MicroRNA-296-5p (miR-296-5p) functions as a tumor suppressor in prostate cancer by directly targeting Pin1

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Upregulation of Pin1 was shown to advance the functioning of several oncogenic pathways. It was recently shown that Pin1 is potentially an excellent prognostic marker and can also serve as a novel therapeutic target for prostate cancer. However, the molecular mechanism of Pin1 overexpression in prostate cancer is still unclear. In the present study, we showed that the mRNA expression levels of Pin1 were not correlated with Pin1 protein levels in prostate cell lines which indicated that Pin1 may be regulated at the post-transcriptional level. A key player in post-transcriptional regulation is represented by microRNAs (miRNAs) that negatively regulate expressions of protein-coding genes at the post-transcriptional level. A bioinformatics analysis revealed that miR-296-5p has a conserved binding site in the Pin1 3′- untranslated region (UTR). A luciferase reporter assay demonstrated that the seed region of miR-296-5p directly interacts with the 3′-UTR of Pin1 mRNA. Moreover, miR-296-5p expression was found to be inversely correlated with Pin1 expression in prostate cancer cell lines and prostate cancer tissues. Furthermore, restoration of miR-296-5p or the knockdown of Pin1 had the same effect on the inhibition of the ability of cell proliferation and anchorage-independent growth of prostate cancer cell lines. Our results support miR-296-5p playing a tumor-suppressive role by targeting Pin1 and implicate potential effects of miR-296-5p on the prognosis and clinical application to prostate cancer therapy.

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1. Introduction

Peptidyl-prolyl isomerase, Pin1, is an enzyme that specifically binds phosphorylated serine or threonine that immediately precedes proline (pSer/Thr-Pro) in a subset of proteins [1]. The conformational regulation catalyzed by Pin1 has a major effect on key proteins involved in regulating cell growth, neuronal differentiation, and survival [2]. Moreover, Pin1 is tightly regulated at multiple levels under physiological conditions. For example, Pin1 expression is subject to E2F or BRCA1-mediated transcriptional regulation in response to growth factors [3, 4]. In addition to being transcriptionally regulated, Pin1 is also regulated by post-translational controls, including phosphorylation [5] and ubiquitylation [6]. Thus, deregulation of Pin1 has an important role in a growing number of pathological conditions, including Alzheimer’s disease (AD), aging, asthma and cancer [7–9]. The link between Pin1 and cancer was originally suggested by the data which showed that Pin1 interacts with a number of phosphoproteins that are cancer-related [10, 11]. Recent studies showed that Pin1 plays an important role in a wide range of human cancers including lung, breast, colon, and prostate cancers, and is considered a biomarker of poor prognosis [10–15]. Furthermore, it was reported that Pin1 can affect cancer cell anchorage-independent growth [16–18]. However, the regulatory mechanism of Pin1 in prostate cancer tumorigenesis has not been well clarified. Thus, increasing our understanding of the molecular mechanisms of Pin1 in prostate cancer tumorigenesis will facilitate our understanding of prostate cancer progression and could also identify important novel therapeutic targets for advanced disease.

MicroRNAs (miRNAs) are small non-coding RNAs, which exert repressive effects on translation by targeting the 3′-untranslated region (UTR) via a 6–8-nucleotide seed region that is critical for coordinating
miRNA–mRNA complexes [19,20]. MiRNAs were also shown to control expressions of an estimated one-third of human protein-coding genes involved in fundamental cellular processes, including metabolism, differentiation, growth, and apoptosis [21–23]. Aberrant expression of miRNAs is closely associated with various cancers, including prostate cancer, breast cancer, gliomas, and lung cancer [24–26]. Therefore, understanding the molecular mechanisms by which these miRNAs play roles in deregulating cellular signaling in prostate cancer cells might help develop better therapeutic strategies for treating this disease. To date, however, the interaction between miRNAs and Pin1 in prostate cancer has not been explored.

In this study, we identified miR296-5p which specifically targets the 3′-UTR of Pin1 and consequently regulates Pin1 expression levels. Furthermore, miR-296-5p decreased the ability of cell proliferation and anchorage-independent growth in prostate cancer cell lines. Restoration of miR-296-5p silenced the expression of the Pin1 protein and depressed prostate cancer cell proliferation. These findings provide evidence that miR-296-5p is a suppressor of tumor formation by directly suppressing oncogenic Pin1 in normal prostate cells and suggest that miR-296-5p might provide a novel clinical application in prostate cancer therapy.

2. Materials and methods

2.1. Cell cultures

PC-3, LNCaP, and 22Rv1 cell lines (ATCC, Manassas, VA, USA) were cultured in RPMI-1640, supplemented with 10% fetal bovine serum (FBS) and antibiotics. The DU145 (ATCC) cell line was cultured in minimum essential Eagle’s medium supplemented with 10% FBS and 2 mM l-glutamine with antibiotics. The PZ-HPV-7, PWR-1E and RWPE-1 (ATCC) human non-tumorigenic prostate epithelial cell lines were cultured in keratinocyte serum-free medium supplemented with 5 mg/ml human recombinant epidermal growth factor and 30 mg/ml bovine pituitary extract (Invitrogen, Carlsbad, CA). Cultures were maintained in a 5% CO₂ humidified atmosphere at 37 °C.

