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Original Article

RNA-seq reveals microRNA-302b as a suppressor of prostate cancer epithelial-mesenchymal transition by targeting RELA/NF-κB

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Abstract: To identify novel biomarker(s) in prostate cancer and demonstrate the mechanistic involvements in this disease, RNA-seq was employed to reveal the differentially expressed genes in the blood samples from prostate cancer patients. Relative expression of miR-302b-3p was evaluated using real-time PCR. The potential regulation of RELA by miR-302b-3p was assessed by luciferase reporter assay. Protein levels of NF-κB, Vimentin, N-cadherin and E-cadherin, were quantified using western blotting. Transwell chamber was employed to measure cell migratory and invasive capacity, while cell attachment/detachment assay was performed to evaluated epithelial-mesenchymal transition (EMT)-related behavior. Xenograft tumor model was adopted to determine the anti-tumor activity of miR-302b-3p in vivo. We demonstrated miR-302b-3p was down-regulated in prostate cancer both in vivo and in vitro. We predicted and identified RELA as directly targeted by miR-302b-3p. Ectopic miR-302b-3p expression in PC-3 cells significantly suppressed cell migration, invasion, attachment, detachment capacity, which was accompanied with a decrease in the expression of N-cadherin and Vimentin, and an increase of E-cadherin expression. MiR-302b-3p-proficiency greatly delayed xenograft tumor growth and associated with favorable overall survival. Co-introduction of RELA completely abolished anti-tumor effects of miR-302b-3p, which indicated a potential genetic interaction between RELA/NF-κB and miR-302b-3p. We characterized the aberrant down-regulation of miR-302b-3p in prostate cancer and unraveled a possible involvement of miR-302b-3p/RELA signaling axis in this scenario.

Keywords: miR-302b-3p, RELA, prostate cancer, epithelial-mesenchymal transition

Introduction

Prostate cancer is the 2nd most common cancer type and positions as the firth leading cause of cancer-related fatalities in men [1]. In 2012, there were an estimated 1.1 million new diagnosis and 305,000 lives claimed by prostate cancer [2]. Epidemiological study revealed the frequent occurrence of prostate cancer in the developed countries while growing tendency in the developing world due to advances in the screening and diagnosis technologies [3]. Recognized risk factors for prostate cancer include hereditary genetic abnormality, race, and older age. The marginal factors involved include diet habit, gonorrhea and especially, BRCA mutations [4]. Diagnosis is usually performed by biopsy and evaluation of metastasis is fulfilled by further medical imaging [5]. Histologically, most prostate cancers are classified as adenocarcinomas or glandular cancer. The therapeutic decision varies depending on the Gleason score and the prostate specific antigen (PSA) level, stage of the disease, and consideration of the balancing the goals of therapies with risk of side effects. The patients at early stage are applicable for regular surveillance, while aggressive cancers may engage surgery, chemotherapy, radiation therapy, hormonal therapy, cryosurgery, high-intensity focused ultrasound, or in combination [6]. Prognosis of prostate cancer is relatively favorable with a 5-year survival rate of 99% in the US. Nonetheless, the in-depth knowledge regarding the molecular basis for this disease is still obligate for clinical success. More importantly, the reliable diagnostic and prognostic biomarkers for specific and sensitive discrimination pros-

tate tumor from benign hyperplasia are still missing currently [7]. To this end, RNA sequencing (RNA-seq) features in *de novo* identification of RNA molecules is widely adopted in number of prospective searching studies [8, 9]. Screening of differentially expressed genes encoding proteins, lncRNAs or microRNAs may generate specific signatures associated with pathological traits. Here, we employed RNA-seq technology to analyze the expression profiles in the blood samples from both prostate cancer patients and health donor and identify the potential miRs with either tumor suppressor or oncogenic properties.

miRs are defined as the short oligonucleotides with master post-transcriptional regulatory functions [10]. By estimation, 30% protein-coding genes are fine-tuned by miRs and with phenotypical diversity. The single miR can simultaneously modulate hundreds of gene expression, vice versa, the single gene can be cooperatively regulated by multiple miRs. The specificity of target gene of miRs is usually determined by the seed region of mature sequences, association of which down-regulated gene expression by diverse mechanisms including mRNA degradation, translation suppression and de-adenylation. Notably, accumulative evidence suggests that miRs are involved in tumor biology of a variety of human malignancies [11].

