

ORIGINAL ARTICLE

Tumor suppressive miR-124 targets androgen receptor and inhibits proliferation of prostate cancer cells

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Although prostate cancer (CaP) is the most frequently diagnosed malignant tumor in American men, the mechanisms underlying the development and progression of CaP remain largely unknown. Recent studies have shown that downregulation of the microRNA *miR-124* occurs in several types of human cancer, suggesting a tumor suppressive function of *miR-124*. Until now, however, it has been unclear whether *miR-124* is associated with CaP. In the present study, we completed a series of experiments to understand the functional role of *miR-124* in CaP. We detected the expression level of *miR-124* in clinical CaP tissues, evaluated the influence of *miR-124* on the growth of CaP cells and investigated the mechanism underlying the dysregulation of *miR-124*. We found that (i) *miR-124* directly targets the androgen receptor (AR) and subsequently induces an upregulation of p53; (ii) *miR-124* is significantly downregulated in malignant prostatic cells compared to benign cells, and DNA methylation causes the reduced expression of *miR-124*; and (iii) *miR-124* can inhibit the growth of CaP cells *in vitro* and *in vivo*. Data from this study revealed that loss of *miR-124* expression is a common event in CaP, which may contribute to the pathogenesis of CaP. Our studies also suggest that *miR-124* is a potential tumor suppressive gene in CaP, and restoration of *miR-124* expression may represent a novel strategy for CaP therapy.

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INTRODUCTION

Prostate cancer (CaP) is the most frequently diagnosed malignant tumor and was the second leading cause of cancer-related death in American men in 2011.¹ Metastatic CaP can be treated effectively with androgen ablation. However, one of the most troubling aspects of this disease is that after hormone treatment, the tumor inevitably progresses from an androgen-dependent to an incurable castration-resistant (CR) form.² The mechanism underlying the progression has been poorly understood. In the past decades, therefore, an important task in CaP research has been to elucidate the molecular alteration occurring in the development of CR tumors. Considerable insight into the progression of CaP has been recently achieved, including the discovery of aberrantly expressed microRNAs (miRNAs).

The human genome may encode over 1000 miRNAs, which negatively regulate ~60% of human genes.^{3,4} Thus, miRNAs are involved in almost all important cellular processes, not only in physiological conditions but also in diseases including cancer.⁵ Indeed, a number of cancer-related miRNAs have been identified recently. Some miRNAs have also been reported to be aberrantly expressed in human CaP.⁶ These miRNAs, which act as tumor suppressor genes (TSGs) or oncogenes, contribute to the pathogenesis of CaP by directly targeting some proliferation-related genes or apoptotic molecules.⁷ In spite of these exciting findings, the role of miRNAs in the development and progression of CaP has been largely unexplored. Thus, identifying CaP-associated miRNAs and investigating their roles in CaP will help us understand the mechanisms related to the development and progression of this disease.

MiRNA-124 (*miR-124*) is a highly conserved miRNA whose *in vivo* function is poorly defined. This small non-coding RNA was first reported to be highly expressed in neuronal cells.⁸ Recent studies revealed that *miR-124* was significantly downregulated in several types of human cancers.^{9–12} Moreover, it regulates some proliferation-related genes such as cyclin-dependent kinase 6,¹³ forkhead box A2¹⁴ and solute carrier family 16, member 1 (SLC16A1).¹³ Thus, *miR-124* is classified as a tumor suppressor miRNA.¹³ Until now, however, the role of *miR-124* in CaP has been totally unknown, although it was reported previously to be undetectable in 22Rv1 CaP cells.¹⁵

In the present study, we aimed to explore the role of *miR-124* in CaP. We found that *miR-124* directly targets the androgen receptor (AR). We also detected the expression level of *miR-124* in clinical CaP tissues and found that a majority of these CaP tissues express low levels of *miR-124*. In addition, we observed that synthetic *miR-124* mimics inhibit the proliferation of CaP cells. As the AR has a crucial role in the pathogenesis of CaP, our studies established a link between dysregulated *miR-124*, overexpressed AR and CaP cell proliferation. Our results imply that *miR-124* is a potential TSG and downregulation of *miR-124* contributes to the development and progression of CaP.

RESULTS

MiR-124 directly targets the AR and inhibits proliferation of CaP cells

Overexpression of the AR is well known to have an important role in the pathogenesis of CaP. We are very interested in determining

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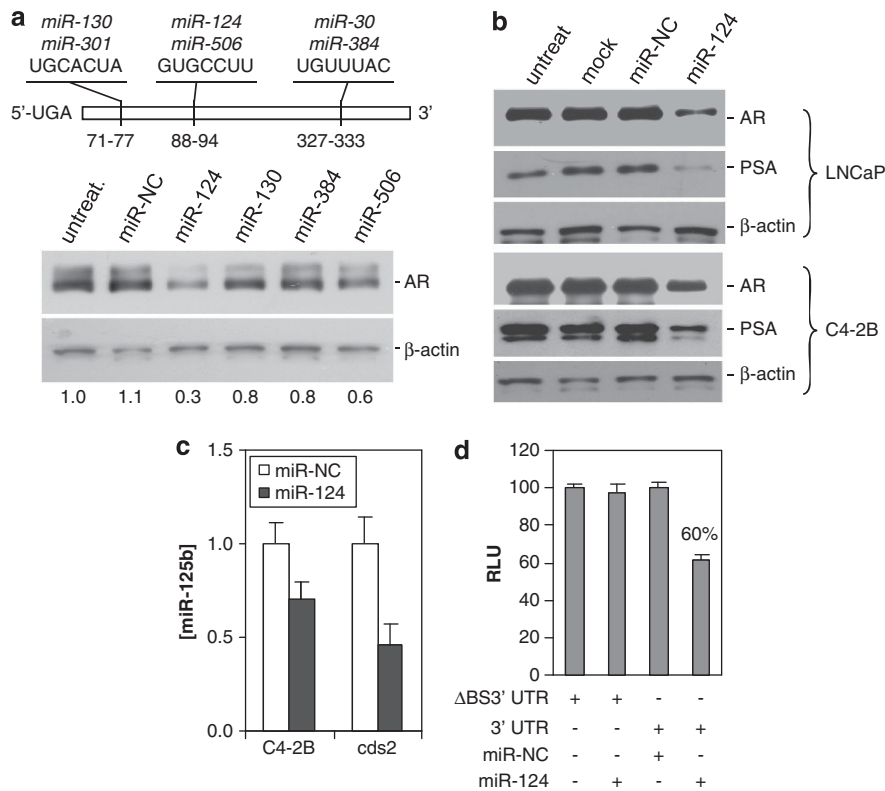