2.2. Clinical specimens

Human prostate specimens were obtained from Kaohsiung Veterans General Hospital (Kaohsiung, Taiwan), National Cheng Kung University Hospital (Tainan, Taiwan) and Wan Fang Hospital, Taipei Medical University (Taipei, Taiwan). All patients gave informed consent, and the protocol was approved by the IRB Committee before a tissue sample was collected during their planned surgery. Carcinoma samples were obtained from palliative transurethral excisions of the prostate, and hematoyxlin and eosin-stained tumor tissue sections were examined by a pathologist. The benign prostate hyperplasia (BPH) samples were obtained from paraffin embedded prostate tissue. The signals were amplified and detected using the Roche LightCycler detection system (Roche) with an NBT/BCIP chromogen at 37 °C using the in situ hybridization kit (Biochain). Finally, sections were counterstained with Nuclear Fast Red. For IHC, the sections were treated with 3% H₂O₂/methanol and incubated with anti-Pin1 antibody (1:1000) for 4 °C overnight after washing with phosphate-buffered saline (PBS). The sections were then allowed to react with the horseradish peroxidase polymer-conjugated secondary antibodies, incubated with aminoethyl carbazole (AEC) chromogen, and then counterstained with hematoxylin.

2.3. Western blot analysis

Cell lines and human prostate cancer specimens were placed in lysis buffer at 4 °C for 1 h. The protein samples were electrophoresed using 12% SDS-polyacrylamide gel electrophoresis (PAGE) and performed as previously described [27].

2.4. Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from clinical tissues and cell lines using the TRIzol reagent (Invitrogen), and complementary DNA (cDNA) was synthesized from 1 μg of total RNA using an M-MLV reverse-transcriptase system kit (Invitrogen) according to the manufacturer’s protocol. After the PCR amplification, the results were analyzed by 1% agarose gel electrophoresis. The primer sequences were: Pin1-F, ATGGCGGACGAGGA GAACTGTCG and Pin1-R, TCACTCAGTGGGAGG-ATGATG; and GAPDH-F, TGTTATCGTGAAGACTCA and GAPDH-R, AG-TGGGTGTCGTTTT GAAG.

2.5. In situ hybridization (ISH) and immunohistochemistry (IHC)

The ISH assay was performed as described previously [28]. In brief, the human prostate specimens were fixed for 24 h in 4% paraformaldehyde. The expression of miR-296-5p was detected by using a digoxigenin (Dig)-conjugated miR-296-5p probe (Exiqon, Denmark) on paraffin embedded prostate tissue. The signals were amplified with an NBT/BCIP chromogen at 37 °C using the in situ hybridization kit (Biochain). Finally, sections were counterstained with Nuclear Fast Red. For IHC, the sections were treated with 3% H₂O₂/methanol and incubated with anti-Pin1 antibody (1:1000) for 4 °C overnight after washing with phosphate-buffered saline (PBS). The sections were then allowed to react with the horseradish peroxidase polymer-conjugated secondary antibodies, incubated with aminoethyl carbazole (AEC) chromogen, and then counterstained with hematoxylin.

2.6. Human protein atlas

In silico approach to analyze Pin1 protein levels in prostate tissues was performed by analyzing the web site of Human Protein Atlas (http://www.proteinatlas.org/). As indicated in the web site, the intensity (fraction of positive cells) was combined into a four-grade scale represented by the colors, white for negative, yellow for weak, orange for moderate, and red for strong level of protein expression, and the quantity for white color is less than 25%, the quantity for yellow or orange color is between 25% and 75%, and the quantity for red color is over 75%.

2.7. Reagents, antibodies, and expression constructs

MiRNA and anti-miR oligonucleotides were purchased from Applied Biosystem (Invitrogen). The Pin1 short hairpin RNA (shRNA) expression vector in the mammalian expression vector, pCIGZ, was provided by Dr. Michael Hsiao. The anti-Pin1, cyclin D1 and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.8. Construction of the 3′-UTR-luciferase plasmid and reporter assays

The Pin1 3′-UTR target site was amplified by PCR using the primers Pin1-WT-UTR-Rev (′-AAGCTTAGTGGTTCTGGGTTTAATTGGGGGTGAA GA-G’)- and Pin1-WT-UTR-Fwd (′-ACTAGTGGGTGGGGAGCCCAGG CCT-3′) cloned downstream of the luciferase gene in the pMIR-REPORT luciferase vector (Invitrogen). This vector was sequenced and named Pin1-WT-UTR. Site-directed mutagenesis of the miR-296-5p target-site in the Pin1 3′-UTR was carried out using a Quick-change mutagenesis kit (Stratagene, Heidelberg, Germany) and named Pin1-Mut1-UTR and Pin1-Mut2-UTR, in which Pin1-WT-UTR was used as a template. For the reporter assays, cells were transiently transfected with a wild-type (WT) or mutant reporter plasmid, miRNA oligonucleotides using Lipofectamine 2000 (Invitrogen). The reporter assay was performed at 48 h post-transfection using the Dual-Luciferase Reporter Assay system (Promega, Madison, WI, USA).

