we measured miRNA expression by qRT-PCR in the immortalized prostate epithelial cell RWPE-1 and the invasive cancer cell line WPE1-NB26 derived from RWPE-1. miR-125b and members of miR-99

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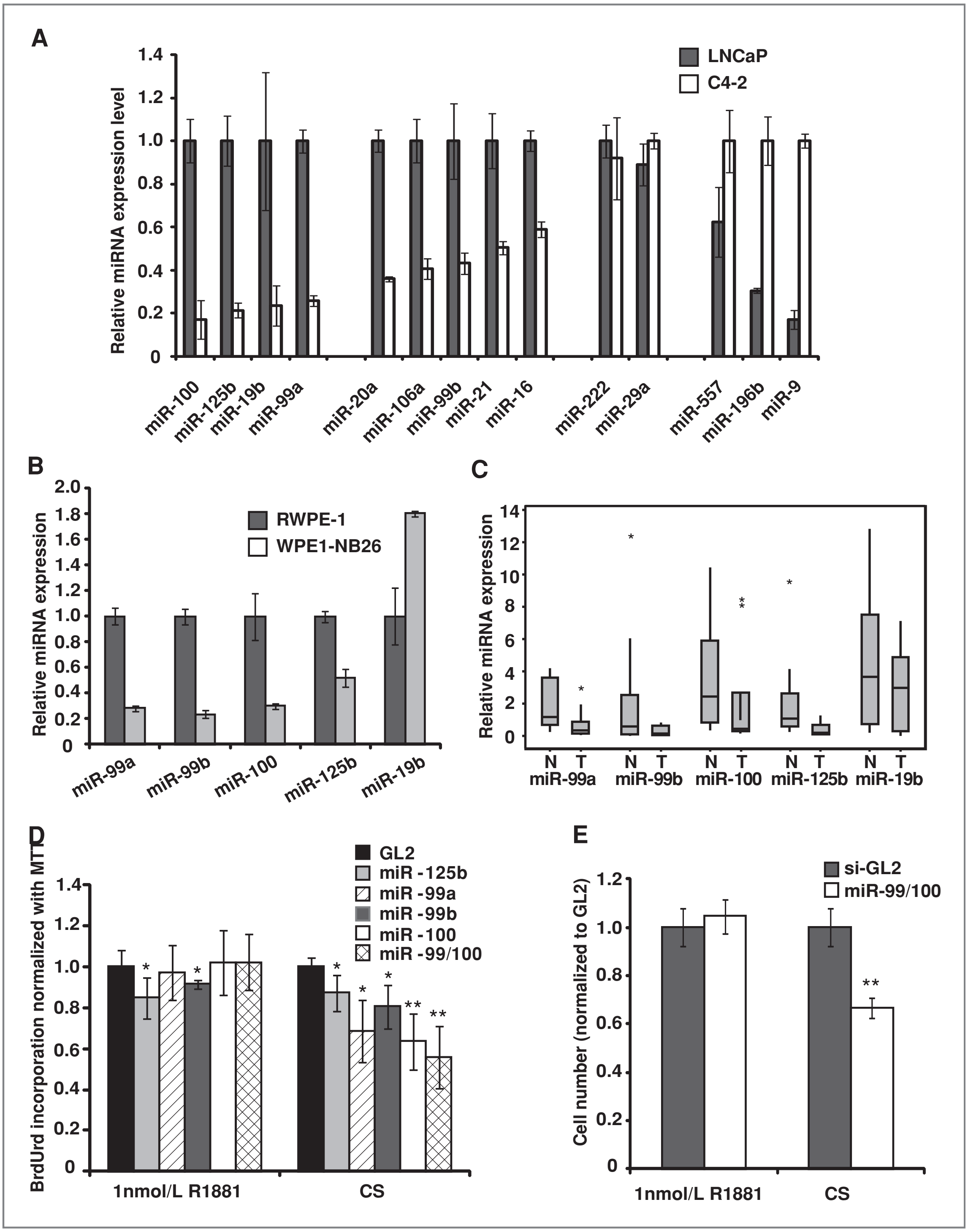

Figure 1. Identifying miR-99a family as potential tumor suppressors. A and B, qPCR was used to measure the expression level of miRNAs. The value was normalized to that of snU6. The mean and SD from triplicate samples are indicated. C, the expression of 5 miRNAs was measured by qPCR in 10 human normal prostate tissue samples (N) and 10 human prostate tumor samples (T). The value was normalized to that of snU6. The data are presented as a box-plot showing quartiles and the median (Minitab). The vertical line indicates the range. Asterisks indicate outliers, which are beyond the outer quartile by more than 3 times the interquartile range (Minitab). The *P* values between normal and tumor for miR-99a, -99b, -100, -125b, and -19b are 0.0086, 0.11, 0.012, 0.015, and 0.13, respectively, (excluding outliers). D and E, on transfection of indicated miRNAs or si-GL2, C4-2 cells were cultured in charcoal-stripped serum with or without 1 nmol/L R1881. D, after 72 hours, BrdUrd incorporation was measured and normalized to cell density from MTT assay (*y*-axis). The mean and SD from triplicate samples are shown. The value of si-GL2 is set as 1. *, $P < 0.05$ difference from si-GL2; **, $P < 0.01$. E, after 72 hours, cell number was counted in a hemacytometer. The mean and SD from triplicate samples are shown. **, $P < 0.01$ difference from si-GL2.

