Original Article
Epigenetic silencing of miR-137 induces resistance to bicalutamide by targeting TRIM24 in prostate cancer cells

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Abstract: Prostate cancer is an important hormone-dependent cancer affecting men. In the initial stages, prostate cancer is often treated using hormone therapy, including bicalutamide. Despite the initial effectiveness of this therapy, the tumor eventually acquires resistance, resulting in recurrence of castration-resistant prostate cancer (CRPC). Dysregulation of microRNA (miRNA) function is one of the putative underlying mechanisms of hormone therapy resistance. Reports have shown that miRNAs act as tumor suppressors in patients with prostate cancer, but the role of these molecules in bicalutamide resistance in prostate cancer cell lines remains unclear. We performed lentiviral miRNA library screening to identify novel miRNAs that modulate the response of human prostate cancer LNCaP cells to the antiandrogen bicalutamide. We found that the tumor suppressor miRNA miR-137 silenced signaling in a spectrum of human cancers and selectively targeted tripartite motif-containing 24 (TRIM24) to suppress tumor proliferation. Silencing of TRIM24 recapitulated the effect of miR-137 on cell proliferation, whereas overexpression of TRIM24 reversed this effect. Real-time reverse transcription PCR analysis revealed a reciprocal relationship between miR-137 and TRIM24 in prostate cancer cell lines and tissues. Mechanistic studies indicated that methyl CpG-binding protein 2 (MeCP2) and DNA methyltransferases (DNMTs) cooperate to promote methylation of the miR-137 promoter and the consequent decreased transcription, leading to enhanced TRIM24 expression and glutamine metabolism. These findings describe a novel mechanism that affects TRIM24 deregulation in human cancers and provide a molecular link between miR-137, TRIM24, and tumor proliferation in CRPC.

Keywords: miR-137, TRIM24, bicalutamide resistance, proliferation, prostate cancer

Introduction

Androgen signaling through the androgen receptor (AR) contributes to the maintenance of prostate function and promotion of castration-resistant prostate cancer (CRPC) [1, 2]. Androgen deprivation via surgical or medical castration is a common treatment modality for prostate cancer [3]. The nonsteroidal antiandrogen bicalutamide is often used in conjunction with androgen deprivation to block AR activity and tumor growth in patients with androgen-responsive prostate cancer [4, 5]. Specifically, bicalutamide (150 mg/day) is regularly prescribed as a monotherapy or an adjuvant to castration for men with locally advanced, non-metastatic prostate cancer [9]. Although bicalutamide treatment is initially effective, prostate cancers eventually become refractory to treatment, develop resistance to bicalutamide, and progress to CRPC [6, 7]. The molecular and non-molecular mechanisms associated with bicalutamide resistance are incompletely understood.

The microRNAs (miRNAs) are a family of small, non-coding RNAs that regulate gene expression by blocking translation or promoting degradation of target mRNAs [10]. These miRNAs function as transcriptional modulators by binding to complementary sequences in the 3'-untranslated region (3'-UTR) of target mRNAs [11].

Deregulation of miRNAs can rewire multiple cellular and biological processes, contributing to the initiation and progression of human cancers via effects on multiple aspects of tumor biology, including proliferation [12]. However, the direct links between miRNA deregulation and proliferation, corresponding regulatory mechanisms, and clinical relevance of these mechanisms in human bicalutamide-resistant prostate cancer remain unknown [13].

Epigenetic alterations, such as DNA methylation and covalent histone modifications, are common features of prostate cancer pathogenesis [14]. Aberrant DNA methylation and chromatin remodeling enzyme activity are associated with altered transcriptional states, which can ultimately impair miRNA expression [15]. Because miRNA deregulation is a common driving force in prostate cancer, we had hypothesized that deregulated miRNA expression may result from epigenetic alterations [16]. Indeed, expression profiling of bicalutamide-resistant prostate cancer revealed widespread downregulation of miRNAs [17]. The expression of some miRNAs was restored following exposure of bicalutamide-resistant prostate cancer cells to a demethylating agent, implicating DNA methylation in miRNA deregulation [18].

Tripartite motif-containing 24 (TRIM24; also known as transcriptional intermediary factor 1-alpha) belongs to the TRIM family of structurally related proteins. These proteins have an N-terminal TRIM domain with potential self-assembly properties, a C-terminal region containing a plant homeodomain (PHD) finger and a bromodomain, and a nuclear receptor interaction box [19]. TRIM24 has been implicated in multiple tumor types because of its ability to interfere with tumor-suppressive and oncogenic pathways [20-24]. Specifically, TRIM24 functions as an oncogenic transcriptional activator in prostate cancer cells and as a proliferation regulator in gastric cancer and hepatocellular carcinoma cells as well [25, 26]. The results of *in vitro* studies suggest that knockdown of TRIM24 suppresses cell proliferation, cell cycle progression, and *in vivo* tumor development, whereas overexpression of TRIM24 promotes cell growth [22]. TRIM24 protein expression progressively increases from primary prostate cancer to CRPC; however, the clinical and biological roles of TRIM24 in human bicalutamide-

resistant prostate cancer and the mechanisms of miRNA regulation of this factor remain incompletely understood.

In this study, we performed lentiviral miRNA library screening to identify novel miRNAs that modulate the resistance to the antiandrogen bicalutamide in androgen-sensitive human prostate adenocarcinoma (LNCaP) cells. We focused our subsequent studies on the effects of one of these identified miRNAs, miR-137, on the growth of resistant cells. The results of these mechanistic studies indicated that methyl CpG-binding protein 2 (MeCP2) and DNA methyltransferases (DNMTs) cooperate to promote active methylation of the miR-137 promoter, decreasing its transcription and leading to enhanced TRIM24 expression and glutamine metabolism. RNA expression analysis confirmed an inverse correlation between miR-137 and TRIM24 in both cell lines and tissues. These findings uncovered a novel, global mechanism underlying TRIM24 deregulation in human cancers, and revealed a molecular link between miR-137, TRIM24, and tumor proliferation in prostate cancer.