2.9. Quantitative RT-PCR assays for mature miRNA

The RT reactions of cell lines or human prostate cancer specimens were performed in a reaction containing 10 ng total RNA. Specific products were amplified and detected using the Roche LightCycler detection system with the cycle profile according to the TaqMan qRT-PCR miRNA Detection Kit (Invitrogen). The relative gene expression was calculated.
by comparing the cycle threshold (Ct) values for each target PCR. The target PCR Ct values were normalized by subtracting the internal control of the RNU6B snoRNA Ct value.

2.10. Cell-cycle analysis

22Rv1 cells were transfected with pre-miR-NC or pre-miR-296-5p molecules for 48 h and cell-cycle analysis was then performed. The distribution of cell-cycle phases was assayed by propidium iodide staining and flow cytometry (FACScan; BD Biosciences, San Jose, CA, USA).

2.11. Cell viability assay

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays as reported previously [29]. Cells were transiently transfected with pre-miR-NC or pre-miR-296-5p oligonucleotide using Lipofectamine 2000 prior to plate onto 96-well plates (10^4 cells/well).

2.12. Soft-agar colony formation assay

The effect of pre-miR-296-5p on anchorage-independent growth was assessed with a soft-agar colony formation assay. For the soft-agar assay, cells seeded on 35-mm dishes at 60–80% confluence were transfected using Lipofectamine 2000 with a concentration of 30 nM of the pre-miR-NC or pre-miR-296-5p oligonucleotide. Agarose (0.5%) containing RPMI/10% FBS medium was added to 6-well plates as the lower layer. Twenty-four hours later, cells were trypsinized, split into 0.35% agarose containing RPMI/10% FBS medium and plated at 5000 cells/well in 6-well dishes. After 10 to 14 days, colonies were stained with 0.1% crystal violet solution and counted.

2.13. Pin1-shRNA preparation and transient transfection

22Rv1 cell line was cultured in 100-mm-diameter antibiotic-free medium. The next day, cells were infected with Pin1-shRNA-carrying virus medium in culture plates (to 60–80% confluence) for 24 h twice, replaced with fresh culture medium and cultured in a 37 °C, 5% CO2 incubator. To select stably transfected cells, cells were transferred to medium containing 4 μg/ml puromycin for selection for 3 weeks, and the stable suppression of Pin1 was checked by Western blot analysis.

2.14. Statistical analysis

Statistical analyses were performed as recommended by an independent statistician. These included unpaired Student’s t-test (miRNA-quantitative RT-PCR, luciferase reporter assay, soft-agar assay, cell proliferation, and Western blot quantification) and paired Student’s t-test (Western blot quantification of NT pair tissues). Relationships between expression levels of Pin1 and prostate cancer were analyzed by a χ² test. The correlation of levels of miR-296-5p and Pin1 was determined using Spearman's test. All statistical analyses were performed using SPSS software (SPSS, Chicago, IL, USA), all values are expressed as the mean ± standard error (SE), and statistical significance was accepted for p < 0.05.

3. Results

3.1. Pin1 is upregulated in prostate cancer specimens

To determine whether Pin1 expression levels were upregulated in clinical prostate cancer, we first analyzed Pin1 expression in paraffin-embedded clinical prostate cancer specimens by IHC. Strikingly, Pin1 levels were significantly upregulated in tumor tissues compared with normal tissues (Fig. 1A and D). Furthermore, as shown in Fig. 1D, positive staining signals of Pin1 were mainly distributed in the cytoplasm and nuclei of cells. In addition, moderate Pin1-positive staining was observed in tissues with hyperplasia (HP) (Fig. 1B) and prostatic intraepithelial neoplasia (PIN) (Fig. 1C). To complement the Pin1 protein expression data of prostate cancer tissues, we utilized an in silico approach to analyze Pin1 protein levels in prostate tissues (Fig. 1E). Results showed a similar trend when looking at IHC staining patterns in normal and prostate cancer tissues via the Human Protein Atlas database [30,31], an antibody-based protein atlas containing histological images. Results of this data showed that there were 22 (95.6%) prostate cancer tissues with >75% positive staining with Pin1 (Fig. 1E) which indicates high expression of the Pin1 protein in prostate cancer tissues compared to benign prostate glands (p < 0.001, by the χ² test). Collectively, these results suggest that overexpression of Pin1 may be associated with prostate tumorigenesis.

