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Original Article

MicroRNA-122 targets δ-catenin to suppress the tumorigenic potential of prostate cancer cells

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Abstract: δ-Catenin is expressed abundantly in various human cancers, including prostate, brain, breast, and lung carcinomas, and is recognized as an oncogene that promotes cancer cell growth and tumorigenesis. Although several transcriptional and post-translational pathways for δ-catenin regulation have been identified in cancer cells, the potential effects of microRNA-mediated regulation remain elusive. Here, we used a δ-catenin 3'-UTR luciferase reporter assay to identify regulatory microRNAs. Subsequent bioinformatics analyses and molecular studies revealed that overexpression of miR-122 downregulated δ-catenin expression significantly via targeted binding to a seed sequence in the 3'-UTR region of δ-catenin, and suppressed the invasion, migration, and proliferation of prostate cancer cells in vitro. In a TRAMP-C2 mouse syngeneic prostate tumor model, stable expression of miR-122 decreased both δ-catenin expression and tumor growth. Mechanistically, overexpression of miR-122 inhibited the expression of δ-catenin-mediated downstream factors significantly in prostate cancer cells, including c-myc and cyclin D1. In cells overexpressing miR-122, there was no additive or synergistic effect of siRNA-mediated knockdown of δ-catenin on cell invasiveness, and overexpression of miR-122 alone had a more pronounced suppressive effect on cell invasion than knockdown of δ-catenin alone. These results suggest that miR-122 acts as tumor suppressor in prostate cancer, mainly by downregulating δ-catenin expression, but also by targeting other factors. Indeed, subsequent experiments showed that overexpression of miR-122 reduced the levels of the mRNAs encoding myc, snail, and VEGF in prostate cancer cells. Overall, our findings demonstrate that targeting of δ-catenin by miR-122 represses the motility and tumorigenesis of prostate cancer cells, indicating a tumor suppressive effect of this miRNA in prostate cancer.

Keywords: MicroRNA, miR-122, δ-catenin, prostate cancer, motility, tumorigenesis

Introduction

Prostate cancer is the most diagnosed cancer in men and the third leading cause of cancer-related deaths in the United States [1, 2]. Surgery and radiation therapy of prostate cancer is successful in approximately 70% of patients; however, 30% of patients experience biochemical recurrence and metastatic progression [3]. Androgen deprivation and other hormone therapies are used to treat patients with recurrent metastatic prostate cancer [4]. However, despite the development of new therapies, treatment options for prostate cancer are still limited. The identification of new biomarkers to predict the prognosis of the disease with high accuracy and specificity is essential. Furthermore, additional research into the molecular mechanisms underlying prostate tumor formation is required to aid the development of new therapeutic targets.

Accumulation of δ-catenin promotes prostate cancer cell growth and tumor formation by altering the cell cycle and expression patterns of survival-related genes [5]. δ-Catenin promotes the cleavage of E-cadherin, thereby increasing the total protein level and nuclear distribution of β-catenin and inducing activation of δ-catenin/lymphoid enhancer-binding factor-1 (LEF-1)-mediated transcription [6]. Furthermore, δ-catenin promotes prostate tumor growth by increasing angiogenesis through upregulation of hypoxia-inducible factor 1-α (HIF-1α) and vas-

cular endothelial growth factor (VEGF) [7]. Although it is well known that δ-catenin functions as an oncogene in prostate cancer, the mechanism of transcriptional regulation is unclear.

MicroRNAs (miRNAs) are noncoding RNAs that can regulate gene expression at transcriptional and translational levels [8]. In prostate cancer, several miRNAs play a vital role in pathogenic cellular processes by targeting multiple transcripts [9]. A recent study demonstrated that miR-214-5 inhibits prostate cancer cell proliferation by specific targeting of S0X4 [10]. In another study, miR-29b inhibited prostate cancer xenograft tumor growth significantly by inducing Bim expression [11]. Moreover, miR-99b-5p acts as a tumor suppressor by targeting the mTOR/androgen receptor axis to induce prostate cancer cell autophagy and inhibit cell proliferation [12].

As several studies have confirmed that miRNAs play an important role in prostate cancer by targeting various oncogenes, we attempted to identify miRNAs that target δ-catenin and modulate its expression in prostate cancer. We used a bioinformatics analysis to identify δ-catenin-targeting miRNAs and found that miR-122 suppressed the migration, invasion, and proliferation of several prostate cancer cell lines via negative regulation of δ-catenin expression. Furthermore, miR-122 inhibited in vivo tumorigenesis in a xenograft mouse model by repressing δ-catenin expression. Our results suggest that miR-122 functions as a tumor suppressor in prostate cancer and may be a potential target for therapeutic intervention.

Materials and methods

Cell culture

Human prostate cancer cell lines CWR22-RV1, DU145, C4-2, and PC3 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI medium (Gen Depot, USA) supplemented with 10% fetal bovine serum (FBS; Gen Depot, USA) and 1% Penicillin-Streptomycin (Gen Depot, USA) at 37°C in a 5% CO_2 in a humidified atmosphere.

MiRNA transfection

Briefly, all miRNA mimics and miRNA inhibitor (anti-miR) were synthesized by Bioneer (Daejeon, Korea) and the transfection was performed using HiPerFect (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The final concentration of miRNA mimics and inhibitors in the transfection system was 30 nmol/L.

RNA isolation and qRT-PCR

Total RNA was extracted by using RNAiso Plus (TaKaRa, Otsu, Shiga 520-2193, Japan) according to the manufacturer's instructions. cDNA was synthesized from total RNA by using a M-MLV reverse Transcriptase kit (Invitrogen, Carlsbad, USA) and SYBR green (Enzynomics, Seoul, Korea). The primers used for qRT-PCR were cyclin-D1 (forward) 5'-ccgtccatgcggaagatc-3' and (reverse) 5'-gaagacctcctcctcgcact-3'; c-myc (forward) 5'-aatgaaaaggcccccaaggtagttatcc-3' and (reverse) 5'-gtcgtttccgcaacaagtcctcttc-3'; GAPDH (forward) 5'-atcaccatcttccaggagcga-3' and (reverse) 5'-agttgtcatggatgaccttggc-3'. Relative miR-122 expression was measured using TaqMan miRNA assay kit (Applied Biosystems, USA). The relative miR-122 expression was normalized to U6. δ-Catenin, c-myc, and cyclin D1 were normalized to GAPDH. qRT-PCR reaction and analysis were performed using CFX (Bio-Rad, Hercules, USA). Related values were calculated with ΔΔCt method [13].

