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Epigenetic repression of miR-31 disrupts androgen receptor homeostasis and contributes to prostate cancer progression

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Abstract

Androgen receptor (AR) signaling plays a critical role in prostate cancer (PCA) pathogenesis. Yet, the regulation of AR signaling remains elusive. Even with stringent androgen deprivation therapy, AR signaling persists. Here, our data suggest that there is a complex interaction between the expression of the tumor suppressor miRNA, miR-31 and AR signaling. We examined primary and metastatic PCA and found that miR-31 expression was reduced as a result of promoter hypermethylation and importantly, the levels of miR-31 expression was inversely correlated with the aggressiveness of the disease. As the expression of AR and miR-31 was inversely correlated in the cell lines, our study further suggested that miR-31 and AR could mutually repress each other. Upregulation of miR-31 effectively suppressed AR expression through multiple mechanisms and inhibited PCA growth *in vivo*. Notably, we found that miR-31 targeted AR directly at a site located in the coding region, which was commonly mutated in PCA. Additionally, miR-31 suppressed cell cycle regulators, including E2F1, E2F2, EXO1, FOXM1, and MCM2. Together,

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our findings suggest a novel AR regulatory mechanism mediated through miR-31 expression. The downregulation of miR-31 may disrupt cellular homeostasis and contribute to the evolution and progression of PCA. We provide implications for epigenetic treatment and support clinical development of detecting miR-31 promoter methylation as a novel biomarker.

Keywords

prostate cancer; androgen receptor; miR-31; DNA hypermethylation; biomarker

Introduction

Prostate cancer (PCA) 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 28,000 deaths in the United States in 2012 (1). PCA depends largely on androgen receptor (AR) signaling for growth and maintenance. Following the seminal observations by Huggins and Hodges over 60 years ago that PCA responded dramatically to castration, androgen deprivation therapy (ADT) has become the standard first-line treatment for advanced hormone naïve PCA (2, 3). By reducing circulating androgen, ADT prevents signaling through AR 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 AR signaling in the face of ADT has been attributed to the acquisition of amplified or mutated *AR*; recent work using next-generation sequencing (NGS) suggests that *AR* gene amplification and mutations occur in up to 44% of CRPC: 24% with copy number gain and 20% with point mutation (4). Perhaps the most important recent finding came when Chen et al. discovered that AR signaling persists under stringent ADT and that AR antagonists act as agonists at high AR levels (5). While these observations have led to the development of more efficacious therapeutic approaches for targeting AR signaling (6), CRPC still persists after treatment; therefore, other interventions are needed for AR regulation.

Epigenetic aberrations arise during PCA initiation and disease progression, which include promoter cytosine-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 PCA (8). Dysregulation of microRNAs (miRNAs) also occurs during PCA pathogenesis (9). MiRNAs are small non-coding RNA molecules that simultaneously regulate the expression of multiple genes by deteriorating messenger RNA (mRNA) 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 AR signaling. We sought to examine the mechanism behind AR-mediated regulation of miRNAs. In this study, we report a novel role for miR-31 in PCA and show that hypermethylation at the *miR-31* promoter occurs in a PCA-specific manner, the extent of which correlates with disease progression, AR regulates miR-31 expression, and miR-31 directly targets AR and other cell cycle regulators and represses PCA growth.

Materials and Methods

Benign and PCA tissue selection

All tissue samples were collected as part of an Institutional Review Board (IRB) approved protocol at WCMC and informed consents were received from participants prior to 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 pathologist (M.A.R./K.P./J.M.M) 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 PCA and benign tissues. False discovery rate (FDR) control was used in multiple hypotheses testing to correct for multiple comparisons. miRNAs with significant changes were chosen based on adjusted $p\text{-}value < 0.05$. 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 EpiTyping

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

Quantitative real-time PCR (qPCR)

cDNA synthesis was carried out using the M-MuLV Reverse Transcriptase (Emzymatics) according to the manufacturer's protocol. Quantitative real-time PCR was performed with the Roche LightCycler480 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 PCA cell lines, VCaP, LNCaP, 22Rv1, PC3, DU145, and HEK293 cells were purchased from American Type Culture Collection (ATCC) and used within 6 months after receipt; authentication of cell lines was performed by ATCC. PC3-neo and PC3-AR cell lines were kind gifts from Dr. David M. Nanus (WCMC) and LNCaP-abl cell line was a kind gift from Dr. Myles Brown (Harvard); 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), AR (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 (ChIP)

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 1nM R1881 for 16~24 hours. For detailed description of methodology, see Supplementary Methods.

