miR-124 and Androgen Receptor Signaling Inhibitors Repress Prostate Cancer Growth by Downregulating Androgen Receptor Splice Variants, EZH2, and Src

Xu-Bao Shi1, Ai-Hong Ma1, Lingru Xue1, Meimei Li1, Hao G. Nguyen2, Joy C. Yang1, Clifford G. Tepper3,4, Regina Gandour-Edwards5, Christopher P. Evans1,4, Hsing-Jien Kung3,4, and Ralph W. de Vere White1,4

Abstract

miR-124 targets the androgen receptor (AR) transcript, acting as a tumor suppressor to broadly limit the growth of prostate cancer. In this study, we unraveled the mechanisms through which miR-124 acts in this setting. miR-124 inhibited proliferation of prostate cancer cells in vitro and sensitized them to inhibitors of androgen receptor signaling. Notably, miR-124 could restore the apoptotic response of cells resistant to enzalutamide, a drug approved for the treatment of castration-resistant prostate cancer. We used xenograft models to examine the effects of miR-124 in vivo when complexed with polycylenimine-derived nanoparticles. Intravenous delivery of miR-124 was sufficient to inhibit tumor growth and to increase tumor cell apoptosis in combination with enzalutamide. Mechanistic investigations revealed that miR-124 directly downregulated AR splice variants AR-V4 and V7 along with EZH2 and Src, oncogenic targets that have been reported to contribute to prostate cancer progression and treatment resistance. Taken together, our results offer a preclinical rationale to evaluate miR-124 for cancer treatment.

Introduction

Prostate cancer is the most frequently diagnosed malignant tumor and the second leading cause of cancer death in American men (1). Our recent study showed that for the past 20 years, the survival for patients presenting in California with metastatic prostate cancer has not improved (2). The androgen receptor (AR) is critical for the development and progression of prostate cancer. Until now, androgen deprivation therapy (ADT), that inhibits AR signaling, represents the primary therapy for patients with hormone-sensitive metastatic prostate cancer. Although it is initially effective, patients invariably relapse and their tumors subsequently affect the signaling pathways involved in a number of physiologic as well as pathologic conditions including cancer. Many miRNAs that act as tumor suppressors or as oncogenes were reported to be aberrantly expressed in various cancer types (11). Of the known aberrantly expressed and cancer-related miRNAs, miR-124 represents an ideal candidate for therapeutic development. Accumulating evidence indicates that miR-124 is a tumor suppressive miRNA in several types of human cancer (13–15).
Materials and Methods

Reagents

Bicalutamide was obtained from AstraZeneca. Enzalutamide (marketed as Xandi and formerly known as MDV3100) was obtained from Medivation, Inc. For in vitro studies, enzalutamide was dissolved in dimethyl sulfoxide. For animal studies, enzalutamide was mixed with 0.5% Methocel A4M suspension (Kremer Pigments Inc.). Ambion pre-miR-124 precursors and mirVana miR-124 mimics are small double-stranded RNA molecules that mimic endogenous miR-124 precursors and are used as negative controls (miR-NC). Pre-miR-124 precursors and mirVana miR-124 mimics are small double-stranded RNA molecules that mimic endogenous miR-124 and upregulate miR-124 activity. Polyethylenimine (jetPEI), a delivery vehicle used in laboratory and clinical trials, was purchased from jetPEI). A delivery vehicle used in laboratory and clinical trials, was purchased from Polyplus-transfection, Inc. PEI-miR-124 complexes were prepared following the manufacturer’s protocol. The anti-AR-V7 monoclonal antibody was purchased from Precision Antibody Store.

Cell lines and culture

Prostate cancer cell lines (LNCaP, C4-2B, 22R\textsubscript{1}, and VCaP) were maintained in RPMI-1640 medium supplemented with 10% FBS (FBS medium) or in RPMI-1640 medium containing 10% charcoal-stripped serum (androgen-deprived medium). To generate the enzalutamide-resistant 22R\textsubscript{1} subline, 22R\textsubscript{1} cells were cultured in androgen-deprived medium containing gradually increased concentrations of enzalutamide (from 5 to 40 μmol/L over 4 months). The resultant enzalutamide-resistant 22R\textsubscript{1} cells were maintained in androgen-deprived medium containing 10 μmol/L enzalutamide.