Luciferase assay

Luciferase assay was performed as previously described [14]. Briefly, Bioinformatic analysis was performed to predict microRNA binding to δ-Catenin using TargetScan (http://www.targetscan.org/) and miRanda (http://www.targetscan.org/). To construct reporter plasmids, δ-catenin 3'-UTR wild type (Wt) and δ-Catenin 3'-UTR mutant (Mut) were prepared by PCR. HEK293T cells (4×10^4 cells/well) were seeded into 24-well plates. After 24 h incubation, the cells were then cotransfected with reporter plasmids (Wt or Mut) and miR-122, miR-429 mimic or a scrambled control miRNA (miR-con). After 48 h incubation, luciferase activity measured using a dual luciferase reporter assay (Promega, Madison, Wis., USA). Renilla luciferase plasmid was used as an internal control.

Cell viability assay

The method for growth and survival of prostate cancer cells is based on the colorimetric quantification of MTT (3-(4, 5-dimethylthiazol-2-yl)-2,

5-diphenyltetrazolium bromide) assay. Briefly, prostate cancer cells (1×10^5 cells/well) were plated into 24-well plates and then transfected with miR-mimic or miR-con for 48 h. The absorbance value at 450 nm was measured with the spectrophotometer.

Invasion assay and migration assay

Transwell migration and invasion assays were performed using an 8 μm-pore polycarbonate membrane Boyden chamber insert in transwell chambers (Corning, New York, USA). For invasion assay, the chamber inserts were pre-coated with 1% gelatin. Briefly, 2×10^6 cultured cells pretreated with miR-con, miR-122, or miR-429 for 48 h were harvested. The cell suspension in RPMI1640 containing 0.2% bovine serum albumin (BSA) was added into the upper chamber. The culture medium containing 10 μg/mL fibronectin was placed as a chemo-attractant in the lower chamber. For migration assay, 2×10^5 cultured cells pretreated with miR-con, miR-122, or miR-429 for 48 h were harvested. The cell suspension in RPMI1640 containing 2% FBS was added into the upper chamber. The culture medium containing 10% FBS was placed as a chemo-attractant in the lower chamber. After 48 h incubation, the cells in the upper chamber were fixed with Diff Quik kit (Sysmex, Kobe, Japan). Then the cells inside the chamber were mechanically removed from the membrane with a cotton swab, and the cells adhering to the under-side of the membrane were stained and counted under light microscope (5 fields per chamber). Each assay was repeated in three independent experiments. The results are expressed as the mean number of cells migrating per high-power field.

Colony formation assay

Rv1 and DU145 cells (1000 cells/well) were seeded with 2.5 mL RPMI1640 medium in 6-well plates and were incubated for attachment. Subsequent to 48 h transfection with the miR-con, miR-mimic, the medium was replaced with RPMI1640+/+ medium for 12 days incubation. Colonies were fixed in 4% paraformaldehyde, stained with 0.5% crystal violet, and counted under a stereomicroscope. The plating efficiency (PE) of untreated cells and the survival fraction (SF) of treated cells were then determined (n = 3).

Western blot analysis

Cells and tissues were lysed in a lysis buffer for 30 mins on ice. After centrifugation, proteins (30 μg) were separated by 10% poly-acrylamide gel electrophoresis containing 0.1% SDS and electrophoretically transferred to nitrocellulose membranes. The membranes were incubated for 1 h at room temperature in a blocking buffer (20 mmol/l Tris-HCl, 137 mmol/l NaCl, pH 8.0, containing 0.1% Tween and 3% nonfat dry milk), and probed with antibodies against δ-catenin (BD Bioscience, San Jose, CA, USA) c-myc (Santa Cruz Biotechnology), cyclin D1 (Calbiochem, SanDiego, CA, USA) and β-actin (Sigma, St Louis, MO). The blots were developed with peroxidase-conjugated secondary antibodies and reacted proteins were visualized using an electrochemiluminescence (ECL) system (Pierce Biotechnology).

In vivo tumor growth

TRAMP-C2 cells (5×10^6 cells/mice) were subcutaneously injected into the right flanks of the C57BL/6 mice (5-week-old) according to the manufacturer's instructions. TRAMP-C2-generated tumors were intratumorally injected with miR-con or miR-122 using in vivo-jetPEL (Polyplus-transfection Inc., New York, NY) after tumors formed at 5 weeks. MiR-122 and miR-con were injected five times for 3 weeks at a concentration of 1.4 μg/μL. Tumor volume was measured twice a week for up to 8 weeks using a caliper. Tumor tissues from miR-122 and miR-con groups were isolated and then detected by qRT-PCR.

Statistical analysis

The differences between the experimental groups were analyzed with multiple comparison correction variances. All statistical data were expressed as mean ± SD, and P values of < 0.05 were considered statistically significant. Statistics was analyzed with SPSS version 23 software.

Results

MiR-122 downregulates δ-catenin expression in prostate cancer cells

Overexpression of δ-catenin reportedly plays an important role in the pathogenesis of pros-