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family (miR-99a, miR-99b, and miR-100) also exhibited a significant decrease in WPE1-NB26 compared with RWPE1 cells (Fig. 1B).

To evaluate whether the differential expression of miRNAs in the cell lines was also seen in human tumor specimens, we conducted miRNA qRT-PCR on 10 human prostate tumor samples and 10 normal prostate tissue samples. miR-125b and miR-99 family members were significantly decreased in the human prostate tumor samples compared with normal tissue (Fig. 1C). Our data are consistent with another study, in which miR-125b and the miR-99 family were decreased in prostate cancer compared with normal tissue (Supplementary Table S3; refs. 18, 19). Therefore, miR-125b and miR-99 family members may play an important role during the genesis and progression of prostate cancer.

miR-99 family as potential tumor suppressors

Having observed a decrease of miR-99 family and miR-125b in human prostate cancer cells relative to normal prostate tissue, we tested whether these miRNAs affect the proliferation of prostate cancer cells. We transfected these miRNAs in C4-2, in which their initial expression was low, and measured the growth of cells by BrdUrd incorporation assay and counting cell numbers. Unlike miR-125b, transfection of miR-99a, -99b, or -100 inhibits the growth of C4-2 cells more markedly in the absence of androgen (CS) than in the presence of 1 nmol/L synthetic androgen R1881 (Fig. 1D and E). This inhibition of androgen-independent growth by the miR-99 family requires the presence of androgen receptor (AR), as the miR-99 family does not affect the growth of PC3 and Du145 cells (Supplementary Fig. S2). Thus, the reduction of miR-99 family, seen during the progression from LNCaP to C4-2, could provide a growth advantage under androgen-depleted condition. This result encouraged us to identify relevant target genes that are regulated by the miR-99 family.

Identification of targets by bioinformatics and microarray

miR-99a, miR-99b, and miR-100 belong to the same family with a shared seed sequence (nt 2–7 of the miRNA) which is known to be the critical determinant in recognition of target mRNAs (Supplementary Fig. S3A). Therefore, miR-99 family members are predicted to target a common list of genes according to the computational target prediction program TargetScan. We employed 2 filtering methods to obtain a shorter list of potential targets of miR-99 family. Intersection of mRNAs downregulated by the miRNA with *in silico* predicted targets was previously shown to yield a significantly shorter list containing *bona fide* targets (12). We conducted a microarray analysis to detect mRNAs decreased after transfection of miR-99a compared with control siRNA (si-GL2) in C4-2 cells. Among the hundreds of targets predicted by TargetScan, 19 were downregulated by at least a third with miR-99a (Table 2).

Filtering targets by polyribosome/monoribosome loading

miRNAs regulate gene expression at posttranscriptional stage. The mechanisms include blocking translational initiation, ribosome loading, or translational elongation (20–24). Therefore, we reasoned that targets of a miRNA would shift from polyribosome to monoribosome fractions if the miRNA blocks translation initiation. To test this, we first measured the ribosome profile of 3 validated targets of miR-206 (DNA Polα, MMD, and CX43; Supplementary Fig. S3B; ref. 12). Compared with the control transfection, miR-206 induced significant accumulation of all the 3 mRNAs in the monoribosome fraction (Supplementary Fig. S3C), encouraging us to add this assay to our filters.

We tested 8 of 19 genes in Table 2 by the ribosome fractionation assay before and after miR-99a transfection. These genes were selected on the basis of previous literature, implicating their involvement in prostate cancer. On the transfection of miR-99a, all 8 genes were accumulated in the monosome fraction (Table 2). Among these genes, *SMARCA5*, *SMARCD1*, *PPFIA3*, and *FRAP1/mTOR* exhibited more than 5-fold accumulations in the monoribosome fraction. For comparison, their mRNA levels were reduced by about 2-fold after introduction of miR-99a (Table 2). Consistent with the decreased loading of ribosomes onto these mRNAs, the protein levels of all 4 genes were all decreased by 1 or more members of the miR-99 family (Fig. 2A and B). Thus, these 4 genes are likely to be direct targets of miR-99 family and were further tested in the following experiments.