Cell proliferation assay

Prostate cancer cells (3 × 10\textsuperscript{5}/well) were seeded in 96-well plates in FBS medium or androgen-deprived medium. After being cultured for 24 hours, cells were transfected with 50 nmol/L miR-124. After 5 hours, cells were treated with fresh medium without or with 10 μmol/L enzalutamide or bicalutamide. A tetrazolium-based cell proliferation assay (WST-1, Promega) was carried out according to the manufacturer’s protocol.

Western blot assay

Total protein was extracted from cultured cells or xenograft tumors, and the concentrations were estimated using the Coomassie (Bradford) Protein Assay Reagent (Pierce). Equal amounts of denatured protein samples were loaded on a 10% SDS-PAGE. After electrophoresis, proteins were transferred to Immobilon PVDF membrane. Immunoblotting was conducted using individual specific primary antibodies and appropriate horseradish peroxidase-conjugated secondary antibodies following standard protocols.

Clonogenic assay

Six-well plates were seeded with 3 × 10\textsuperscript{4} 22R\textsubscript{1}-EnzR cells per well and incubated overnight. Cells were transfected with miR-124 or transfected with miR-124 for 3 days followed by treatment with 10 μmol/L enzalutamide. After 2 weeks, cells were stained with crystal violet (0.4% crystal violet in 20% methanol). For quantitative clonogenic assays, the colonies were solubilized with 30% acetic acid, and the absorbance was read at a wavelength of 540 nm.

Animal experiments

Animal studies were performed according to the protocols approved by the Institutional Animal Care and Use Committee of the University of California, Davis (Sacramento, CA). Male athymic nude mice (4~6 weeks old) were purchased from Harlan Laboratories and housed in pressurized, ventilated cages with standard rodent chow and water and a 12-hour light/dark cycle. The CWR22 xenograft tumor was a gift from Dr. Thomas A. Pretlow (Case Western Reserve University, Cleveland, OH). Xenografts were implanted by subcutaneous injection into the flanks of the mice with CWR22 cell suspensions (~2 × 10\textsuperscript{6} cells) in a mixture (1:1 vol/vol) of culture medium and Matrigel (Becton Dickinson). When tumor volume reached about 50 mm\textsuperscript{3}, mice were randomized into four treatment groups (n = 8 mice per group): negative control, miR-124, enzalutamide, and miR-124 + enzalutamide. Treatment was conducted by intravenous injection of 10 μg jetPEI/miR-NC or 10 μg jetPEI/miR-124 complexes (three times weekly for 5 weeks) or by oral administration of enzalutamide (20 mg/kg/wk, once weekly for 5 weeks). During
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the treatment period, tumor volumes were monitored twice weekly and tumor volume was calculated according to the following formula: \( \frac{1}{2} \times \text{length} \times \text{width} \times \text{height} \). Mice were either sacrificed before or on day 35 after the first treatment due to having tumors that reached the upper limit for acceptable size according to the criteria of the IACUC or euthanized on day 42 because of planned termination of the experiment. Tumors were removed and immediately snap-frozen for RNA isolation or Western blot analysis.

**TUNEL assay**

Apoptosis was detected on 4-μm-thick formalin-fixed, paraffin-embedded tumor specimens using the TUNEL Assay Kit (Roche) following the manufacturer's protocol. In brief, paraffin sections were dewaxed in xylene, rehydrated in serially graded ethanol steps, and treated with proteinase K and H2O2. After washing with PBS, the slides were incubated in buffer containing TUNEL-peroxidase for 1 hour at 37°C. The reaction was stopped by rinsing the slides in stop wash buffer. The sections were then incubated with 3,3'-diaminobenzidine solution. After counterstaining with hematoxylin, the sections were covered and apoptosis was assessed by light microscopic examination. Apoptosis was quantitated by measuring the TUNEL-positive areas in three randomly chosen fields in each of three tumors.