MiRNA reporter Luciferase Assays

LNCaP cells were transfected in triplicate with 30 nM 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 (MREs) or the 3′UTR region containing the MREs of indicated genes by DharmaFECT Duo transfection reagent, according to the manufacturer's protocol (Dharmacon). After 48 hours, cell were lysed and luciferase activity was measured using the Dual 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). 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 (IACUC) at Weill Cornell Medical College 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 performed with GraphPad Prism 4.0 (Graph Pad software). Two-sided and p values <0.05 were 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 PCA

Global miRNA expression profiling in PCA has been performed 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 PCA and matched benign prostate tissue. 105 miRNAs were identified as significantly altered in PCA (FDR-adjusted $p\ value < 0.05$, Supplementary Fig. S1A, Supplementary Table S1), including 25 miRNAs with at least 1.5-fold expression change (Fig. 1A; Supplementary Table S2). Consistent with the study by Schaefer et al. that 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 PCA.

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 PCA 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 PCA 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 PCA specimens was significantly lower as compared to 15 benign prostate tissues (*p-value* < 0.0001, Fig. 1C). Taken together, our data demonstrated the downregulation of miR-31 in primary PCA.

PCA-specific downregulation of miR-31 is mediated by promoter hypermethylation

To delineate the mechanism behind the downregulation of miR-31 in PCA, 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 PCA 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 PCA. 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 PCA 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 PCA.

Epigenetic alterations, such as promoter DNA hypermethylation, can result in silencing of miRNA expression. Therefore, we examined if 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 four pairs of primers (Supplementary Fig. S1D; 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-value* < 0.001, Fig. 1E). PCA samples that displayed significantly higher levels of promoter methylation as compared to matched benign prostate tissues had lower miR-31 levels (ratio < 1.0 in Fig. 1B). Interestingly, the PCA sample with high miR-31 expression (ratio > 1) had similar levels of promoter methylation as its benign counterpart (Fig. 1F and Supplementary Fig. S1E). DNA methylation levels between PCA and benign prostate tissue of the first 11 cases were significantly different across the whole region (*p-value* < 0.001) as well as in each of the four subdivided regions (*p-values* < 0.006) (Supplementary Table S4). Furthermore, three of individual CpG units showed cancer-specific DNA methylation changes (*p-values* < 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 PCA cases.

These observations were also examined in common *in vitro* PCA models. A previous study observed that miR-31 was downregulated in the advanced cell line WPE1-NA26 as compared to the benign cell line WPE1-NA22; however, no explanation was provided (20). We examined benign prostate and PCA cell lines for promoter hypermethylation and expression of miR-31. The immortalized human prostate epithelial cell line, RWPE1, and human PCA 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 PCAs (Fig. 1G–H, and Supplementary Fig. S1F–G). The expression levels of DNA methyltransferases (DNMTs), 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 and Supplementary Fig. S1I), supporting the role of promoter hypermethylation in downregulating miR-31 expression in PCA.

MiR-31 promoter hypermethylation correlates with aggressiveness of PCA

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

AR and H3K27 trimethylation negatively regulate miR-31 expression

We then sought to identify other factors that could regulate miR-31 expression. We found that AR expression levels were also inversely correlated with miR-31 expression levels in the prostate cell lines (Fig. 1H) and in primary PCA ($r = -0.173097$, $p < 0.42$, Supplementary Fig. S2A). AR-positive cells expressed much lower miR-31. VCaP cells with *AR* amplification and the highest AR expression showed the lowest expression level of miR-31. Activation of AR signaling with synthetic androgen, R1881, led to increasing expression of AR-targeting genes, NDRG1, PSA, and TMPRSS2, and downregulation of miR-31, while knocking down AR by siRNA interference reversed the repression on miR-31 (Fig. 2A and Supplementary Fig. S2B). Additionally, PC3AR cells, which are PC3 cells engineered to express AR (21), and HEK293 cells transiently overexpressing AR also showed a decreased expression of miR-31 (Fig. 2B and Supplementary Fig. S2C). Chromatin immunoprecipitation (ChIP) assays in LNCaP cells showed AR enrichment at the *miR-31* promoter after androgen treatment, indicating a potential direct regulation of miR-31 expression by AR (Fig. 2C). To evaluate the binding of AR to the *miR-31* promoter, we conducted luciferase assays by using the *miR-31* promoter-driven luciferase reporter system. Expression of AR in HEK293 cells resulted in the inhibition of luciferase activity with constructs containing regions of the *miR-31* promoter, suggesting that AR 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 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* while EZH2 was recruited to these regions after androgen stimulation (Fig. 2C). Knocking down AR and

EZH2 alone or simultaneously in LNCaP cells increased miR-31 expression, suggesting that AR and EZH2 concurrently regulate the expression of miR-31 (Fig. 2E). Collectively, our data suggest that AR binding and repressive H3K27me3 coexist with promoter hypermethylation to downregulate miR-31 expression.