Confirming targets as directly repressed by miR-99 family in luciferase reporter assay

TargetScan predicted one recognition site of miR-99 family in the 3′-UTR region of SMARCD1, SMARCA5, mTOR, and PPFIA3 (Supplementary Fig. S4A). We inserted the 3′-UTR fragments downstream of luciferase open reading frame (ORF) in a reporter plasmid to test whether they are directly repressed by the miR-99 family. For FRAP1/mTOR, SMARCA5, and SMARCD1, the 3′-UTRs conferred repression of the heterologous luciferase ORF after transfection of miR-99a, miR-99b, or miR-100 (Fig. 2C–E). In all 3 cases, the repression by miR-99 family was abolished when we mutated the predicted target sites (Supplementary Fig. S4B; Fig. 2C–E). In case of PPFIA, we did not observe any significant reduction of luciferase expression by miR-99 family (Fig. 2F). Thus, the reduction of PPFIA3 mRNA and protein by miR-99 family (Table 2 and Fig. 2A and B) was either due to an indirect effect or due to a target site in the ORF.

Together with the data from mRNA expression microarray, ribosome profiling, and protein measurements, the luciferase results clearly show that FRAP1/mTOR, SMARCA5, and SMARCD1 are direct targets of miR-99 family.

The miR-99 family decreases expression of PSA

miR-99a, -100, and -125b were downregulated in C4-2 relative to LNCaP. Interestingly, the expression of these 3 miRNAs was repressed by an androgen analogue, R1881, in LNCaP cells in a dose-dependent manner (Fig. 3A). As a positive control for androgen activity, we checked that R1881 stimulated the level of PSA mRNA, an androgen-responsive gene (Supplementary Fig. S6A). All 3 miRNAs are repressed by androgen and during the progression of prostate

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Table 2. Genes downregulated at mRNA level and/or blocked at translational initiation by miR-99a

Target gene	Gene name	miR-99a/si-GL2 (micorarray)	miR-99a monosome/polysome / si-GL2 monosome/polysome
TRIB1	Tribbles homolog 1 (*Drosophila*)	0.17	1.41
HOXA1	Homeobox A1	0.28	2.74
INSM1	Insulinoma-associated 1	0.29	
ADCY1	Adenylate cyclase 1 (brain)	0.42	
CTDSPL	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase-like	0.49	
PPFIA3	Protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 3	0.5	5.29
SMARCA5	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 5	0.51	6.83
SMARCD1	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily d, member 1	0.51	6.5
KBTBD8	Kelch repeat and BTB (POZ) domain containing 8	0.52	
C4orf16	Chromosome 4 open reading frame 16	0.52	
FRAP1	FK506 binding protein 12-rapamycin associated protein 1/mTOR	0.54	5.31
SLC44A1	Solute carrier family 44, member 1	0.57	
BMPR2	Bone morphogenetic protein receptor, type II (serine/threonine kinase)	0.61	
BAZ2 A	Bromodomain adjacent to zinc finger domain, 2 A	0.65	
ICMT	Isoprenylcysteine carboxyl methyltransferase	0.66	3.64
MBNL1	Muscleblind-like (*Drosophila*)	0.66	
FZD8	Frizzled homolog 8 (*Drosophila*)	0.66	3.34
C1orf34	Chromosome 1 open reading frame 34	0.67	
ZZEF1	Zinc finger, ZZ-type with EF-hand domain 1	0.67	

cancer, which suggests that prostate cancer progression is accompanied by the cells spontaneously phenocopying the effect of androgen. It is also possible that the reduction of these miRNAs in C4-2 relative to LNCaP may be due to hyperactivation and/or constitutive activation of the AR in C4-2, and conversely, these miRNAs may play an active role in androgen refractoriness in C4-2.