**Results**

**miR-124 inhibits proliferation of prostate cancer cells alone or in combination with ARSIs**

Our previous studies revealed that miR-124 was downregulated in clinical prostate cancer samples and lentivirally expressed miR-124 (lenti-miR-124) inhibited growth of prostate cancer cells, indicating that miR-124 acts as a tumor suppressor (16). Hence, to explore miR-124 as a potential therapeutic modality, we determined whether synthetic miR-124 is able to reduce resistance of prostate cancer cells to enzalutamide. For this purpose, both androgen-independent 22Rv1 and C4-2B cells grown in androgen-deprived medium and androgen-dependent LNCaP cells in FBS medium were treated with miR-124 and enzalutamide, alone or in combination. As shown in Fig. 1A–C, combination treatment resulted in significant inhibition of proliferation compared with the single-agent treatment (\( P < 0.01 \)). In addition, C4-2B cells were treated with miR-124 and bicalutamide. Similarly, the combination treatment significantly increased growth inhibition of C4-2B cells (\( P < 0.01 \), Fig. 1D), which is accompanied with obvious cell morphologic changes, characterized by cellular shrinking or displaying an appearance of dying cells (Supplementary Fig. S1). These in vitro data provide evidence that miR-124 increases therapeutic efficacy of ARSIs.

**miR-124 restores the response of prostate cancer cells to enzalutamide**

In our previous study, we found that miR-124 directly targets full-length AR and downregulates the level of truncated ARs (16) that are derived from AR transcript splice variants (18). Because expression of AR variants mediates the development of resistance to enzalutamide (19), we tested whether miR-124 is able to reduce resistance of prostate cancer cells to enzalutamide. We established an enzalutamide-resistant subline (22Rv1-EnzR) that displays an equivalent growth rate as the parental 22Rv1 cells (data not shown), and an 8-fold increase in the expression of AR-regulated miR-125b (Supplementary Fig. S2A). As miR-124 downregulates miR-125b (16), we first evaluated the effect of miR-124 on miR-125b levels. Treatment with miR-124 induced a 52% reduction of miR-125b (Supplementary Fig. S2B). The effect of miR-124 on proliferation of 22Rv1-EnzR cells was then assessed. Consistent with the results for the parental 22Rv1 cell line, in which miR-124 reduced clonogenic cells by 43%, treatment of 22Rv1-EnzR cells with miR-124 resulted in a clonogenic inhibition by 36% when compared with the miR-NC control (\( P < 0.01 \), Fig. 2A and B). Next, we tested whether miR-124 was able to decrease the resistance of 22Rv1-EnzR cells to enzalutamide. In this regard, 22Rv1-EnzR cells were first transfected with miR-124 for 3 days followed by enzalutamide treatment for 5 days. It was found that miR-124 treatment significantly increased the efficacy of enzalutamide (10% in enzalutamide vs. 68% in enzalutamide + miR-124, \( P < 0.01 \), Fig. 2C). Data shown in Fig. 2 strongly suggest that miR-124 is able to restore the responsiveness of 22Rv1-EnzR cells to enzalutamide.

**Identification of miR-124 targets**

To further understand the role of miR-124 in prostate cancer, we performed miR-124 target prediction using the BIBISERV and TargetScan programs and focused on prostate cancer metastasis- and castration resistance–related genes. Three genes were predicted to be potential miR-124 targets on the basis of the presence of miR-124–binding sites in their 3’UTRs: EZH2, Src, and STAT3, which function as oncogenes and contribute to metastasis and castration resistance of prostate cancer. To validate the in silico predictions, luciferase assays of their 3’UTRs were completed. Cotransfection of a luciferase-EZH2 3’UTR reporter and synthetic
miR-124 (50 nmol/L) in C4-2B cells resulted in a reduction of luciferase activity by 46% (Fig. 3A). Similarly, cotransfection of the luciferase-Src 3′UTR reporter and synthetic miR-124 induced a 42% decrease of the enzyme activity (Fig. 3B) when compared with transfection with the negative control (miR-NC). Similar results were obtained in PC3 cells (data not shown). We also examined the levels of EZH2 and total Src proteins in miR-124–transfected 22Rv1 and C4-2B cells and found that miR-124 downregulated these two oncogenic proteins (Fig. 3C and D). Although STAT3 was also identified as a high potential target of miR-124, experimental data did not support this, at least in the context of prostate cancer cells tested (data not shown).

Figure 2.
The inhibitory effect of miR-124 on colony formation in enzalutamide-resistant 22Rv1 (22Rv1-EnzR) cells. A, influence of miR-124 on colony-forming cells, as evaluated by quantitative clonogenic assay. B, representative dishes are shown for colony-forming assays of 22Rv1 cells (left) and 22Rv1-EnzR cells (right). C, miR-124 decreases the resistance of 22Rv1-EnzR cells to enzalutamide (Enz). The clonogenic assays were repeated three times, with similar results obtained each time. The results are shown as mean ± SE (n = 3). * P < 0.01, miR-124 treatment versus miRNA-negative control (miR-NC) treatment. untreat., untreated.