MiR-31 represses AR expression by targeting AR 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 AR at both the transcript and protein levels (Fig. 3A, Supplementary Fig. S3A and S3B). qPCR assays also showed that miR-31 suppressed AR signaling, which was abrogated by overexpression of AR (Fig. 3B). Therefore, we posited that miR-31 might in turn modulate AR expression. Although miRNA target-prediction algorithms provided by TargetScan, microRNA.org and PicTar did not list AR as a miR-31 target, we identified four putative miRNA recognition elements (MREs) of AR transcript variant 1 (RefSeq NM_000044) and transcript variant 2 (RefSeq NM_001011645) by RNA22 (23) (Fig. 3C and Supplementary Fig. S3C). AR MRE1 and MRE4 were located at the 5′UTRs of AR variant 1 and 2, respectively. AR MRE2 and MRE3 were located at the coding sequence (CDS): MRE2 in the ligand-binding domain and MRE3 near the DNA binding domain. Interestingly, four previously reported AR mutations were located within MRE2 and MRE3, including three point mutations: two transitions (A>G and G>A), one transversion (G>T), and one deletion (ΔG) (Fig. 3D) (24–27), suggesting that these sites may be important in regulating AR.

To determine whether reduced AR expression was directly mediated by miR-31, we cloned the four predicted wild-type (WT) MREs as well as the four mutations identified previously in human tumor samples into a luciferase reporter system and performed co-transfection 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 one of the three known mutations at MRE2 (G>T), suggesting that this mutation might lead to loss of AR 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 AR 3′UTR (28), but inhibition of luciferase activity was not detected (Supplementary Fig. S3D). Consistently, inhibition of AR expression by miR-31 occurred in 293HEK cells transfected with the construct containing the entire CDS of WT AR, but not the mutant construct (Fig. 3F). PC3AR cells, expressing the AR coding region and consequently MRE2, showed reduced AR expression upon overexpression of miR-31, while the miR-31 inhibitor increased AR expression (Fig. 3G). These results indicate that miR-31 can directly repress AR expression through the AR 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 analysis (GO) 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 to 4C; Supplementary Fig. S4A). The decreased cell proliferation was likely due to cell cycle arrest, since 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 AR 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 AR through direct repression of E2F1, in addition to directly targeting the AR 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 4H). 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. 4I to 4K; Supplementary Fig. S4D and S4E).

MiR-31 represses PCAgrowth

To evaluate the anti-tumor 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 *in vitro* data, miR-31 attenuated tumor growth over time (Fig. 5A to 5C). Additionally, tumors treated with miR-31 showed a marked reduction in AR expression (Fig. 5D and 5E). Xenografts established with VCaP cells expressing miR-31 also showed smaller tumor sizes, decreased growth rates, and reduced AR levels (Supplementary Fig. S5A–E). These data supported a model in which miR-31 represses PCA growth, in part through the downregulation of AR.

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, *LATS2* and *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 AR and cell cycle. Different from breast cancer cell lines, metastatic PCA 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 two 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).

MiRNAs have previously been implicated in the regulation of AR signaling. MiR-130a, miR-203, and miR-205 interfere with AR signaling by repressing AR coactivators, CDK1, PSAP, PSMC3IP, and PARK7, as well as by inhibiting the MAPK signaling pathway, which facilitates ligand-independent AR activation (33). There are miRNAs that downregulate AR expression. Let-7c inhibits AR transcription through targeting c-MYC (34), while miR-488* directly targets AR mRNA (35). Our study reveals a complex regulatory pattern between miR-31 and AR. For the first time, we show that miR-31 directly targets and destabilizes AR

mRNA through its coding sequence. MiR-31 can also repress AR indirectly through E2F1. Finally, we find that miR-31 can decrease CDK1, which may indirectly contribute to AR downregulation, as CDK1 stabilizes AR and contributes to AR activation (36).

AR regulates a number of genes at transcription level, including miRNAs (37). Extensive studies have characterized AR transcriptional coregulators, and several of which, p300/CBP, p/CAF, TIP60, class I and class II HDACs, and p160/SRC proteins, have histone acetylase/deacetylase activity (38). More recently, histone demethylases have also been shown to be part of a regulatory complex with AR (39). AR utilizes these coregulators to achieve either transcriptional activation or suppression. Using ChIP, we detected that AR 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 AR (40), AR 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 ATL 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 PCA. Moreover, low-frequency germline deletions of 10–20 kilobase pairs spanning the *MIR31HG* locus were found in a previously published dataset (44) and in about 1% of individuals with PCA of our dataset (data not shown). Germline deletion might represent yet another type of regulation of miR-31.