Materials and methods

Patients and sample collection

Prostate cancer tissues were collected from 28 prostate cancer patients (mean age, 60.33 ± 11.23 years), who received non local or systemic treatment before surgery at the NO1 hospital affiliated with Xinjiang medical university (Urumqi, China) between 18 July 2015 and 30 December 2017. Prostate cancer (stage I, II, or III) diagnosis was based on histopathological evaluation [27]. All tissue samples were frozen in liquid nitrogen immediately after removal and stored until use in the experiments described below. The study protocol was approved by the ethics committee of the NO1 hospital affiliated with Xinjiang medical university, and written informed consent was obtained from each patient.

Cell culture

Human LNCaP, 22Rv1, 1013L, ARCaP, DU-145, MPC-3-10, ND-1, PC-3, PPC-1, PSK-1, UM-SCP-1, and VCaP cell lines were purchased from the Chinese academy of sciences (Shanghai, China). LNCaP cells were cultured in Ros-

well Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2. Bicalutamide-resistant prostate cancer (resistant) cells were derived from an AR-positive, bicalutamide-sensitive LNCaP prostate cancer cell line (parental). Cells were treated with vehicle (control) or bicalutamide (10 µM) for at least 8 d in phenol red-free RPMI medium supplemented with 10% charcoal dextran-treated fetal bovine serum, penicillin (50 U/mL), and streptomycin (50 µg/mL) at 37°C in a humidified atmosphere containing 5% CO_2 [28].

Lentiviral miRNA library screening and microarray analysis

The screening method was performed as previously described [29]. Briefly, a human miRNA precursor lentivirus library consisting of a pool of 445 human miRNA precursor clones coexpressing GFP (System Biosciences, Palo Alto, CA, USA) was transduced into bicalutamide-resistant and parental cells. The library was labeled using the genome DNA enzymatic labeling Kit (Agilent Technologies, Santa Clara, CA, USA) and then subjected to microarray hybridization (Oligo cDGH/ChIP-on-ChIP Hybridization Kit, Agilent Technologies). Agilent feature extractor software was used to scan the microarray images and normalize signal intensities.

Bioinformatics analysis

The miRNA targets were predicted using the TargetScan (http://www.targetscan.org/), TargetMiner (www.isical.ac.in/), and TarBase (http://mirtarbase.mbc.nctu.edu.tw/) applications. The predicted targets were assessed using the functional annotation tools of the database for annotation, visualization, and integrated discovery (DAVID; http://david.abcc.Ncifcrf.gov/). The terms for gene ontology (GO) enrichment analysis were selected using a cut-off of $P<0.05$.

Plasmid construction

Pre-miR-137 and specific shRNAs against EZH2 and MeCP2 were cloned into a pLKO.1 lentiviral vector using synthesized DNA oligonucleotides. The TRIM24 open reading frame was amplified using specific primers and cloned into

a pCDH-CMV-GFP vector (System Biosciences, Johnstown, PA, USA). A TRIM24-3'UTR sequence containing the miR-137-binding sites and another sequence lacking these sites were generated using PCR amplification. The amplified sequences were then cloned into the psiCHECK-2 vector (Promega, Madison, WI, USA). The vectors were designated TRIM24-3'UTR and TRIM24-3'UTR-mut. The DNA oligonucleotide and primer sequences are presented in Supplementary Table 1.

mRNA and miRNA quantitative PCR

Total RNA was isolated from cells using the TRIzol reagent (Ambion, Austin, TX, USA) according to the manufacturer's protocol. RNA (10 ng) was reverse transcribed to cDNA using a TaqMan miRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). The miRNAs were purified using a miniRNA fast extraction kit (Aidlab, Beijing, China), and reverse transcription was performed using a Taqman microRNA reverse transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). The relative levels of miRNA were calculated by normalizing the target gene signal to the signal for U6B small nuclear RNA. Relative mRNA levels were quantified using SYBR Green-based real-time reverse transcription PCR (qRT-PCR) and were normalized to that of GAPDH. The primer sequences are presented in Supplementary Table 1.

siRNA transfection

We purchased stealth RNAi siRNAs (Life Technologies, Waltham, MA, USA) targeting TRIM24, and a negative control siRNA. Cells were transfected with siRNAs using siGene (Promega) 48-72 h before each experiment. The primer sequences are presented in Supplementary Table 1.