3.2. Identification of miRNA candidates targeting the Pin1 3’-UTR of prostate cancer cells

To understand the molecular mechanism causing the upregulation of Pin1 and its possible role in oncogenesis of prostate cancer, we first analyzed the expression levels of Pin1 protein and miRNA in several cancer cell types (prostate cancer, lung cancer, esophagus cancer, and breast cancer) by Western blot and RT-PCR analyses, respectively. Compared to three nontumorigenic prostate epithelial cell lines (PZ-HPV-7, RWPE-1, and PWR-1E), the Pin1 protein was highly expressed in prostate cancer cell lines, including DU145, 22Rv1, PC-3, and LNCaP (Fig. 2A, upper panel). Interestingly, mRNA and protein levels of Pin1 did not show any similar trend between nontumorigenic prostate epithelial cell lines and prostate cancer cell lines (Fig. 2A, lower panel). However, this correlation between mRNA and Pin1 protein levels was not observed in cell lines of other cancer types, including lung, esophagus, and breast cancers (Supplementary Fig. 1A–C). Inconsistent changes between the mRNA and protein strongly imply that the upregulation of the Pin1 protein in prostate cancer cell lines may be regulated at the post-transcriptional level. A key player in post-transcriptional regulation is represented by miRNA that controls approximately 60% of all protein-coding genes to be predicted to contain miRNA-binding sites within their 3’-UTR [32]. To identify candidate miRNAs targeting the Pin1 3’-UTR, the PicTar [33], TargetScan [34], miRnada [35], microRNA.org [36] and microcosm [37] databases were used which identified ten candidate miRNAs with the potential to interact with the Pin1 3’-UTR which were predicted by at least two of the five bioinformatics databases (Supplementary Table 1). Among these ten candidates, the miR-200 family of miRNAs (miR-200b/200c/429) and miR-296-5p was predicted by at least three databases and found to be frequently dysregulated in prostate cancer [38–40]. To determine whether these candidate miRNAs were involved in regulating Pin1 expression and serve as putative tumor suppressors in prostate cancer, we first analyzed the structural symmetry of the predicted miRNA–mRNA binding complexes. An RNAhybrid software [41] analysis revealed that miR-296-5p contained two target sites of the 3’-UTR of Pin1 (Fig. 2B) with minimal free energy, ΔG = −32.5 kcal/mol for site 1 and ΔG = −30.4 kcal/mol for site 2, whereas miR-200c and miR-429 contained only one target site of the 3’-UTR which were predicted by at least two of the five bioinformatics databases (Supplementary Table 1). Among these ten candidates, the miR-200 family of miRNAs (miR-200b/200c/429) and miR-296-5p was predicted by at least three databases and found to be frequently dysregulated in prostate cancer [38–40]. To determine whether these candidate miRNAs were involved in regulating Pin1 expression and serve as putative tumor suppressors in prostate cancer, expression levels of miR-200c, miR-429, and miR-296-5p in three nontumorigenic prostate epithelial cell lines and four prostate cancer lines were detected by the miRNA-quantitative RT-PCR analysis. Among these three miRNAs, miR-296-5p was the only miRNA that exhibited high expressions in nontumorigenic prostate epithelial cell lines and was diminished in four prostate cancer cell lines (Fig. 2C, Supplementary Fig. 2A and B, lower panel). Across all seven cell lines tested, we found an inverse correlation between Pin1 protein levels
and miR-296-5p (Fig. 2A, upper panel, 2C, Spearman correlation coefficient \( r = -0.703 \)); however, there was no association between Pin1-mRNA and miR-296-5p (Fig. 2A, lower panel, 2C, Spearman correlation coefficient \( r = 0.847 \)). These experiments suggested that miR-296-5p might negatively regulate Pin1 at the post-transcriptional level.

3.3. MiR-296-5p was downregulated in prostate cancer tissues and showed an inverse correlation with Pin1 expression

To evaluate the correlation between Pin1 and miR-296-5p in prostate cancer tissues, we first analyzed the miR-296-5p expression levels from a publicly available dataset, deposited in the NCBI Gene Expression Omnibus (GEO) under accession no. GSE21032 [42]. As shown in Fig. 3A, we observed that the miR-296-5p expression was significantly downregulated in the clinical T2 stage of prostate cancer tissues compared to the normal group (Fig. 3A, middle panel) \( (p = 0.03) \). However, there was no statistically significant (NS) difference between the clinical T1c stage group and normal group (Fig. 3A, left panel) or the clinical T3 stage group and normal group (Fig. 3A, right panel). Individual expression values of miR-296-5p in different clinical stages of prostate cancer tissues are shown in Supplementary Fig. 3. These results reveal that prostate cancer tissues had low miR-296-5p expression, especially in the clinical T2 stage group. Moreover, the mRNA level of Pin1 was also analyzed in the same GEO dataset. As shown in Fig. 3B, we observed that Pin1 mRNA levels did not have the significant difference between normal group and different clinical stage groups. Next, we performed ISH and IHC staining on consecutive tissue sections to investigate whether there was an inverse correlation between the miR-296-5p and Pin1 protein in prostate cancer tissues. Fig. 3C shows the representative images of the miR-296-5p and Pin1 protein expressions in prostate cancer tissues and reveals low levels of the miR-296-5p in clinical specimens (Fig. 3C, upper panels I, II and III) and relatively high Pin1 protein expression (Fig. 3C, bottom panels IV, V and VI). Staining scores were obtained and defined from the immunoreactivity of IHC and ISH (Supplementary Fig. 4) and indicated a highly inverse correlation between miR-296-5p and Pin1 expressions in prostate tumor tissues.