MiR-302b-3p has been unraveled involving in several human cancers. For example, Xie et al. found that miR-302b suppressed invasion and migration of osteosarcoma cells via Runx2 [12]. Wang et al. reported that in human liver carcinoma SMMC-7721 cells, miR-302b repressed cell propagation through inhibiting EGFR [13]. Liu et al. showed that miR-302b targeted CDK2 through ERK signaling pathway and modulated cell cycles in gastric cancer [14]. Cai et al. suggested that miR-302b improved the response of hepatocellular carcinoma cells to 5-FU treatment through Mcl-1 and DPYD [15]. In respect to prostate cancer, three studies so far focused on miR-302b with contradictory conclusions. Here we identified miR-302b-3p as differentially expressed gene in prostate cancer patients and sought to elucidate its roles in prostate cancer.

Materials and methods

Patient blood tissues

Blood samples were collected from 10 patients with prostate cancer and 10 health individual donors in Longhua Hospital Shanghai University of Traditional Chinese Medicine. Written informed consents were provided by all enrolled subjects. The human related study was approved by the Ethics Committee of Longhua Hospital Shanghai University of Traditional Chinese Medicine and in strict compliance with the reviewed protocol.

RNA-seq

For differential expression analysis, total RNA was prepared from samples with Trizol reagent (Invitrogen, CA, USA), and from whole blood using the RiboPure Blood RNA Purification Kit (AM1928, Invitrogen, CA, USA) following the provided protocol. RNA samples were subjected to quality check prior to RNA-seq on Illumina HiSeq platform (Illumina, CA, USA). The resultant reads were annotated according to miRBase microRNA reference (R21). Read count was calculated as relative expression value and differential expression genes were identified. The fold change >2 between health donors and cancer patients was considered significant with FDR-p <0.05.

Real-time PCR

RNA samples prepared from either blood samples or indicated cell samples were reversely transcribed into cDNA using miScript II RT kit (Qiagen, Hilden, DE). Real-time PCR was conducted with CFX96 Touch System (Bio-Rad, CA, USA) using miScriptR SYBR Green MasterMix (Qiagen, Hilden, DE). U6 was employed as endogenous reference and the $2^{-\Delta\Delta Ct}$ method was employed to calculate relative expression.

Cell culture

PC-3 and DU145 cell lines, which are human prostate cancer cell lines, as well as RWPE-2 and WPE1-NB14, both normal prostate epithelial cell lines, were obtained from the American Type Culture Collection (ATCC, MD, USA). The cell identities and mycoplasma-free states were authenticated by Abace (Beijing, China). All cells were cultured in RPMI modified medium containing 1% streptomycin+penicillin (Gibco, MA, USA) plus 10% FBS (Gibco, MA, USA) in humidified CO_2 incubator (5%).

Luciferase reporter assay

The sequence of miR-302b-3p precursor was constructed and sub-cloned into the pMXs-miR

plasmid. For luciferase reporter assay, 3'UTR sequence of RELA was amplified and fused into pGL4 vector. Mutagenesis PCR was adopted for generating mutation in the putative recognizing sites. miR-302b-3p was co-transfected with RELA-luciferase reporter plasmids into PC-3 cell with Lipofectamine 2000 (Invitrogen, MA, USA). Luciferase activities were assessed 48 h later with Dual-Luciferase Reporter Kit (Promega, WI, USA).

Western blotting

The indicated cells were collected and subjected to lysis in RIPA buffer (30 min on ice) with allcell debris removed. Proteins (20 μg per lane) were run on a 10% SDS-polyacrylamide gel and then transferred onto a PVDF membrane. Immunoblotting was performed with the following primary antibodies: anti-NF-κB, #3034, 1:1000; anti-Vimentin, #5741, 1:1000; anti-N-cadherin, #13116, 1:1000; anti-E-cadherin, #3195, 1:1000; anti-GAPDH, #2118, 1:1000; Cell Signaling Technology, MA, USA). Blots were visualized after incubation with the recommended secondary antibody (anti-rabbit, #7074, 1:5000; Cell Signaling Technology, MA, USA) using enhanced chemiluminescence (ECL, Millipore, CA, USA). Scanned images were processed and analyzed using ImageJ software.