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

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 PCA, 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 non-invasive blood and urine tests, which may enhance current clinical strategies for PCA 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 AR through direct targeting of AR mRNA at the CDS. The majority of miRNA 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 demonstrated that miRNAs target CDS for inhibition of gene expression (48, 49). 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 AR, which involves mutual gene repression, and furthermore, suggests that loss of miR-31 may play a critical role in the dysregulation of AR in PCA. We propose a model for the mutual regulation of miR-31 and AR in the context of PCA (Fig. 6). In normal prostate epithelium, miR-31 controls cell proliferation and cell cycle progression through targeted inhibition of AR and key cell cycle components, thereby maintaining tissue homeostasis. In PCA, however, miR-31 is downregulated and therefore no longer able to regulate AR expression. Conversely, AR 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 AR expression, or alternatively, through initial epigenetic/genetic changes that cause increased AR expression and subsequent miR-31 silencing, resulting in blockage of this regulatory loop.

In summary, the mutual regulation between miR-31 and AR maintains prostate cellular homeostasis and loss of miR-31 contributes to PCA progression. Promoter hypermethylation during prostate tumorigenesis results in the downregulation of miR-31 and diminishes its ability to regulate AR. AR and H3K27me3 are also involved in the regulation of miR-31. Additionally, 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 AR regulation. Finally, the frequent hypermethylation of the *miR-31* promoter in PCA suggests that epigenetic therapy could complement existing therapeutic strategies to block AR activity. Such combinatorial treatment might decrease the emergence of CRPC, which represents a major cause of progression and mortality in PCA patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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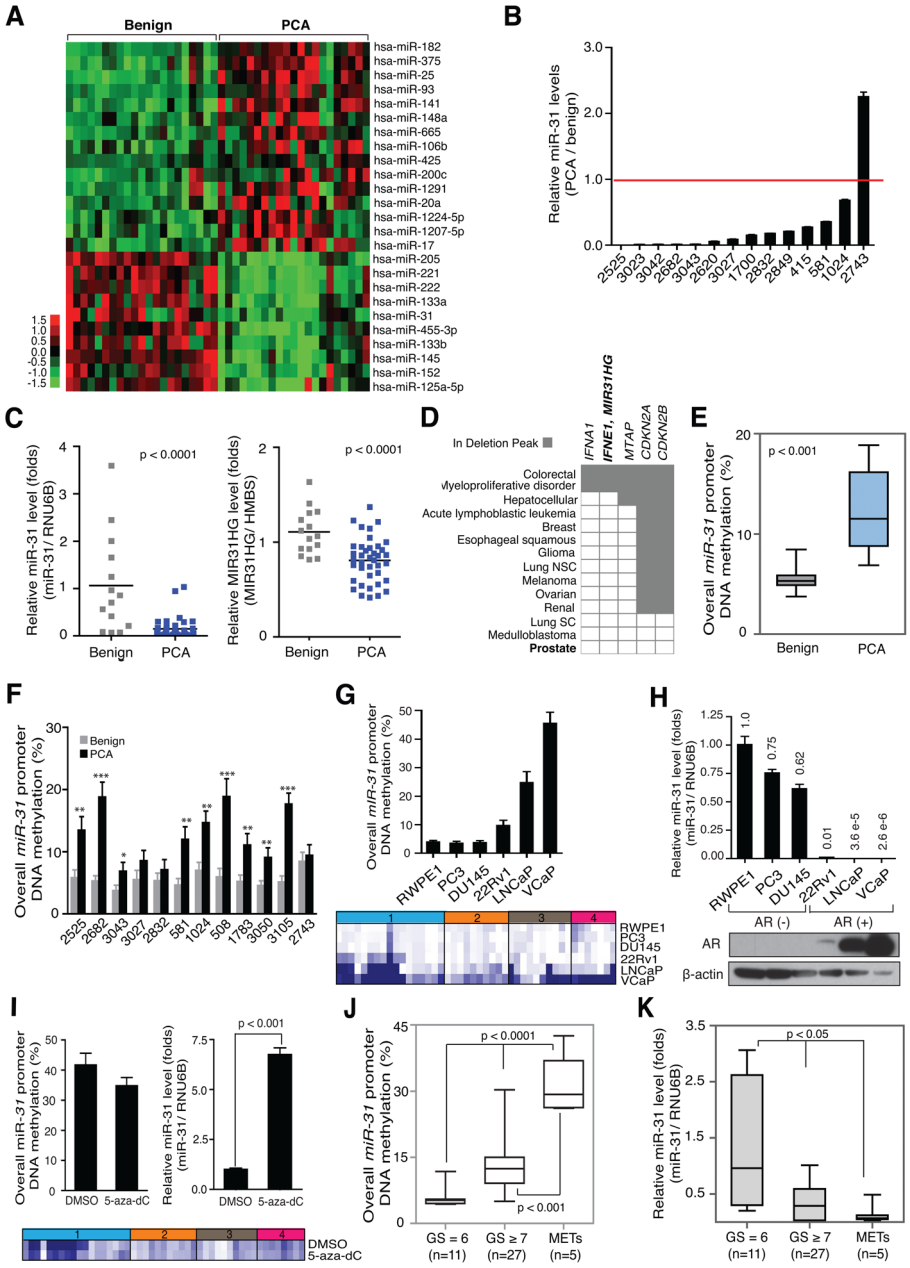

Figure 1.