Fig. 1. Expression of Pin1 in human prostate cancer tissues. (A) Normal prostate tissue negatively stained for Pin1 expression. (B and C) Moderate staining of Pin1 in HP and PIN tissues. (D) Prostate tumor tissue with overexpression of Pin1 protein showing strong staining of the majority of cells (brown) in the tumor areas. (E) Pin1 protein expression levels obtained from the Human Protein Atlas database. The percentage of cases is indicated on the y-axis, whereas the type of sample is shown on the x-axis.
(Fig. 3D, Spearman correlation coefficient \( r = -0.638, p = 0.0002, n = 27 \). To further confirm the lower miR-296-5p level in prostate cancer cells with higher Pin1 expression, the five NT paired tissues were used for miR-296-5p and Pin1 expression examination. The tumors had lower levels of miR-296-5p than their adjacent nontumor tissues (Fig. 3E, bottom panel, \( p < 0.01 \)) and had higher levels of Pin1 protein than the nontumor tissues (Fig. 3E, upper panel, \( p < 0.05 \)). Collectively, these results indicated that high miR-296-5p levels in normal prostate cells played a tumor-suppressor role by negatively regulating Pin1 expression and downregulation of miR-296-5p may be involved in prostate cancer tumorigenesis.

### 3.4. MiRNA-296-5p directly targets the 3′-UTR of Pin1

To further verify the targeting of Pin1 by miR-296-5p, the WT 3′-UTR of Pin1 was cloned downstream of the luciferase open reading frame (referred to as Pin1-WT-UTR) (Fig. 4A). In a parallel experiment, the conserved miR-296-5p targeting sequence, GGGGCCC, of sites 1 and 2 within the 3′-UTR of Pin1 was individually mutated to GGUCAGA (referred to as Pin1-Mut1-UTR and Pin1-Mut2-UTR, respectively) (Fig. 4A). Negative control miRNA (pre-miR-NC) or the precursor of miR-296-5p (pre-miR-296-5p) was cotransfected with the Pin1-WT-UTR luciferase construct. As shown in Fig. 4B, transfection with the pre-miR-296-5p led to a significant decrease in luciferase activity compared to the pre-miR-NC (Fig. 4B, a 50% decrease compared to lanes 2 and 3, \( p < 0.05 \)). In contrast, co-transfection of anti-miRNA of miR-296-5p (anti-miR-296-5p) with the Pin1-WT-UTR plasmid in HEK-293T cells significantly increased luciferase activity compared to co-transfection of the negative control anti-miRNA (anti-miR-NC) (Fig. 4B, lanes 4 and 5, \( p < 0.01 \)). The same result of luciferase assay on 22Rv1 cell line was also observed (Fig. 4D). Next, to identify which miR-296-5p-binding site of the Pin1 3′-UTR is critical for miR-296-5p binding, the Pin1-Mut1-UTR and Pin1-Mut2-UTR were used in the luciferase reporter assay. As shown in Fig. 4C and E, the luciferase activity of...
Fig. 3. Expression level of Pin1 was inversely correlated with miR-296-5p in prostate cancer tissues. (A) Relative expression levels of miR-296-5p and mRNA levels of Pin1 in different clinical stages of prostate cancer tissues analyzed using the public GEO database. (B) Photomicrographs illustrating serial sections to compare Pin1 (immunoreactivity) and miR-296-5p (in situ hybridization) expressions in paraffin sections of human prostate cancer tissues. The nucleus is stained with hematoxylin (blue) or Nuclear Fast Red (red). The cytoplasm is stained with eosin (purple). Pin1 was detected using a Pin1 monoclonal antibody (brown). MiR-296-5p was detected using a miR-296-5p probe (blue). Black bars are the actual size of magnification. (D) Expression levels indicating an inverse correlation between Pin1 and miR-296-5p expressions in prostate tumor tissues (r = −0.638, p < 0.001, n = 27). NS: No statistically significant. (E) The level of Pin1 protein expression was inversely correlated with miR-296-5p expression in the representation of five NT pair prostate cancer specimens. *p < 0.05, **p < 0.01, ***p < 0.001.
the reporter in HEK-293T and 22Rv1 cell lines that carried Pin1-Mut1-UTR reporter construct was abolished by miR-296-5p that mediated the Pin1 3′-UTR luciferase activity that was suppressed by the simultaneous transfection of pre-miR-296-5p (Fig. 4C and E, lanes 2 and 3). However, the inhibitory effect of pre-miR-296-5p on luciferase activity remained in Pin1-Mut2-UTR-transfected HEK-293T and 22Rv1 cell lines (Fig. 4C and E, lanes 5 and 6). These results indicated that site 1 of the Pin1 3′-UTR was the major target for miR-296-5p to suppress Pin1 protein expression. Taken together, these data suggest that miR-296-5p binds to the 3′-UTR of Pin1 and regulates Pin1 protein expression.
Fig. 5. MiR-296-5p inhibits growth of 22Rv1 prostate cancer cells in vitro. (A) Validation of the miR-296-5p expression level after transfection with the miR-296-5p precursor for 48 and 72 h in the 22Rv1 cell line. (B) Pin1 protein level after overexpression of miR-296-5p in the 22Rv1 cell line. After transfection, the total protein was extracted and used for Western blot analysis. Quantification of Pin1 expression by the ratio of Pin1/β-actin. (C) The proliferative ability of 22Rv1 cells after miR-296-5p transfection was significantly reduced compared to the control anti-miR or Mock. (D) Soft agar colony formation assays were performed 24 h after transfection (upper panel). Quantification of the soft-agar assay in transient transfected 22Rv1 cells (lower panel). (E) After 48 h of transfection, endogenous Pin1 was suppressed by ectopic miR-296-5p transfection by Western blot analysis. (F) After transfection, cell growth was measured in the indicated time points by MTT assay. Anti-growth effect of ectopic miR-296-5p was attenuated by co-transfection of Pin1-expressing plasmid. (G) Suppressed cell proliferation rate by miR-296-5p was rescued by ectopic Pin1 expression by soft agar colony formation assays. *p < 0.05, **p < 0.01.
3.5. Growth inhibitory effect of miR-296-5p on prostate cancer cells by directly inhibiting Pin1