Figure 1. *MiR-124* negatively regulates AR. **(a)** Western blot analysis of the AR expression in C4-2B CaP cells that were treated with 100 nM of synthetic *miR-124*, *miR-130*, *miR-384* or *miR-506*. The numbers under the gels are the fold changes of AR protein relative to the untreated C4-2B cells (untreat). Fold changes were calculated by scanning the AR bands and normalizing for β -actin bands. The upper portion is a schema of the first 436 bases of the AR 3' UTR (based on the RefSeq NM_000044.2). The digital numbers indicate the predicted three miRNA-binding sites targeted by six potential AR-targeting miRNAs. **(b)** Western blot analysis of the expression of AR and PSA in LNCaP and C4-2B cells treated with 100 nM of *miR-124* mimic. Both mock and miRNA-NC were used as controls. **(c)** qPCR assay of *miR-125b* levels in C4-2B and *cds2* cells treated with 100 nM of *miR-124* mimic or miR-NC. The values shown as mean \pm s.e. ($n = 3$) are from three independent experiments performed in triplicate. **(d)** Luciferase analysis in C4-2B cells. The assay was repeated three times with each assay being performed in four wells, and similar results were obtained each time. The representative results are shown as mean \pm s.d. ($n = 4$). The percentage represents enzyme activity in 100 nM *miR-124* mimic-transfected cells relative to that in 100 nM miR-NC-transfected cells. RLU, relative luciferase unit. Δ BS3' UTR, AR 3' UTR fragment lacking the *miR-124* binding site.

whether certain miRNA(s) contribute to upregulation of the AR. From two algorithms, TargetScan (Release 5.2) and miRanda (August 2010 Release), that predict miRNA-binding sites in the first 436 base of the AR 3' UTR (NM_000044.2), we identified three broadly conserved miRNA-binding sites for six miRNAs (*miR-130/miR-301*, *miR-124/miR-506* and *miR-30/miR-384*). In order to assess the ability of these miRNAs to regulate AR expression, AR-positive C4-2B CaP cells cultured in androgen-deprived medium were separately treated with four chemically modified miRNA mimics (*miR-124*, *miR-130*, *miR-384* or *miR-506*, purchased from Ambion, Austin, TX, USA). As shown in Figure 1a, compared with miRNA-negative control (miR-NC), treatment with *miR-124* mimic resulted in a reduction of AR protein by $\sim 70\%$, while the other three miRNA mimics induced 20–40% decrease in AR expression, suggesting a potent downregulation of the AR by *miR-124*. To determine the influence of these miRNAs on proliferation of CaP cell, AR-positive CaP cell lines (LNCaP, C4-2B and 22Rv1) were transiently transfected with each of these miRNA mimics. Consistent with its effect on AR level, transfection of *miR-124* mimic inhibited proliferation to a greater extent than the other miRNA mimics tested (Supplementary Figure 1). We thus focused on *miR-124* in this study.

To determine whether *miR-124*-mediated downregulation of the AR affects the AR activity, both AR-positive LNCaP and C4-2B were treated with *miR-124* mimic. Western blot analysis demonstrated that *miR-124* induced downregulation of the AR, which was concomitant with a reduced PSA level (Figure 1b). As AR regulates *miR-125b*,¹⁶ we used C4-2B and *cds2* cells that express

increased *miR-125b*¹⁶ to examine the effect of *miR-124* mimic on endogenous *miR-125b*. We found that *miR-124* induced downregulation of *miR-125b* by 30% in C4-2B and 54% in *cds2* (Figure 1c). To verify that the putative *miR-124* binding site in the 3' UTR of AR mRNA is responsible for regulation by *miR-124*, the 3' UTR was cloned into a luciferase reporter vector and then cotransfected with *miR-124* mimic into C4-2B cells. Luciferase activity was measured 2 days after the transfection. As shown in Figure 1d, transfection of *miR-124* mimic resulted in 40% reduction of the enzyme activity, indicating a direct interaction between *miR-124* and AR mRNA. Taken together, these data indicate that *miR-124* targets the AR and affects its activity.

MiR-124 is significantly downregulated in CaP cells

We determined the expression level of *miR-124* in CaP cells. The abundance of *miR-124* was first examined in seven prostate cell lines (two benign and five malignant) using quantitative PCR (qPCR). We found a reduced expression of *miR-124* in the malignant cell lines compared with the benign cell lines (Figure 2a). We also performed northern blot analysis of these cell lines and similar results were obtained (Supplementary Figure 2a). Next, we tested whether *miR-124* is downregulated in clinical prostate samples. We reviewed the miRNA expression profiling of our initial clinical tissue specimens, and found that the abundance of *miR-124* is markedly reduced in four CaP samples relative to that in one benign prostatic hyperplasia (BPH) tissue

