Systematic Analysis of MicroRNAs Targeting the Androgen Receptor in Prostate Cancer Cells

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Abstract

Androgen receptor (AR) is expressed in all stages of prostate cancer progression, including in castration-resistant tumors. Eliminating AR function continues to represent a focus of therapeutic investigation, but AR regulatory mechanisms remain poorly understood. To systematically characterize mechanisms involving microRNAs (miRNAs), we conducted a gain-of-function screen of 1129 mRNA molecules in a panel of human prostate cancer cell lines and quantified changes in AR protein content using protein lysate microarrays. In this way, we defined 71 unique miRNAs that influenced the level of AR in human prostate cancer cells. RNA sequencing data revealed that the 3′UTR of AR (and other genes) is much longer than currently used in miRNA target prediction programs. Our own analyses predicted that most of the miRNA regulation of AR would target an extended 6 kb 3′UTR. 3′UTR-binding assays validated 13 miRNAs that are able to regulate this long AR 3′UTR (miR-135b, miR-185, miR-297, miR-299–3p, miR-34a, miR-34c, miR-371–3p, miR-421, miR-449a, miR-449b, miR-634, miR-654–5p, and miR-9). Fifteen AR downregulating miRNAs decreased androgen-induced proliferation of prostate cancer cells. In particular, analysis of clinical prostate cancers confirmed a negative correlation of miR-34a and miR-34c expression with AR levels. Our findings establish that miRNAs interacting with the long 3′UTR of the AR gene are important regulators of AR protein levels, with implications for developing new therapeutic strategies to inhibit AR function and androgen-dependent cell growth. Cancer Res 71(5): 1–12. ©2011 AACR.

Introduction

Androgen receptor (AR) is critical for the development and progression of prostate cancer (PCa) and AR inhibition represents the first-line therapeutic modality for patients with advanced disease (1–3). In normal prostate, androgens promote survival and differentiation, but during PCa development, AR becomes an inducer of uncontrolled cell growth (2). Endocrine therapies directed toward reducing serum androgens and inhibition of AR initially block PCa growth, but often fatal castration-resistant disease develops (4).

The molecular mechanisms responsible for this lethal transition in PCa are not completely understood. AR is known to be expressed throughout PCa progression (5). The most consistent change associated with castration-resistant growth in global gene expression profiles of PCa xenografts was a small increase in the AR mRNA levels (6). Chen and colleagues have shown that the increase of AR mRNA and protein was necessary and sufficient for the conversion from a hormone-sensitive to a hormone-refractory state (6). Central to the AR response are the specific downstream target genes, including the oncogenic ETS fusion genes (7). Recent findings suggest that in the castration-resistant stage of the disease, AR controls a distinct transcriptional program linked to cell-cycle regulation (8). Taken together, these results suggest that AR signaling plays a central role in all stages of PCa and therefore, it is critically important to understand the regulation of its expression and to find novel ways of inhibiting this pathway.

MiRNAs are small (~22 nucleotides), endogenously occurring untranslated RNAs that control gene expression post-transcriptionally by inhibiting protein translation and/or degrading target mRNA (9, 10). Each miRNA can control hundreds of target genes and up to 60% of all transcripts are potentially modulated by miRNAs (11). Strong links between miRNA deregulation and human disease, particularly cancer, have been established (12–15). Altered miRNA expression profiles have been shown in a large number of cancer types, including PCa (16) and altered miR-34c expression has...
been shown to inversely correlate with aggressiveness of PCa (17).

To understand the role of miRNAs in PCa, we carried out a systematic study of their role in regulating the AR protein in 5 PCa cell lines. A gain-of-function analysis of 1129 miRNA molecules was combined with AR protein quantitation using reverse-phase protein lysate microarray (LMA). RNA sequencing was used to determine the length of the AR 3′UTR and custom bioinformatic prediction of miRNAs targeting AR was done. With 3′UTR luciferase reporter assays, 13 miRNAs were identified as regulators of AR 3′UTR. Several miRNAs affecting AR protein levels were also potent reducers of androgen-induced proliferation. Taken together, our data show that AR protein levels were also potent reducers of androgen.