Figure 3.
Validation of EZH2 and Src as miR-124 targets. A and B, luciferase reporter assay analyses of the 3′UTRs of EZH2 and Src in C4-2B cells. The top sequences are the miR-124 seed sequence and the predicted miR-124–binding sites in the EZH2 and Src 3′UTR target regions. The 3′UTRs of EZH2 and Src lacking the miR-124 binding site were used as controls in these experiments. The assays were repeated three times, with each assay being performed in triplicate wells and similar results being obtained each time. Representative results are shown as mean ± SD (n = 3). MBS, miR-124–binding site; miR-NC, miRNA-negative control; RLU, relative luciferase units. The numbers (25 and 50) are the concentrations (nmol/L) of miR-124 used in these assays. C and D, Western blot analyses of EZH2 and Src expression levels in C4-2B cells, treated, and untreated controls.
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Previous study demonstrated that miR-124 downregulated truncated AR (16), suggesting a regulatory link between miR-124 and AR splice variants that lack the AR ligand–binding domain (LBD). In this study, we observed an increased expression of AR-V7 in 22Rv1 and enzalutamide-resistant 22Rv1 (22Rv1-EnzR) cells. A, top, map of AR-V3, -V4, and -V7 3′UTRs (black) with exon 2 (E2) and/or exon 3 (E3; white) plus an identical miR-124–binding site in their 3′UTRs and the miR-124 seed sequence. TGA is the stop codon. Bottom, luciferase reporter assay analysis of the 3′UTR of AR-V7 in 22Rv1 cells. The assay was repeated three times, with each assay being performed in three wells and similar results were obtained each time. Representative results are shown as mean ± SD (n = 3). MBS, miR-124–binding site; miR-NC, miRNA-negative control; RLU, relative luciferase units. C, Western blot analysis of AR-V7 expression in miR-124-treated 22Rv1 cells. E, WST proliferation analysis of AR-V7 in 22Rv1 and VCaP cell lines that were treated with siAR-V7 for 3 days, followed by treatment with 50 nmol/L miR-124.

miR-124 targets. A, Western blot analysis of AR-V7 expression level in 22Rv1 and enzalutamide-resistant 22Rv1 (22Rv1-EnzR) cells. B, top, map of AR-V3, -V4, and -V7 3′UTRs (black) with exon 2 (E2) and/or exon 3 (E3; white) plus an identical miR-124–binding site in their 3′UTRs and the miR-124 seed sequence. TGA is the stop codon. Bottom, luciferase reporter assay analysis of the 3′UTR of AR-V7 in 22Rv1 cells. The assay was repeated three times, with each assay being performed in three wells and similar results were obtained each time. Representative results are shown as mean ± SD (n = 3). MBS, miR-124–binding site; miR-NC, miRNA-negative control; RLU, relative luciferase units. C, Western blot analysis of AR-V7 expression in miR-124-treated 22Rv1 cells. E, WST proliferation analysis of AR-V7 in 22Rv1 and VCaP cell lines that were treated with siAR-V7 for 3 days, followed by treatment with 50 nmol/L miR-124.

