Epigenetic Repression of miR-31 Disrupts Androgen Receptor Homeostasis and Contributes to Prostate Cancer Progression

Pei-Chun Lin1,4, Ya-Lin Chiù2, Samprit Banerjee2, Kyung Park1, Juan Miguel Mosquera1, Eugenia Giannopoulou3,5, Pedro Alves8, Ashutosh K. Tewari6, Mark B. Gerstein8, Himisha Beltran7, Ari M. Melnick7, Olivier Elemento3,5, Francesca Demichelis1,5,9, and Mark A. Rubin1,4

Abstract

Androgen receptor signaling plays a critical role in prostate cancer pathogenesis. Yet, the regulation of androgen receptor signaling remains elusive. Even with stringent androgen deprivation therapy, androgen receptor signaling persists. Here, our data suggest that there is a complex interaction between the expression of the tumor suppressor miRNA, miR-31, and androgen receptor signaling. We examined primary and metastatic prostate cancer and found that miR-31 expression was reduced as a result of promoter hypermethylation, and importantly, the levels of miR-31 expression were inversely correlated with the aggressiveness of the disease. As the expression of androgen receptor and miR-31 was inversely correlated in the cell lines, our study further suggested that miR-31 and androgen receptor could mutually repress each other. Upregulation of miR-31 effectively suppressed androgen receptor expression through multiple mechanisms and inhibited prostate cancer growth in vivo. Notably, we found that miR-31 targeted androgen receptor directly at a site located in the coding region, which was commonly mutated in prostate cancer. In addition, miR-31 suppressed cell-cycle regulators including E2F1, E2F2, EXO1, FOXM1, and MCM2. Together, our findings suggest a novel androgen receptor regulatory mechanism mediated through miR-31 expression. The downregulation of miR-31 may disrupt cellular homeostasis and contribute to the evolution and progression of prostate cancer. We provide implications for epigenetic treatment and support clinical development of detecting miR-31 promoter methylation as a novel biomarker. Cancer Res; 1–13. ©2012 AACR.

Introduction

Prostate cancer represents a major public health problem among the aging Western population. It has the highest incidence rate of all noncutaneous malignancies in men, accounting for more than 241,000 new cases and 29,000 deaths in the United States in 2012 (1). Prostate cancer depends largely on androgen receptor (AR) signaling for growth and maintenance. Following the seminal observations by Huggins and Hodges over 60 years ago that prostate cancer responded dramatically to castration, androgen deprivation therapy (ADT) has become the standard first-line treatment for advanced hormone-naïve prostate cancer (2, 3). By reducing circulating androgen, ADT prevents signaling through androgen receptor and limits cancer growth. Unfortunately, the beneficial effect of ADT is short-lived and patients progress to castration-resistant prostate cancer (CRPC). The continued dysregulation of androgen receptor signaling in the face of ADT has been attributed to the acquisition of amplified or mutated androgen receptor; recent work using next-generation sequencing (NGS) suggests that androgen receptor gene amplification and mutations occur in up to 44% of CRPCs; 24% with copy number gain and 20% with point mutation (4). Perhaps the most important recent finding came when Chen and colleagues discovered that androgen receptor signaling persists under stringent ADT and that androgen receptor antagonists act as agonists at high androgen receptor levels (5). While these observations have led to the development of more efficacious therapeutic approaches for targeting androgen receptor signaling (6), CRPC still persists after treatment; therefore, other interventions are needed for androgen receptor regulation.

Epigenetic aberrations arise during prostate cancer initiation and disease progression, which include promoter...
guanine (CpG) island hypermethylation at specific gene loci and changes in chromatin structure (7). Promoter hypermethylation at certain genes, such as glutathione-S-transferase gene (GSTP1), has been proposed as a biomarker for early detection and prognosis of prostate cancer (8). Dysregulation of miRNAs also occurs during prostate cancer pathogenesis (9). MiRNAs are small noncoding RNA molecules that simultaneously regulate the expression of multiple genes by dete

tion and repression of transcription, miRNA stability and/or interrupting translation. As miRNAs are involved in critical cellular functions in a tissue-specific manner, aberrant expression of miRNAs can contribute to tumorigenesis by inducing oncogenes, inhibiting tumor suppressor genes, or disrupting important signaling pathways (10). While silencing miRNAs with tumor suppressor features by DNA hypermethylation is linked to human cancer, little is known about the association between DNA methylation, miRNA expression, and androgen receptor signaling. We sought to examine the mechanism behind androgen receptor-mediated regulation of miRNAs. In this study, we report a novel role for miR-31 in prostate cancer and show that hypermethylation at the miR-31 promoter occurs in a prostate cancer-specific manner, the extent of which correlates with disease progression, androgen receptor regulates miR-31 expression, and miR-31 directly targets androgen receptor and other cell-cycle regulators and represses prostate cancer growth.