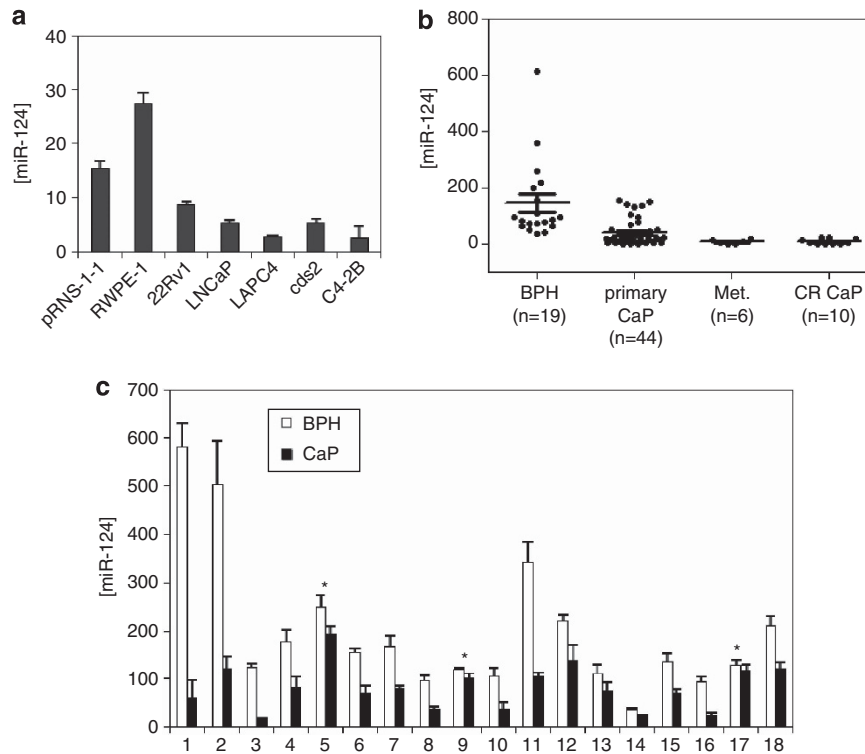


Figure 2. qPCR assays of *miR-124* expression levels in CaP cells. **(a)** *miR-124* abundance in two benign prostate cell lines (pRNS-1-1 and RWPE-1) and five CaP cell lines (22Rv1, LNCaP, LAPC-4, cds2 and C4-2B). **(b)** *miR-124* expression levels in 19 BPH tissues, 44 primary CaPs, 6 lymph node metastases and 10 CR tumors. **(c)** *miR-124* levels in 18 matched benign and malignant prostate samples. The levels of *miR-124* of five pairs of prostate tissues (5, 8, 9, 11 and 16) were assayed using northern blot analysis and similar results were obtained. The northern blot results are shown in Supplementary Figure 2b. *, no statistical difference ($P > 0.05$) between BPH and CaP tissues. In **(a, b)** and **(c)**, qPCR assays were repeated three times with each assay being performed in triplicate, and similar results were obtained each time. The values are shown as mean \pm s.e. ($n = 3$).

(Supplementary Figure 2B). We then determined whether downregulation of *miR-124* is common in clinical CaP tissues. The levels of *miR-124* were detected in 79 clinical prostatic tissues, including 19 BPHs, 44 primary CaPs, 6 lymph node metastases and 10 CR tumors. Although *miR-124* abundances have overlap in a small number of benign and primary tumor tissues, the average *miR-124* level was 2.7-fold less in primary CaPs than in BPHs (39 in CaPs vs 146 in BPHs, $P < 0.01$) (Figure 2b). Interestingly, 10 CR tumors and 6 metastases expressed extremely low levels of *miR-124*. Compared with the primary CaP samples, the average *miR-124* level decreases by 77% in these advanced tumors (39 in CaPs vs 9 in CR and metastatic tumors, $P < 0.05$) (Figure 2b), suggesting a potential involvement of *miR-124* in CaP progression. To confirm the downregulation of *miR-124*, 18 CaP samples and matched benign adjacent tissues were analyzed using qPCR. In comparison with matched benign tissues, 15 of 18 CaP samples (83%) expressed significantly reduced *miR-124* (Figure 2c). In addition, we performed northern blot analysis in five matched prostate tissues in which sufficient quantities of RNA were available. As expected, the three CaPs (8, 11 and 16) having low *miR-124* levels detected by qPCR exhibit lower signal intensity in northern blots (Supplementary Figure 2C). Taken together, these data provide strong evidence that *miR-124* is significantly reduced in CaP cell lines and in a majority of clinical CaP samples, and also suggest that decreased expression of *miR-124* is common in human CaP tissues.

miR-124 and AR are negatively correlated in human CaP tissues
Having demonstrated that *miR-124* targets the AR and is significantly downregulated in CaP cells, we therefore asked

whether clinical CaP samples having a low level of *miR-124* overexpressed the AR. To address the issue, eight additional matched pairs of CaP and BPH were examined for expression of AR protein using immunohistochemical analysis. In these samples, *miR-124* level detected by qPCR was significantly decreased in CaP samples compared with that in BPH. Conversely, immunohistochemical results demonstrated that AR immunostaining was more intense in seven of eight CaP samples than that in BPH matches. These results reveal an inverse relationship between *miR-124* and AR expression levels in prostatic tissues. Figure 3 shows the representative results from four matches. In CaP samples, AR protein staining is much higher than that in matched BPH tissues (Figure 3a), while the expression of *miR-124* is significantly decreased in CaP tissue compared with that in BPH tissues (Figure 3b). Therefore, our data suggest that downregulation of *miR-124* results in increased expression of the AR in clinical CaP tissues.

miR-124 is methylated in CaP cells

The question remained: why is *miR-124* downregulated in CaP cells? We first confirmed that androgens do not regulate *miR-124*, based on our observation that *miR-124* levels were similar in androgen R1881-treated and untreated CaP cell lines (data not shown). A previous study has shown that DNA methylation regulates the expression of miRNAs.¹⁷ Moreover, computer analysis demonstrated that the *miR-124-1* at 8p23.1 and the *miR-124-3* at 20q13.33 are embedded within CpG islands and the *miR-124-2* at 8q12.3 is 760 bp downstream of a CpG island.¹³ In order to determine that DNA methylation downregulates *miR-124* in CaP cells, we performed a demethylation experiment in three

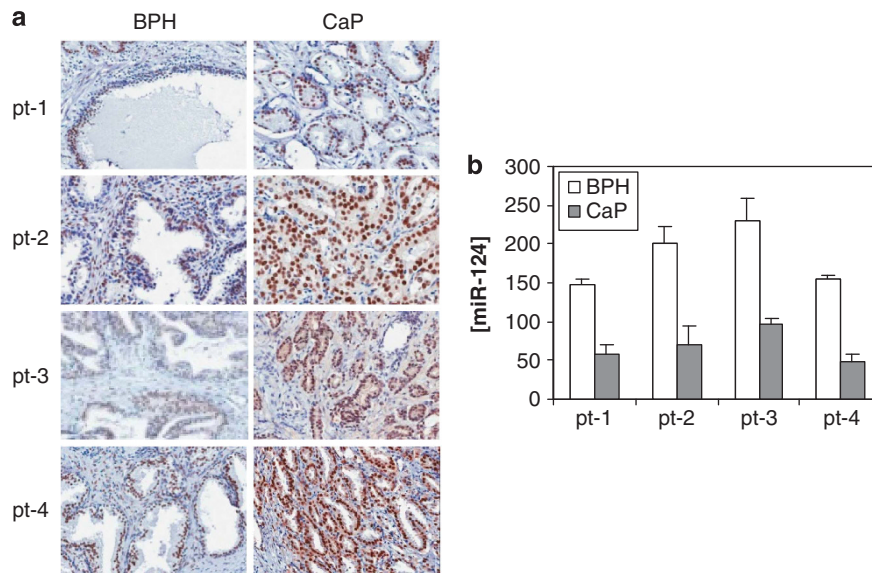


Figure 3. The expression levels of AR and *miR-124* in four matched prostate tissues (pt-1 to pt-4). **(a)** Immunohistochemical analysis of the AR protein in four human CaP samples (right) and their benign prostate tissue (left). **(b)** qPCR detection of the abundance of *miR-124* in both BPH and CaP tissues. The values are shown as mean \pm s.e. from three independent experiments.