Western blot

Cell lysates were separated on 12% polyacrylamide gels and transferred onto a nitrocellulose membrane using a semi-dry transfer method. The membrane was incubated for 1 h in blocking buffer (5% nonfat dry milk) and incubated overnight at 4°C with the following antibodies: TRIM24 (ab211300, Abcam, London, UK), DNMT3B (ab2851, Abcam), MeCP2 (ab-
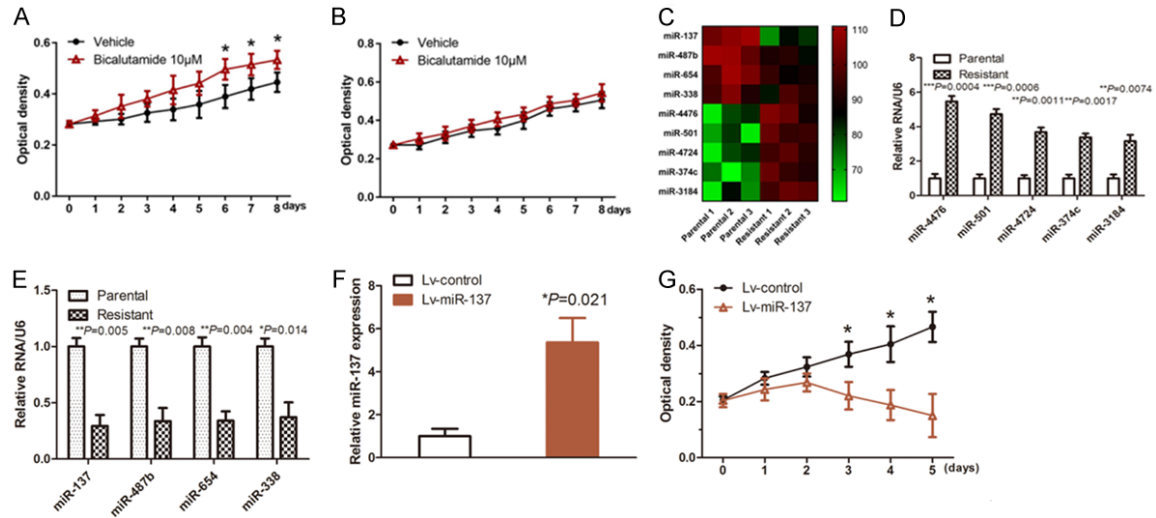
Figure 1. Identifcation of androgen-downregulated miRNAs using microarray analysis of bicalutamide-resistant cells. Cell proliferation of LNCaP parental cells (A) and bicalutamide-resistant cells (B). Cells were treated with 10 µM bicalutamide or a control, and the viability of the indicated cells was determined using an MTS assay. (C) Distinct changes in miRNA expression in bicalutamide-resistant cells and parental cells. Red indicates upregulation, and green indicates downregulation. qRT-PCR of the top five upregulated (D) and four downregulated (E) miRNAs identified in (C) in bicalutamide resistant cells. (F) Results of qRT-PCR analysis of miR-137 expression in bicalutamide-resistant cells infected with Lv-control or Lv-miR-137. (G) MTS assay of cell proliferation in bicalutamide-resistant cells infected with Lv-control or Lv-miR-137.

2828, Abcam), and β-actin (sc-47778, Santa Cruz, Dallas, TX, USA). The original whole films/membranes in figures are presented in Supplementary Figure 1.

Luciferase assay

The potential miR137-binding sites (wild-type or mutant) in the 3'-UTR of human TRIM24 were synthesized by GENEWIZ Inc. (China) and then cloned into a pGL3-promoter vector (Promega). Luciferase activity was measured 24 h post-transfection using a dual luciferase kit (Promega). Firefly luciferase activities were normalized to renilla luciferase control values and presented as the mean of three replicate values. Luciferase activity was assessed using the secrete-pair™ dual luminescence assay kit (GeneCopoeia, Rockville, MD, USA) according to the manufacturer's instructions.

Genome-wide chromatin immunoprecipitation (ChIP)

ChIP was performed as previously described [28] using antibodies to MeCP2 (ab2828, Abcam), DNMT3B (ab2851, Abcam), and IgG (sc-2025, Santa Cruz). The enrichment of DNMTs on gene promoters was determined using the ChIP-qPCR assay [29]. DNA fragments were

precipitated with antibodies against DNMT1, DNMT3A, or DNMT3B, using genomic DNA as the positive input. The primer sequences are presented in Supplementary Table 1.

Cell proliferation assay

The cells (3 × 10³ cells/well) were plated in 96-well plates. The MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, inner salt) assay was performed using the cell titer 96 aqueous MTS reagent (Promega) according to the manufacturer's instructions. The experiment was repeated five times.

Statistical analyses

For the cell line experiments, differences among groups were obtained using the two-sided Student's *t*-test. The Pearson correlation coefficient was calculated to assess the correlation between miR-137 and TRIM24. All experiments were performed at least twice, and similar results were obtained for each experiment. A *P*-value <0.05 was considered to indicate a statistically significant difference. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA).
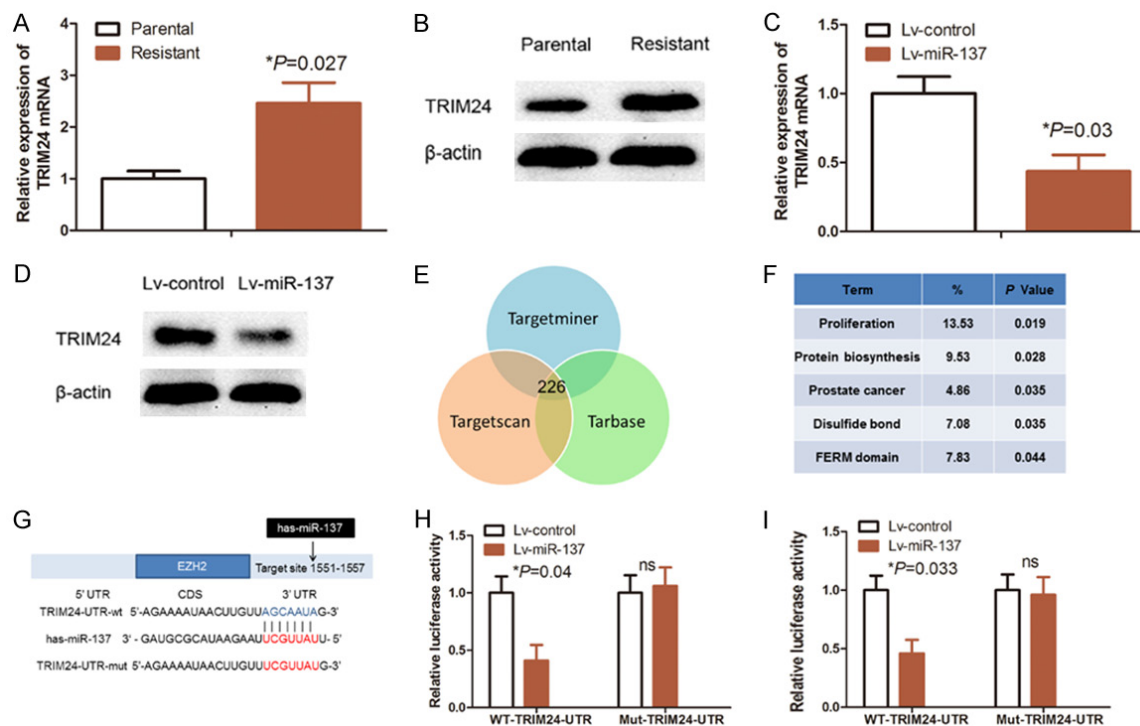
Figure 2. miR-137 directly targets TRIM24 3'-UTR. (A) qRT-PCR and (B) western blot analysis of TRIM24 expression in bicalutamide-resistant cells. (C, D) miR-137 mimics selectively reduce TRIM24 mRNA (C) and protein (D) levels in bicalutamide-resistant cells. Actin was used as a loading control. (E) GO classification of miR-137 potential targets using TargetMiner, TargetScan, and TarBase algorithms. (F) Pathway enrichment analysis of 226 genes from (D). (G) The putative miR-137 target site in the 3'-UTR of TRIM24 transcript was predicted using bioinformatics analysis. (H, I) Luciferase assays of lentiviral TRIM24 3'-UTR constructs with intact and mutated binding sequences for miR-137 in 293 T (H) and bicalutamide-resistant prostate cancer (I) cells.