Materials and Methods

Benign and prostate cancer tissue selection

All tissue samples were collected as part of an Institutional Review Board-approved protocol at Weill Cornell Medical College (WCMC; New York, NY), and informed consents were received from participants before inclusion in this study. Hematoxylin and eosin (H&E) slides were prepared from frozen tissue blocks and evaluated for cancer extent and tumor grade by the study pathologists (M.A. Rubin/K. Park/J.M. Mosquera), and 1.5-mm biopsy cores of desired regions were taken from frozen tissue blocks for RNA/DNA extraction. For more details, see Supplementary Methods.

MiRNA profiling

Asuragen Inc. processed samples for miRNA profiling studies according to the company’s standard operating procedures. Total RNA (100 ng) from each sample was run with GeneChip miRNA Array (Affymetrix). The two-sample Wilcoxon rank-sum test was applied to evaluate the difference between prostate cancer and benign tissues. False discovery rate (FDR) control was used in multiple hypotheses testing to correct for multiple comparisons. The miRNAs with significant changes were chosen based on adjusted $P < 0.05$. To make the selection more stringent, fold change more than 1.5 and difference more than 100 were applied.

Quantitative DNA methylation analysis by MassARRAY EpiTYPER

Measurement of DNA methylation levels was conducted at WCMC Epigenomics core facility by matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF) mass spectrometry (MS) using EpiTYPER assays by MassARRAY (Sequenom) on bisulfite-converted DNA according to the manufacturer’s protocol. For EpiTYPER primer sequences and analysis, see Supplementary Methods.

Quantitative real-time PCR

cDNA synthesis was carried out using the M-MuLV Reverse Transcriptase (Enzymatics) according to the manufacturer’s protocol. Quantitative real-time PCR (qPCR) was carried out with the Roche LightCycler 480 with SYBR Green I Master Mix or Probe Master Mix for TaqMan Assay (Roche). Each sample was run in triplicate for every experiment. TaqMan MicroRNA Assays (Life technologies) were used to quantify mature miRNA expression, carried out with TaqMan MicroRNA Reverse Transcription Kit, hsa-miR-31 (AB Assay ID: 002279), and RNU6B (AB Assay ID: 001093) according to the manufacturer’s protocol. Primer sequences are listed in Supplementary Methods.

Cell lines

The benign prostate epithelial cell line, RWPE-1, and prostate cancer cell lines, VCaP, LNCaP, 22Rv1, PC3, DU145, and HEK293, were purchased from American Type Culture Collection (ATCC) and used within 6 months after receipt; authentication of cell lines was conducted by ATCC. PC3-neo and PC3-AR cell lines were kind gifts from Dr. David M. Nanus (WCMC) and LNCaP-abi cell line was a kind gift from Dr. Myles Brown (Harvard University, Cambridge, MA); they were characterized by short-tandem repeat profiling by Genetica DNA Laboratories Inc. and authenticated. Cells were maintained according to manufacturer and providers’ protocols.

Small RNA interference and miRNA transfection

Cells were treated with DharmaFECT2 transfection reagent (Dharmacon) for RNA interference and microRNA transfection, according to the manufacturer’s protocol: non-targeting siRNA (D-001810-01), siRNA specific to EZH2 (11), androgen receptor (L-003400), miR-31 (C-300507-05), miR-31 inhibitor (IH-300507-06), miR mimic Negative Control/NC (CN-001000-01), and miR inhibitor NC (IN-001005-01).

Chromatin immunoprecipitation

LNCaP cells were grown in phenol red-free RPMI-1640 media supplemented with 5% charcoal-stripped serum for 3 days, then treated with ethanol or 1 nmol/L R1881 for 16 to 24 hours. For detailed description of methodology, see Supplementary Methods.

miRNA reporter luciferase assays

LNCaP cells were transfected in triplicate with 30 nmol/L miR-31 or control miRNA-NC mimic together with psiCHECK2 vector (Promega: 0.4 μg/well, 24-well plate) containing 21-bp miRNA recognition elements (MRE) or the 3’untranslated region (UTR) region containing the MREs of indicated genes by DharmaFECT Duo transfection reagent, according to the manufacturer’s protocol (Dharmacon). After 48 hours, cells were lysed and luciferase activity was measured using the Dual
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### Figure A

Heatmap showing expression levels of miRNAs in benign and PCA tissues.

### Figure B

Graph showing relative miR-31 levels in PCA vs benign tissues.

### Figure C

Bar graph comparing relative miR-31 levels in benign and PCA tissues.

### Figure D

Volcano plot illustrating significant differences in miR-31 levels between benign and PCA tissues.

### Figure E

Box plot showing overall miR-31 promoter DNA methylation in benign and PCA tissues.

### Figure F

Bar graph showing overall miR-31 promoter DNA methylation in different conditions.

### Figure G

Bar graph showing overall miR-31 promoter DNA methylation in different conditions.

### Figure H

Graph showing relative miR-31 level (folds) in different cell lines.

### Figure I

Graph showing effects of DMSO and 5-aza-dC on overall miR-31 promoter DNA methylation.

### Figure J

Graph showing effects of GS and METs on overall miR-31 promoter DNA methylation.