To measure the androgen-response on modulation of the miR-99 family, miR-99a, -99b, and -100 duplex were transfected to C4-2 cells in the presence of 1 nmol/L R1881. PSA is an androgen-responsive secreted protein and important marker for prostate cancer detection. Its secretion was measured by ELISA and normalized to cell numbers assessed by MTT assay. When miR-99 family was ectopically expressed in C4-2, the PSA level was significantly repressed to the level of LNCaP (Fig. 3B). Conversely, the PSA level was upregulated in LNCaP cells on inhibition of miR-99 family by treating with 2′-*O*-methyl antisense oligonucleotides against them (Fig. 3B). To test whether the change in the secreted PSA level was due to the impaired AR activity, we tested the mRNA expression of 2 AR-regulated genes *PSA* and *SARG*. We observed a similar decrease in mRNA level of both PSA and SARG after transfection of miR-99 family miRNAs (Fig. 3C; Supplementary Fig. S6B). The protein level of PSA also showed a decrease after overexpression of miR-99/100 (Fig. 3D). We next tested whether repression of the targets of these miRNAs phenocopied the effects of the miRNAs. The siRNA-mediated knockdown of SMARCD1 or SMARCA5 in C4-2 cells specifically decreased the PSA protein without affecting the mRNA level, suggesting a posttranscriptional regulation on PSA expression (Fig. 3B–D). Knockdown of mTOR by siRNA decreases both the PSA mRNA and the protein level (Fig. 3B–D). Thus, repression of these targets could contribute to PSA repression by the miR-99 family, though the chromatin-remodeling factors SMARCD1 and SMARCA5 seem to be required for the expression of PSA protein at a posttranscriptional step. To test which of the 3 targets was rate limiting after miR-99/100 expression, we ectopically expressed the ORF of each of the 3 targets and then transfected the miRNAs of the miR-99 family. The absence of the 3′-UTRs makes these exogenous genes resistant to the repression by the miR-99 family. Ectopic expression of the ORF region of SMARCA5 alone rescued the repression of miR-99/100 on the PSA protein level (Fig. 4A). miRNA-resistant SMARCD1 or mTOR expression did not rescue the effect of miR-99 family on PSA expression (Fig. 4A; Supplementary Fig. S6C and D). Additional targets of miR-99 family may contribute to the selective repression of AR activity by miR-99 family, as none of the 3 identified targets rescued the repression of PSA mRNA (Supplementary Fig. S6C and D). Our results suggest that loss of miR-99 family affects

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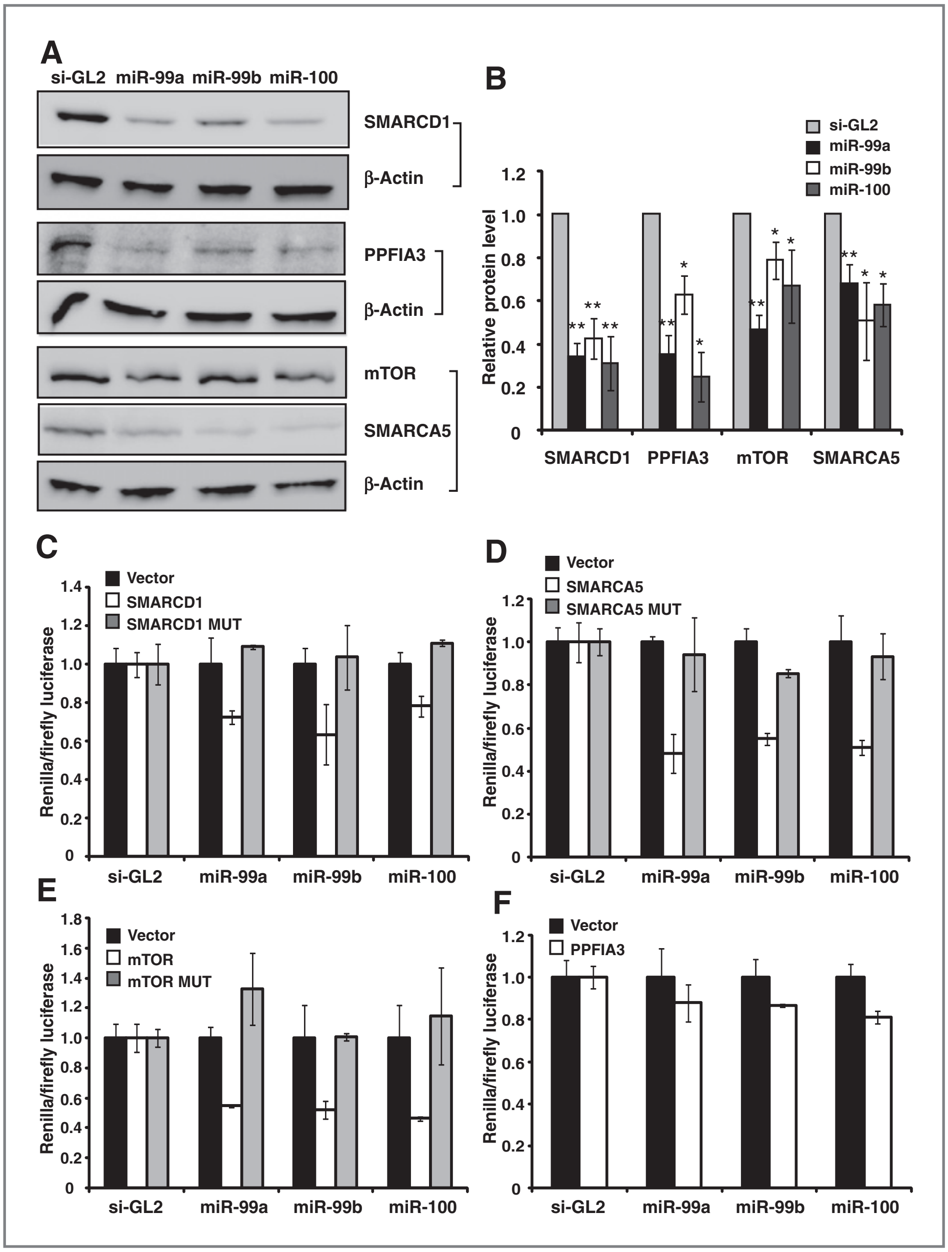