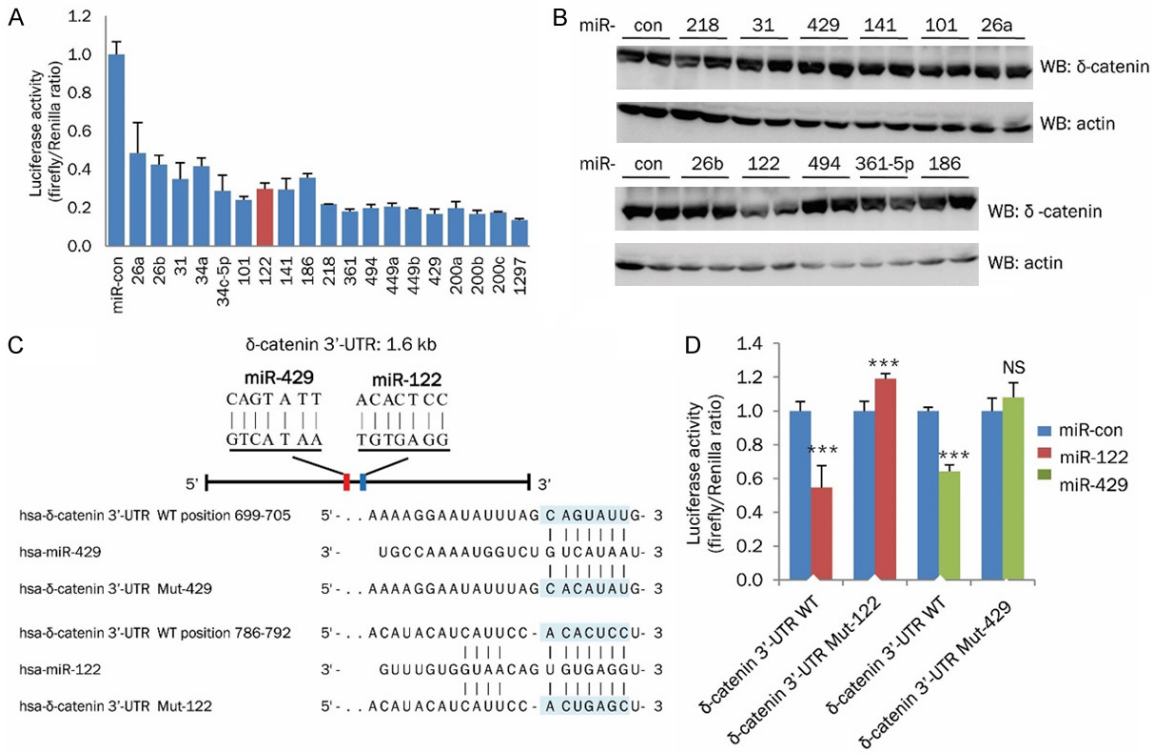

Figure 1. MiR-122 modulates δ-catenin expression by binding to its 3'-UTR. A. Luciferase assay to screen for miRNAs affecting the 3'-UTR activity of δ-catenin. The 3'-UTR of the mRNA encoding human δ-catenin was cloned into the pSYC-31 vector downstream of a luciferase reporter. HEK293T cells were co-transfected with the indicated miRNAs (each 30 nM) and the reporter plasmid. Data are presented as the mean ± SEM. B. Western blotting analyses of the effects of the indicated miRNA mimics on δ-catenin protein expression in Rv-1 cells. Actin was used as an internal control. C. TargetScan analysis to identify putative miR-122 and miR-429 (negative control) binding sites and seed sequences in the 3'-UTR of the mRNA encoding human δ-catenin. Point mutations of the seed sequences were made as indicated. WT: wild type; Mut: mutated. D. Luciferase assay to confirm the seed sequence-dependent interaction of miR-122 and miR-429 with the 3'-UTR of the mRNA encoding δ-catenin. HEK293T cells were co-transfected with a miR-122 or miR-429 mimic (each 30 nM) and the indicated reporter plasmid. Values are presented as the mean ± SEM (triplicates of three independent experiments); ***P < 0.001; NS, no significant difference compared with the control group.

tate cancer. Here, we used a 3'-UTR luciferase reporter system to examine the miRNA-mediated regulation of δ-catenin expression in prostate cancer cells. An in silico analysis predicted that the 3'-UTR of the mRNA encoding δ-catenin is targeted by 19 miRNAs (Supplementary Figure 1), and overexpression of these miRNAs in HEK293T cells reduced the activity of a δ-catenin 3'-UTR luciferase reporter (**Figure 1A**). However, we only examined the 11 individual miRNAs which shared the same seed region with others, such as miR-200bc/429, miR-141/200a, miR-26ab/1297, miR-361/361-5p. The miRNA gene cluster which produced miR-34a/34b-5p/34c/34c-5p/449/449abc/699 was not tested because these miRNAs have the same seed region [15, 16], resulting in probably functional redundancy [17, 18]. Among the 11 miRNAs examined, only miR-122 reduced the level of the δ-catenin protein in the Rv-1 human prostate cancer cell line (**Figure 1B**).

A bioinformatics analysis revealed that the region of the δ-catenin 3'-UTR spanning nucleotides 786-792 contains a miR-122 seed sequence (**Figure 1C**). A binding site for miR-429 was also identified at position 699-705 and was used as a negative control in subsequent experiments. Co-transfection of a miR-122 or miR-429 mimic suppressed the luciferase activity of the δ-catenin 3'-UTR reporter in HEK293 cells. By contrast, this inhibitory effect was abrogated by point mutations in the miR-122 and miR-429 seed sequences (**Figure 1D**).

Next, we examined the endogenous expression levels of miR-122 (**Figure 2A**) and δ-catenin

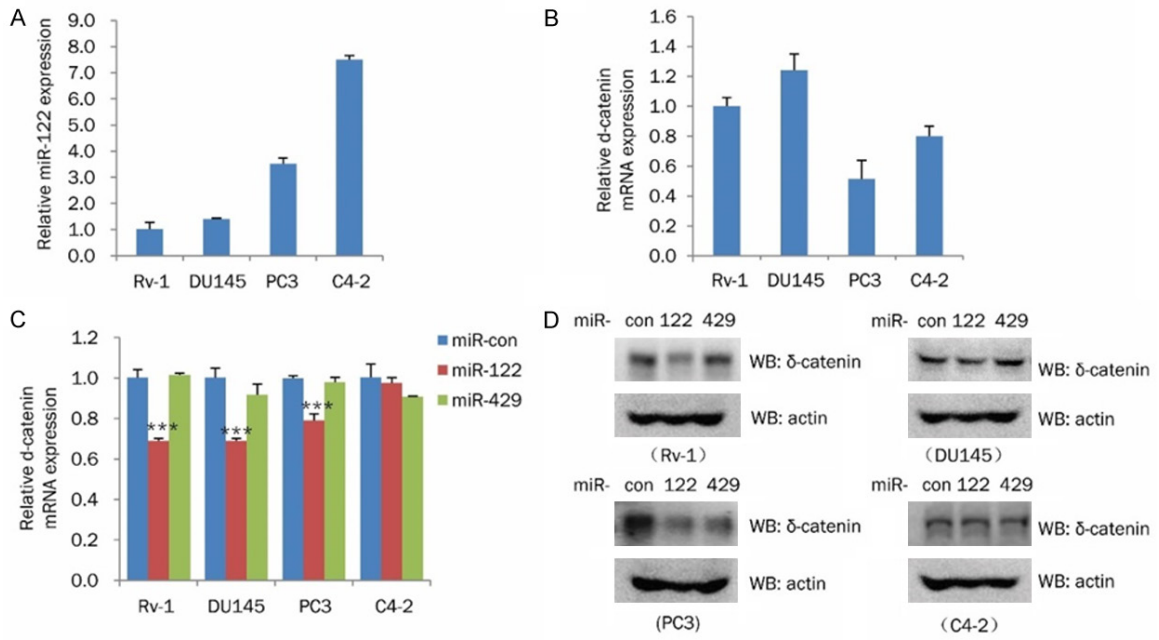

Figure 2. MiR-122 suppresses δ-catenin expression in prostate cancer cells. A. The expression levels of miR-122 in Rv-1, DU145, PC3, and C4-2 prostate cancer cell lines. The U6 snRNA was used as an internal control for quantitative analysis of miR-122. Data are represented as the mean ± SEM. B. The expression levels of the mRNA encoding δ-catenin in Rv-1, DU145, PC3, and C4-2 cells. Data are represented as the mean ± SEM. C. A qRT-PCR analysis of the effects of miR-122 and miR-429 on expression levels of the mRNA encoding δ-catenin in prostate cancer cells. Synthetic miR-122 and miR-429 mimics (each 30 nM) were transfected into Rv-1, DU145, PC3, and C4-2 cells. Data are represented as the mean ± SEM (n = 3); *P < 0.05, **P < 0.01, ***P < 0.001 versus miR-con. D. Western blotting analysis of the effects of miR-122 and miR-429 on δ-catenin protein levels in prostate cancer cells. Synthetic miR-122 and miR-429 (each 30 nM) were transfected into Rv-1, DU145, PC3, and C4-2 cells. Actin was used as an internal control.