androgen-independent cell lines (22Rv1, C4-2B and cds2). Cells were treated with 50 μ M of 5'-Aza-2'-deoxycytidine (Aza) for 4 days and *miR-124* was detected by qPCR. It was found that Aza treatment induced a 2- to 5-fold increase in the *miR-124* level compared with that in the untreated cells (Figure 4a). As *miR-124* targets the AR as shown in Figure 1, we tested whether Aza downregulates expression of the AR. It was found that treatment with Aza reduced the AR level by \sim 40% in LNCaP and \sim 60% in C4-2B cells (Figure 4b). We then assessed the methylation status of two benign prostate cell lines (pRNS-1-1 and RWPE-1) and five CaP cell lines using methylation-specific PCR (MSP). The 5'-DNA fragments of three *miR-124* genes were strongly amplified by MSP primers in all cell lines except RWPE-1 whose *miR-124-2* fragment was not amplified by the MSP primers (Figure 4c). The fragments of *miR-124-1* and *miR-124-2* were also amplified weakly by unmethylated DNA primers in some of these cell lines (Figure 4c). MSP data suggest different extents of DNA methylation at the 5' regions of *miR-124* genes. In order to validate DNA methylation, the 5'-DNA fragments of three *miR-124* genes in six cell lines were amplified using COBRA (combined bisulfite restriction analysis) primers, cloned and sequenced. It was found that 41–82% of CpG sites were methylated in *miR-124-1*, 17–57% in *miR-124-2* and 15–31% in *miR-124-3*, in five CaP cell lines, and the CpG methylation are 18%, 13% and 12%, respectively, in benign RWPE-1 cells (Figure 4d).

We next determined the methylation status in 9 BPH tissues and 14 CaP tissues using COBRA. The 5'-DNA fragments of three *miR-124* genes that contain two, five and six CGCG sites, respectively, were amplified with COBRA primers and digested with the restriction enzyme BstU1, which only cleaves methylated CGCG sites. It was found that BstU1 was able to cut the *miR-124-1* PCR products in 13 of 14 (93%) CaP samples, *miR-124-2* in 11 of 14 (79%) samples and *miR-124-3* in 12 of 14 (86%) samples, while the BstU1-digested BPH DNA fragments of *miR-124* genes are 11% (1/9), 22% (2/9) and 44% (4/9), respectively (Figure 4e), indicating that significant *miR-124* methylation occurs in clinical CaP specimens. Taken together, our data suggest that DNA methylation occurring at the 5' regions of *miR-124* genes causes, at least in part, the reduced expression of *miR-124* in CaP cells.

MiR-124 induces apoptosis

Previous studies demonstrated that the AR upregulates *miR-125b*, while *miR-125b* represses the expression of p53¹⁸ and facilitates the proliferation of CaP cells.^{16,19} As *miR-124* targets the AR and downregulates *miR-125b*, we asked whether increased *miR-124* level increases apoptotic cell death. To address the issue, LNCaP and C4-2B cells were transfected with *miR-124* mimic and then cultured for 4 days. Apoptotic dead cells were quantitatively detected by Annexin V binding assay. We observed that *miR-124* mimic induced 10.4% of LNCaP cells and 9.7% of C4-2B cells to undergo apoptotic cell death (Figure 5a). The comparative figures for the miR-NC cells was 0.7% and 1.1%, respectively ($P < 0.01$). A representative Annexin V assay is shown in Supplementary Figure 3, in which *miR-124* mimic-induced apoptosis (upper-right and lower-right squares in quadrant gates) was 11.4% in LNCaP cells and 8.4% of C4-2B cells. To provide biochemical evidence for the occurrence of apoptosis, we determined whether *miR-124* mimic increases the expression of p53 and caspase 3. As expected, treatment of C4-2B cells with *miR-124* mimic induced an approximately ninefold-enhanced expression of p53 (Figure 5b) and an approximately twofold increase in caspase 3 (Figure 5c). These data indicate that *miR-124*, at least in part, induces apoptosis by activating the p53 signaling pathway.

MiR-124 inhibits xenograft growth of CaP cells

Having demonstrated that *miR-124* inhibits proliferation of AR-positive CaP cells and induces apoptosis, we tested whether this miRNA exerts an inhibitory effect on xenograft growth of CaP cells *in vivo*. We used 22Rv1 CaP cells to address this question owing to their expression of low (Figure 2a) or undetectable¹⁵ level of *miR-124*, as well as their ability to efficiently form tumors in nude mice. Overexpression of *miR-124* in 22Rv1 CaP cell lines was established using a lentiviral vector system that expresses 28-fold elevation of *miR-124* relative to controls (data not shown). Xenograft tumors were generated by subcutaneously inoculating intact male nude mice with 22Rv1-lenti-*miR-124* cells or 22Rv1-lenti-vector cells as a control. Tumors in both the *miR-124* and control groups became palpable 18 days after inoculation, suggesting that *miR-124* did not affect the onset of 22Rv1 tumors. However, overexpression of