Figure 2. Confirming 3 direct targets of miR-99 family. A, Western blot was used to detect changes of 4 target proteins after transfecting miR-99a, -99b, or -100 in C4-2 cells. β-Actin was used as a loading control. See Supplementary Figure S7 for full Western blot. B, Western blot quantification. The level of the indicated protein is normalized to β-actin. The mean and SEM (error bar) of Western blots are presented. *, $P < 0.05$; **, $P < 0.01$. C–F, luciferase assay was carried out with control luciferase vector, vector with 3′-UTR of 4 targets (indicated by gene names), or 3′-UTR with mutation in the predicted target sites (indicated by MUT). The ratio of the Renilla luciferase to firefly luciferase (transfection control) was normalized to that in the si-GL2 transfection.

AR-driven gene expression, particularly the expression of *PSA* at both mRNA and protein level (Fig. 4B). The derepression of SMARCA5 by the decrease of miR-99 family in C4-2 clearly contributes to the elevated expression of PSA in this more advanced prostate cancer cell line.

Discussion

In the last few years, the miRNA expression profiles have been studied by several groups in prostate cancer cell lines and clinical samples, mainly using miRNA microarrays and qPCR analysis (4, 11, 19, 25–28). In this study, we applied cloning and deep sequencing method, besides the LNA-based miRNA microarray to profile the miRNAs. We also avoided heterogeneity between prostate cancer samples by first doing the comparison between the two cell lines LNCaP and C4-2 and then following the validated changes in other cell lines and human tumors. On the basis of this conservative strategy with multiple iterative loops, we determined that the miR-99 family members (miR-99a, -99b, and -100) were decreased in most advanced prostate cancer, relative to normal prostate epithelium. Our results were supported by other profiling studies in which miR-99a and miR-100 were shown to be reduced in prostate carcinomas and more aggressive and metastatic prostate tumors (27). The consistent changes of these miRNAs in prostate cancer in several independent studies suggest that decrease of miR-99 family is a signature for the genesis and progression of prostate cancer. Moreover, a