(**Figure 2B**) in four prostate cancer cell lines and found high levels of miR-122 in PC3 and C4-2 cells, which corresponded with lower δ-catenin levels. Contrarily, high δ-catenin expression corresponded to low miR-122 levels in Rv-1 and DU145. Compared with those in cells expressing a scrambled control miRNA (miR-con) or a miR-429 mimic, the expression levels of the δ-catenin mRNA (**Figure 2C**) and protein (**Figure 2D**) were downregulated in Rv-1, DU145, and PC3 cells expressing a miR-122 mimic. However, overexpression of miR-122 in C4-2 cells was resistant to suppress δ-catenin levels (**Figure 2D**), probably due to higher endogenous expression of miR-122 in this cell line (**Figure 2A**). Taken together, these results suggest that miR-122 binds to the 3'-UTR of the mRNA encoding δ-catenin and inhibits its expression significantly.

Downregulation of δ-catenin by miR-122 inhibits the motility and proliferation of prostate cancer cells

Next, we examined whether targeting of δ-catenin by miR-122 plays a role in regulating the motility and proliferation of prostate cancer cells. An MTT assay revealed that the viabilities of Rv-1, DU145, and PC3 cells (but not C4-2 cells) were reduced after transfection of a miR-122 mimic (**Figure 3A**). We selected Rv-1 and DU145 cells for further study. A transwell assay revealed that overexpression of miR-122 reduced the invasion and migration abilities of both cell types significantly (**Figure 3B** and **3C**). Moreover, overexpression of miR-122 suppressed colony formation by Rv-1 and DU145 cells (**Figure 3D**). Overall, these results indicate that targeting of δ-catenin by miR-122 inhibits the tumorigenicity of prostate cancer cells in vitro.

MiR-122 suppresses the tumorigenicity of TRAMP-C2 cells in vivo

To support our findings that targeting of δ-catenin by miR-122 has an anti-cancer effect in vitro, we used a xenograft model of TRAMP-C2 prostate cancer cells in which C57BL/6 mice, TRAMP-C2-generated tumors were intratumorally injected with miR-con or miR-122 (1.4 mg/

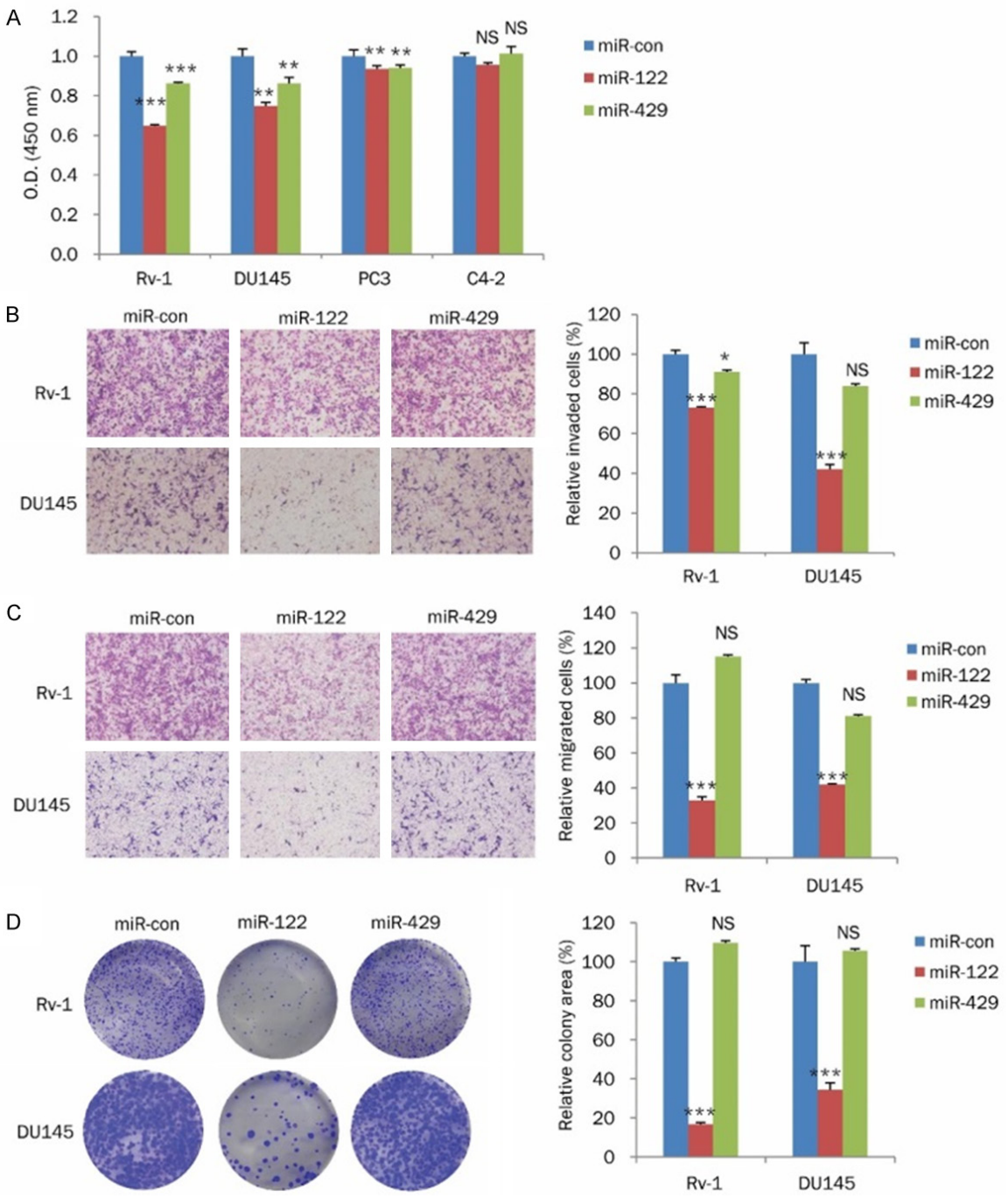

Figure 3. Overexpression of miR-122 inhibits the motility and proliferation of Rv-1 and DU145 cells. A. MTT assay to examine the viabilities of Rv-1, DU145, PC3, and C4-2 cells after transfection of miR-con, miR-122, or miR-429. B. Invasion assay of Rv-1 and DU145 cells after transient transfection of miR-con, miR-122, or miR-429. C. Cell migration assay of Rv-1 and DU145 cells transfected with miR-con, miR-122, or miR-429. D. Cell colony numbers 2 weeks after transfection of Rv-1 and DU145 cells with miR-con, miR-122, or miR-429. A-D. The mean values and SEMs were calculated from triplicates of a representative experiment; *P < 0.05; **P < 0.01; ***P < 0.001; NS, no significant difference compared with miR-con.