Custom miRNA target prediction

BLAT search was used to acquire human transcript AR-001 (ENST000003574690) and orthologous UTRs for 10 mammalian species that were aligned using ClustalW. Predictions for all the miRNAs covered by TargetScan 5.0 algorithm were obtained (11).

Immunoblotting

SDS-PAGE and immunoblotting were done as described previously (18). AR, PSA, and β-actin, specific primary antibodies were used (#1-280, Santa Cruz Biotechnology; #A0562 Dako Cytomation; #A1978 Sigma-Aldrich, respectively). The signals were obtained with Alexa Fluor 680 tagged secondary IgG antibodies (Invitrogen) and Odyssey Licor scanner (LI-COR Biosciences).

Quantitative real-time RT–PCR analysis

RNA isolation and reverse transcription were done as described previously (18). Specific primers for AR, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β-actin (ACTB) were designed by Universal Probe Library (Roche Applied Biosciences; Supplementary Table II). Fluorescent Taqman probes were from Roche Human Probe Library (AR: no. 14, GAPDH no. 60, ACTB no. 64). The expression of AR mRNA was determined by the relative quantitation method using GAPDH or ACTB as controls. Data were collected from 2 separate biological experiments, run twice with triplicates.

AR 3′UTR reporter constructs and luciferase assays

Seven separate fragments of the AR 3′-UTR region were amplified from LNCaP genomic DNA using specific primers for AR 3′UTR (Supplementary Table III). The AR 3′UTR fragments were cloned into Mlu/HindIII sites of pMIR-REPORT luciferase vector (Ambion Inc.). Luciferase assays were done in 22Rv1 cells as previously described (18).

Microarray miRNA expression analyses in prostate cell lines

MiRNA expression were profiled with Agilent human miRNA microarrays (V1). RNA was isolated from LNCaP, LNCaP, LNCaP-C4–2B, LNCaP-C4–2B4, MDA-PCa-2b, PC-3, PC-3-MP40, parental PC-3, VCaP and 22Rv1, EP156T, PWR-1E, and RWPE-1 cells with mirVana miRNA Kit (Applied Biosystem) and labeled according to the Agilent protocol (version 1.0, April 2007). The arrays were scanned with Agilent Microarray G2565, and Agilent Feature Extraction Software (version 9.5) was used to extract the data. The data were normalized using the vsn R-package (22) and generalized log2 variance stabilization.

Pca tissue specimens and analysis of mir-34c levels by qRT-PCR

Prostatic tissues obtained by transurethral resection of the prostate (TURPs) were collected 1990–1999 in Malmö,
Swedish. The materials were fixed in 4% buffered paraformaldehyde and paraffin-embedded. Results were based on histopathological diagnosis in randomly selected cases with evidence of prostate adenocarcinoma in 47 patients. The age range at the time of TURP was 63–89, with a mean of 76 years. Appropriate ethical approval has been obtained from the Ethics’ Committee, Lund University, and we have adhered to the Helsinki Declaration. Analyses of miR-34a/c levels by the Ethic’s Committee, Lund University, and we have adhered to the Helsinki Declaration. Analyses of miR-34a/c levels by the Ethic’s Committee, Lund University, and we have adhered to the Helsinki Declaration.

**Immunohistochemistry and scoring of AR on prostate tissue slides**

Detection of AR in the prostate tissue sections was done by Dako EnVision FLEX detection system (Dako) on a Dako Autostainer. Briefly, 4-μm sections were deparaffinized with xylene, rehydrated through a graded series of ethanol followed by heat-induced epitope retrieval using EnVision FLEX target retrieval solution high pH. Endogenous peroxidase was blocked by EnVision FLEX peroxidase-blocking reagent. AR (#AR 441, NeoMarkers, Thermo Scientific) was used as a primary antibody. Detection was done by Dako EnVision FLEX/HRP detection system using EnVision FLEX DAB+ Chromogen, diaminobenzidine (DAB) solution and EnVision FLEX substrate buffer. The slides were mounted with organo-limonene Mount (Santa Cruz Biotechnology). The stained sections were scored independently, based on the AR staining intensity, by 1 pathologist and 2 researchers. Overall staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate to strong), and 3 (intense). For each patient, the AR intensity was scored in malignant epithelium, benign epithelium and stroma.