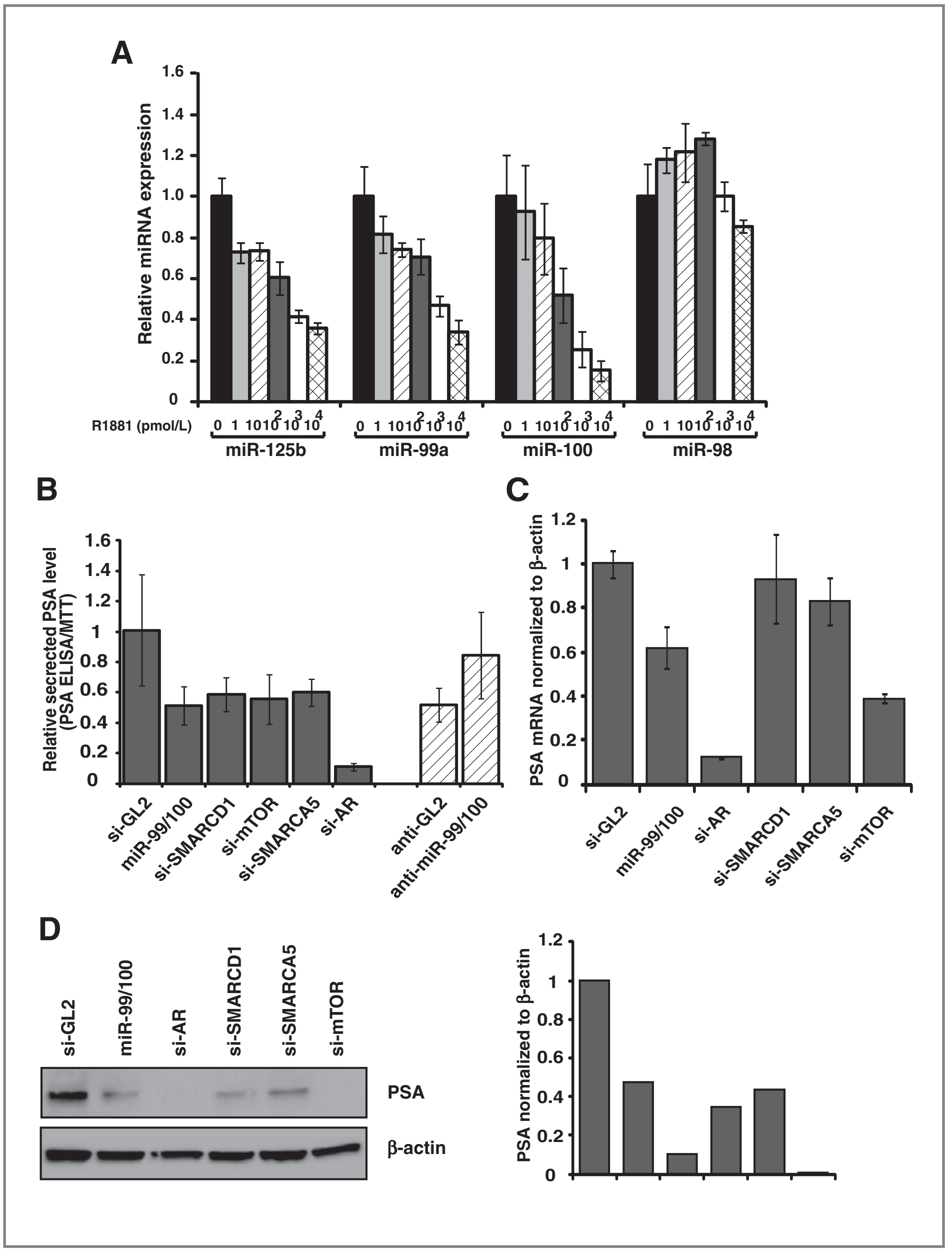

Figure 3. miR-99 family decreases PSA level. A, qPCR assays of selected miRNAs after treating LNCaP with the androgen analogue R1881 at indicated concentrations. The miRNA level at no R1881 is set as 1. B, 72 hours after transfection of indicated siRNAs, miR-99 family, or si-GL2 in C4-2 cells (first 6 bars), or transfection of 2′-*O*-methyl antisense oligonucleotide in LNCaP cells (the last 2 bars), PSA ELISA was measured using the culture supernatant and normalized to cell density from MTT assay. The average and SD from triplicate samples are shown. C, qRT-PCR was used to determine the mRNA of PSA after transfecting the indicated miRNA or siRNA in C4-2 cells. Results were normalized to β-actin. D, Western blot of PSA was carried out in C4-2 cells after transfection of indicated miRNA or siRNA. β-Actin was used as a loading control. Western blot quantification: the value is normalized to β-actin.

frequent downregulation of miR-99a and miR-100 was also seen in other types of cancer such as ovarian cancer, lung cancer, and squamous cell carcinoma of tongue (29–31), suggesting the potential involvement of miR-99 family in the genesis and progression of cancers.

Identifying *bona fide* miRNA targets has been a difficult step in studying miRNAs. Bioinformatics methods utilize the sequence complement to identify targets and often produce hundreds of predicted targets. On experimental validation, the majority of the predicted targets seem not to be suppressed significantly by miRNAs, yielding a high false-positive rate. The Bartel group reported that genes whose proteins were repressed more than 50% by miRNAs also exhibit mRNA degradation (32). Therefore, mRNA microarray is widely used to determine the actual targets of the miRNA. In many cases, however, the modest change in mRNA level is not due to a direct degradation by the miRNA and not accompanied by a decrease in protein. In this study, we thus added a polyribosome fractionation method to filter targets that were repressed at translational initiation stage. Binding of the miRNA to the 3′-UTR of the target mRNA shifted the majority of target mRNAs from polyribosome to monoribosome fraction without affecting the nontarget mRNAs (23, 24). By using this method, we focused on 4 genes *SMARCD1*, *SMARCA5*, *mTOR*, and *PPFIA3*. Of the 4 genes we tested further by luciferase reporter assay, 3 proved to be direct targets of the miR-99 family. The exception, *PPFIA3*, was decreased at the protein level by the miR-99 family but not in the luciferase