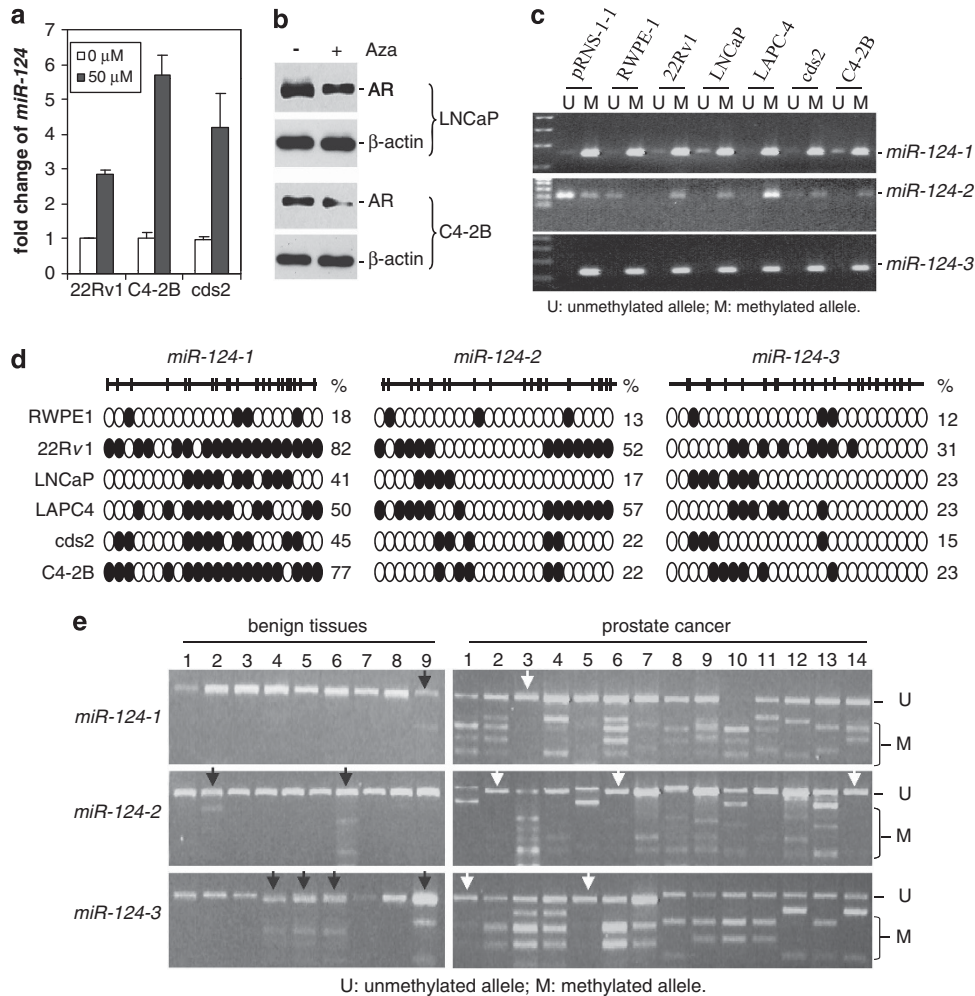


Figure 4. Methylation of *miR-124* in CaP cells. **(a)** Expression levels of *miR-124* in 22Rv1, C4-2B and cds2 CaP cell lines before and after treatment with 50 μ M of Aza. Results are expressed as fold change of *miR-124* relative to the untreated control. The assay was repeated three times, with each assay being performed in three wells, and similar results were obtained each time. The representative results are shown as mean \pm s.d. ($n = 3$). **(b)** Western blot analysis of the AR expression in LNCaP and C4-2B cells treated with 50 μ M of Aza. **(c)** MSP assay of the 5' CpG islands of *miR-124-1*, *miR-124-2* and *miR-124-3* in seven prostatic cell lines: two benign lines (pRNS-1-1 and RWPE-1) and five malignant lines (22Rv1, LNCaP, LAPC-4, cds2 and C4-2B). **(d)** Bisulfite sequencing analysis of *miR-124* CpG island methylation in RWPE-1 cell line and five CaP cell lines. The top schematic view represents individual amplified 5' DNA fragments of three *miR-124* genes, which are located at -1895 to -1655 upstream of pre-*miR-124-1*, -1456 to -1188 of pre-*miR-124-2* and -1465 to -1246 of pre-*miR-124-3*. The vertical bars denote individual CpG dinucleotides. Methylation profiles of *miR-124* CpG island fragments in six cell lines tested were demonstrated in the bottom of the schema. CpGs are represented by open circles if unmethylated, and by black circles if methylated. Each row exhibits methylated CpGs from at least three clones. The numbers on the right of each row are the percentage of methylated-CpG dinucleotides. **(e)** COBRA analysis of methylation of *miR-124-1*, *miR-124-2* and *miR-124-3* in 9 BPH tissues and 14 CaP samples. Purified PCR product was digested with BstUI that cuts methylated CGCG. The black arrows indicate the BPH samples having detectable methylation, and the white arrows indicate the CaP samples without methylation.

miR-124 significantly inhibited the growth of 22Rv1 tumors after 3 weeks when compared with controls (Figure 6a). At the end of the experiments, qPCR analysis of *miR-124* levels was performed in three lenti-*miR-124* tumors. It was found that *miR-124* levels were increased 23-fold in the *miR-124* tumors compared with the control tumors (Figure 6b), similar to that in cultured 22Rv1-lenti-*miR-124* cells, revealing a stable expression of *miR-124* *in vivo*. In addition, one lenti-vector tumor and two lenti-*miR-124* tumors were examined by western blot analysis for their AR expression. As shown in Figure 6c, lenti-*miR-124* tumors express moderate reduction of the full-length AR. Interestingly, overexpression of *miR-124* in 22Rv1 cells induced a downregulation of the truncated AR that may contribute to the aggressive features of 22Rv1 cells.²⁰ Collectively, our results suggest that *miR-124* inhibits growth of 22Rv1 xenograft tumors by repressing AR expression.

DISCUSSION

Although *miR-124* has been reported to be involved in several other cancer types,⁹⁻¹¹ its role in CaP is largely unknown. To date, only one study has reported that *miR-124* was undetectable in 22Rv1 CaP cells.¹⁵ The present study explored the role of *miR-124* in CaP. We found that *miR-124* directly targets the AR, suggesting that it is a potential TSG in CaP. In addition, our expression analysis revealed that *miR-124* was significantly downregulated in a majority of clinical CaP specimens tested, which suggests that dysregulation of *miR-124* may be a common event in patients with CaP, and loss of *miR-124* may contribute to the progression of CaP.