**Cell viability assays**

MDA-PCa-2b cells in 5% charcoal-stripped serum were reverse-transfected with 20 nmol/L miRNAs and treated with 0.1 nmol/L R1881 (NLP005005, Perkin Elmer Inc). Control cells were treated with 10 μmol/L Casodex (Bicalutamide, B9061, Sigma-Aldrich). Viability was measured 7 days after transfection using CellTiter-Glo Luminescence Assay (Promega) with Envision Plate-reader (Perkin Elmer Inc.). All measurements were done in quadruplicates with 2 biological repetitions (n = 8). Students t-test was used to determine P-values.

**Results**

**Systematic analyses of miRNA impacting on AR protein levels**

We conducted LMA screens to identify miRNAs that influence protein levels of AR in PCa cell lines (LNCaP, LAPC-4, CWR-R1, 22Rv1, MDA-PCa-2b). The cells were transfected with 2 miRNA overexpression libraries containing a total of 1129 miRNAs. We aimed at identifying miRNAs that affect AR in all 5 cell lines (Fig. 1A). The results were subjected to rank-based meta-analysis using the RankProd function from the R/Bioconductor (20). We identified a total of 77 miRNAs, of which 71 were unique mature miRNA sequences influencing the levels of AR in all 5 cell lines (Fig. 1B, Supplementary Table IV), 52 decreasing and 19 increasing AR protein.

**Characterization of the AR 3’UTR length**

Next, we studied whether these miRNAs were predicted to bind to the AR 3’UTR. The prediction programs such as TargetScan and microRNA.org use RefSeq sequences (http://www.ncbi.nlm.nih.gov/RefSeq/) in their predictions (11, 23). The RefSeq for AR (NM_000044) has a transcript length of 4314 bp and a 3’UTR of 436 bases. However, a much longer transcript with a length ranging from 4.7 to 11 kb has been detected (24, 25). To investigate the length of the AR 3’UTR, we obtained RNA-Sequencing data from untreated VCaP cells which have a very high expression of AR (26, 27). The 8 exons present in our sequencing analysis produce a transcript >10 kb matching the sequence of the transcript AR-001 (ENST00000374690, http://wwwensembl.org). Specifically, the last exon (Fig. 2A, right panel) is longer than expected from the RefSeq annotation (NM_000044) and in the transcript AR-001 (ENST00000374690) ends at position 66 950 461 in GCRh37/hg19. Multiple short reads in the RNA-Sequencing data end around position 66 950 388 (data not shown), thereby producing a 3’UTR region of approximately 6680 b in VCaP cells. The presence of the long 3’UTR was confirmed by RT-PCR in VCaP, LNCaP, LAPC-4, 22Rv1, and MDA-PCa-2b cells (Supplementary Figure 1). This vastly increases the region for putative miRNA regulation of the AR transcript. Intrigued by this finding, we returned to our RNA-Sequencing data to study whether additional 3’UTR regions in our data were extended compared with current annotations. Interestingly, the data contained a total of 633 3’UTR regions that were calculated to be at least 100 b longer compared with current annotations in the Ensembl database (Supplementary Table V).

**Custom prediction of miRNA-target interactions on the AR 3’UTR**

The long AR 3’UTR sequence was analyzed for putative miRNA binding sites using the TargetScan algorithm (v. 5.0) (11) resulting in a total of 550 predicted binding sites where 664 human miRNAs were predicted to bind (Supplementary Table VI). Since predictions contain a vast amount of false positives, we compared these predictions to the results from the LMA screen (Fig. 1B). Thirty-five unique miRNAs predicted to bind one or several sites changed the protein levels of AR in the LMA screen (Supplementary Table VII). This number is probably an underestimation since TargetScan ignores star miRNAs, which were not included in our prediction. In general, the miRNAs predicted to target the AR 3’UTR were enriched among the LMA results (Fig. 2B). The 2 sample Wilcoxon test P-value for the enrichment was highly significant as calculated for all cell lines at the 48 hours and 72 hours timepoint (P = 7.5e−06). Importantly, most of the predicted miRNA regulation on the AR 3’UTR region occurs on the extended 3’UTR region and would not have been detected using the RefSeq annotation (NM_000044).