Migration and invasion

The invasion and migration were evaluated using Transwell chamber with or without precoated Matrigel (BD Biosciences, MD, USA), respectively. Briefly, the indicated single-cell suspension was prepared in serum-free medium. 1×10^5 cells were loaded into the upper compartment, and lower chamber was supplied with the complete culture medium. After 12 h of culturing, the invaded cells were treated using 4% PFA to be fixed and then subjected to crystal violet staining.

Cell attachment and detachment assay

For attachment, the indicated cells were loaded into 24-well plate at concentration of 5×10^4 cells per well. One hour later, the unattached cells were washed off and the attached ones were counted after trypsinization. For detachment assay, after being cultured for 24 h, the cells were partially digested and detached with 0.05% trypsin for 4 min, which was terminated by the addition of complete culture medium. After removal the detached cells, the remaining cells were harvested and counted.

Xenograft tumor model

PC-3 cells were subcutaneously inoculated into the lower flanks of nude mice (3~4-week old, Vital River, Beijing, China). The xenograft tumor progression was regularly monitored using digital caliper and tumor volumes were estimated as: Volume = $(\text{width})^2 \times \text{length}/2$. Mice were sacrificed at post-injection day 15 and tumor was weighed. All animal study obtained the approval from the Ethical Committee of Longhua Hospital Shanghai University of Traditional Chinese Medicine and in strict compliance with the Animal Use and Care Guideline of NIH.

Statistical analysis

The Prism GraphPad software was employed for processing and analyzing the data. Multiple comparisons were conducted with one-way ANOVA followed by Tukey's test. The *P* value was generated and P less than 0.05 was regarded as significant.

Results

MiR-302b-3p is downregulated in prostate cancer

Here we set out to apply RNA-seq for the patient blood samples to discover the novel microRNAs with differential expression and association with prostate cancer. Hence, we collected serum samples from 10 prostate cancer patients and 10 health donors. As shown in **Figure 1A**, our RNA-seq data unambiguously identified that miR-302b-3p was markedly and consistently reduced in cancer group in comparison with control across three independent repeats, which suggested the potential tumor suppressing function of miR-302b-3p in prostate cancer. The sequencing data was validated by quantitative rt-PCR as well (**Figure 1B**). The miR-302b-3p downregulation in prostate cancer cell lines was further examined and marked down-regulation was observed in both PC-3 and DU145 when compared with the normal human prostate cell lines PWPE-2 and WPE1-NB14 (**Figure 1C**). Therefore, our results demonstrated the downregulated miR-302b-3p in prostate cancer both in vivo and in vitro.

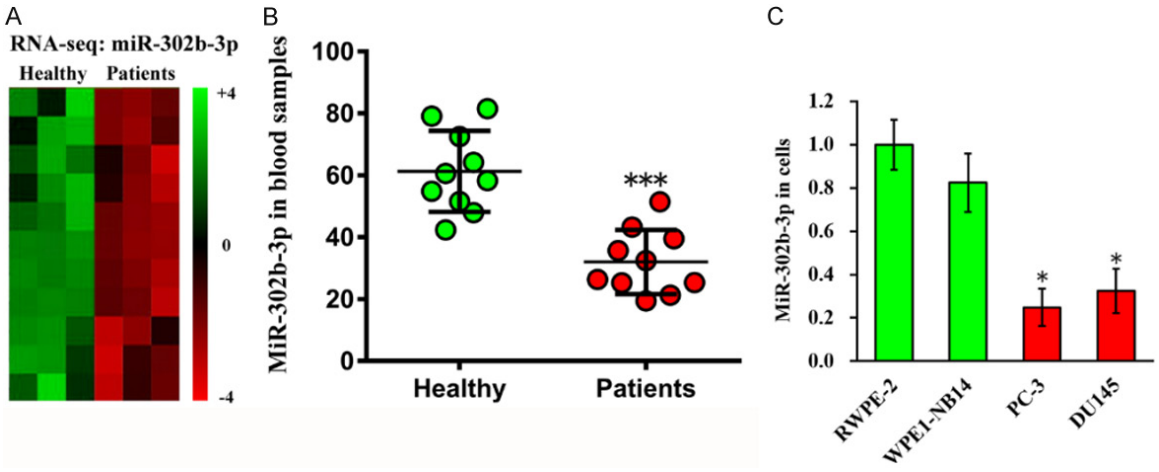

Figure 1. MiR-302b-3p is downregulated in prostate cancer patients' blood samples and prostate cancer cell lines. A. RNA-seq was performed in prostate cancer patients and healthy controls (n=10 each group, 3 independent repeats), where miR-302b-3p was consistently downregulated in prostate cancer patients. B. Mature microRNA assay for miR-302b-3p was conducted in the same prostate cancer patients and healthy control blood samples. $^{***}P<0.001$, compared to normal. C. Mature microRNA assay for miR-302b-3p was conducted in normal human prostate cell lines RWPE-2, WPE1-NB14, and human prostate cancer cell lines PC-3, DU145. Values presented as mean ± SD. $^{*}P<0.05$, compared to both RWPE-2 and WPE1-NB14.