Results

Bicalutamide-resistant prostate cancer cells exhibit proliferation advantages over parental cells

AR-positive, bicalutamide-sensitive LNCaP prostate cancer cells were cultured continuously for 8 d with 10 μM bicalutamide, resulting in the acquisition of bicalutamide resistance in the surviving cell population [28]. To assess the proliferation of these cells, we performed an MTS assay with both LNCaP parental cells and bicalutamide-resistant cells. As expected, bicalutamide inhibited the proliferation of LNCaP cells but not that of the bicalutamide-resistant cells (**Figure 1A**).

To identify miRNAs that are differentially expressed in bicalutamide-resistant prostate cancer cells compared with parental cells, 1275 miRNAs were profiled. Of the miRNAs that exhibited a >1.5-fold difference in expression,

64 and 10 miRNAs were found to be downregulated and upregulated in bicalutamide-resistant prostate cancer cells, respectively, compared to parental cells (**Figure 1B**). Among the upregulated miRNAs, miR-4476, miR-501, miR-4724, miR-374c, and miR-3184 had the greatest variations (**Figure 1C**). Furthermore, miR-137, miR-487b, miR-654, and miR-338 were the most greatly downregulated (**Figure 1D**).

Compared with the parental LNCaP cells, miR-137 was the most downregulated in response to androgen treatment in resistant cells. We used a lentivirus-mediated delivery system to stably transfect resistant cells with an miR-137 precursor (**Figure 1E**). We then performed an MTS assay to evaluate the effect of miR-137 on the proliferation of laryngeal cancer cells (**Figure 1F**). Ectopic expression of miR-137 significantly reduced the growth of resistant cells compared with the parental cells. Taken together, these results suggest that miR-137 is a negative regulator of bicalutamide-resistant prostate cancer cell proliferation.
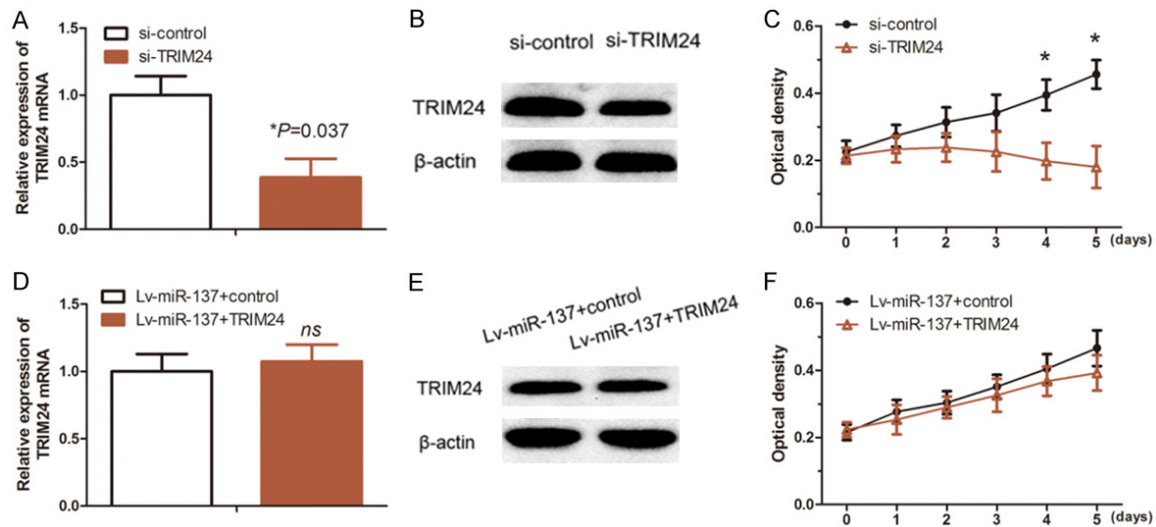
Figure 3. TRIM24 plays a critical role in miR-137-induced proliferation in bicalutamide-resistant prostate cancer cells. (A) Expression levels of TRIM24 following transfection of cells with a TRIM24-targeting siRNA or control siRNA were detected using qRT-PCR. β-actin served as a reference gene. (B) Western blot analysis of endogenous TRIM24 expression in bicalutamide-resistant prostate cancer cells stably transfected with a TRIM24-targeting siRNA or control siRNA. (C) The MTS assay was used to evaluate cell viability in bicalutamide-resistant prostate cancer cells stably transfected with a TRIM24-targeting siRNA or control siRNA. qRT-PCR (D) and western blot (E) analysis of adenovirus-mediated TRIM24 expression in bicalutamide-resistant, miR-137-overexpressing cancer cells. TRIM24 levels are compared to those in control adenovirus transduced cells. (F) Cell proliferation in Lv-miR-137 bicalutamide-resistant, miR-137-overexpressing cancer cells that overexpressed TRIM24, as determined by a CCK-8 assay.