MiR-31 is downregulated in PCA due to promoter hypermethylation. A, heatmap of the 25 differentially expressed miRNAs in PCA as compared to 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 PCA 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 PCA (n=12) and Benign (n=12). F, comparison of overall DNA methylation at the *miR-31* promoter in 12 matched pairs (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$). 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 (DMSO) or 5-aza-dC. Left panel and heatmap: DNA methylation levels, right panel: miR-31 levels (n=3). J–K, comparisons of DNA methylation levels at the *miR-31* promoter and miR-31 levels between three groups: Gleason score (GS) 6, ≥ 7, and metastatic cancer (METs). All bar graphs are shown with mean ± SEM.

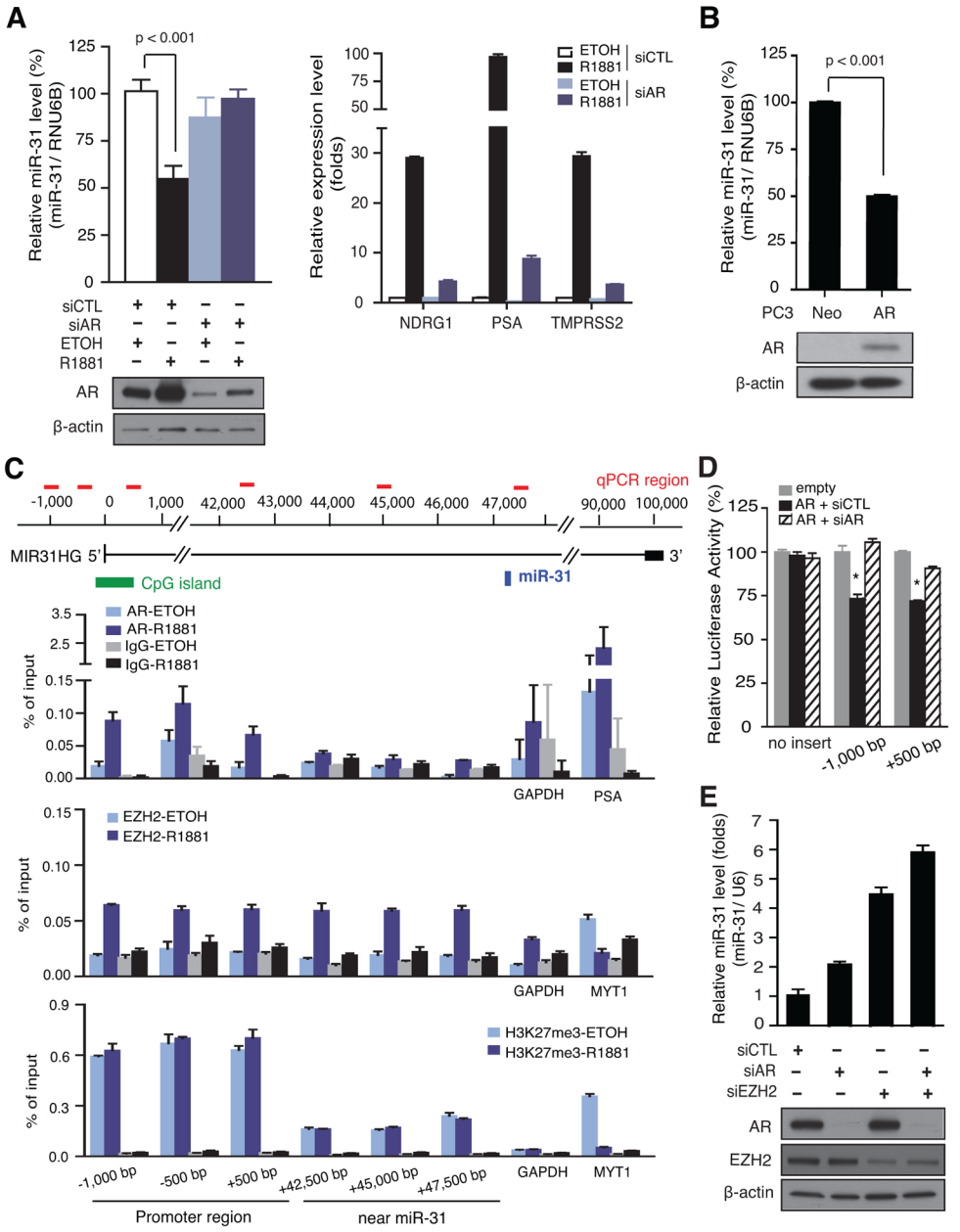

Figure 2.