We then investigated the inhibitory effects of overexpression of miR-296-5p on the development of prostate cancer. First, to determine whether the overexpression of miR-296-5p in cancer cells with a low miR-296-5p level can decrease the Pin1 protein level, the 22Rv1 prostate cancer line was transiently transfected with pre-miR-296-5p or pre-miR-NC. MiR-quantitative RT-PCR and Western blot experiments were performed to detect the mature miR-296-5p level and Pin1 protein level. Compared to pre-miR-NC transfection, the results showed that pre-miR-296-5p transfection resulted in about a 10-fold increase in mature miR-296-5p in the 22Rv1 cell line examined at a time course of 48 and 72 h (Fig. 5A). As expected, there was a significant reduction in the Pin1 protein amount in pre-miR-296-5p-transfected 22Rv1 cells (Fig. 5B). Quantification of Pin1 protein expression showed that it was reduced by about 50% at 48 h and by 60% at 72 h after transfection (Fig. 5B). Similar results were also observed in another prostate cancer cell line, PC-3 (Supplementary Fig. 5A and B). These data suggest that miR-296-5p specifically downregulates Pin1 at the post-transcriptional level. It was demonstrated that Pin1 plays a pivotal role in cellular proliferation of prostate cancer [43]. We next examined the effect of miR-296-5p on the cell proliferation ability with the MTT proliferation assay. 22Rv1 and PC-3 cells were plated onto a 96-well culture plate (10^3 cells/well), and the proliferation rate was measured at the indicated times (24, 48, 72, and 96 h). Transient transfection of pre-miR-296-5p resulted in a significant decrease in miR-296-5p-transfected 22Rv1 or PC-3 cells growth compared to the groups of mock and pre-miR-NC-transfected 22Rv1 or PC-3 cell lines, respectively (Fig. 5C, Supplementary Fig. SC). To investigate the effect of miR-296-5p-mediated suppression of Pin1 on the tumorigenic phenotypes of prostate cancer cells, we examined the effect of miR-296-5p-mediated suppression of Pin1 on the anchorage-independent colony formation. Strikingly, we found that enforced expression of miR-296-5p in 22Rv1 (Fig. 5D) or PC-3 (Supplementary Fig. 5D) prostate cancer cell lines drastically inhibited their anchorage-independent growth ability, as shown by decreased colony numbers and sizes. To make a sufficient functional link between miR-296-5p and Pin1, we performed the rescue experiment by introducing Pin1-expressing plasmid (pEGFP-Pin1) or empty vector in the presence or absence of ectopic pre-miR-296-5p expression in prostate cancer cells. Ectopic pre-miR-296-5p expression attenuated endogenous Pin1 protein expression, thereby led to the suppression of cellular growth and proliferation in both MTT and anchorage-independent colony formation assay (Fig. 5E–G for 22Rv1 cells, Supplementary Fig. SE–G for PC-3 cells). Importantly, this anti-growth effect of ectopic pre-miR-296-5p was attenuated by co-transfection of Pin1-expressing plasmid. Note that miR-296-5p suppresses endogenous Pin1 protein expression in both 22Rv1 and PC-3 cell lines (Fig. 5E, lanes 2 and 4, Supplementary Fig. 5E, lanes 2 and 4). Moreover, anti-growth effect of miR-296-5p was significantly attenuated by ectopic GFP-Pin1 expression (Fig. 5F and G of 22Rv1 cells, Supplementary Fig. 5F and G of PC-3 cells). This result clarifies that miR-296-5p directly targets and suppresses Pin1, and that overexpression of Pin1 can abolish pre-miR-296-5p-mediated growth suppression. In addition, when we assessed the effect of miR-296-5p on cell cycle distribution, we observed that miR-296-5p induced a significant change in G2/M arrest to compare to pre-miR-NC (Supplementary Table 2 and Supplementary Fig. 6A, p < 0.05). The levels of cyclin D1 in the G2/M phase of cell cycle are crucial for the cells to decide if they have to undergo another round of replication [44]. Moreover, Pin1 has been demonstrated to positively regulate cyclin D1 by transcriptional activation and posttranslational stabilization [3]. We then examined the expression of cyclin D1 in miR-296-5p-expressing cells by Western blot analysis. As we expected, ectopic expression of pre-miR-296-5p suppressed expression of Pin1 and cyclin D1 proteins (Supplementary Fig. 6B). Taken together, this result indicated that miR-296-5p induced anti-growth effect through Pin1-mediated regulation of cyclin D1 expression in prostate cancer cells. To confirm the function of miR-296-5p in nontumorigenic prostate epithelial cells, anti-miR-296-5p was used to investigate whether downregulation of endogenous miR-296-5p expression in nontumorigenic prostate epithelial cells could increase the Pin1 protein level and promote cell growth. The anti-miR-296-5p oligonucleotides was transiently transfected into PZ-HPV-7 cell line. The expression of miR-296-5p was significantly downregulated in anti-miR-296-5p-transfected PZ-HPV-7 cells (Supplementary Fig. 7A, with about 70% and 90% reductions at 48 and 72 h, respectively) compared to anti-miR-NC. Expression levels of the Pin1 protein increased by about 50% at 48 h after transfection (Supplementary Fig. 7B), and statistical analysis of Pin1 protein quantification showed that there was a significant difference between anti-miR-NC-transfected and anti-miR-296-5p-transfected cells in three independent experiments (Supplementary Fig. 7C, p < 0.05). In addition, cell viability results showed that there was a 30% increase in anti-miR-296-5p-transfected PZ-HPV-7 cells compared to the mock and anti-miR-NC-transfected groups at 72 and 96 h after transfection (Supplementary Fig. 7D, p < 0.01). These results suggest that upregulation of miR-296-5p can downregulate the Pin1 protein level and inhibit prostate cancer cell proliferation and prostate cancer cell tumorigenicity in vitro.