mL) and tumor progression was examined for up to 8 weeks. Compared with that in the miR-con group, in vivo tumor growth was inhibited significantly in the miR-122 group (**Figure 4A**). In addition, the tumor volume and weight in the miR-122 group were significantly lower than

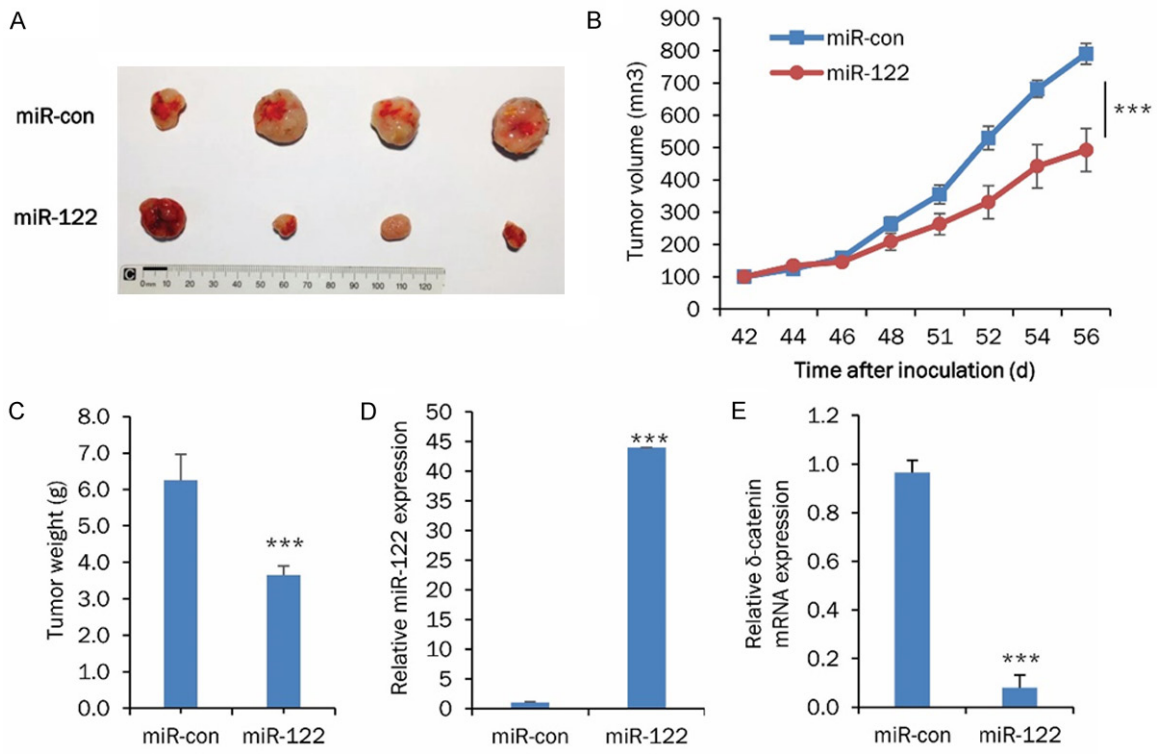

Figure 4. Overexpression of miR-122 attenuates TRAMP-C2 xenograft tumor growth in C57BL/6 mice. (A) Isolated tumor tissues (representative of five mice per group). C57BL/6 mice with TRAMP-C2-generated tumors were intratumorally injected with miR-con or miR-122. Tumor volume was measured twice a week for 3 weeks using calipers. (B) The effect of overexpressing miR-122 on TRAMP-C2 tumor formation in a syngeneic mouse xenograft model. Each data point represents the mean ± SEM of five tumors. (C) The mean tumor weight for each group. (D, E) Expression levels of mature miR-122 (D) and the mRNA encoding δ-catenin (E) in isolated tumor tissues from both groups, obtained 56 days after injection of TRAMP-C2 cells. GAPDH was used as an internal control. (C, E) The mean values and SEMs were calculated from triplicates of a representative experiment. (B-E) ***P < 0.001 compared with miR-con.

those in the miR-con group (**Figure 4B** and **4C**). Next, the expression levels of miR-122 and the mRNA encoding δ-catenin in tumor tissues isolated 21 days after injection were examined by qRT-PCR. Compared with those in the miR-con group, the expression level of miR-122 was increased significantly (by ~35-fold) in the miR-122 group (**Figure 4D**), whereas the expression level of the δ-catenin mRNA was decreased significantly (by ~8-fold) (**Figure 4E**). These results suggest that miR-122 targeted δ-catenin in TRAMP-C2 cells to suppress tumor growth in the xenograft mouse model.

MiR-122 inhibits the expression of downstream genes in the δ-catenin signaling pathway in prostate cancer cells

A previous study showed that δ-catenin promotes the cleavage of E-cadherin, thereby inducing activation of β-catenin/LEF-1 mediated transcription and subsequent upregulation of downstream targets such as cyclin D1 and c-myc [19]. Therefore, we examined whether miR-122 affects the expression levels of cyclin D1 and c-myc in prostate cancer cells. Western blot analyses showed that the expression levels of the cyclin D1 and c-myc proteins in Rv-1 and DU145 cells harboring a miR-122 mimic were lower than those in cells harboring miR-con (**Figure 5A**). By contrast, these changes were reversed in cells expressing a synthetic miR-122 inhibitor (**Figure 5A**). In addition, compared with those in cells harboring miR-con, the expression levels of the mRNAs encoding cyclin D1 and c-myc were significantly lower in Rv-1 and DU145 cells expressing the miR-122 mimic, with a corresponding increase (albeit not statistically significant) after transfection with the miR-122 inhibitor (**Figure 5B** and **5C**). Moreover, in the xenograft tumor samples, the protein levels of both of downstream targets,