### Figure K

Graph showing effects of GS and METs on relative miR-31 level (folds) in different conditions.
Luciferase Assay System (Promega) and GloMax-Multi Detection System (Promega). Data were normalized to firefly luciferase. Individual wild-type and mutant MREs were cloned into psiCHECK2 vector as previously described (12). psiCHECK2-E2F1 3′UTR was a kind gift of Dr. Judy Lieberman (Addgene plasmid 29468; Harvard University). Site-directed mutagenesis was carried out by the QuikChange Site-Directed Mutagenesis Kit (Agilent). Primer sequences are shown in Supplementary Methods.

Prostate tumor xenograft model
All procedures involving mice were approved by the Institutional Animal Care and Use Committee at WCMC and were in compliance with regulatory standards. For detailed description of methodology, see Supplementary Methods.

Data analysis and statistical methods
Statistical analysis of expression data was conducted with GraphPad Prism 4.0 (Graph Pad software). Two-sided P < 0.05 was considered statistically significant.

Accession number
All microarray data are deposited in the GEO database under accession number GSE36803.

Additional methods
Detailed methodology is described in the Supplementary Methods.

Results
miR-31 expression is suppressed in prostate cancer
Global miRNA expression profiling in prostate cancer has been conducted previously with highly variable results from study to study (13). Newly discovered miRNAs and improved detection platforms prompted us to re-examine this topic. Using Affymetrix microarray technology, we interrogated 21 pairs of primary prostate cancer and matched benign prostate tissue. One hundred and five miRNAs were identified as significantly altered in prostate cancer (FDR-adjusted P < 0.05; Supplementary Fig. S1A and Supplementary Table S1), including 25 miRNAs with at least 1.5-fold expression change (Fig. 1A; Supplementary Table S2). Consistent with the study by Schafer and colleagues who had used matched samples (14), our data showed upregulation of miR-182 and miR-375 and downregulation of miR-31, miR-145, miR-205, miR-221, and miR-222 in prostate cancer.

Aberrant miR-31 expression has been reported in various cancer types, including adult T-cell leukemia (ATL), bladder cancer, breast cancer, colon cancer, gastric cancer, lung cancer, serous ovarian cancer, and urothelial carcinoma, suggesting its involvement in tumorigenesis and cancer progression (15–17). We thus focused on miR-31, as its role in prostate cancer disease progression is largely unknown. We verified miR-31 expression in 14 of the 21 matched pairs, and 93% (13/14) showed decreased miR-31 expression in prostate cancer with respect to matched benign prostate tissue (Fig. 1B). miR-31 is located in the intronic region of its host gene MIR31HG (RefSeq NR_027054). The overall expression of miR-31 and MIR31HG in a cohort of 40 primary prostate cancer specimens was significantly lower than 15 benign prostate tissues (P < 0.0001; Fig. 1C). Taken together, our data showed the downregulation of miR-31 in primary prostate cancer.

Prostate cancer–specific downregulation of miR-31 is mediated by promoter hypermethylation
To delineate the mechanism behind the downregulation of miR-31 in prostate cancer, we first examined whether genomic (i.e., somatic) loss was responsible. miR-31 is adjacent to a region containing CDKN2A/2B, a known hotspot of genomic loss in cancer (Supplementary Fig. S1B). By examining somatic copy number alterations across a variety of tumor types from a previously published dataset (18), we found that prostate cancer did not have any deletion peaks at the MIR31HG locus (Fig. 1D). The genomic area spanning the MIR31HG locus and adjacent genes was deleted in only a small fraction (2%–4%) of individuals with localized prostate cancer. In contrast, genomic regions spanning the same area were frequently deleted in up to 35% of other tumor types (Supplementary Fig. S1C). In another independent prostate cancer dataset, focal deletion at MIR31HG was also rarely observed (19). Altogether, the low rate of somatic copy number losses cannot account for the high frequency of miR-31 downregulation in prostate cancer.

Epigenetic alterations, such as promoter DNA hypermethylation, can result in silencing of miRNA expression. Therefore, we examined whether epigenetic alterations might account for the regulation of miR-31 expression. The promoter region of MIR31HG/miR-31 harbors a CpG island; we evaluated DNA methylation of this region on 12 of the 21 matched samples by a direct quantitative DNA methylation assay (MassARRAY Epityping), with 4 pairs of primers (Supplementary Fig. S1D and Supplementary Table S3; we did not have enough DNA for the remaining 9 samples). We found that the miR-31 promoter showed cancer-specific hypermethylation (P < 0.001; Fig. 1E).