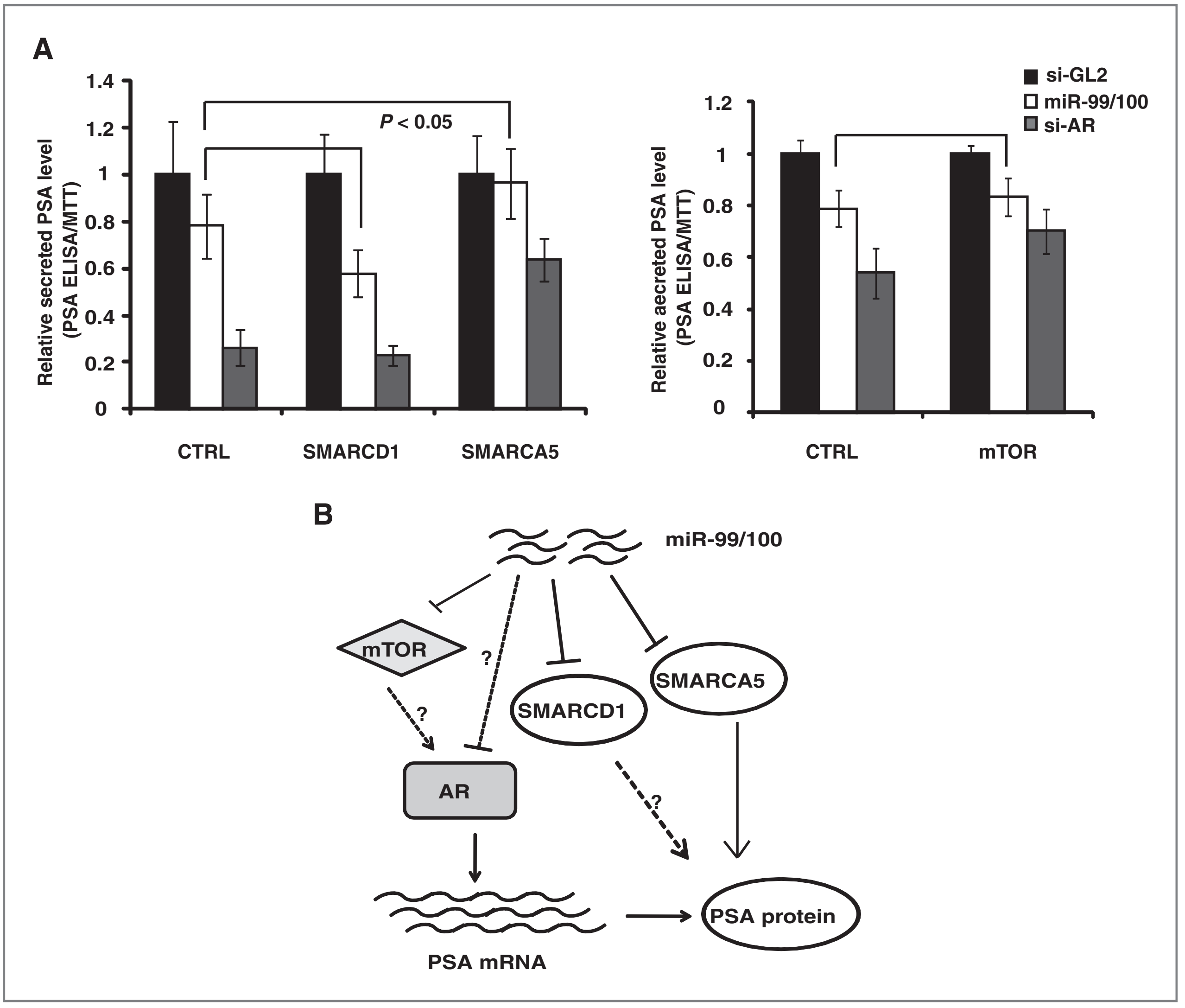

Figure 4. SMARCA5 rescues the miR-99 family–induced reduction of PSA. A, PSA ELISA assay was carried out using culture supernatant of C4-2 cells stably expressing miRNA-resistant form of SMARCD1, SMARCA5, or mTOR 72 hours after transfection of indicated miRNA or siRNA. Square brackets, *P* values of the differences: <0.05 or >0.05 (unlabeled). CTRL, control. B, schematic to show the regulation of miR-99 family on AR and PSA.

reporter with the 3′-UTR of PPFIA3. A potential explanation is that the target site of the miRNA is in the ORF of the gene. Indeed, the ORF region has a site with perfect match with the seed sequence of miR-99a and miR-100, which could be the primary binding site of miR-99 family members responsible for reducing PPFIA3 mRNA and protein. By adding the polyribosome fractionation screen, we were able to increase the true positive rate of candidate genes to more than 75%. A large-scale screening of candidate genes by subjecting the RNAs from polyribosome and monoribosome fractions to sequencing or microarray would be very useful to identify target genes of miRNAs in a future study.

Hyperactivity of AR is one of the reasons that prostate cancer cells become androgen independent (5, 6, 33). C4-2 cells possess a higher activity of AR and respond to much lower concentration of androgen than in LNCaP cells (34, 35). Restoring the level of miR-99 family members significantly reduced the AR activity in C4-2 cells, as measured on the *PSA* and *SARG* promoter. Thus, repression of the miR-99 family may promote the hyperactivity of AR, which may, in turn, lead to the androgen independence of advanced prostate cancer. The growth effect of the miR-99 family in prostate cancer cells was tested by ectopically expressing them in C4-2 cells, in which their initial expression level was low. Restoring the expression of miR-99 family members reduced the cell growth in androgen-depleted media, suggesting a potential tumor-suppressive role of miR-99 family in prostate cancer cells. The targets through which miR-99 family inhibits cell proliferation are not clear yet and need to be further examined. Taken together, the miR-99 family represses both AR responsiveness and cell growth in the absence of androgen. This raises the possibility of treating prostate cancer with antiandrogen along with gene therapy vectors overexpressing the miR-99 family of miRNAs.