MiR-137 targets TRIM24 by directly binding to its 3'-UTR

Because TRIM24 functions as an oncogenic transcriptional activator and proliferation regulator in cancer cells, we hypothesized that the function of TRIM24 in proliferation may underlie the ability of miR-137 to induce proliferation inhibition in bicalutamide-resistant prostate cancer cells. To test this hypothesis, we analyzed the expression of TRIM24 in bicalutamide-resistant prostate cancer cells and parental cells. qRT-PCR and western blot analyses showed an increase TRIM24 mRNA and protein levels, respectively, in bicalutamide-resistant prostate cancer cells (**Figure 2A** and **2B**). Furthermore, levels of TRIM24 mRNA and protein were clearly decreased in Lv-miR-137-infected bicalutamide-resistant prostate cancer cells compared with control cells (**Figure 2C** and **2D**). These findings suggest that miR-137 negatively regulates the expression of TRIM24.

We next performed a computer-aided algorithmic analysis using TargetMiner, TargetScan, and TarBase and found that miR-137 targets 518 genes in the human genome (**Figure 2E**). GO analysis revealed that miR-137-targeted

genes function in important cellular processes and conditions, including proliferation and prostate cancer (**Figure 2F**). Moreover, TRIM24 was identified as a potential target gene of miR-137. To determine whether TRIM24 is a direct target of miR-137, we examined the predicted miR-137 binding site in the TRIM24 mRNA 3'-UTR (**Figure 2G**). When the TRIM24 3'-UTR was used to drive expression of a luciferase reporter in 293T or bicalutamide-resistant prostate cancer cells co-transfected with miR-137 mimics, the wild-type TRIM24 3'-UTR inhibited luciferase activity, but mutation of the predicted binding site significantly reversed this inhibition under similar conditions (**Figure 2H** and **2I**). Taken together, these results indicate that TRIM24 is a direct target of miR-137 in bicalutamide-resistant prostate cancer cells.

Overexpression of TRIM24 rescues miR-137-induced proliferation inhibition in bicalutamide-resistant prostate cancer cells

Overexpression of TRIM24 is associated with recurrence and poor survival in patients with prostate cancer [30]; however, the mechanism underlying this effect in bicalutamide-resistant prostate cancer cells has not been well-charac-
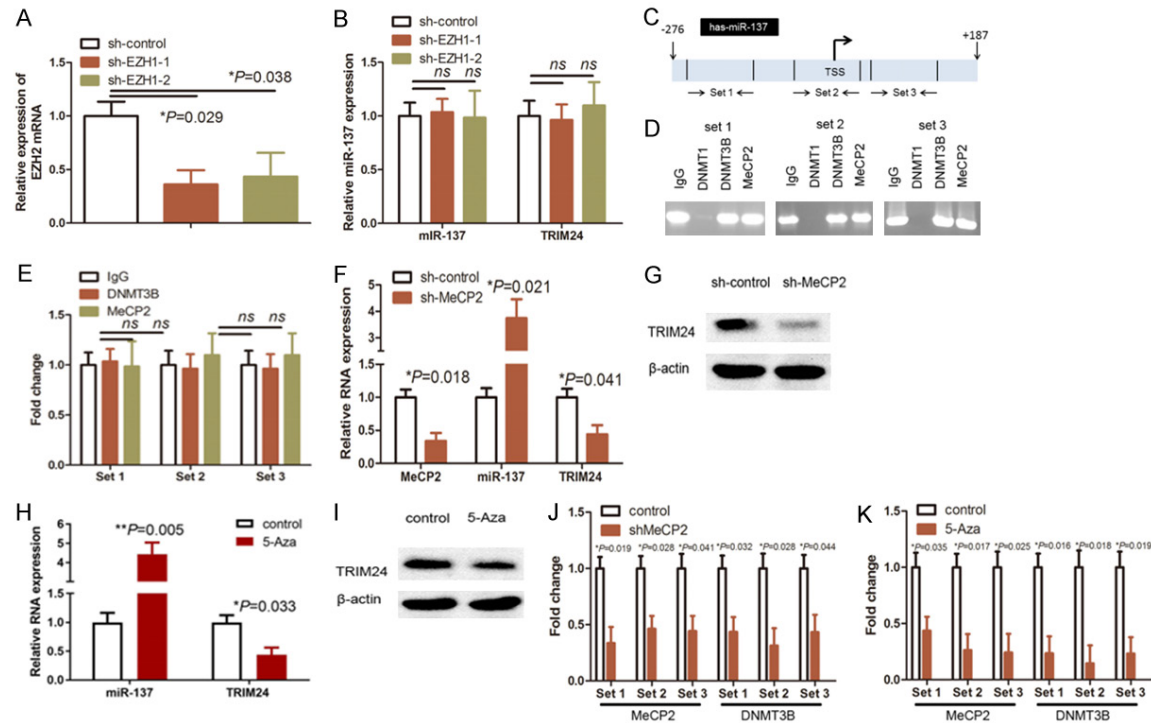
Figure 4. Epigenetic silencing of miR-137 by MeCP2 and DNMT reactivates TRIM24 expression. qRT-PCR analysis of EZH2 (A) and miR-137 (B) levels upon depletion of EZH2 expression in bicalutamide-resistant prostate cancer cells. (C) Binding of MeCP2 and DNMTs to the miR-137 CpG island as determined using a ChIP assay in bicalutamide-resistant prostate cancer cells. The actin promoter was used as a negative control. Results are presented as average fold-change in MeCP2, DNMT1, and DNMT3B levels compared to isotype IgG controls. P1, primer set 1; P2, primer set 2; P3, primer set 3. Methylation (D) and fold-change (E) of the three sites (MeCP2, DNMT1, and DNMT3B) in bicalutamide-resistant prostate cancer cells. (F) qRT-PCR analysis of MeCP2, miR-137, and TRIM24 RNA levels upon depletion of MeCP2 expression in bicalutamide-resistant prostate cancer cells. (G) Western blot analysis of MeCP2 after depletion of MeCP2 expression in bicalutamide-resistant prostate cancer cells. (H) Real-time qRT-PCR analysis of miR-137 and TRIM24 RNA levels after 5'-Aza-CdR treatment in bicalutamide-resistant prostate cancer cells. (I) Western blot analysis of TRIM24 after 5'-Aza-CdR treatment in bicalutamide-resistant prostate cancer cells. (J, K) Binding of MeCP2 and DNMT3B to the miR-137 CpG island as determined by a ChIP assay in bicalutamide-resistant prostate cancer cells following MeCP2 depletion (J) or 5'-Aza-CdR treatment (K). Results are presented as average fold-change in MeCP2 and DNMT3B levels compared to isotype IgG controls.