AR and PRC2-mediated repressive histone modification in regulation of miR-31 expression. A, expression of miR-31 (left panel) and NDRG1, PSA, and TMPRSS2 (right panel) in LNCaP cells transfected with AR siRNA (siAR) or control siRNA (siCTL), and treated with 1 nM 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 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 (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 +500bp co-transfected 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 ± SEM.

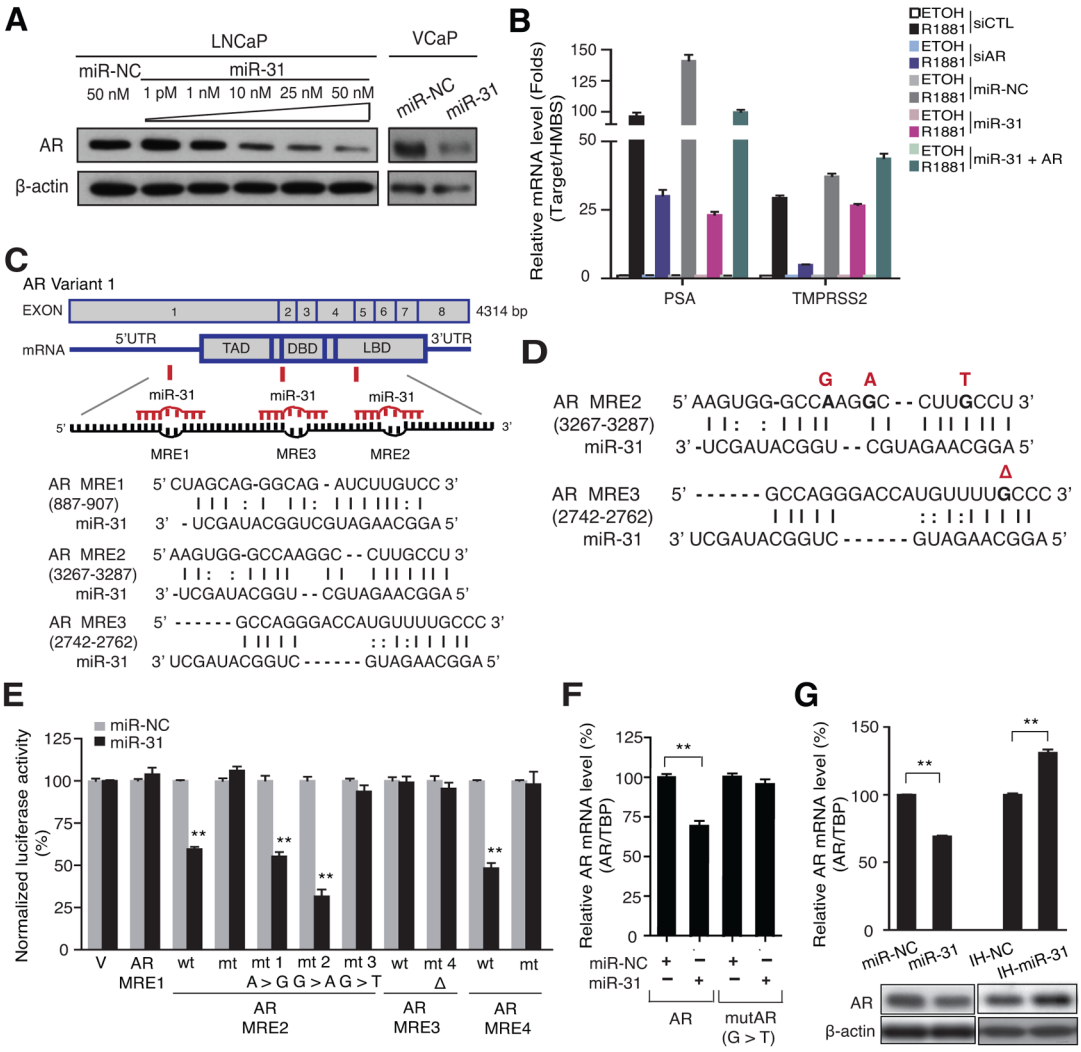

Figure 3.

Downregulation of 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 1nM R1881 or vehicle (ethanol) for 24 hours. C, schematic graph illustrating predicted locations of three miR-31 MREs within the transcript of AR variant 1. Numbers in parenthesis 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 co-transfected 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 co-transfected 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.