MiR-302b-3p directly targets the 3'UTR of RELA mRNA and inhibits RELA mRNA/NF-κB protein expression

Next, we aimed to identify the molecular events downstream aberrant suppressed miR-302b-3p in prostate cancer. Aided by a well-recognized bioinformatic online tool, we predicted RELA as the potential candidate gene to be targeted by miR-302b-3p. The alignment between 3'UTR region of RELA and miR-302b-3p was illustrated in **Figure 2A**. To experimentally interrogate the regulatory role of miR-302b-3p in RELA expression, here we generated luciferase reporter gene with fused 3'UTR of RELA transcript. The putative recognizing site was disrupted via mutagenesis PCR in our mutation construct in comparison with wild type one (**Figure 2B**). Co-transfection with miR-302b-3p significantly inhibited the relative activities of luciferase, which was abolished by the introduction of mutation in the seed region (**Figure 2C**). Furthermore, we determined the endogenous levels of RELA mRNA and NK-κB protein in response to miR-302b-3p introduction in PC-3 cells. Dramatic decreases of both RELA mRNA and NK-κB were detected in the miR-302b-3p-proficient cells (**Figure 2D** and **2E**). Taken together, we identified RELA as directly targeted by miR-302b-3p in prostate cancer.

MiR-302b-3p inhibits migration, invasion, proliferation and suppresses EMT phenotypes of prostate cancer cells in vitro

Our foregoing results suggested potential anti-tumor properties of miR-302b-3p in prostate cancer, which prompted us to investigate this probability in vitro. To address this, we employed a stable cell line in PC-3 overexpressing miR-302b-3p. High levels of miR-302b-3p significantly suppressed cell migrative and invasive capacity in our system (**Figure 3A**, **3B**). Of note, cell proliferation was also inhibited by miR-302b-3p (Figure S1A and S1B). The EMT phenotype was evaluated by cell attachment and detachment as well. As demonstrated in **Figure 3C** and **3D**, miR-302b-3p over-expression greatly inhibited cell attachment and detachment in PC-3 cells. Furthermore, molecular markers related to the EMT, including Vimentin, E-cadherin, and N-cadherin, were evaluated. Consistent with compromised migrative, invasive, attachment and detachment capacities, we observed remarkable down-regulation of both N-cadherin and Vimentin, and up-regulation of E-cadherin upon miR-302b-3p over-expression in PC-3 cells (**Figure 3E**). Therefore, we presented evidence supporting the suppressive effect of miR-302b-3p on cell migration, invasion and EMT-related behaviors.