terized. Using a previously reported TRIM24-specific RNAi method, we established stable cell clones that expressed low levels of TRIM24 (**Figure 3A** and **3B**) [30]. This knockdown of TRIM24 suppressed cell proliferation (**Figure 3C**), which was consistent with the effects of lentiviral overexpression of miR-137 in bicalutamide-resistant prostate cancer cells. These results indicate that downregulation of TRIM24 is essential for miR-137-induced cell proliferation inhibition.

If TRIM24 is a functional target of miR-137 in bicalutamide-resistant prostate cancer cells, then reintroduction of TRIM24 into cells that express high levels of miR-137 should antagonize the effect of miR-137 on proliferation. To

test this hypothesis, we transfected exogenous TRIM24 into Lv-miR-137-infected cancer cells using a previously described adenovirus delivery system [25]. Expression of TRIM24 in miR-137-overexpressing cells significantly increased the subsequent adenovirus-mediated expression of exogenous TRIM24 (**Figure 3D**). Ectopic overexpression of TRIM24 significantly counteracted the defect in cell proliferation induced by miR-137 in bicalutamide-resistant prostate cancer cells (**Figure 3E** and **3F**). The results of this rescue experiment demonstrate that reintroduction of TRIM24 abrogates the miR-137-induced reduction in cell proliferation, suggesting that TRIM24 is a functional mediator of miR-137 in bicalutamide-resistant prostate cancer cells.
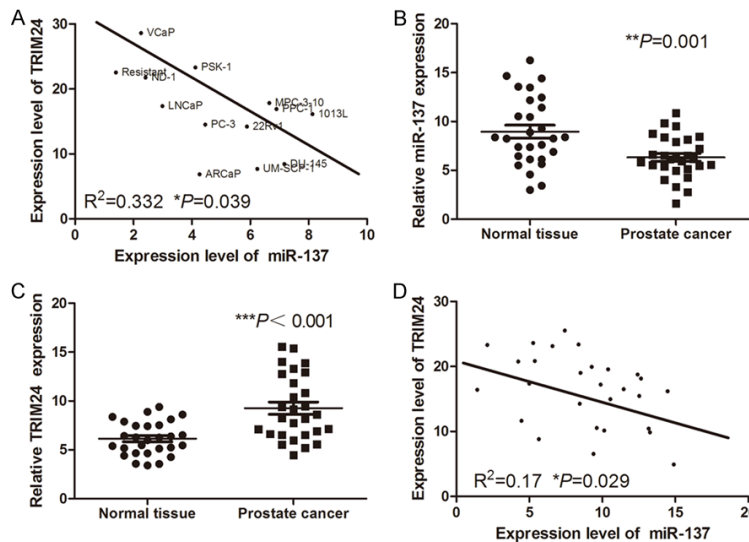
Figure 5. Expression of miR-137 and TRIM24 mRNA are negatively correlated in prostate cancer cells. (A) Negative correlation of miR-137 with TRIM24 mRNA levels in prostate cancer cell lines. Each data point represents a prostate cancer cell line. (B) Expression levels of miR-137 and (C) TRIM24 mRNA were analyzed by qRT-PCR for randomly selected human prostate cancer and normal tissue samples. (D) Pearson's correlation analysis of the relative expression levels of miR-137 (normalized to U6) and the relative expression levels of TRIM24 mRNA (normalized to GAPDH) determined using qRT-PCR in 28 human bicalutamide-resistant prostate cancer cell tissue samples.

Epigenetic silencing of miR-137 by MeCP2 and DNMTs reactivates TRIM24 expression

Previous studies suggest that the epigenetic silencing of miR-137 involves the Polycomb group proteins EZH2/SUZ12, DNA hypermethylation, or both [31]; however, the molecular mechanism underlying the epigenetic silencing of miR-137 remain to be determined. We depleted EZH2 expression in bicalutamide-resistant prostate cancer cells and found that this change had little effect on miR-137 or TRIM24 expression (**Figure 4A** and **4B**). Importantly, miR-137 contains an obvious CpG island flanking the transcriptional start site (**Figure 4C**). DNMTs frequently methylate the cytosine within CpG dinucleotides in CpG islands, and these methylated CpGs then recruit MeCPs and MBDs [32]. Using ChIP assays, we detected the presence of DNMT1, DNMT3B, and MeCP2 at the pre-miR-137 chromatin regions that harbor CpG islands with no detectable reactivity at the actin promoter (**Figure 4E**). We next depleted MeCP2 using specific shRNAs in bicalutamide-resistant prostate cancer cells. As expected, MeCP2 depletion signifi-

cantly increased endogenous miR-137 expression, and conversely, depleted TRIM24 accumulation (**Figure 4F** and **4G**). Treatment of the cells with the DNMT inhibitor 5-aza-2-deoxycytidine (5-Aza-CdR) also resulted in a significant increase in endogenous miR-137 expression and conversely depleted TRIM24 accumulation (**Figure 4H** and **4I**). Concordantly, knockdown or 5-Aza-CdR treatment significantly reduced MeCP2 and DNMT3B occupancy at these CpG islands (**Figure 4J** and **4K**). Altogether, these results suggest that MeCP2 and DNMTs cooperate to reactivate TRIM24 expression.