Figure 1. miR-31 is downregulated in prostate cancer due to promoter hypermethylation. A, heatmap of the 25 differentially expressed miRNAs in prostate cancer (PCA) as compared with matched benign tissues (Benign). Red, high expression; green, low expression. B, expression ratio of miR-31 in PCA to matched benign; red line for ratio 1. C, expression of miR-31 and MIR31HG in 40 prostate cancer and 15 benign as evaluated by qPCR. D, deletion analysis of chromosome region 9p21.3 in various cancer types; gray indicates genes that fall within the deletion peak. E, DNA methylation levels at the miR-31 promoter in prostate cancer (n = 12) and benign (n = 12). F, comparison of overall DNA methylation at the miR-31 promoter in 12 matched pairs (n = 0.05; n = 0.01; n = 0.0001), G, DNA methylation at the miR-31 promoter in indicated cell lines. Top, comparison of overall DNA methylation levels; bottom, heatmap of DNA methylation levels. Each row corresponds to an individual sample, and each column corresponds to an individual CpG unit, which is a single CpG site or a combination of CpG sites. H, expression of miR-31 and AR in indicated cell lines by qPCR and immunoblot (n = 3). I, VCaP cells treated by vehicle (dimethyl sulfoxide [DMSO]) or 5-aza-dC. Left and heatmap, DNA methylation levels; right panel, miR-31 levels (n = 3). J and K, comparisons of DNA methylation levels at the miR-31 promoter and miR-31 levels between 3 groups: Gleason score (GS) 6, ≥7, and metastatic cancer (MET). All bar graphs are shown with mean ± SEM.
Figure 2. Androgen receptor (AR) and PRC2-mediated repressive histone modification in regulation of miR-31 expression. A, expression of miR-31 (left) and NDRG1, PSA, and TMPRSS2 (right) in LNCaP cells transfected with AR siRNA (siAR) or control siRNA (siCTL) and treated with 1 nmol/L R1881 or vehicle (ethanol), evaluated by qPCR, and AR expression by immunoblot (n = 3). B, expression of miR-31 and AR in PC3neo cells versus the AR-expressing PC3AR
Prostate cancer samples that displayed significantly higher levels of promoter methylation than matched benign prostate tissues had lower miR-31 levels (ratio < 1.0 in Fig. 1B). Interestingly, the prostate cancer sample with high miR-31 expression (ratio > 1) had similar levels of promoter methylation as its benign counterpart (Fig. 1F; Supplementary Fig. S1E). DNA methylation levels between prostate cancer and benign prostate tissue of the first 11 cases were significantly different across the whole region \((P < 0.001)\) as well as in each of the 4 subdivided regions \((P < 0.006;\) Supplementary Table S4). Furthermore, 3 of individual CpG units showed cancer-specific DNA methylation changes \((P < 0.05)\). Taken together, DNA methylation levels at the miR-31 promoter were inversely correlated with miR-31 expression, suggesting that promoter hypermethylation accounts for miR-31 downregulation in the majority of prostate cancer cases.

These observations were also examined in common in vitro prostate cancer models. A previous study observed that miR-31 was downregulated in the advanced cell line WPE1-NA26 as compared with the benign cell line WPE1-NA22; however, no explanation was provided \((20)\). We examined benign prostate and prostate cancer cell lines for promoter hypermethylation and expression of miR-31. The immortalized human prostate epithelial cell line, RWPE1, and human prostate cancer cell lines, PC3 and DU145, had high expression of miR-31 with little DNA methylation at the miR-31 promoter. In contrast, 22Rv1, LNCaP, LNCaP-abl, and VCaP cancer cells had low expression of miR-31 with concurrent high DNA methylation levels at the miR-31 promoter, consistent with what was observed in primary prostate cancers (Fig. 1G and H; Supplementary Fig. S1F and S1G). The expression levels of DNA methyltransferases (DNMT), however, did not parallel the DNA methylation patterns in the cell lines (Supplementary Fig. S1H). Importantly, VCaP cells treated with the DNA methylation inhibitor 5-aza-2-deoxycytidine (5-aza-dC) showed decreased DNA methylation levels at the miR-31 promoter and increased expression of miR-31 (Fig. 1I; Supplementary Fig. S1I), supporting the role of promoter hypermethylation in downregulating miR-31 expression in prostate cancer.

miR-31 promoter hypermethylation correlates with aggressiveness of prostate cancer

We next explored for an association between miR-31 promoter methylation and prostate cancer disease progression. Prostate cancer is graded using the Gleason score. A Gleason score ranges from 2 to 10, and higher scores (i.e., 7–10) are associated with a more aggressive clinical course. We examined 38 primary prostate cancer cases with Gleason scores ranging from 6 to 9. We also evaluated 5 metastatic CRPC cases from patients who failed endocrine therapy and/or developed a predominantly androgen independent prostate cancer associated with lack of androgen receptor expression and extensive neuroendocrine differentiation (Gleason scores are not assigned to metastatic prostate cancers). DNA methylation at the miR-31 promoter was positively correlated with prostate cancer progression (Supplementary Fig. S1J and Supplementary Table S5). The overall DNA methylation at the miR-31 promoter showed significant differences among 3 groups: Gleason scores 6, \(\geq 7\), and metastatic cancer (Fig. 1J), and it was inversely correlated with miR-31 expression levels (Fig. 1K). Thus, our data showed a close association between the extent of DNA methylation at the miR-31 promoter and the aggressiveness of prostate cancer, and both promoter hypermethylation and downregulation of miR-31 could serve as indicators for aggressive behaviors in prostate cancer.