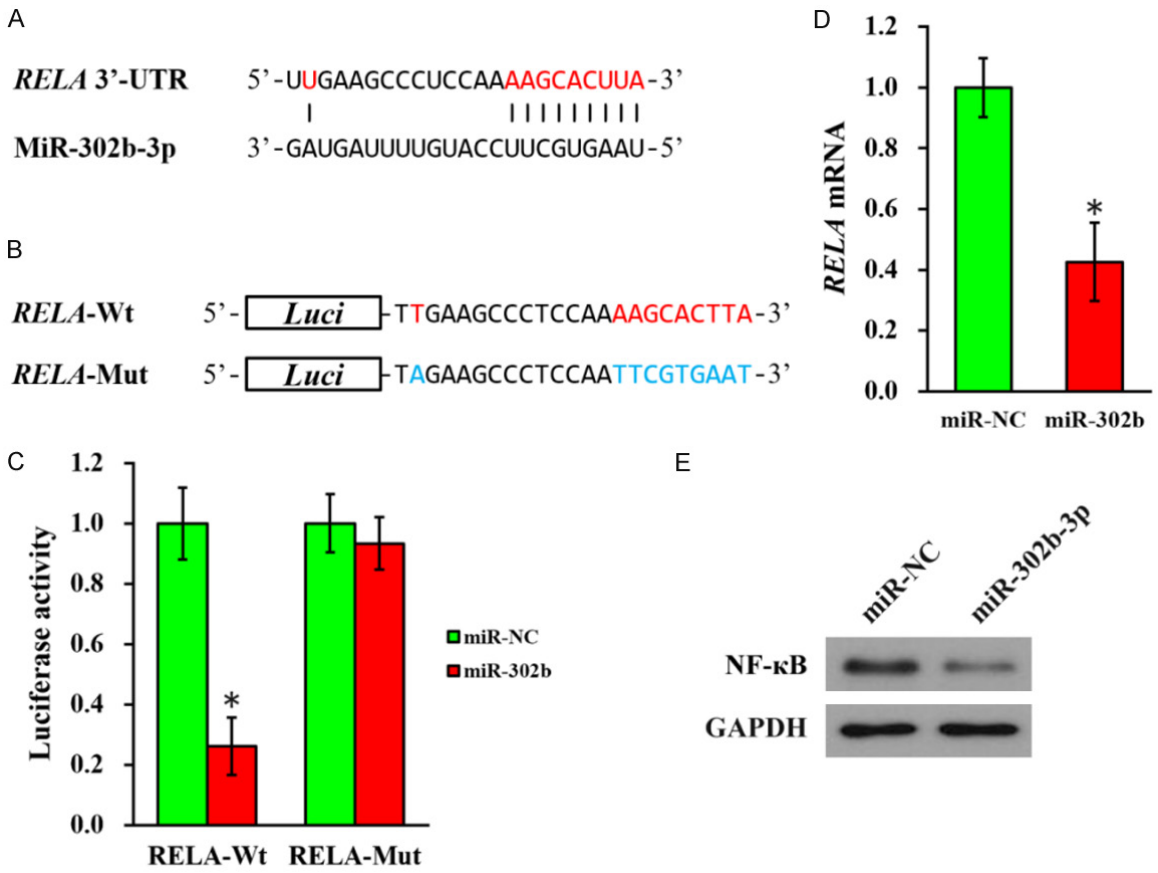

Figure 2. MiR-302b-3p directly targets the 3'UTR of *RELA* mRNA and inhibits *RELA* mRNA/NF-κB protein expression. (A) Sequence of the putative miR-302b-3p targeting site (red nucleotides) on the 3'UTR of *RELA* mRNA. (B) Putative targeting sequences from the 3'UTR of *RELA* mRNA, wild type (*RELA*-Wt, red nucleotides) or mutated (*RELA*-Mut, blue nucleotides), were constructed to the downstream of a luciferase reporter open reading frame (Luci). (C) Luciferase activity levels of *RELA*-Wt and *RELA*-Mut constructs were assessed in PC-3 cells co-transfected with either miR-302b-3p mimic (miR-302b) or negative control miR (miR-NC). (D) *RELA* mRNA and (E) NF-κB protein were analyzed in PC-3 cells co-transfected with either miR-302b-3p or miR-NC. Values were mean ± SD obtained from at least three independent experiments. $^{*}P<0.05$, compared to miR-NC.

MiR-302b-3p inhibits growth of xenograft tumor from inoculated prostate cancer cells, and promotes survival of xenograft mice

To exclude the possibility that in vitro cell cultures may generate artifacts, we further evaluated the anti-tumor activity of miR-302b-3p in vivo using a xenograft mouse model. The miR-302v-3p-proficient PC-3 cells were inoculated subcutaneously into immunodeficient mice, after which tumor development was regularly checked and recorded. As shown in **Figure 4A**, ectopic expression of miR-302b-3p significantly delayed the xenograft tumor progression in comparison with scramble control. Consistently, the average weight of the xenograft tumors resected from sacrificed miR-302b-3p mice was remarkably less than the control group (**Figure 4B**). Consequently, the favorable prognosis was noticed in the miR-302b-3p-proficient mice (**Figure 4C**), which further consolidated our preliminary observations in respect to the anti-tumor activity of miR-302b-3p in vivo. Notably, we validated that miR-302b-3p affected NF-κB and EMT markers as previously demonstrated in vitro. The relevant proteins were quantified by western blotting showed the persistent inhibition on NF-κB, N-cadherin, Vimentin and stimulation on E-cadherin expression in response to ectopic introduction of miR-302b-3p (**Figure 5**), which molecularly underlay the impacts on tumor progression.