**Validation of miRNAs decreasing AR**

To validate the top ranking miRNAs decreasing AR protein and predicted to bind to the 3’UTR, we reverse-transfected 21 miRNAs (Table I) into LNCaP and 22Rv1 cells, which are
Figure 1. LMA screen for miRNAs targeting AR. A Overexpression of 1129 mature human miRNAs in PCa cells by reverse-transfection on 384-well plates. Screens were done in duplicate and with 2 time points (48 hours and 72 hours) for 5 AR positive PCa cell lines grown in full media. The lysates were printed on nitrocellulose-covered glass slides and stained with AR antibody and Sypro Ruby Blot solution to detect total protein content. Rank-based meta-analysis and correction for multiple testing ($q < 0.01$) was used to find miRNAs that regulate AR in all 5 cell lines. B For visualization, data were standardized using median and MAD from R/Bioconductor (v.2.9) with default options. Red denotes increases in AR, whereas blue represents decrease. The scale indicates Z-score standardized values ($\pm 4$ SD).
optimally transfectable PCa cell lines, and analyzed AR by Western blotting (WB) and qRT-PCR (Fig. 3A and B). Our WB results show that all selected miRNAs decreased AR protein when compared with scrambled controls (Fig. 3A). The LNCaP cells were analyzed for PSA levels and most of the AR-decreasing miRNAs downregulated the PSA protein (Fig. 3A, left panel). In contrast, overexpression of miR-299–3p, which clearly downregulated AR, led to an increase in PSA protein levels in LNCaP cells. MiR-30d, predicted to target AR by TargetScan increased AR and PSA protein in LNCaP cells, an effect also observed in the LMA screen. Although small in magnitude, the decrease of AR with miR-9 transfection in LNCaP was consistently observed in repeated experiments. The 22Rv1 cells express an AR isoform produced by alternative

Figure 2. Characterization of the AR 3’UTR by RNA-Sequencing. A RNA-Sequencing data from untreated VCaP cells on the AR locus. Sequencing coverage (red dots) was normalized by the number of uniquely mapped short reads. The boxes below the coverage plots represent exons in the AR transcript, exon 2 being present only in a shorter transcript variant (AR-201: ENST00000396043, NM_001011645). On the right a close-up of the last exon showing coverage for a much longer sequence of the 3’UTR (6680 b) than the RefSeq annotation (NM_000044, 436 b). B A histogram of LMA screen hits, which are either predicted (red line) or not predicted (blue line) to target AR. Significant enrichment of the predicted miRNA hits (P-value 7.5e-06) was observed with 2 sample Wilcoxon test.
splicing (28, 29). This alternative transcript is downregulated by miR-135b, miR-147, miR-299–3p, miR-34a, and miR-644 (Fig. 3A, right panel).

Next, we conducted qRT-PCR to investigate the effect of miRNAs on AR mRNA (Fig. 3B). Compared with the scrambled control, miR-147, miR-297, miR-298, miR-299–3p, miR-421, miR-449a reduced AR mRNA levels in both LNCaP and 22Rv1 cells. Cell-type specific effects were observed for miR-371–3p, miR-449b and miR-491–5p, which were more efficient in downregulating AR mRNA in LNCaP cells whereas miR-876–3p had this effect only in 22Rv1 cells. No clear cell-type specific differences were evident for these miRNAs at the protein level (Fig. 3A). On the other hand, miR-488 had a cell-type specific effect at the protein level and displayed only a minor difference at the mRNA level. Taken together, these data highlight distinct patterns of miRNA regulation. The results in Fig. 3B suggest that miR-644 increased AR mRNA. The data were normalized to GAPDH and miR-644 is predicted by TargetScan to target this gene. We noticed that miR-644 decreases GAPDH mRNA levels (data not shown) and we thus used β-actin for normalization for miR-644 (Fig. 3D, right panel) to show that miR-644 decreases AR mRNA by 20%.

**Table 1.**

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<th>FC</th>
<th>FDR q-value</th>
<th>Predicted binding sites</th>
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<td>0.63</td>
<td>0.000</td>
<td>7mer-m8 (x3), 8mer</td>
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<tr>
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<td>0.000</td>
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*RP/Rsum; RankProduct/Ranksum, FC: Fold Change, FDR q-value; False Discovery Rate q-value.