Androgen receptor and H3K27 trimethylation negatively regulate miR-31 expression

We then sought to identify other factors that could regulate miR-31 expression. We found that androgen receptor expression levels were also inversely correlated with miR-31 expression levels in the prostate cell lines (Fig. 1H) and in primary prostate cancer \((r = -0.173097, P < 0.42\); Supplementary Fig. S2A). Androgen receptor–positive cells expressed much lower miR-31. VCaP cells with androgen receptor amplification and the highest androgen receptor expression showed the lowest expression level of miR-31. Activation of androgen receptor signaling with synthetic androgen, R1881, led to increasing expression of androgen receptor–targeting genes, NDRG1, PSA, and TMPRSS2, and downregulation of miR-31, whereas knocking down androgen receptor by siRNA interference reversed the repression on miR-31 (Fig. 2A; Supplementary Fig. S2B). In addition, PC3AR cells, which are PC3 cells engineered to express androgen receptor \((21)\), and HEK293 cells transiently overexpressing androgen receptor also showed a decreased expression of miR-31 (Fig. 2B; Supplementary Fig. S2C). Chromatin immunoprecipitation (ChIP) assays in LNCaP cells showed androgen receptor enrichment at the miR-31 promoter after androgen treatment, indicating a potential direct regulation of miR-31 expression by androgen receptor (Fig. 2C). To evaluate the binding of androgen receptor to the miR-31 promoter, we conducted luciferase assays by using the miR-31 promoter–driven luciferase reporter system. Expression of androgen receptor in HEK293 cells resulted in the inhibition of luciferase activity with constructs containing regions of the miR-31 promoter, suggesting that androgen receptor might associate with the miR-31 promoter and inhibit its expression (Fig. 2D).

EZH2, a methyltransferase involved in epigenetic silencing through H3K27 trimethylation (H3K27me3), has been shown

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cells evaluated by qPCR and immunoblot \((n = 3)\). C, quantitative ChIP analysis with AR, EZH2, and H3K27me3 antibodies at the miR-31 promoter and regions near miR-31 in LNCaP cells treated with 1 nM R1881 or vehicle \((\text{ethanol}; n = 3)\). Red bars represent qPCR regions. D, luciferase activity of reporter constructs containing the miR-31 promoter region of \(-1,000\) bp and downstream region \(-500\) bp cotransfected with constructs containing empty vector or AR CDS with siCTL, or siAR in HEK293 cells \((n = 3); P < 0.01\). E, LNCaP cells in regular medium, miR-31 levels in response to knockdown of AR, EZH2, or both, evaluated by qPCR, and AR expression by immunoblot \((n = 3)\). All bar graphs are shown with mean \(\pm\) SEM.
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Figure 3. Downregulation of androgen receptor (AR) by miR-31. A, AR protein level was examined by immunoblot. LNCaP and VCaP cells were transfected with miR-31 or miR-NC (n = 3). B, expression of PSA and TMPRSS2 evaluated by qPCR (n = 3). LNCaP cells transfected with siCTL, siAR, miR-NC, miR-31, and miR-31 with AR CDS for 48 hours, followed by treatment with 1 nmol/L R1881 or vehicle (ethanol) for 24 hours. C, schematic graph illustrating predicted locations of 3 miR-31 MREs within the transcript of AR variant 1. Numbers in parentheses correspond to the position in the whole transcript (NM_000044). Perfect matches are shown by a line; G:U pairs by a colon (:). D, previously reported mutations are shown in red and the original sequence in bold. Three point mutations, A>G, G>A, and G>T, were located within MRE2 and one deletion, ΔG, was located within MRE3. E, luciferase activity of LNCaP cells cotransfected with reporter constructs containing WT, mutant (mt), or empty vector (v) and either miR-31 or miR-NC (n = 3). F, AR expression levels in HEK293 cells cotransfected with AR CDS WT or mutant containing the G>T mutation in MRE2 and either miR-31 or miR-NC evaluated by qPCR (n = 3). G, AR expression in PC3AR cells transfected with miR-31, miR-NC, inhibitor negative control (IN-NC), or miR-31 inhibitor (IH-miR-31) evaluated by qPCR and immunoblot (n = 3). **, P < 0.01. All bar graphs are shown with mean ± SEM.

to negatively regulate the expression of miR-31 in ATL and melanoma (15, 22). Complementary to these observations, we found that H3K27me3 was steadily enriched at the miR-31 promoter and regions near miR-31, whereas EZH2 was recruited to these regions after androgen stimulation (Fig. 2C). Knocking down androgen receptor and EZH2 alone or simultaneously in LNCaP cells increased miR-31 expression, suggesting that androgen receptor and EZH2 concurrently regulate the expression of miR-31 (Fig. 2E). Collectively, our data suggest that androgen receptor binding and repressive H3K27me3 coexist with promoter hypermethylation to down-regulate miR-31 expression.
Figure 4. Genes in cell-cycle regulation are direct targets of miR-31. A, proliferation assay of LNCaP cells transfected with miR-31 or miR-NC (n = 6; *, P < 0.01). B, colony formation analysis of VCaP cells overexpressing miR-31 or vector alone (n = 3). C, cell-cycle analysis of LNCaP cells transfected with miR-31 or miR-NC by fluorescence-activated cell sorting (n = 3). D, caspase-3/7 activity in LNCaP cells transfected with miR-31 or miR-NC (n = 6). E, expression of genes involved in cell cycle in LNCaP cells transfected with miR-31 or miR-NC evaluated by qPCR (n = 3). F, immunoblot
miR-31 represses androgen receptor expression by targeting androgen receptor directly