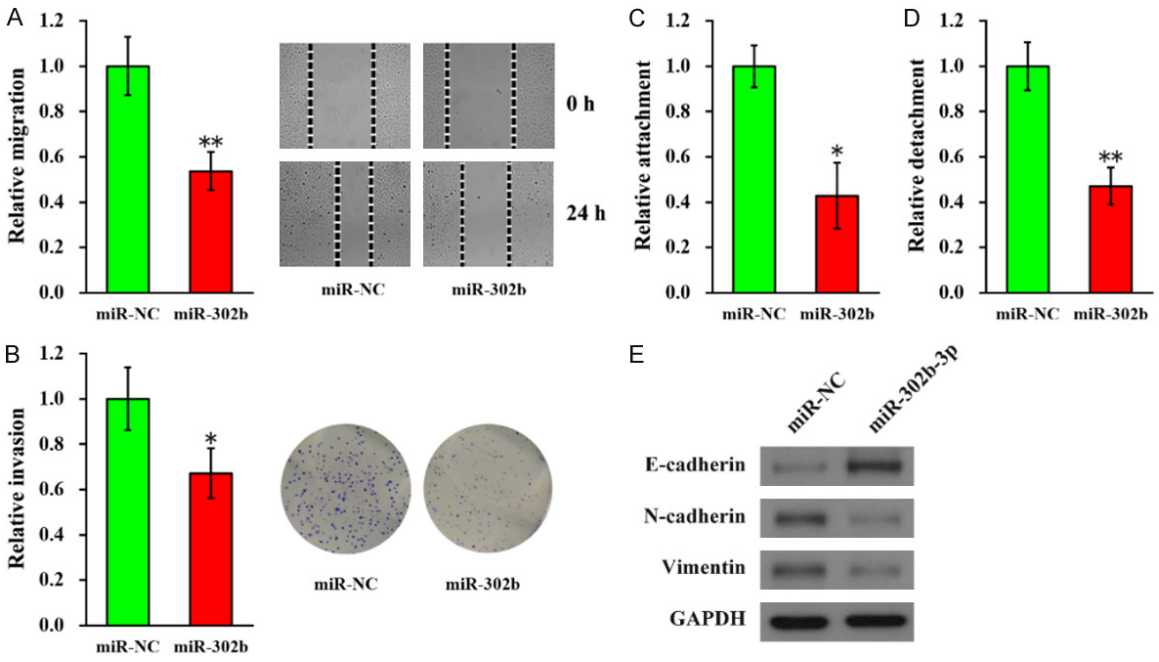

Figure 3. MiR-302b-3p inhibits migration, invasion and suppresses EMT phenotypes of prostate cancer cells *in vitro*. PC-3 cells stably expressing miR-302b-3p mimic (miR-302b) or negative control miR (miR-NC) were subjected to *in vitro* assays to analyze their (A) migration, (B) invasion, (C) attachment and (D) detachment abilities. Values were mean ± SD from at least three independent experiments. $^{**}P<0.01$, $^{*}P<0.05$, compared to miR-NC. (E) Protein expressions of epithelial marker E-cadherin, and mesenchymal markers N-cadherin and vimentin, were assessed using Western blot in PC-3 cells stably expressing negative control miR (miR-NC) or miR-302b-3p mimic (miR-302b).