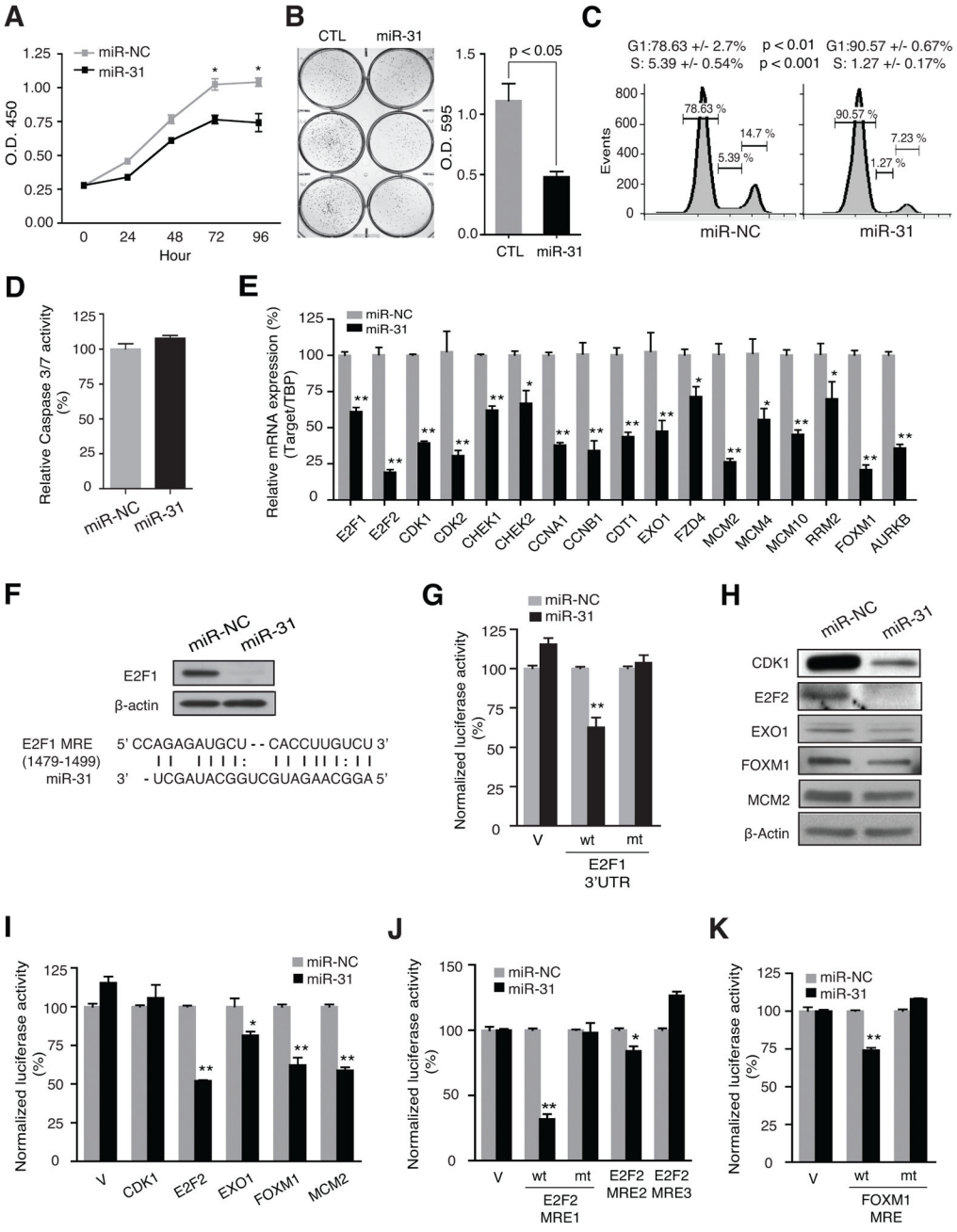

Figure 4.

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, * $p < 0.001$). 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 FACS (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 of E2F1 with lysates from LNCaP cells transfected with miR-31 or miR-NC (top). Schematic graph illustrates the miR-31 MRE within the 3′UTR of E2F1 (bottom). G, luciferase activity of LNCaP cells co-transfected with reporter constructs containing WT

or mutant (mt) E2F1 3′UTR or vector alone (v) with either miR-31 or miR-NC (n = 3, **p < 0.01). H, expression levels of indicated proteins from LNCaP cells transfected with miR-31 or miR-NC by immunoblot. I, luciferase activity of LNCaP cells transfected with reporter constructs containing 3′UTRs of CDK1, E2F2, EXO1, FOXM1, or MCM2 in conjunction with miR-31 or miR-NC (n = 3, *p < 0.05, **p < 0.01). J–K, luciferase activity of LNCaP cells transfected with reporter constructs containing WT or mutant MREs of E2F2 and FOXM1 in conjunction with miR-31 or miR-NC (n = 3, *p < 0.05, **p < 0.01). All bar graphs are shown with mean ± SEM.