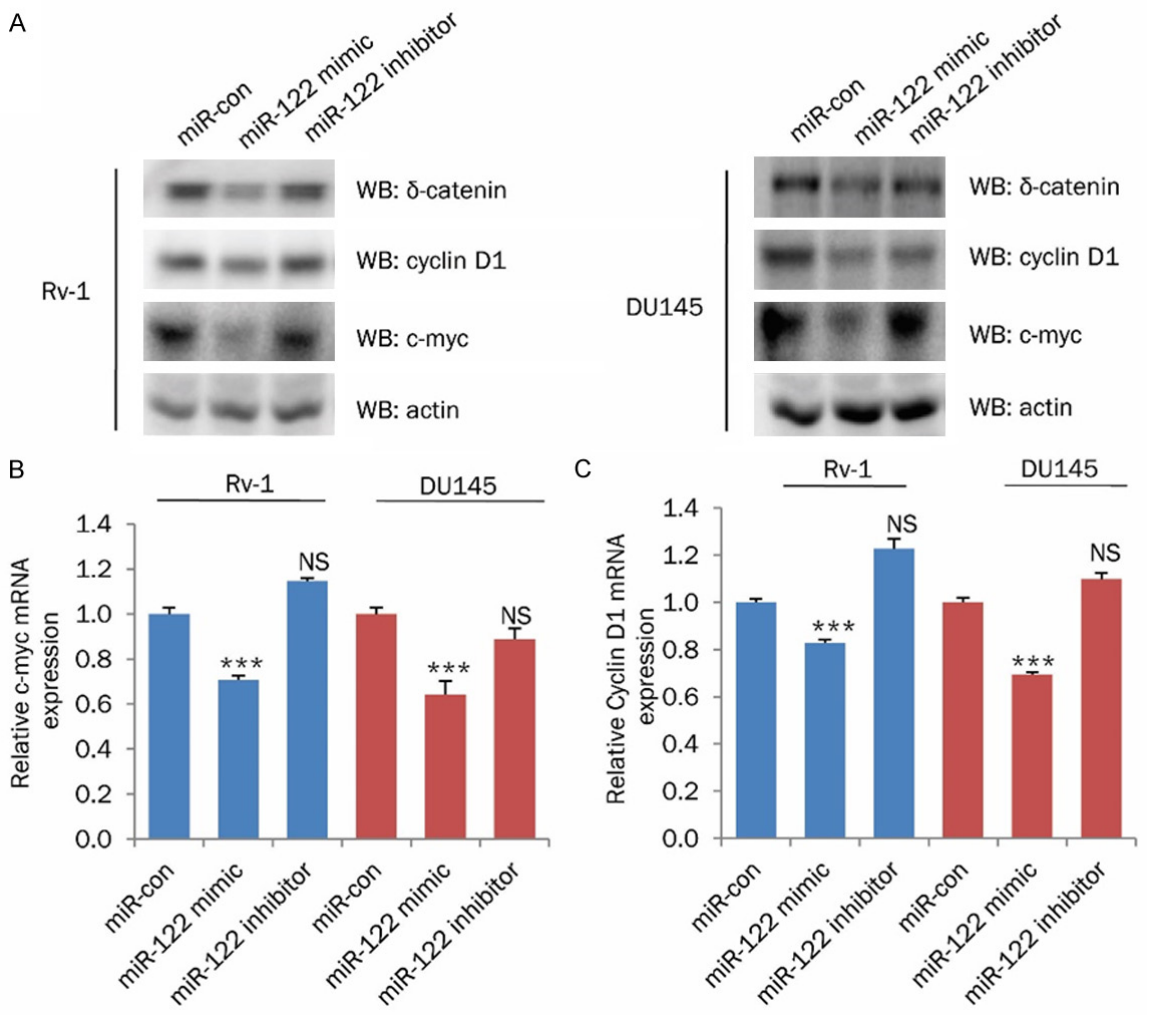

Figure 5. MiR-122 suppresses the expression of downstream factors in the δ-catenin signaling pathway. (A) Western blotting analyses of the δ-catenin, cyclin D1, and c-myc proteins in Rv-1 and DU145 cells 48 h after transfection with miR-con, a miR-122 mimic, or a miR-122 inhibitor. Actin was used as an internal control. (B, C) A qRT-PCR analysis of the expression levels of the mRNAs encoding c-myc (B) and cyclin D1 (C) in Rv-1 and DU145 cells 48 h after transfection with miR-con, a miR-122 mimic, or a miR-122 inhibitor. (B, C) The mean values and SEMs were calculated from triplicates of a representative experiment; ***P < 0.001; NS, no significant difference compared with miR-con.

c-myc and cyclin D1, were significantly lower in the miR-122 group compared with those in the miR-con group (Supplementary Figure 2A and 2B). Collectively, these data indicate that the miR-122-mediated reduction in δ-catenin expression inhibits β-catenin-mediated oncogenic signaling.

MiR-122 suppresses prostate cancer tumorigenicity by targeting multiple oncogenes

Since a single miRNA can regulate the expression of several genes, it is possible that targeting of multiple mRNAs by miR-122 contributes to the suppression of prostate cancer tumorigenicity. To investigate this possibility, we performed siRNA-mediated knockdown of δ-catenin (si-δ-catenin) in Rv-1 cells to examine whether the inhibition of prostate cancer cell invasion caused by overexpression of miR-122 was due to suppression of δ-catenin expression only. The effects of overexpression of miR-122 and/or knockdown of δ-catenin were examined using a transwell invasion assay. Compared with that of cells expressing miR-con, the invasive ability of Rv-1 cells expressing si-δ-catenin was reduced by ~40%, whereas the invasive abilities of cells expressing miR-122 alone or miR-122 and si-δ-catenin were reduced by ~60% (**Figure 6A** and **6B**). The expres-