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Previous study demonstrated that miR-124 downregulated truncated AR (16), suggesting a regulatory link between miR-124 and AR splice variants that lack the AR ligand–binding domain (LBD). In this study, we observed an increased expression of AR-V7 in 22Rv1-EnzR cells compared with 22Rv1 cells (Fig. 4A). We thus asked whether miR-124 directly targets AR-V7. Using the BIBSBERV program, we analyzed the 3′UTR sequences of five AR variants deposited in the NCBI database: AR-V1 (also termed AR4, GI:224181615), AR-V3 (also termed AR6 or AR17/22b, GI:224181621), AR-V4 (also termed AR5, ARV6 or AR17/21/26b, GI:224181619), AR-V7 (also termed AR3, GI:224181613), and AR-V12 (also termed ARv567w, GI:270358641). One miR-124–binding site was identified in the 3′UTR of AR-V3, V4, and V7, which contain an identical sequence of 1.3 kb in length (Fig. 4B, top). However, an miR-124–binding site was not identified in AR-V1, or in AR-V12 in which the 3′UTR length is 219 bases without a poly-A tail, indicating an incomplete 3′UTR. To validate this 3′UTR element in AR-V3, -V4, and -V7 as being responsible for their regulation by miR-124, a luciferase reporter vector containing the miR-124–binding site was cotransfected with synthetic miR-124 into 22Rv1 cells. As shown in Fig. 4B (bottom), cotransfection resulted in a 62% reduction of the enzyme activity. Similar results were obtained when the assay was performed in 293 cells (data not shown). Using immunoblot analysis with the anti-AR-V7 antibody, we evaluated the effect of miR-124 on the regulation of AR-V7 protein level. Transfection of 22Rv1 cells with miR-124 for 3 days downregulated the expression of AR-V7 by 60%, whereas enzalutamide treatment for 3 days did not alter AR-V7 level (Fig. 4C). In addition, immunostaining of miR-124–treated 22Rv1-EnzR cells for AR-V7 expression demonstrated a dramatic reduction of AR-V7 intensity (Supplementary Fig. S3). Because AR-V3 lacks exon 3 that is necessary for the AR binding to DNA, transactivational function of AR-V3, -V4, and -V7 was assessed using a yeast AR functional assay that can be used to detect constitutive activity of AR spliced variants (Supplementary Fig. S4A and S4B). Both AR-V4 and -V7, but not AR-V3, were able to constitutively activate the expression of the reporter gene ADE2 (Supplementary Fig. S4C), suggesting that miR-124–regulated AR-V4 and -V7, or lack thereof, contribute to prostate cancer pathogenesis. Next, we determined whether miR-124 inhibits growth of prostate cancer cells via its regulation of AR-V7. To this end, both 22Rv1 and VCaP cell lines that express high level of AR-V7 were transfected with 100 nmol/L siAR-V7 for 3 days, followed by treatment with 50 nmol/L miR-124. Western blotting demonstrated that siAR-V7 was able to dramatically downregulate the expression of AR-V7 protein but not the level of full-length AR (Fig. 4D). WST proliferation analysis was then performed on days 3 and 6 after miR-124 treatment. It was found that knockdown of AR-V7 truncates miR-124–mediated growth inhibition in both 22Rv1 and VCaP cell lines (Fig. 4E). Taking the results from Figs. 3 and 4 together, miR-124 directly targets EZH2 and Src, as well as AR-V4 and -V7.
miR-124 inhibits growth of prostate cancer xenografts and sensitizes prostate cancer tumors to enzalutamide

Having validated that miR-124 negatively regulates the expression of AR variants and multiple oncopogens, we next investigated whether systemic administration of synthetic miR-124 could inhibit growth of CWR22 xenograft tumors. This xenograft model was selected as (i) we detected the expression of AR-V7 in frozen CWR22 tumors (data not shown) and (ii) Sirotnak and colleagues found that the antiandrogen bicalutamide does not inhibit the growth of the CWR22 tumors (20). In this experiment, miR-124 was delivered into CWR22 tumors by using the linear polyethyleneimine derivative jetPEI that has been reported to efficiently deliver miRNAs into tumor xenografts (21). In the mice of the negative control treatment group (miR-NC), tumors rapidly grew and all mice were sacrificed before day 35 due to tumors reaching the maximal-acceptable size. In contrast, treatment with miR-124 significantly inhibited tumor growth, with almost a 50% reduction of tumor volume at day 35 compared with that of miR-NC–treated tumors (P < 0.05, Fig. 5A and B). We next determined whether combined administration of miR-124 and enzalutamide would result in enhanced inhibition of prostate cancer cell growth. When compared with enzalutamide treatment, combination treatment inhibited tumor growth by 54% on day 35 posttreatment (P < 0.05, Fig. 5A and B), indicating that miR-124 increased anti–prostate cancer efficacy of enzalutamide. In addition, there were no safety concerns of PEI-miR-124 complexes based on observations that mouse body weights were unchanged throughout the course of the study (data not shown). Therefore, synthetic miR-124 is able to inhibit growth of prostate cancer tumors when given by itself or in combination with enzalutamide.

miR-124 downregulates AR-V7, EZH2, and Src and induces apoptosis in CWR22 xenografts

Upon termination of the in vivo experiments described above, immunohistochemistry (IHC) was performed to determine the levels of the AR-V7 protein in the xenograft tumors. Treatment with miR-124 alone or in combination with enzalutamide caused a marked decrease in the immunostaining intensity compared with treatment with miR-NC or enzalutamide alone (Supplementary Fig. S5A). We examined protein levels of AR-V7, EZH2, and total Src in tumor tissues using Western blot analysis. It was found that these three miR-124 targets were significantly downregulated in miR-124 + enzalutamide–treated tumors compared with tumors treated with enzalutamide alone (Fig. 6A). Therefore, combined repression of constitutively active AR variants and downregulation of EZH2 and Src contribute to miR-124–mediated tumor inhibition. The observation of miR-124–induced downregulation of these targets provides evidence that PEI nanoparticles can efficiently deliver miR-124 into prostate cancer xenograft cells.