Previous reports suggest that dysregulation of critical miRNA–protein regulatory networks is involved in cancer. In fact, LNCaP and VCaP cells transfected with increasing amounts of miR-31 showed decreased expression of androgen receptor at both the transcript and protein levels (Fig. 3A; Supplementary Fig. S3A and S3B). qPCR assays also showed that miR-31 suppressed androgen receptor signaling, which was abrogated by overexpression of androgen receptor (Fig. 3B). Therefore, we posited that miR-31 might in turn modulate androgen receptor expression. Although miRNA target prediction algorithms provided by TargetScan, microRNA.org, and PicTar did not list androgen receptor as a miR-31 target, we identified 4 putative MREs of androgen receptor transcript variant 1 (RefSeq NM_000044) and transcript variant 2 (RefSeq NM_00101645) by RNA22 (ref. 23; Fig. 3C; Supplementary Fig. S3C). Androgen receptors MRE1 and MRE4 were located at the 5′UTRs of androgen receptor variants 1 and 2, respectively. Androgen receptors MRE2 and MRE3 were located at the coding sequence (CDS): MRE2 in the ligand-binding domain and MRE3 near the DNA-binding domain. Interestingly, 4 previously reported androgen receptor mutations were located within MRE2 and MRE3, including 3 point mutations: 2 transitions (A> G and G>A), 1 transversion (G>T), and 1 deletion (ΔG) (refs. 24–27; Fig. 3D), suggesting that these sites may be important in regulating androgen receptor.

To determine whether reduced androgen receptor expression was directly mediated by miR-31, we cloned the 4 predicted wild-type (WT) MREs as well as the 4 mutations identified previously in human tumor samples into a luciferase reporter system and conducted cotransfection with either miR-31 or a negative control miR-NC in LNCaP cells (Fig. 3E).

Inhibition of luciferase activity was shown with constructs containing MRE2 and MRE4 but not with constructs containing MRE1 or MRE3. Resistance to miR-31 repression was observed as a result of 1 of the 3 known mutations at MRE2 (G>T), suggesting that this mutation might lead to loss of androgen receptor regulation by miR-31. As MRE3 was not a bona fide target site for miR-31, the deletion at MRE3 had no effect on luciferase activity. We also examined the putative miR-31 target site identified in a recently characterized longer androgen receptor 3′UTR (28), but inhibition of luciferase activity was not detected (Supplementary Fig. S3D). Consistently, inhibition of androgen receptor expression by miR-31 occurred in 293HEK cells transfected with the construct containing the entire CDS of WT androgen receptor but not the mutant construct (Fig. 3F). PC3AR cells, expressing the androgen receptor coding region and consequently MRE2, showed reduced androgen receptor expression upon overexpression of miR-31, whereas the miR-31 inhibitor increased androgen receptor expression (Fig. 3G). These results indicate that miR-31 can directly repress androgen receptor expression through the androgen receptor CDS.

Genes involved in cell-cycle regulation are direct targets of miR-31

To gain insights into the cellular mechanism through which miR-31 exerts its effect, we analyzed whole-genome gene expression data from miR-31–overexpressing experiments in LNCaP cells. The top cellular processes that were enriched by gene ontology (GO) analysis included cell cycle, mitosis, DNA replication, microtubule-based process, and DNA repair (Supplementary Table S6). Consistent with this analysis, overexpression of miR-31 inhibited cell proliferation and colony formation and arrested cell-cycle progression (Fig. 4A–C; Supplementary Fig. S4A). The decreased cell proliferation was likely due to cell-cycle arrest, as little apoptosis was observed as indicated by a minimal change in caspase-3/7 activity (Fig. 4D; Supplementary Fig. S4B).

Expression levels of several genes involved in cell-cycle regulation were decreased in the presence of miR-31 (Fig. 4E). Among them, transcription factor E2F1, which has been previously shown to regulate androgen receptor expression via transcriptional regulation (29), was decreased at both transcript and protein levels (Fig. 4F; Supplementary Fig. S3A and S3B). One putative miR-31 MRE was identified at the 3′UTR of E2F1. Inhibition of luciferase activity was observed in cells expressing the WT construct (12) but not with the mutant (Fig. 4G), confirming that miR-31 could target E2F1 directly. These data suggested that miR-31 could regulate androgen receptor through direct repression of E2F1, in addition to directly targeting the androgen receptor mRNA.