In this study, we address the potential mechanisms underlying *miR-124* downregulation in CaP cells. As methylation of TSGs is very common in both early and advanced stages of CaP,²¹⁻²⁴ *miR-124* acting as a potential TSG in CaP may be susceptible to

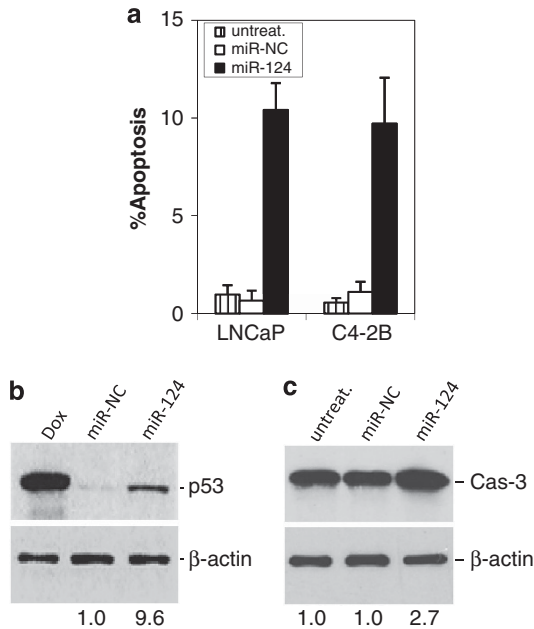


Figure 5. *MiR-124* induces apoptosis and upregulates p53. **(a)** Annexin V assay of apoptosis. LNCaP and C4-2B cells were treated with 100 nM *miR-124* mimic or miR-NC for 4 days and stained with Annexin V and propidium iodide. Both early and late apoptotic cells are combined. The values are shown as mean \pm s.e. from the three independent experiments. **(b)** Western blotting analysis of p53 expression in 100 nM *miR-124* mimic-transfected C4-2B cells. Doxorubicin (Dox)-treated cells were used as positive control. **(c)** Western blotting analysis of caspase 3 (Cas-3) in 100 nM *miR-124* mimic-transfected C4-2B cells. In both **(b)**, **(c)**, the numbers under the gels are the fold changes of p53 and Cas-3 in *miR-124* mimic-treated C4-2B cells relative to miR-NC-treated cells.

epigenetic modification. Indeed, we observed DNA methylation of three *miR-124* genes in CaP cell lines and clinical CaP samples, which provides an explanation for the reduction of *miR-124* levels. This is supported by the fact that demethylation treatment with Aza increased the abundance of *miR-124* in CaP cells. These data provide strong evidence that *miR-124* is downregulated in CaP in part as a result of DNA methylation. Our results are consistent with those reported in other cancer types in which *miR-124* loci are aberrantly methylated.^{9,11,13} However, methylation of *miR-124* loci was not detected in a recent report.²⁵ In that study, Rauhala *et al.*²⁵ treated CaP cells with 1 μ M of Aza for 2 days followed by 300 nM of trichostatin A (a histone deacetylase inhibitor) for 24 h. They identified 38 methylated miRNAs but not *miR-124*. Mazar *et al.*²⁶ reported that Aza upregulates *miR-34b* expression in a dose-dependent manner, and 1 μ M of Aza did not induce elevation of *miR-34b* in melanoma cells. As up to 100 μ M of Aza failed to induce cytotoxicity in CaP cell lines,²⁷ we used 50 μ M of Aza to treat CaP cells for 4 days, which induced significant elevation of *miR-124*. It is thus likely that our ability to detect the increased level of *miR-124* is due to the higher concentration of Aza used. Besides the epigenetic regulation, the expression level of *miR-124* can be affected by genetic factors, such as mutation and heterozygosity (LOH). Indeed, we detected a G to C point mutation (or single-nucleotide polymorphism) in the 5' region of *miR-124-2* (Supplementary Figure 4). Mutation (or single-nucleotide polymorphism) in the 5' region can reduce expression of mature miRNA by altered miRNA processing.^{28,29} In addition, a previous study revealed that tumor suppressive miRNAs are frequently located in LOH regions.³⁰ We know that *miR-124-1* is located at 8p23.1 and *miR-124-2* at 8q12.3. LOH in these two regions were reported

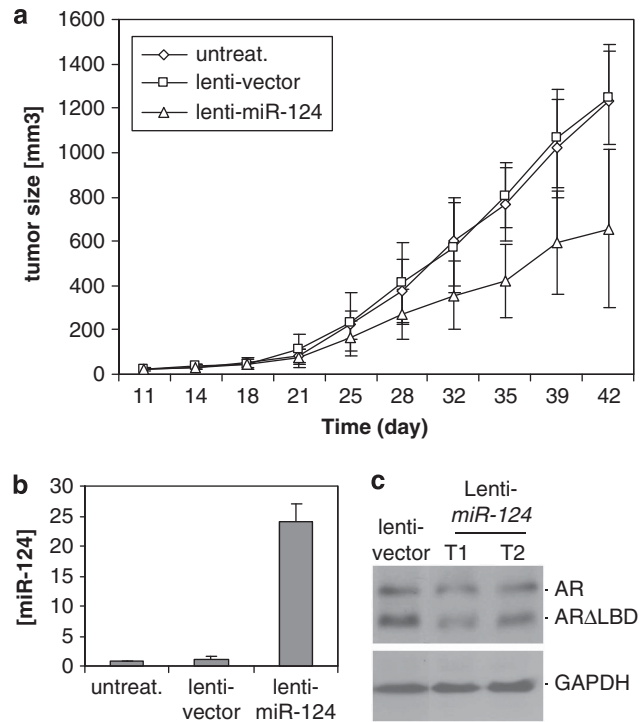


Figure 6. Inhibition of xenograft tumor growth by *miR-124*. **(a)** Five nude mice per group were injected subcutaneously with 2×10^6 22Rv1-*miR-124* cells, 22Rv1-vector cells or untreated 22Rv1 cells. The growth of xenograft tumors were measured twice per week. Each time point represents mean \pm s.d. of five independent values (mm³). **(b)** qPCR assay of *miR-124* level in xenograft tumors. The assay was repeated twice and similar results were obtained each time. The representative results are shown as mean \pm s.d. ($n = 3$). **(c)** Western blot assay of the AR expression in two 22Rv1-*miR-124* tumors that express the full-length AR and a truncated AR.²⁰

to occur in an $\sim 50\%$ of CaPs.³¹⁻³³ Thus, LOH may affect the levels of *miR-124-1* and *miR-124-2*. In order to elucidate the mechanism underlying the downregulation of *miR-124*, further study is needed to detect the frequencies of DNA mutation (or single-nucleotide polymorphism) and LOH in the three *miR-124* loci.