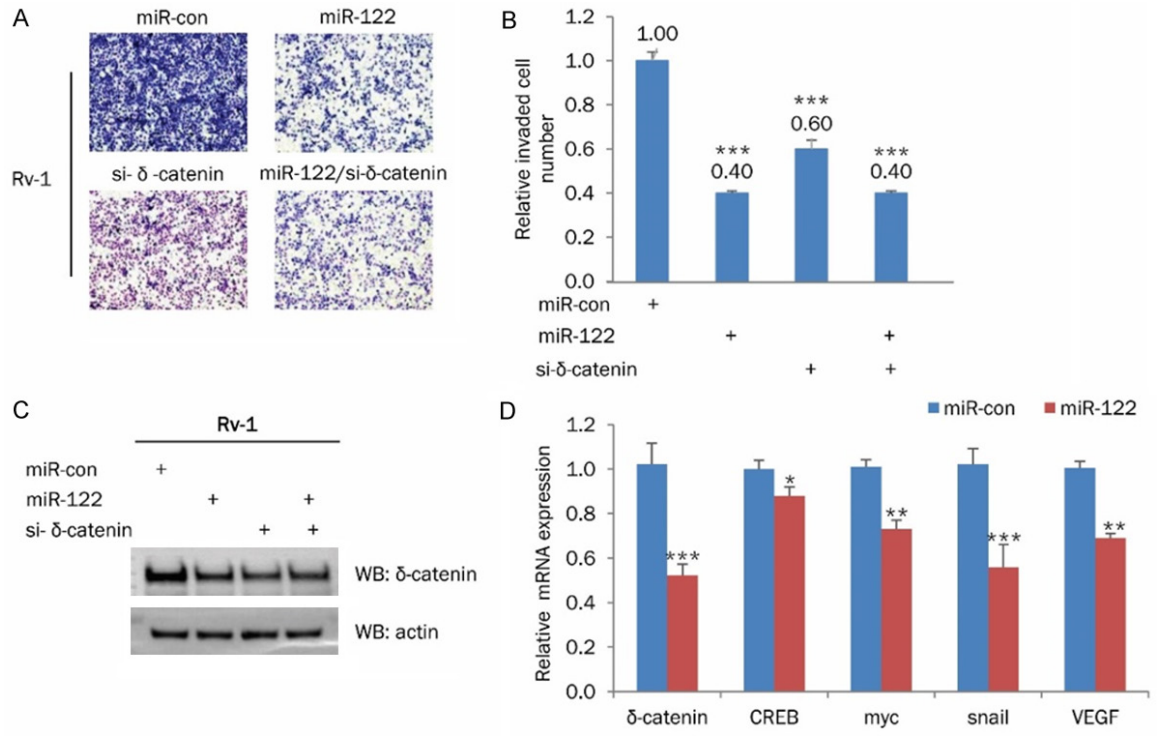

Figure 6. MiR-122 suppresses the invasiveness of Rv-1 cells by downregulating δ-catenin, snail, and VEGF. A, B. The effects of transfection with miR-con, miR-122, si-δ-catenin, or miR-122 plus si-δ-catenin on the invasive ability of Rv-1 cells. C. Western blotting analysis of δ-cadherin expression in Rv-1 cells transfected with miR-con, miR-122, si-δ-catenin, or miR-122 plus si-δ-catenin. Actin was used as an internal control. D. A qRT-PCR analysis of the expression levels of the mRNAs encoding δ-catenin, CREB, myc, snail, and VEGF in Rv-1 cells transfected with miR-con or miR-122. B-D. The mean values and SEM were calculated from triplicates of a representative experiment; *P < 0.05; **P < 0.01; ***P < 0.001; NS, no significant difference compared with miR-con.

sion level of the δ-catenin protein was reduced similarly in Rv-1 cells expressing miR-122, si-δ-catenin, or miR-122 plus si-δ-catenin (**Figure 6C**). These findings suggest that miR-122 inhibits prostate cancer cell invasion by targeting δ-catenin and other factors.

To support our claim, we examined the effects of miR-122 on the expression levels of the mRNAs encoding CREB, myc, snail, and VEGF, which were predicted as potential targets of miR-122 by bioinformatic analyses. These targets were chosen because previous studies have shown that miR-122 inhibits gastric cancer cell proliferation by targeting CREB [20] and induces gastric cancer cell apoptosis by targeting MYC [21], inhibits bladder tumor growth and angiogenesis by targeting VEGF [22], and inhibits the epithelial-mesenchymal transition (EMT)/snail signaling pathway in hepatocellular carcinoma [23]. As shown in **Figure 6D**, the expression levels of the mRNAs encoding δ-catenin, myc, snail, and VEGF were suppressed in Rv-1 cells after transfection of a mature miR-122 mimic. Expression of the mRNA encoding CREB was also reduced but the change was not statistically significant (**Figure 6D**). Taken together, our results indicate that miR-122 suppresses prostate cancer tumorigenesis mainly by negative regulation of δ-catenin expression, but also via suppression of other oncogene targets.

Discussion

MiRNAs play a role in the pathogenesis of most types of cancers by modulating the expression of oncogenes and tumor suppressors [24]. In prostate cancer, several miRNAs play an important role in the regulation of basic biological processes, including cell growth, apoptosis, metastasis, and drug resistance. MiR-29b suppresses prostate cancer metastasis by inhibiting snail expression in the EMT pathway [25]. In addition, miR-128 targets BMI-1 to suppress prostate cancer tumor growth [26] and miR-

34a inhibits prostate cancer cell stemness and metastasis by suppressing CD44 [27]. Despite these findings, the regulation and potential mechanisms of action of miRNAs in prostate cancer are not fully understood.

δ-Catenin was initially reported to play a regulatory role in neurodevelopment [28]; however, we reported previously that it is expressed at high levels in human prostate cancer cells. The accumulation of δ-catenin advances the growth and progression of prostate cancer cells by affecting the cell cycle [19, 29], and promotes prostate cancer cell angiogenesis by modulating HIF-1α and VEGF expression [7]. Despite these findings, the mechanisms controlling transcription and post-transcriptional regulation of the δ-catenin gene remained unclear. It was previously reported that miR-218-5p directly targeted δ-catenin and decreased δ-catenin 3'-UTR luciferase reporter, consistent with our results (**Figure 1A**). Overexpression of miR-218-5p suppressed IL-13-induced δ-catenin transcripts and protein levels in lung epithelial Cells (BEAS-2B) [30]. Interestingly, miR-218 did not suppress δ-catenin protein level in prostate cancer cells as shown in **Figure 1B**. In our study, we found that miR-122 acts as a tumor suppressor that inhibits the metastasis and proliferation of prostate cancer cells by repressing δ-catenin expression. Of the 19 miRNAs that were able to decrease the activity of a δ-catenin 3'-UTR luciferase reporter, only miR-122 reduced the level of the δ-catenin protein in prostate cancer cells (**Figure 1**). In addition, high endogenous expression of miR-122 corresponded to low endogenous expression of δ-catenin in prostate cancer cell lines (**Figure 2A** and **2B**). Overexpression of miR-122 inhibited the migration and invasion abilities of prostate cancer cells, suggesting that it acts as an endogenous inhibitor of prostate cancer cell motility (**Figure 3**). Furthermore, a colony formation assay confirmed that miR-122 inhibited the proliferation of prostate cancer cells. Using a mouse xenograft tumor model, we also confirmed that miR-122 functions as a tumor suppressor in vivo by suppressing the expression of δ-catenin (**Figure 4**). Notably, previous studies demonstrated that δ-catenin promotes prostate cancer progression via activation of the Wnt/β-catenin pathway [19, 31], and the accumulation of β-catenin leads to the upregulation of downstream factors such as c-myc and cyclin D1 [32]. Here, the levels of the c-myc and cyclin D1 mRNAs and proteins were lower in prostate cancer cells expressing a miR-122 mimic than in cells expressing miR-control or a miR-122 inhibitor (**Figure 5**), suggesting that miR-122-mediated downregulation of δ-catenin inhibits β-catenin-mediated oncogenic signaling.