We previously observed that miR-124 downregulates miR-125b in cultured prostate cancer cells (16). Herein, the expression of miR-125b was measured by qPCR. Similarly, systemic administration of miR-124 induced a reduction of miR-125b abundance in miR-124–treated tumors (Supplementary Fig. S5C). Because miR-125b targets p53 (22, 23), we evaluated the effects of miR-124 on p53 level. As expected, a profound increase in p53 protein was detected in miR-124–treated tumors (Fig. 6A). Having demonstrated that miR-124 inhibits the growth of prostate cancer xenografts and upregulates p53, we next investigated whether systemic administration of miR-124 induces apoptosis in the mouse tumors. TUNEL staining was performed. Positive staining was rare in tumors treated with miR-NC. Different extents of TUNEL-positive areas were detected in tumors treated with enzalutamide and/or miR-124, particularly in combined treated tumors, in which large TUNEL-positive areas were detected. The nuclei of apoptotic cells are small and contain condensed and/or fragmented chromatins. Representative results are shown in Fig. 6B. Quantitative TUNEL assays demonstrated that the combination of miR-124 and enzalutamide resulted in apoptosis in 37% of the cell population, whereas the single-agent treatment induced only 17% apoptosis (P < 0.05, Fig. 6B).

Discussion

Tumor-suppressive miR-124 is a highly conserved miRNA and has been reported to be downregulated in various human cancers,
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Like most other miRNAs, prostate cancer–related targets of miR-124 have not been validated experimentally. In our earlier report, full-length AR was identified and confirmed as a bona fide target of miR-124 (16). In the present study, EZH2 and Src were validated as targets of miR-124. EZH2 functions not only as a histone methyltransferase, silencing tumor suppressor genes and antimetastatic genes (30), but also as an AR coactivator to support prostate cancer growth (31). Overexpression of EZH2 is a common occurrence in prostate cancer and is associated with a poor clinical outcome of patients with prostate cancer (32). Similarly, Src is highly involved in prostate cancer, and inhibition of Src activity inhibits prostate cancer tumor growth (33, 34). Thus, their activities are required for the development of metastasis and castration resistance of prostate cancer. Perhaps, the most striking finding of this study is the detection of miR-124 target sites at the 3′UTRs of AR-V4 and AR-V7 transcripts. AR-V7 is the most studied AR variant. Its expression was obviously enhanced in CRPC cells due to intragenic AR gene rearrangement and altered RNA splicing (35, 36). In this study, restoration of miR-124 level led to significant downregulation of AR-V7 protein in xenograft tumors. The potential mechanisms for decreasing the levels of AR-V7 protein include: (i) miR-124 directly binds to the 3′UTR of AR-V7, leading to degradation of AR-V7 mRNA and inhibition of AR-V7 translation; (ii) miR-124 directly represses the full-length AR (16), whereas AR-V7 RNA splicing is coupled with the full-length AR gene transcription (35). Therefore, miR-124–induced downregulation of the full-length AR accompanies a reduction of AR-V7; and (iii) RNA splicing factor ASF that is a potential miR-124 target is specifically important for AR-V7 splicing (35). Taken together, the finding that miR-124 mediates downregulation of at least three oncoproteins relevant to prostate cancer progression and therapy resistance provides a firm molecular foundation for using miR-124 as novel adjunctive therapeutic agent.