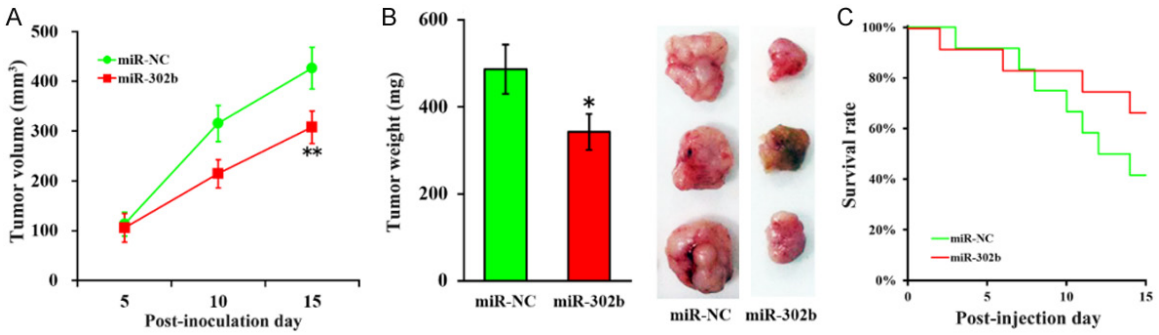

Figure 4. MiR-302b-3p inhibits growth of xenograft tumor from inoculated prostate cancer cells, and promotes survival of xenograft mice. A. PC-3 cells stably expressing negative control miR (miR-NC) or miR-302b-3p mimic (miR-302b) were inoculated into mice (n=12 each group), and tumor size was measured on indicated post-inoculation days. B. On day 15, mice were sacrificed to extract the xenograft and the tumor were resected and weighed, with representative tumor images shown on the left. Values were mean ± SD (n=12 each group). $^{*}P<0.05$, $^{**}P<0.01$, compared to miR-NC. C. Survival rates of mice from both groups (n=12 each group) were continually monitored for up to 15 days.

Re-introducing RELA plasmid abolishes miR-302b-3p inhibitory effect on migration, invasion and EMT phenotypes of prostate cancer cells in vitro

Our previous study identified that RELA as directly targeted by miR-302b-3p. Next, we sought to evaluate the importance of RELA for the anti-tumor properties in prostate cancer. We thus re-introduced RELA into miR-302b-3p-proficient PC-3 cells, and the potential effects on migration, invasion, and EMT phenotypes of cells were investigated. As shown in **Figure 6A** and **6B**, the suppressed migration as well as

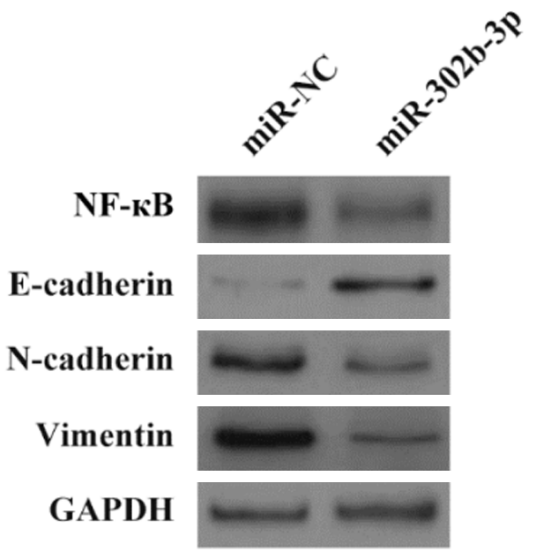

Figure 5. MiR-302b-3p inhibits protein expressions of NF-κB and EMT markers in xenograft tumors. All mice were sacrificed on day 15 to extract the xenograft, followed by Western blot analysis for NF-κB, mesenchymal markers N-cadherin and vimentin, and epithelial marker E-cadherin.

invasion by miR-302b-3p was readily and completely restored by introduction of RELA. Similarly, supplementation with RELA evidently reversed the decrease in cell attachment and detachment elicted by miR-302b-3p over-expression (**Figure 6C**, **6D**). The up-regulated epithelial marker E-cadherin was significantly decreased and down-regulated mesenchymal markers including N-cadherin and Vimentin were prominently increased by RELA in the miR-302b-3p-expressing PC-3 cells (**Figure 6E**). Therefore, our data suggested a potential genetic interaction between RELA/NF-κB and miR-302b-3p in the setting of prostate cancer cells *in vitro*.

Re-introducing RELA abolishes the anti-tumor properties of miR-302b-3p in vivo

To confirm our observations that RELA predominantly mediated the anti-tumor features of miR-302b-3p in vivo, here we further established stable cell line in miR-302b-3p-proficient PC-3 cells with over-expression of RELA. Consistent with our in vitro conclusion, we noticed significant increase of tumor growth in RELA-mice in comparison with control group (**Figure 7A**). Similarly, the xenograft tumor weight was greatly increased by RELA introduction in the miR-302b-3p-proficient PC-3 cells (**Figure 7B**). More importantly, the overall survival was remarkably compromised by RELA re-introduction as well (**Figure 7C**), which unambiguously underlined the oncogenic properties of RELA in prostate cancer as well as its predominance in mediating the anti-tumor activities of miR-320b-3p. Furthermore, we validated the restoration of NF-κB, N-cadherin, Vimentin and decrease of E-cadherin in xenograft tumor tissues (**Figure 7D**). These above data further verified that a potential genetic interaction between RELA/NF-κB and miR-302b-3p might also occur *in vitro*.