We also identified putative miR-31 MREs at 3′UTRs of CDK1, E2F2, EXO1, FOXM1, and MCM2, which are critical players in cell-cycle regulation (Supplementary Fig. S4C). The transcript and protein levels of these genes were decreased in the presence of miR-31 (Fig. 4E and H). Even though a previous study in serous ovarian carcinoma had suggested that E2F2 was a predicted direct target of miR-31 (30), it did not provide experimental data to validate this relationship. To address it, we used luciferase reporter assays to show that miR-31 could directly repress the expression of E2F2, EXO1, FOXM1, and MCM2 but not CDK1 (Fig. 4L–K; Supplementary Fig. S4D and S4E).

miR-31 represses prostate cancer growth

To evaluate the antitumor effect of miR-31 in vivo, we established murine xenograft experiments with LNCaP cells and treated tumors with miR-31 or control miR-NC mimics.
Consistent with the \textit{in vitro} data, miR-31 attenuated tumor growth over time (Fig. 5A–C). In addition, tumors treated with miR-31 showed a marked reduction in androgen receptor expression (Fig. 5D and E). Xenografts established with VCaP cells expressing miR-31 also showed smaller tumor sizes, decreased growth rates, and reduced androgen receptor levels (Supplementary Fig. S5A–S5E). These data supported a model in which miR-31 represses prostate cancer growth, in part, through the downregulation of androgen receptor.

\section*{Discussion}

There is an increasing appreciation for the role of miRNAs in maintaining cellular homeostasis and for their tissue-specific dysregulation in tumorigenesis. miRNAs, depending on the cellular context, can act as oncogenes or tumor suppressors. miR-31, the focus of this study, exemplifies this paradigm being implicated in both tumor promotion and suppression. In lung adenocarcinoma, miR-31 acts as an oncogene by repressing the tumor suppressor genes, \textit{LATS2} and \textit{PPP2R2A} (17), whereas in breast cancer, it serves as a tumor suppressor by inhibiting tumor metastasis through inhibition of RhoA, Fzd3, ITGA5, and RDX (31). Our data suggest a tumor-suppressive role for miR-31 in prostate tissue through the modulation of androgen receptor and cell cycle. Different from breast cancer cell lines, metastatic prostate cancer cell lines PC3 and DU145 contain high expression levels of miR-31. This may suggest that miR-31 has a different role in those cells. A recent study showed that...
overexpression of miR-31 in these 2 cell lines could further inhibit cell proliferation, cell invasion, and migration, and in silico analysis of genome-wide gene expression data suggested that miR-31 has other functions in PC3 cells (32).

The miRNAs have previously been implicated in the regulation of androgen receptor signaling. miR-130a, miR-203, and miR-205 interfere with androgen receptor signaling by repression androgen receptor coactivators, CDK1, PSAP, PSMC3IP, and PARK7, as well as by inhibiting the mitogen-activated protein kinase (MAPK) signaling pathway, which facilitates ligand-independent androgen receptor activation (33). There are miRNAs that downregulate androgen receptor expression. Let-7c inhibits androgen receptor transcription through targeting c-MYC (34), whereas miR-488 directly targets androgen receptor mRNA (35). Our study reveals a complex regulatory pattern between miR-31 and androgen receptor. For the first time, we show that miR-31 directly targets and destabilizes androgen receptor mRNA through its coding sequence. miR-31 can also repress androgen receptor indirectly through E2F1. Finally, we find that miR-31 can decrease CDK1, which may indirectly contribute to androgen receptor downregulation, as CDK1 stabilizes androgen receptor and contributes to androgen receptor activation (36).

Androgen receptor regulates a number of genes at transcription level, including miRNAs (37). Extensive studies have characterized androgen receptor transcriptional coregulators, and several of which, p300/CBP, p/CAF, TIP60, class I and class II HDACs, and p160/SRC proteins, have histone acetylase/demethylase activity (38). More recently, histone demethylases have also been shown to be part of a regulatory complex with androgen receptor (39). Androgen receptor uses these coregulators to achieve either transcriptional activation or suppression. Using ChIP, we detected that androgen receptor is associated with the miR-31 promoter. Occupation of H3K27me3 was also found at the miR-31 promoter, which was consistent with the induced expression of miR-31 when knocking down EZH2 in LNCaP cells. As EZH2 has been recently shown to occupy genes repressed by androgen receptor (40), androgen receptor binding and H3K27me3 might work together to repress miR-31.

Association of histone modification and DNA methylation for gene silencing is well established for H3K9 methylation and implicated for H3K27me3 through the interaction between EZH2 and DNMTs (41, 42); however, these repressive histone modifications are not always accompanied by DNA methylation. Studies in breast cancer cell lines and in ATLLs have suggested that miR-31 is downregulated through promoter hypermethylation and the Polycomb repressive complex 2 (PRC2), respectively (15, 43). In this study, both mechanisms are implicated in the inhibition of miR-31 in prostate cancer. Moreover, low-frequency germline deletions of 10 to 20 kbp spanning the MIR31HG locus were found in a previously published dataset (44) and in about 1% of individuals with prostate cancer of our dataset (data not shown). Germline deletion might represent yet another type of regulation of miR-31.