Data presented in this study strongly suggest that miR-124 holds great promise as a therapeutic target. In our previous study, lentivirally expressed miR-124 exhibited significant growth inhibition of prostate cancer tumors (16). Although lentiviral vectors are efficient delivery agents, virus-based miRNA therapy may raise serious safety problems related to toxicity and immunogenicity. In this study, synthetic miR-124 mimics were used because they have the same physical and chemical characters as endogenous nucleic acid–based therapies, a major challenge is encountered for the delivery of miR-124 into prostate cancer cells. In this study, we elected to use PEI–based delivery, which has a considerable advantage over lentiviral-based vectors and has been widely studied for gene delivery. The PEI nanoparticles, or ‘polyplexes,’ used in this study are composed of linear polymers that have sufficient stability, favorable pharmacokinetic properties, and higher biocompatibility (e.g., low toxicity relative to virus-based vectors). Under physiologic conditions, PEI molecules are partially protonated and easily form complexes with miRNA. After systemic injection, PEI–miRNA complexes interact with the tumor cell surface and enter cells by endocytosis (37). Notably, PEI increases internalization and facilitates the release of internalized miRNAs from endosomes due to a so-called “proton sponge effect” (21). Our data obtained in animal experiments demonstrated that PEI is able to efficiently deliver miR-124 to prostate cancer xenografts, which is shown by PEI–miR-124 complex–induced antitumor effects and the inhibition of several specific targets. Although PEI as a delivery agent still has...
limitations (e.g., lower efficiency relative to virus-based vectors), the observed antitumor effects and lack of unwanted side effects suggest that this method of systemic delivery might be more clinically relevant and worthwhile to pursue.

Treatment of advanced prostate cancer has proven extremely challenging, especially in light of the fact that the newest ARSI enzalutamide provides only a modest improvement in survival of patients. Thus, while we search for new agents, improving response to presently available agents could be beneficial in lowering mortality in this disease. Because one miRNA can target multiple cancer-related genes or pathways, combined use of miRNA and enzalutamide may represent a novel and viable therapeutic strategy to prevent or delay recurrence of castration resistance. Over the last several years, we have investigated the role of miRNA in prostate cancer and discovered several miRNAs (miR-124, miR-125b, miR-30, and let-7c) that contribute to the AR signaling network. Using the data presented in this study combined with those in our previous publications (23, 38—41), an miR-124—regulated oncopathway is depicted in Fig. 7. This oncopathway regulates several proapoptotic genes and oncogenes and miR-124 is a key regulator. In this study, we targeted this oncopathway by using miR-124 and enzalutamide. Indeed, we observed that combination treatment inhibited proliferation of cultured CRPC cells in an additive manner and caused significantly greater antitumor activity in mice, when compared with the single treatment. Therefore, our results establish miR-124 as a prostate cancer—relevant miRNA with the promise of being an adjuvant therapeutic agent. Even so, the question can be asked, what will be the mechanism of resistance of CRPC against miR-124? One potential mechanism is through altering its AR-V pattern. On the basis of the fact that AR-V12 is constitutively active in prostate cancer and that the 3′UTR in AR-V12 is different from AR-V7 (42), it will be a prime target for bypassing miR-124.

Future studies to define the mechanism of resistance need to be conducted.

In summary, we found that tumor-suppressive miR-124 is an important modifier of an oncopathway in prostate cancer that regulates the expression of EZH2, Src, and AR variants that contribute to pathogenesis and treatment resistance of prostate cancer and that restoration of miR-124 inhibited the growth of enzalutamide-resistant prostate cancer cells and sensitized these cells to enzalutamide. Importantly, we observed that PEI—mediated systemic administration of synthetic miR-124 inhibited the growth of androgen-dependent and -independent and enzalutamide-resistant prostate cancer cells. Therefore, our data presented in this study suggest that miR-124—based therapies have great potential in the design of combination therapy for prostate cancer treatment.

Disclosure of Potential Conflicts of Interest
J. G. Yang reports receiving commercial research grant from Medivation. C. P. Evans reports receiving commercial research grant and speakers bureau honoraria from Medivation/Astellas; has ownership interest (including patents) in Medivation; and is also a consultant/advisory board member for Medivation/Astellas. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X.-B. Shi, A.-H. Ma, L. Xue, M. Li, H. G. Nguyen.
R. Gandour-Edwards.
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X.-B. Shi, A.-H. Ma, L. Xue, M. Li, H. G. Nguyen, C. G. Tepper, C. P. Evans.
Writing, review, and/or revision of the manuscript: X.-B. Shi, A.-H. Ma, C. G. Tepper, C. P. Evans, H.-J. Kung.
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miR-124 and Androgen Receptor Signaling Inhibitors Repress Prostate Cancer Growth by Downregulating Androgen Receptor Splice Variants, EZH2, and Src

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