An interesting and clinically relevant discovery is that *miR-124* directly targets the AR. The AR contributes to the initiation and progression of CaP. A study has demonstrated that a moderately altered AR expression is sufficient to convert androgen dependent CaP cells to a CR form.³⁴ The AR has been reported to be upregulated in clinical CaPs, particularly those that are CR. Why AR is upregulated in CaP remains poorly understood. AR gene amplification appears to account for the AR upregulation in an $\sim 30\%$ CR CaPs;³⁵ however, it is unknown how the AR is upregulated in the other CR tumors. As the 3' UTR of the AR mRNA contains a conserved *miR-124* binding site, we performed several experiments to validate the regulation of the AR by *miR-124*. We also observed an inverse correlation between *miR-124* abundance and the AR expression level in clinical CaP samples tested. Therefore, our study demonstrates that dysregulation of *miR-124* may elevate the expression of the AR, which is a new mechanistic explanation for the overexpression of the AR in CaP. In this present study, identification of *miR-124* as an AR-targeting miRNA was based on the TargetScan and miRanda prediction programs. We noted that *miR-124*, as well as the other three predicted miRNAs as shown in Figure 1a, were not selected as AR-targeting miRNAs in a recent study.³⁶ In that study, the authors transfected five AR-positive CaP cell lines with 20 nM of human miRNA library, and selected 71 miRNA candidates that affect the level of AR in all the five cell lines. However, miRNA repression of

target molecular expression is dose dependent.³⁷ The dose of *miR-124* mimic used in this study is 100 nM. In addition, miRNA functions in a cell type- and context-dependent manner,³⁸ and some AR-targeting miRNAs may not equally affect the AR level in all the five CaP cell lines. Therefore, different treatment conditions and selection criteria may account for the differential results.

Although we focus on the AR in this study, we realize that *miR-124* targets hundreds of human genes, and other potential targets may also be relevant for the tumor suppressive function of *miR-124* in CaP. We have identified a putative *miR-124* binding site in the 3' UTR of high-mobility group AT-hook 1 gene (*HMGA1*). In a pilot study, transfection of C4-2B cells with *miR-124* mimic resulted in the reduction of *HMGA1* expression by 60%; furthermore, two clinical CaP specimens having low level of *miR-124* did overexpress cytoplasmic *HMGA1* (see Supplementary Figure 5), suggesting a link between low *miR-124* and overexpression of *HMGA1* in CaP. Previous studies revealed that *HMGA1* was highly expressed in advanced CaPs,^{39,40} and cytoplasmic *HMGA1* directly interacts with p53, leading to p53 inactivation.^{41,42} Therefore, functional characterization of this *miR-124* target will provide new insight into elucidating the molecular alteration occurring in advanced CaP. In addition, cyclin-dependent kinase 6 and forkhead box A2 have been identified as promising targets of *miR-124*.^{13,14} Both cyclin-dependent kinase 6 and *Foxa2* were previously reported to be elevated in CaP cells and correlate with tumor grade.⁴³⁻⁴⁶ Moreover, these two proteins interact with the AR and enhance the AR activity.^{47,48} Further studies are required to understand whether upregulation of these two molecules in CaP cells are the result of decreased expression of *miR-124*.

Consistent with a previous report in nerve cells,⁴⁹ restoration of *miR-124* level by transfecting CaP cells with synthetic *miR-124* induces increased apoptosis. We also found that treatment of CaP cells with *miR-124* upregulated the expression of p53, which is due at least partially to *miR-124*-mediated inhibition of AR/*miR-125b* signaling.¹⁶ Thus, upregulation of p53 signaling may have a key role in *miR-124*-induced apoptosis. As *miR-124* induces apoptosis, we evaluated its effect on proliferation of CaP cells. Restoration of *miR-124* level significantly inhibited the growth of CaP cells. More interestingly, when lentiviral-transduced 22Rv1 CaP cells that stably express *miR-124* were injected subcutaneously into intact male nude mice, xenograft tumor growth was also significantly inhibited. Therefore, further testing of *miR-124* in pre-clinical models of CaP will help define its ultimate therapeutic potential for the treatment of CaP.

In conclusion, this study has shown that loss of *miR-124* expression may be a common event in CaP. As *miR-124* negatively regulates the AR, downregulation of *miR-124*, as found in CaP, increases expression of the receptor. Therefore, deregulation of *miR-124* as well as other AR-targeting miRNA candidates³⁶ contributes to high levels of AR expression in clinical CaP. In addition, our data suggest that downregulation of *miR-124* may be involved in the pathogenesis of CaP. Our evidence supporting the ability of *miR-124* to induce apoptosis in CaP cells encourages the hope that restoring *miR-124* function in CaP cells, either by itself or in conjunction with other therapies, will offer improved survival, which will be through delayed development or treatment of CR CaP.

MATERIALS AND METHODS

Cell lines

Two immortalized benign prostatic epithelial cell lines (RWPE-1 and pRNS-1-1) and five CaP cell lines (LNCaP, C4-2B, *cds2*, 22Rv1 and LAPC-4) were used in this study. RWPE-1 (provided by Dr Mukta Webber, Michigan State University, East Lansing, MI, USA) and pRNS-1-1 (provided by Dr John Rhim, University of the Health Sciences, Bethesda, MD, USA) were maintained in keratinocyte serum-free medium supplemented

with 50 mg/ml bovine pituitary extract and 5 ng/ml epidermal growth factor. CaP cell lines were maintained in RPMI 1640 medium containing 10% fetal bovine serum. Both C4-2B and *cds2* cell lines are derivatives of LNCaP.^{50,51}

Clinical samples

Collection and use of CaP patient specimens were approved by the Institutional Review Board (UCD IRB#: 200312072-6). Primary CaP samples from radical prostatectomy, metastatic pelvic lymph nodes, CR tumor specimens and BPH tissues were obtained fresh from surgical excision by the Department of Urology, University of California, Davis, Sacramento, USA. These CR tumors were described in our previous publication:⁵² six from stage D patients and four from patients receiving combined androgen blockade therapy before radical prostatectomy. To ensure that portions of CaP tissues were enriched for tumor cells, a surgeon and a pathologist together sectioned the excised tissue, and a fresh piece was taken from the palpated cancer and rapidly frozen to -80°C until use. A permanent section was then cut from the immediately adjacent area for hematoxylin and eosin staining. Based upon a histological review of hematoxylin and eosin section, it was confirmed that these CaP samples used in this study were tumor cell enriched.