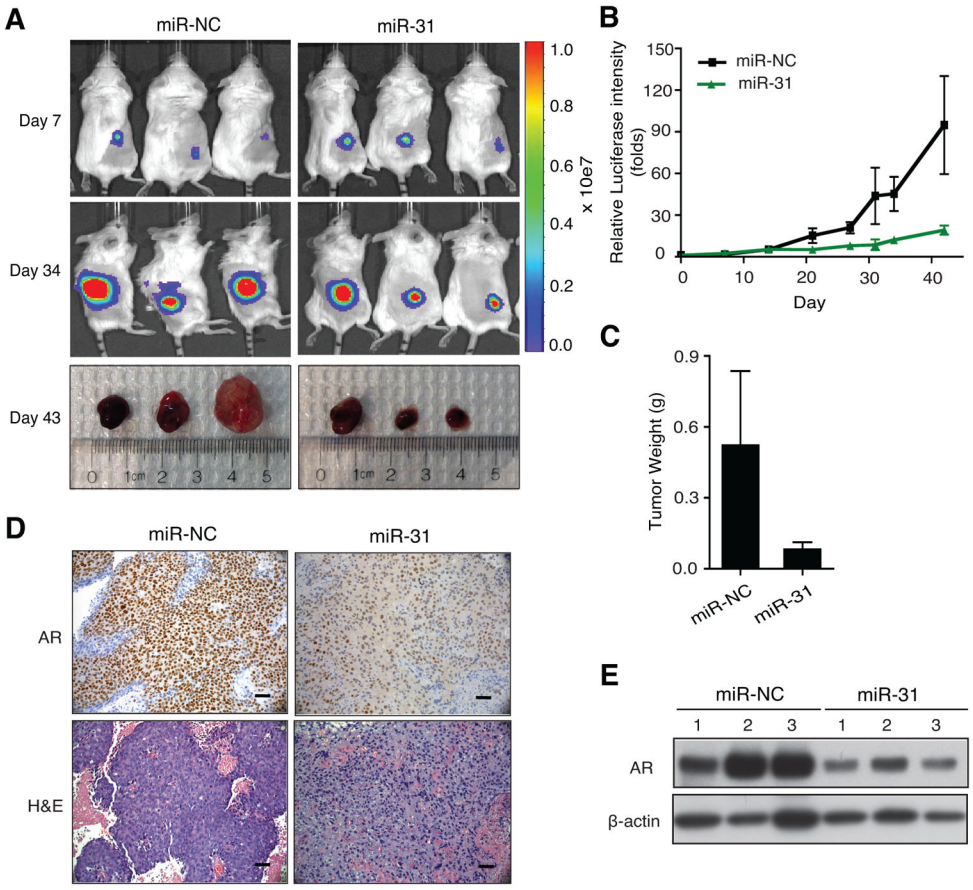

Figure 5.

MiR-31 represses PCA growth *in vivo*. A–B, luciferase imaging in mice with LNCaP xenografts treated with miR-31 or miR-NC intratumorally. The experiment was terminated after 43 days of initial treatment. C, tumors were removed on Day 43 and weighed. D, representative immunohistochemistry images of AR (top) and Hematoxylin and eosin staining (H&E) (bottom) in LNCaP xenografts treated with miR-31 or miR-NC. Scale bar: 100 μm. E, expression of AR protein levels in LNCaP xenografts treated with miR-31 or miR-NC, evaluated by immunoblot.

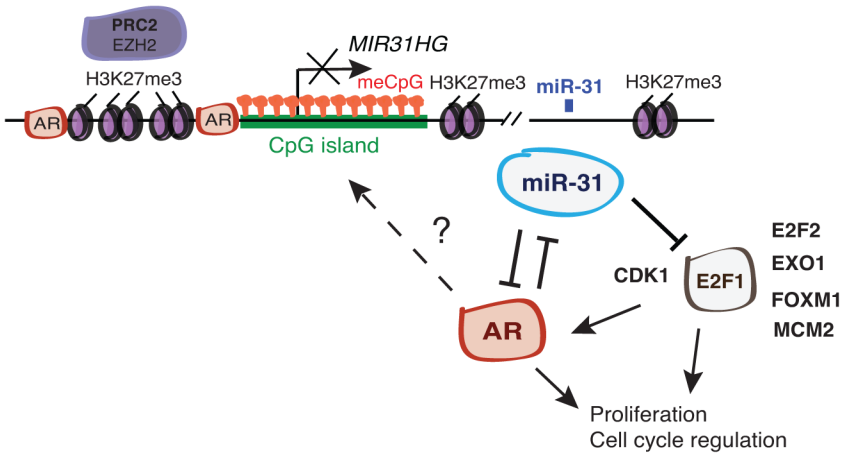

Figure 6.

Mutual regulatory model of miR-31 and AR. MiR-31 inhibits the expression of AR and several proteins involved in cell cycle regulation and proliferation. On the other hand, AR and H3K27 trimethylation can repress miR-31 expression. During PCA pathogenesis, increased promoter methylation leads to the loss of miR-31 expression. Downregulation of miR-31 in PCA may occur as an early event in PCA resulting in increased AR expression. Alternatively, increased AR expression or activity may be a preliminary event that leads to miR-31 silencing.