**Thirteen miRNAs decrease AR 3' UTR-containing reporter activity**

To investigate whether the 21 miRNAs interact with the AR 3' UTR region, we cloned 7 different fragments covering the predicted areas of interaction in luciferase reporter constructs (Fig. 4A). The reporters were transfected into 22Rv1 cells together with the miRNAs and a Renilla luciferase control. Transfection of miR-135b, miR-185, miR-297, miR-299–3p, miR-34a, miR-34c, miR-371–3p, miR-421, miR-449a, miR-449b, miR-634, miR-654–5p, and miR-9, significantly inhibited the luciferase activity of the constructs containing their predicted binding sites, as compared with scrambled transfected controls (Fig. 4B). Surprisingly miR-147, -491–5p, -644, and -876 increased the reporter activity which is most likely due to indirect effects. Our results confirmed that 13 miRNAs were able to interact with the extended 3' UTR of the AR gene. To determine whether the 3' UTR is the main regulatory region for these miRNAs, we overexpressed AR lacking the 3' UTR in PC-3 cells that are devoid of endogenous AR (Supplementary Figure 2). Our WB results showed that the majority of the 13 miRNAs exert regulation on AR primarily through the 3' UTR whereas miR-421, miR-449a, miR-449b, and miR-9 are also able to decrease the levels of the exogenous AR.

**miRNA expression in prostate cell lines**

We analyzed the expression of the AR targeting miRNAs in PCa cells and in nonmalignant cells. The data were normalized and SAM analysis (30) was used to find differentially expressed miRNAs between the malignant and control cell lines. This analysis revealed miR-34c as having higher expression in the nonmalignant lines as compared with the malignant ones (Fig. 5A, Supplementary Figure 3).

**Inverse correlation of miR-34a and miR-34c expression to AR protein levels in malignant prostate epithelium**

Reduced miR-34c expression has previously been linked to PCa aggressiveness, WHO grade, PSA levels, and occurrence of...
Figure 3. Western blot and qRT-PCR analysis of miRNAs decreasing AR. A. miRNAs were transfected into LNCaP (left panel) and 22Rv1 (right panel) cells grown in the presence of 10% FBS, lysed (72 hours) and analyzed by WB using AR, PSA and β-actin antibodies. mRNA levels were analyzed by qRT-PCR 24 h after transfection and normalized to GAPDH or ACTB levels. The bars represent averages of 2 biological and 2 technical repeats (±SEM, n = 4) compared with scrambled (scr) controls which were arbitrarily set to 1.
Therefore, we analyzed whether miR-34c and miR-34a inversely correlate with AR levels in PCA patients. The levels of miR-34c and miR-34a were measured with qRT-PCR in 47 prostatic tumors. The AR protein content was determined by immunostaining and the staining was scored by overall intensity (1; weak, 2; moderate to strong, 3; intense, Figure 5B, lower panel). When correlating miR-34c expression levels in the malignant epithelial cells with the semiquantitative assessment of AR immunostaining, we found a statistically significant inverse association (Fig. 5B, left panel, \( P = 0.0082 \) for intensity score 1 + 2 versus 3, and \( P = 0.0046 \) when comparing intensity 2 versus 3; Mann–Whitney test). No
correlation was found for miR-34c expression and AR staining in the benign epithelium nor in stromal tissue (data not shown). There was also a significant inverse correlation between AR staining intensity and miR-34a expression (Figure 5B, right panel, \( P = 0.0085 \) for intensity score 1 + 2 versus 3, and \( P = 0.0058 \) when comparing intensity 2 versus 3; Mann–Whitney test). These data together with the results from Figure 4, showing interaction of miR-34a/c on the AR 3’ UTR, suggest that these miRNAs are important regulators of AR levels during PCa progression.

**AR-inhibiting miRNAs reduce androgen-induced PCa cell viability**

To determine how androgen-induced proliferation is influenced by the miRNAs decreasing AR, we transfected the 21 validated miRNAs into MDA-PCa-2b cells, treated these with a
synthetic androgen analog R1881 and measured viability 7 days later. Casodex was able to reduce proliferation of MDA-PCa-2b cells by 60% whereas a positive control siRNA (siCellDeath) showed a reduction of up to 80% (Fig. 6). The siRNAs against AR decreased viability by 20%–40%, indicating that this cell line is not completely dependent on AR for sustaining cell growth. Most miRNAs (15/21) reduced viability to at least the same extent as the AR siRNAs (Fig. 6). This suggests that miRNAs present a novel and potent way of influencing not only AR levels but also the more complex phenomena of androgen-induced prostate cell proliferation.