PSA is a serine protease belonging to the kallikrein family. As an androgen-regulated gene, it has been used as a biomarker for prostate cancer diagnosis. Previous studies showed that PSA might assist the invasion of prostate cancer cells by degrading the extracellular matrix components fibronectin and laminin (36). PSA is also known to release IGF-I (insulin like growth factor I) and TGF-β from their binding partners and thus involved in osteoblastic lesions (37). C4-2 has higher metastatic capacity than LNCaP in the mouse model (38). Intrafemoral injection of C4-2 forms PSA-producing osteoblastic tumors (38). In this study, we found that miRNAs of miR-99 family decrease PSA expression at both mRNA and protein levels. A decrease of miR-99 family may thus contribute to the elevation of PSA production in C4-2 compared with LNCaP, suggesting the potential involvement of miR-99 family in the bone metastasis of prostate cancer. This, of course, will need experimental testing in the future. Although siRNA against mTOR also decreased the PSA mRNA level, the miRNA-resistant forms of none of the 3 targets rescued PSA mRNA level (Supplementary Fig. S6C and D). Thus, at least one other unknown target of miR-99/100 must be important for decreasing AR activity at the *PSA* promoter.

SMARCA5 (hSNF2H) is a member of SWI/SNF family, containing helicase and ATPase activities. As part of a chromatin-remodeling complex, it facilitates ATP-dependent nucleosome remodeling and transcription initiation (39). It is overexpressed in ovarian cancer and promotes tumor growth in ovarian cancer through interacting with remodeling and spacing factor 1 (Rsf1; ref. 40). In this article, we showed that as a direct target of miR-99 family, SMARCA5, regulates the PSA protein level, which contributes to the elevated expression of PSA in C4-2 cells. No direct interaction between SMARCA5 and AR, or effect of SMARCA5 on translation or protein stability, has been shown. Our data reveal a novel mechanism in which SMARCA5 seems to regulate the expression of PSA posttranscriptionally in prostate cancer cells. Most likely, this is through the regulation of expression of genes whose products are involved in the translation and/or stability of PSA protein.

SMARCD1 (BAF60a) is a member of SWI/SNF family of proteins and known to interact with the ligand-binding domain of AR through its FxxFF motif in an androgen-dependent manner (41). It is also known to interact with glucocorticoid receptor and provides the docking site for chromatin-remodeling BRG1 complex (42). SMARCD1 is repressed by hepatocyte-specific miRNA miR-122 (43). In this article, we showed that the miR-99 family also represses the expression of SMARCD1 at both mRNA and protein levels. SMARCD1 is required for PSA protein expression but is not sufficient by itself to restore PSA expression in miR-99/100 overexpression cells.

FRAP1 (mTOR) is a phosphatidylinositol kinase–related kinase known to mediate cellular responses to growth factors and regulate cell proliferation, metabolism, and angiogenesis. AKT/mTOR signaling was shown to be important during the development of androgen independence of prostate cancer. Inhibition of mTOR along with antiandrogen additively represses the prostate tumor growth in PTEN-null mouse, suggesting there might be cross-talk between mTOR and AR pathways (44). mTOR promotes translation through phosphorylation of S6K1 and 4EBP1, which may also enhance the expression of AR-responsive genes. Several studies suggest that the inhibition of mTOR combined with antiandrogen could be useful in prostate cancer treatment (45, 46). The repression of mTOR signaling by miR-100 was previously reported in clear cell ovarian cancer model (47). In this study, we showed that mTOR is repressed not only by miR-100 but also by its family members miR-99a and miR-99b. mTOR is required for the expression of PSA mRNA (Fig. 3B) but not for maintaining AR levels (Supplementary Fig. S5A). It will be interesting to elucidate how mTOR impact on AR activity at the *PSA* promoter.

In summary, we implicated that the miRNAs of miR-99 family are repressed and 3 validated targets are derepressed during the genesis and progression of prostate cancer. We also showed that miR-99 family represses AR activity and independently repress PSA protein level with SMARCA5 (Fig. 4B). The consistent decrease of miR-99 family in the human prostate tumors increases the possibility of using them as a signature of prostate cancer progression. Our study also underlines the possible treatment of prostate cancer by restoring the level of miR-99 family members. Finally, in pursuing the targets of these tumor-suppressive miRNAs, we make the exciting discovery that the SMARCA5 chromatin-remodeling factor is important for posttranscriptional regulation of a metastatic factor, PSA.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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