Reporter plasmid construction and luciferase assay

To construct reporter plasmids, a 0.45-kb DNA fragment of the AR 3' UTR containing the putative *miR-124* binding site was prepared by PCR. The AR 3' UTR fragment lacking the *miR-124* binding site was used as negative control. DNA fragments were cloned into the pMIR-REPORT luciferase vector (Ambion) downstream of the reporter gene. The sequences and cloning direction of these PCR products were validated by DNA sequencing. For luciferase assay, cells (4×10^4 per well) were seeded into 24-well plates and cultured for 24 h. The cells were then cotransfected with reporter plasmids and 100 nM chemically-modified *miR-124* mimic or miRNA-negative control (miR-NC). The pRL-SV40 Renilla luciferase plasmid (Promega, Madison, WI, USA) was used as an internal control. Two days later, cells were harvested and lysed with passive lysis buffer (Promega). Luciferase activity was measured using a dual luciferase reporter assay (Promega). Luciferase activity was normalized by Renilla luciferase activity.

Western blot analysis

Cells were grown to 70–80% confluence and lysed in lysis buffer consisting of 25 mmol/l Tris-HCl (pH 7.5), 150 mmol/l NaCl, 1 mmol/l EDTA, 1% Triton X-100, 0.1% SDS and $1 \times$ proteinase inhibitor cocktail, and equal concentrations of denatured protein samples were loaded on a 10% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to an Immobilon PVDF membrane (Millipore, Bedford, MA, USA) and immunoblot analysis was conducted by the standard method.

Quantitative-PCR

For quantitative expression analysis of miRNA, total RNA was isolated from fresh-frozen prostatic tissues, cultured cells or xenograft tumors using TRIzol reagent (Life Technologies, Inc., Carlsbad, CA, USA). The miRNA level was measured using a TaqMan miRNA assay kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instruction and PCR amplification was carried out in a 7900HT Fast Real-time PCR system (Applied Biosystems). Real-time PCR was performed in triplicate for each sample. The abundance of miRNA was measured using C_t (threshold cycle) following the approach as described previously.⁵³ ΔC_t was calculated by subtracting the C_t of *U6* RNA from the C_t of the miRNA tested. $\Delta\Delta C_t$ was calculated by subtracting the ΔC_t of a reference sample from the ΔC_t of each sample. For clinical tissues, the reference sample is one CaP specimen tested that expresses the lowest level of miRNA. Fold change was generated using the equation $2^{-\Delta\Delta C_t}$.

Methylation assay

Genomic DNA was isolated from prostate cell lines using standard procedures and from CaP cell-enriched tumor tissues following our previous method.⁵⁴ Bisulfite modification of DNA (1.0 μg) was carried out by using an EZ DNA methylation-direct kit (Zymo Research, Irvine, CA, USA). For the prostate cell lines, the methylation of 5'-CpG island regions was detected by MSP using the primers specific for either methylated or unmethylated DNA. The methylated CpG dinucleotides were validated using bisulfite DNA sequencing. Amplified CpG islands were separately cloned into pCR

2.1 vector using TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA), and at least three clones per PCR product were picked up for DNA sequencing. In clinical prostate tissues, the methylation status was assessed by COBRA assay. COBRA primer-amplified PCR products were purified using Amicon 30 filter (Millipore, Billerica, MA, USA), digested by BstUI and separated on a 3% agarose gel (1% agarose plus 2% NuSieve GTG low-melting temperature agarose). The primers used for MSP and COBRA were designed based on "The Li Lab" program (<http://www.urogene.org/methprimer/index1.html>). The primer sequences and amplified size are listed in Supplementary Table 1.

Annexin V binding assay

miR-124-induced apoptosis was analyzed using a FACS Annexin V assay kit (Trévigen, Inc, Gaithersburg, MD, USA) following the protocol provided by the manufacturer. Briefly, cells were transfected with miR-124 mimic and incubated for 4 days. The cells were harvested, washed once with PBS, re-suspended in 100 µl of 1 × Annexin V binding buffer containing 1 µl of Annexin V-FITC conjugate and 10 µl of propidium iodide solution, and then incubated for 30 min at room temperature in the dark. Subsequently, 400 µl 1 × Annexin V binding buffer was added and samples were analyzed on a FACScan flow cytometer. Data analysis was performed using FACScan software (Becton Dickinson, Franklin Lakes, NJ, USA).

Animal experiment

A lentiviral miR-124 expression vector that expresses an ~500-base human pre-miR-124-1 and the empty lentiviral vector were purchased from System Biosciences (SBI, Mountain View, CA, USA). Pseudovirus production and cell transduction were performed following the manufacturer's protocol. The resulting 22Rv1-miR-124 and 22Rv1-vector cells were selected through fluorescence-activated cell sorting (FACS) and were maintained in the medium described above. Overexpression of miR-124 in infected 22Rv1 cells was confirmed by qPCR. Animal studies were performed in male athymic nu/nu mice (4- to 6-week-old; Harlan Laboratories, Indianapolis, IN, USA). Mice were injected subcutaneously with suspensions of 2 × 10⁶ 22Rv1-miR-124 cells or 22Rv1-vector cells in a mixture (1:1 vol/vol) of culture medium and Matrigel (Becton Dickinson). Tumor growth was monitored and tumor dimensions were measured twice per week. Tumor volumes were calculated according to the following formula: ½ (length × width × height). Animal studies were performed according to the protocols approved by the Animal Care and Use Committee at University of California, Davis, Sacramento, CA, USA.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software 5.0 (GraphPad Software, La Jolla, CA, USA). Student's *t*-test was used to analyze the difference in miR-124 levels between different groups. Statistical significance was set up to *P* < 0.05 in each test.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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