Discussion

We report here a systematic gain-of-function screening to discover AR regulating miRNAs in PCa cell lines. By focusing on miRNAs that influence AR across all the cell lines, we reduced cell-type specific bias, and identified 71 unique miRNAs influencing AR protein levels, 52 decreasing and 19 increasing. To date there is only one report, published during revision of this manuscript, showing miR-488* regulation of AR through the proximal 3'UTR region (31). Thus, our screen vastly enhances the understanding on the role of posttranscriptional regulation of this key player in PCA.

The 3’UTR sequence of a gene is often considered the primary region of miRNA regulation (10), although regulatory sites may exist also in other regions of the transcript (32–36). Our RNA-Sequencing results show that for hundreds of genes, including the AR, the 3’UTR regions are much longer than currently annotated in the genome databases. Therefore, the commonly used miRNA target prediction algorithms will miss such miRNA regulation. For example, in the case of the AR, only the short 436 b 3’UTR is used in such analyses, while our custom prediction, validated by experimental data, was based on the 6680 b 3’UTR region. Understanding of miRNA regulatory networks in cancer will thus be critically dependent on better characterization of 3’UTRs. Interestingly, a recent RNA-sequencing study in *C. elegans* resulted in a revision of approximately 40% of gene models (37).

Our study is also one of only a handful of reports showing comprehensive analysis of multiple miRNAs targeting the same gene (18, 38). All the selected miRNAs from the LMA screen were validated in LNCaP and 22Rv1 cells, showing the robustness of the LMA technology. Only 6 of the miRNAs decreased the mRNA significantly after 24 hours suggesting that most miRNAs decrease AR protein through translational inhibition.

The introduction of miRNA mimics made it possible for us to systematically identify miRNAs capable of regulating AR. However, to explore, whether endogenous levels of these miRNAs impact on AR protein levels, we conducted expression analyses of 13 different prostate cell lines and 47 clinical tumors. These analyses showed an inverse correlation in clinical patient samples between the AR levels and miR-34a and miR-34c expression validating the functional cell line data. Previously, reduced miR-34c expression has been linked to PCa aggressiveness (17).

MiR-34a and miR-34c belong to a family of evolutionary conserved miRNAs that are regulated by the tumor-suppressor TP53 (39, 40). *mir-34a* is encoded by its own gene, whereas *mir-34b* and *mir-34c* share a common primary transcript. Although the seed of miR-34b is very similar to miR-34c, we did not observe any effect of miR-34b in our LMA screen. *Mir-34* genes have been suggested to be potent mediators of tumor suppression by p53 and are implicated in the negative control of the cell cycle, senescence, and apoptosis (39). Our results on miR-34a and miR-34c downregulating AR, represent an additional explanation by which PCa cells could benefit from reduced expression of these cancer-relevant miRNA genes.

Fifteen AR-regulating miRNAs were potent regulators of androgen-induced proliferation of PCa cells. In our analyses, miR-541, miR-631, miR-634, miR-644, miR-654–5p, and miR-876–3p were more efficient inhibitors of cell growth than AR
siRNAs or treatment with Casodex. The strong effects of these miRNAs suggest that they may target additional important players in PCa proliferation.

Almost 70 years after the initial finding of the important role of androgen in PCa (41), reducing AR activity by anti-androgen remains the primary treatment mode for advanced PCa. Recently, novel drugs aimed at targeting androgen signaling have been reported including abiraterone, RD162, and MDV3100 (42–44). An important feature of MDV3100 a second-generation anti-androgen is that it inhibits AR activity despite increased levels of AR (44). The miRNAs identified in our study reduced AR and were able to efficiently reduce androgen-induced proliferation. In conclusion, our results show that miRNAs binding to the long 3′UTR of the AR gene need to be considered as one mechanism for how PCa cells regulate the levels of AR. This provides future opportunities and starting points to explore the applications of miRNAs and their derivatives in PCa therapy.

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