Expression of endogenous miR-137 inversely correlates with expression of TRIM24 mRNA in prostate cancer cells

TRIM24 is frequently overexpressed in several types of cancer, including prostate cancer [25, 26]. Because the overexpression of miR-137 resulted in downregulation of TRIM24 mRNA, we postulated that miR-137 expression may be inversely correlated with TRIM24 mRNA expression in prostate cancer cell lines and tissues. To test this hypothesis, we employed qRT-PCR to assess the expression levels of TRIM24 mRNA and mature miR-137 in cell lines and 28 human prostate cancer tissues. The expression levels of endogenous miR-137 were negatively correlated with those of TRIM24 mRNA in 14 human prostate cancer cell lines (P=0.039; **Figure 5A**). Next, we measured the expression levels of miR-137 and TRIM24 mRNA in paired tumor and normal tissue samples from 28 patients with prostate cancer using qRT-PCR. miR-137 RNA levels were significantly lower in the prostate cancer samples than in the normal prostate tissue samples (**Figure 5B**), whereas TRIM24 mRNA levels were significantly higher in the prostate cancer samples than in the normal prostate tissue samples (**Figure 5C**). Pearson correlation analysis showed that miR-137 levels were negatively correlated with TRIM24 mRNA levels (P=0.029, **Figure 5D**).

These results provide further evidence of a functional link between miR-137 and TRIM24 in prostate cancer cells.

Discussion

Although both oncogenic and tumor-suppressive functions have been ascribed to specific miRNAs, global miRNA downregulation is a common feature of human cancers. We performed a functional screen using a lentiviral miRNA library to identify miRNAs associated with acquired resistance to endocrine therapy in patients with prostate cancer. Based on comparison with control cells, we identified two miRNAs in the bicalutamide-treated cells that might be involved in the modulation of bicalutamide resistance in LNCaP cells. Examination of one of these downregulated miRNAs, miR137, revealed that overexpression of this miRNA significantly inhibited bicalutamide-mediated growth promotion in LNCaP cells. Our study also uncovered a novel mechanism in which miR-137 directly targets TRIM24, and levels of endogenous miR-137 expression are negatively correlated with the expression levels of TRIM24 mRNA. Mechanistic studies also showed that MeCP2 and DNMTs cooperate to promote the active methylation of the miR-137 promoter and its decreased transcription, leading to enhanced TRIM24 expression and glutamine metabolism. This suggests that the epigenetic silencing of miR-137 may lead to TRIM24 upregulation, and may at least partially underlie the bicalutamide resistance of prostate cancer cell lines.

miRNAs contribute to basic biological processes such as development, differentiation, apoptosis, and cell proliferation; it is therefore not surprising that dysregulation of miRNA expression contributes to the pathogenesis of human malignancies [33]. Aberrant miRNA expression profiles in tumor cells provide valuable insights into the molecular pathways of oncogenesis. Although miR-137 is one of the most prominent miRNAs implicated in tumorigenesis, its role in tumor progression is controversial [34]. miR-137 is frequently upregulated in cancers, functioning as an oncogene in some, such as bladder cancer [35]. The expression of this miRNA is reduced in other human cancers, including glioblastoma, malignant melanoma, and pancreatic cancer, and thus miR-137 functions as

a tumor suppressor gene in these cases as a result of promoter methylation [36-39]. The precise role and methylation status of miR-137 in bicalutamide-resistant prostate cancer cell lines remains unclear. However, the results of our study highlight the importance of global gene regulation by miR-137 in determining the proliferation of bicalutamide-resistant prostate cancer cell lines. Our bioinformatics analysis demonstrated that miR-137 was downregulated due to methylation at three different sites. Based on these findings, we hypothesized that miR-137 is a potential tumor suppressor gene in prostate cancer cells. As expected, enforced expression of miR-137 inhibited the proliferation of bicalutamide-resistant prostate cancer cells, suggesting a critical role of miR-137 in regulating the proliferation of bicalutamide-resistant prostate cancer cell lines and providing a potential diagnostic and predictive biomarker for prostate cancer.

TRIM24 is important in tumor development and progression [40]. The oncogenic role of TRIM24 in tumorigenesis is not restricted to AR signaling, as evidenced by the fact that TRIM24 overexpression is detected in a wide variety of tumors [41, 42]. Genetic alterations and malignant phenotypes of tumors from bicalutamide-resistant prostate cancer cells suggest that factors, such as TRIM24, that feed into these oncogenic pathways may drive prostate cancer progression from primary disease to CRPC [43, 44]. Our results indicated that TRIM24 drives the proliferation of bicalutamide resistance in prostate cancer cell lines. This observation aligns with the concept that AR co-activators, such as TRIM24, drive CRPC by sustaining AR signaling under conditions of low hormone availability [45-47]. Here, using a cell model, we have demonstrated that this "AR reprogramming" may depend on TRIM24, which ultimately results in a marked dependency of CRPC cells on TRIM24. This relationship is also apparent in patients with prostate cancer: the expression levels of these AR/TRIM24-stimulated genes increase with disease progression. The discrepancies between our study findings and those of Groner *et al.* are associated with upstream relationships that are relevant to bicalutamide resistance in prostate cancer cell lines [48]. Identification of the signaling pathways regulated by miRNAs, and miR-137 in particular, would facilitate the elucidation of the mecha-

nisms responsible for prostate cancer progression.