3.6. Effect of Pin1 knockdown on prostate cancer cells

A previous study [43] and our data showed that overexpression of Pin1 contributes to the promotion of cell proliferation. In order to verify the Pin1-regulated cell proliferation of prostate cancer cells, the 22Rv1 prostate cancer cell line was infected with lentiviral particles carrying shRNA of Pin1. As shown in Fig. 6A, Pin1 protein expression levels were significantly reduced in cells expressing Pin1 shRNA-7-22Rv1 and shRNA-12-22Rv1 (about 78% and 41% reductions, respectively). In our study, we showed that the Pin1 protein level was modulated by miR-296-5p in prostate cancer cells (Fig. 5B). To investigate whether the phenotype of Pin1-specific knockdown is similar to miR-296-5p’s function, we performed the MTT proliferation and soft agar colony formation assays of the 22Rv1 cell line. The proliferation index of Pin1 stably knocked-down 22Rv1 cells significantly decreased in both shRNA-7 and shRNA-12 groups compared to the parental group (Fig. 6B, p < 0.01). Moreover, anchorage-independent growth of 22Rv1 cells in both shRNA-7 and shRNA-12 stably knocked-down Pin1 was also significantly reduced (Fig. 6C, p < 0.001). The same results were also observed in PC-3 cells that were transiently transfected with Pin1 shRNA (Supplementary Fig. 8). These data indicate that the effect of Pin1-specific knockdown concurs with the effect of miR-296-5p, and Pin1 is a critical regulator of prostate cancer oncogenesis.