MiR-122 inhibits gastric and bladder cancer cell proliferation by targeting CREB1 [20, 33] and inhibits breast cancer tumorigenesis by targeting IGF1R [34]. In addition, miR-122 targets VEGF in bladder cancer to inhibit tumor growth and angiogenesis [22], and inhibits EMT by targeting snail in hepatocellular carcinoma cells and regulating P4HA1 in ovarian cancer cells [35, 36]. Furthermore, miR-122-mediated downregulation of Rho-associated protein kinase 2 expression inhibits the proliferation of prostate carcinoma cells [37]. These reports indicate that miR-122 is able to inhibit the proliferation, invasion, and angiogenesis of various cancers by targeting multiple oncogenes. Therefore, we examined whether the suppressive role of miR-122 in prostate cancer progression involves targeting of genes other than δ-catenin, focusing on those encoding CREB, myc, snail, and VEGF. Targeting of CREB1 by miR-122 inhibits gastric cancer cell invasion and proliferation [20]. In hepatocellular carcinoma, the myc/miR-122-5p/lactate dehydrogenase axis modulates glycolysis [38] and miR-122 inhibits EMT by targeting snail, thereby suppressing the Wnt/β-catenin signaling pathway [35]. In addition, miR-122 targets VEGF to inhibit the growth and angiogenesis of various tumors, such as human bladder and liver cancers [22, 39]. Here, we provide the first evidence that miR-122 suppresses expression of the mRNAs encoding CREB, myc, snail, and VEGF in prostate cancer cells. Nonetheless, the inhibitory effect of miR-122 on δ-catenin expression was more pronounced than those on CREB, myc, snail, and VEGF expression, suggesting that miR-122 inhibits prostate cancer cell invasiveness and tumorigenesis mainly via downregulation of δ-catenin.

In conclusion, our results show that miR-122 targets the 3'-UTR and decreases the expression of δ-catenin, as well as its related downstream factors, in prostate cancer cells. Downregulation of δ-catenin by miR-122 inhibits

prostate cancer cell metastasis, proliferation, and tumor growth. Overall, these findings suggest that miR-122 may serve as a potential therapeutic target for prostate carcinoma.

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Disclosure of conflict of interest

None.

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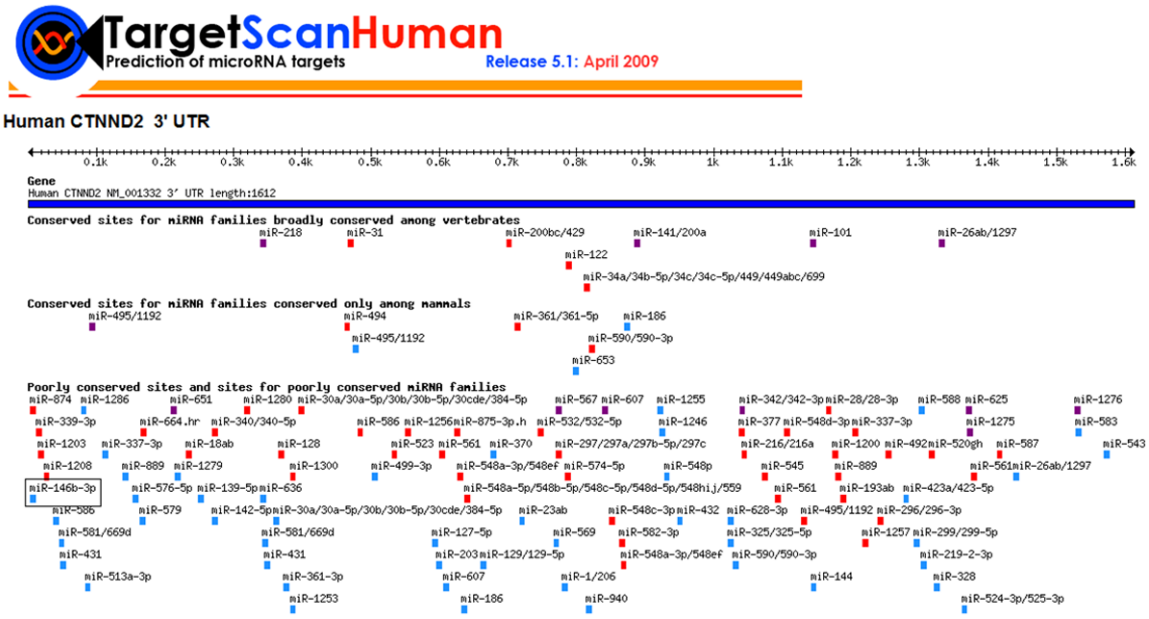
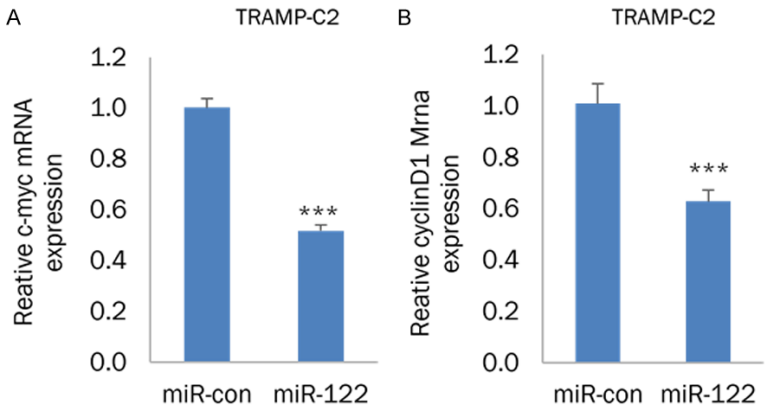


Supplementary Figure 1. An online program Target-Scan (release human 5.1) was used for predicting miRNAs that might target δ-catenin.



Supplementary Figure 2. MiR-122 suppresses the expression of downstream factors on TRAMP-C2 tumor formation in mouse xenograft model. A, B. Expression levels of c-myc and cyclin D1 in isolated tumor tissues from both groups, obtained 56 days after injection of TRAMP-C2 cells. GAPDH was used as an internal control. The mean values and SD were calculated from triplicates of a representative experiment. ***P < 0.001 compared with miR-con.