In summary, we found that miR-137 was significantly downregulated in a bicalutamide-resistant prostate cancer cell line, and selectively targeted TRIM24. Furthermore, we uncovered a reciprocal relationship between miR-137 and TRIM24 expression in prostate cancer cell lines and tissues. Mechanistic studies indicated that MeCP2 and DNMTs cooperate to promote active methylation of the miR-137 promoter and its decreased transcription, leading to enhanced TRIM24 expression and glutamine metabolism. Thus, our study results suggest that CRPC has a marked dependency on TRIM24 for enhanced cellular proliferation. This relationship could be used to identify new targets for the diagnosis and treatment of advanced prostate cancer.

Acknowledgements

Disclosure of conflict of interest

None.

Abbreviations

TRIM24, tripartite motif-containing 24; miRNA, microRNA; BR, bicalutamide resistance; CRPC, castration-resistant prostate cancer.

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Epigenetic inhibition of miR-137 promotes prostate cancer cell proliferation

Supplementary Table 1. Sequences list for gene transfection, vector constructing, CHIP and qRT-PCR

|	
Gene transfection	
TRIM24 siRNA	5'-GCUGGACUCUCUAAACAAUTT-3'
siRNA control	5'-UUCUCCGAACGUGUC ACGUTT-3'
Vectors constructing	
TRIM2-UTR	5'-GAAATTTCTGTTGGGATTGTTAAT-3'
	5'-AATTCATACCTGCTGTCACTT-3'
Lv-miR-137	5'-GACGGGTATTCTTGGGTGGATAATACGG-3'
	5'-CCGCTGGTACTCTCCTCGACTACG-3'
TRIM24	5'-ATGGAGGTGGCGGTGGAGAAGGCGG-3'
	5'-TTATTTAAGCAACTGGCGTTCTTCAA-3'
sh-EZH2	5'-CCGGGCTAGGTTAATTGGGACCAAACT-3'
sh-MeCP2	5'-GGGAAACTTGTTGTCAAGATGCC-3'
Primers for ChIP	
Primer set 1	5'-CATTTGGATTTGGGCAGGAAGC-3'
	5'-GAACTCTTGCTGCTCGCTGA-3'
Primer set 2	5'-TCAGCGAGCAGCAAGAGTTC-3'
	5'-CCAAGAATACCCGTCACCGAAG-3'
Primer set 3	5'-TCCTCTGACTCTCTTCGGTGAC-3'
	5'-CTTGGCAACCACGGGCGTTTAG-3'
qRT-PCR	
TRIM24	5'-TGTGAAGGACACTACTGAGGTT-3'
	5'-GCTCTGATACACGTCTTGCAG-3'
MeCP2	5'-TGACCGGGGACCCATGTAT-3'
	5'-CTCCACTTTAGAGCGAAAGGC-3'
DNMT3b	5'-AGGGAAGACTCGATCCTCGTC-3'
	5'-GTGTGTAGCTTAGCAGACTGG-3'
GAPDH	5'-TCACCAGGGCTGCTTTTAAC-3'
	5'-GACAAGCTTCCCGTTCTCAG-3'
miR-137	5'-TTATTGCTTAAGAATACGCGTAG-3'
miR-4476	5'-CAGGAAGGATTTAGGGACAGGC-3'
miR-501	5'-AATCCTTTGTCCCTGGGTGAGA-3'
miR-4724	5'-AACTGAACCAGGAGTGAGCTTCG-3'
miR-374c	5'-ATAATACAACCTGCTAAGTGCT-3'
miR-3184	5'-TGAGGGGCCTCAGACCGAGCTTTT-3'
miR-487b	5'-GTGGTTATCCCTGTCCTGTTCG-3'
miR-654	5'-TGGTGGGCCGCAGAACATGTGC-3'
miR-338	5'-AACAATATCCTGGTGCTGAGTG-3'

1
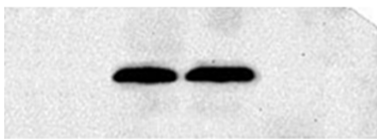
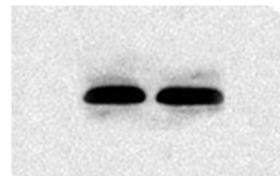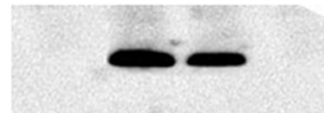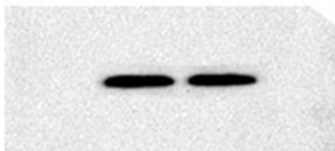Figure 2 B

Figure 2 D
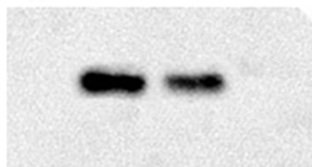
Figure 3 B

Figure 3 E

Figure 4 G
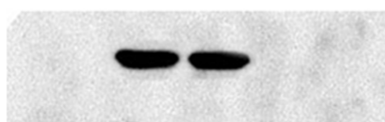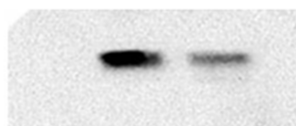
Figure 4 I
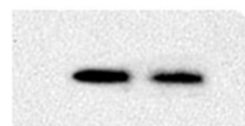
Supplementary Figure 1. The original whole films/membranes in the **Figures 2B**, **2D**, **3B**, **3E**, **4G** and **4I** in the manuscript.