Interestingly, our data showed that androgen receptor expression was correlated with promoter hypermethylation at the miR-31 promoter. To our knowledge, this is the first report linking androgen receptor signaling and DNA methylation. It is unclear whether androgen receptor mediates DNA methylation globally or at specific gene loci. EZH2 is known to interact with DNMTs and, therefore, it is plausible that androgen receptor might be involved in DNA methylation through EZH2 or through interactions with DNMTs. Overexpression of androgen receptor was found to enhance its association with chromatin under low androgen conditions (45), suggesting that the increased accessibility might facilitate androgen receptor to epigenetically modify global gene expression and possibly contribute to the development of CRPCs. Further investigation is warranted as to the involvement of androgen receptor in DNA methylation.

The miRNAs are believed to be more stable than mRNA in serum and urine, making them ideal for biomarker development. However, it may not be feasible to detect the reduced miR-31 levels in such specimens for the diagnosis of prostate...
cancer, due to background normal prostate and other types of cells that have high levels of miR-31 expression, as recently suggested. Alternatively, our current study suggests that one could detect miR-31 promoter hypermethylation in noninvasive blood and urine tests, which may enhance current clinical strategies for prostate cancer risk prediction using PCA3, TMPRSS2-ERG, and PSA (46).

A potentially important observation in the current study is that miR-31 mediates the repression of androgen receptor through direct targeting of androgen receptor mRNA at the CDS. The majority of mirRNA target prediction methods solely take the 3' UTR into consideration; however, about 50% of miRNA-binding sites have been identified at CDS by PAR-CLIP (47). Although the effectiveness of RNA degradation or translational interference by targeting the 3' UTR or CDS remains unknown, prior studies have shown that miRNAs target CDS for inhibition of gene expression (48, 49). The miRNA binding to the 5' UTR has also been shown to be as effective at repressing gene transcription as targeting the 3' UTR (50). Seedless miRNA recognition sites for miR-24 are yet another way miRNAs identify their targets (12). Taken together, these findings suggest considering beyond the canonical 3' UTR miRNA recognition sites for the identification of target genes.

This study reveals a previously unknown relationship between miR-31 and androgen receptor, which involves mutual gene repression, and furthermore, suggests that loss of miR-31 may play a critical role in the dysregulation of androgen receptor in prostate cancer. We propose a model for the mutual regulation of miR-31 and androgen receptor in the context of prostate cancer (Fig. 6). In normal prostate epithelium, miR-31 controls cell proliferation and cell-cycle progression through targeted inhibition of androgen receptor and key cell-cycle components, thereby maintaining tissue homeostasis. In prostate cancer, however, miR-31 is downregulated and therefore no longer able to regulate androgen receptor expression. Conversely, androgen receptor binds to miR-31 and recruits epigenetic machinery, resulting in miR-31 silencing. In this model, prostate carcinogenesis may progress through the initial accumulation of epigenetic alterations at the miR-31 locus, leading to miR-31 silencing and unfettered androgen receptor expression, or alternatively, through initial epigenetic/genetic changes that cause increased androgen receptor expression and subsequent miR-31 silencing, resulting in blockage of this regulatory loop.

In summary, the mutual regulation between miR-31 and androgen receptor maintains prostate cellular homeostasis and loss of miR-31 contributes to prostate cancer progression. Promoter hypermethylation during prostate tumorigenesis results in the downregulation of miR-31 and diminishes its ability to regulate androgen receptor. Androgen receptor and H3K27me3 are also involved in the regulation of miR-31. In addition, miR-31 targets cell-cycle regulators and modulates cell proliferation and cell-cycle progression. This miR-31-AR regulatory mechanism provides not only a rationale for the tissue-specific nature of miR-31 but also a deeper understanding of androgen receptor regulation. Finally, the frequent hypermethylation of the miR-31 promoter in prostate cancer suggests that epigenetic therapy could complement existing therapeutic strategies to block androgen receptor activity. Such combinatorial treatment might decrease the emergence of CRPC, which represents a major cause of progression and mortality in patients with prostate cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: P.-C. Lin, M.A. Rubin
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.K. Tewari, H. Beltran, M.A. Rubin
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P.-C. Lin, Y.-L. Chu, S. Banerjee, E. Giamnopooulou, P. Alves, M.B. Gerstein, A.M. Melnick, O. Elemento, F. Demichelis, M.A. Rubin
Writing, review, and/or revision of the manuscript: P.-C. Lin, Y.-L. Chu, S. Banerjee, J.M. Mosquera, P. Alves, A.K. Tewari, H. Beltran, A.M. Melnick, O. Elemento, F. Demichelis, M.A. Rubin
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P.-C. Lin, K. Park
Study supervision: M.B. Gerstein, M.A. Rubin

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Pei-Chun Lin, Ya-Lin Chiu, Samprit Banerjee, et al.


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