4. Discussion

It was reported that Pin1 is strikingly overexpressed in a subset of human tumors, and high Pin1 expression is correlated with cancer development and a poor prognosis in patients with prostate cancer [12, 45]. However, the mechanism of Pin1 overexpression in prostate cancer is still unclear. The present study established for the first time the important role played by miR-296-5p in inhibiting expression of the Pin1 oncprotein in prostate cancer. There are several lines of evidence that support this conclusion which are listed below. First, Pin1 was significantly upregulated in prostate tumor tissues compared to normal tissues from our IHC results and the Human Protein Atlas database. Second, significantly decreased expression of miR-296-5p in prostate cancer tissues at clinical T2 stage compared to the normal group was determined from the Taylor dataset [42]. Third, expressions of miR-296-5p and Pin1 were found to be significantly inversely correlated in resected prostate cancer tissues. Fourth, overexpression of miR-296-5p in 22Rv1 prostate cancer cells decreased Pin1 expression, and the cell proliferative ability and tumorigenic activity. Fifth, targeting miR-296-5p by
anti-miR-296-5p oligonucleotides in nontumorigenic prostate epithelial cells dramatically augmented the cell growth ability. Collectively, this study is the first to report that Pin1 is negatively regulated by miR-296-5p and also showed that miR-296-5p is a significant suppressor of prostate cancer tumorigenesis, as evidenced by studies using cell lines, clinical samples, and GEO prostate cancer dataset analyses.

Typically, miRNAs exert their function by affecting multiple genes. Thus, one miRNA may have different functions when interacting with different targets [46]. Among these miRNAs, miR-296-5p was recently found to be progressively lost during tumor progression and was correlated with metastatic disease in colorectal, breast, lung, parathyroid, liver, and bile duct cancers [47–49]. Furthermore, miR-296-5p was found to be located on chromosome 20q13.32, and it was reported that the 20q13.32–13.33 chromosome region is deleted in 20% of prostate cancer patients [50]. In a recent study, it was demonstrated that miR-296-5p modulates tumor invasiveness by modulating HMGA1 expression in prostate cancer cells [40]. Overexpression of HMGA1 in prostate cancer cell lines was found to be associated with highly aggressive growth and a relatively high degree of metastatic potential [51]. MiR-296-5p has an especially negative impact on HMGA1 translation and also enhances HMGA1 degradation. As a result, tumor progression is markedly attenuated [40]. Importantly, these observations concur with our analysis of the Taylor dataset which showed significantly decreased expression of miR-296-5p in clinical T2 stage but not in T1 stage prostate cancer tissues compared to the normal group. Moreover, there is no statistical significance of miR-296-5p expression in clinical T3 stage of prostate cancer tissues compared to the normal group due to small sample sizes. In addition, overexpression of miR-296-5p in prostate cancer cells was found to significantly suppress cell proliferation and anchorage-independent growth through inhibition of Pin1 protein expression. Collectively, these results showed that miR-296-5p acts as a suppressor of malignant transformation and progression by attenuating its target transcription in different types of cancer.

The prostate-specific antigen (PSA) is commonly used for population screening, diagnosis, and monitoring of patients with prostate cancer [52]. Recent research suggests that the rate of increase of PSA is not a more-specific marker for prostate cancer [53] and is not a good biomarker for distinguishing BPH from prostate cancer. Hence, a new diagnostic marker to replace or complement PSA needs to be identified. Pin1 expression is associated with clinicopathologic features of different types of tumors [17,54,55], and these results indicate that Pin1 may have a role in tumor development and metastasis.

Fig. 6. Stable knockdown of Pin1 suppresses cell proliferation and anchorage-independent growth of prostate cancer cells. (A) Expression of Pin1 was detected in Pin1 stably knocked-down 22Rv1 cells by Western blot. Quantification of Pin1 expression by the ratio of Pin1/ß-actin. (B) MTT assay of Pin1 stably knocked-down 22Rv1 cells. (C) Soft-agar assay of Pin1 stably knocked-down 22Rv1 cells (upper panel). Quantification of soft-agar assay in Pin1 stably knocked-down 22Rv1 cells (lower panel). *p < 0.05, **p < 0.01, ***p < 0.001.
Ayala et al. [14] reported that patients with high Pin1 expression had worse survival than those without Pin1 expression, and they concluded that Pin1 is an excellent prognostic marker for prostate cancer. Our clinical studies indicated that there was an inverse correlation between the expressions of miR-296-5p and Pin1 in prostate cancer clinical specimens. MiR-296-5p was lower in resected tissues of prostate cancer patients and is a negative regulator of Pin1. Thus, a combination of the expression levels of miR-296-5p and Pin1 may provide potential clinical applications in the diagnosis and prognosis of prostate cancer.

In conclusion, we demonstrated that the loss of miR-296-5p and concomitant elevation of Pin1 are most pronounced in prostate cancer. We also demonstrated that Pin1 is negatively regulated by miR-296-5p at the post-transcriptional level through a specific target site within the 3′-UTR. Thus, we postulated that the loss of miR-296-5p may represent a progressive molecular lesion in the development of more-aggressive disease. This study suggests that targeting the miR-296-5p/Pin1 interaction or perturbing miR-296-5p expression may open a new therapeutic avenue for the development of anticancer therapies for prostate cancer patients.

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Supplementary data

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References