Discussion

The next-generation sequencing has been increasingly adopted for diagnostic and prognostic exploitations in various human malignancies [16]. Here we aimed to analyze the blood samples from both prostate cancer patients and health individual donors using RNA-seq technology and notably identified the miR-302b-3p as among the most significantly differentially expressed genes in this disease. The aberrant miR-302b-3p reduction was further confirmed by quantitative rt-PCR. Consistent low abundance of miR-302b-3p was observed in prostate cancer cell lines PC-3 and DU145, as compared to normal prostate cell lines RWPE-2 and WPE1-NB14 as well. Our data supported potential anti-tumor capacities of miR-302b-3p in prostate cancer. Noting worthily, the previous investigation by Mathieu et al. reported that hypoxia in prostate cancer cell line induced a human embryonic stem cells (hESC)-like transcriptional program including miR-302b-3p via hypoxia-inducible factor (HIF) [17]. On the contrary, Poliseno et al. found that miR-302 family was barely detected in prostate cancer but highly expressed and consequently associated with decrease of PTEN in breast cancer [18]. Recently, Guo et al. characterized miR-302/367/LATS2/YAP pathway was crucial for the propagation of prostate tumor cells and promoted castration resistance [19]. In sharp contrast to the archived oncogenic roles, here we uncovered novel anti-tumor properties of miR-302b-3p in prostate cancer both in vivo and in vitro. Along this direction, we used bioinformatics to predict and experimentally validated that RELA was directly targeted by miR-302b-3p in prostate cancer in this scenario.

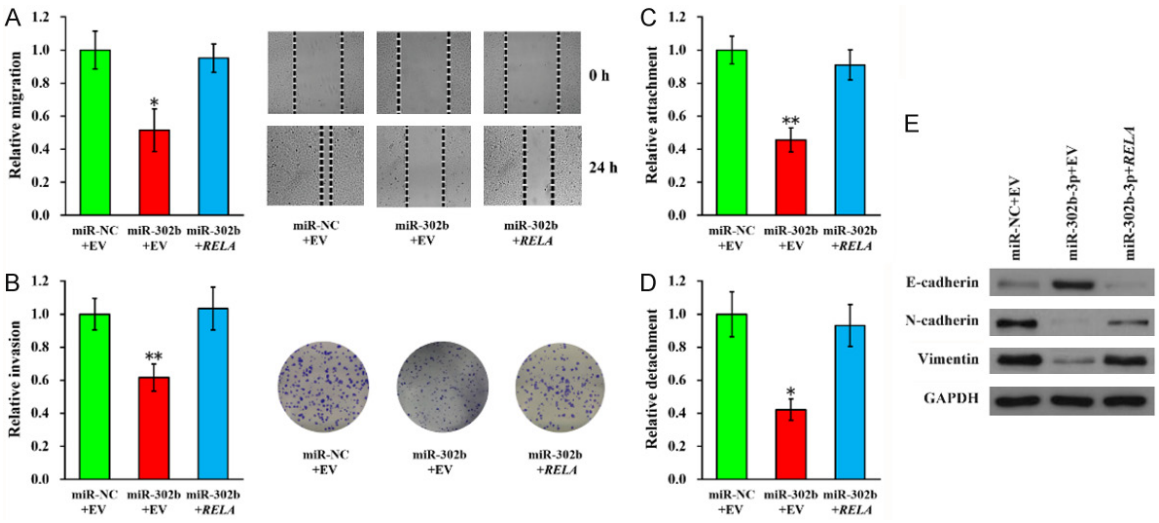

Figure 6. Re-introducing RELA plasmid abolishes miR-302b-3p inhibitory effect on migration, invasion and EMT phenotypes of prostate cancer cells *in vitro*. PC-3 cells stably expressing miR-302b-3p mimic (miR-302b) and negative control miR (miR-NC) were transfected with empty vector (+EV) or a plasmid containing the RELA open reading frame without its 3'UTR (+*RELA*), then subjected to *in vitro* assays to analyze their (A) migration, (B) invasion, (C) attachment and (D) detachment abilities. Values were mean ± SD from at least three independent experiments. $^{**}P<0.01$, $^{*}P<0.05$, compared to miR-NC. (E) Protein expressions of epithelial marker E-cadherin, and mesenchymal markers N-cadherin and vimentin, were analyzed using Western blot in the above-mentioned PC-3 cells.

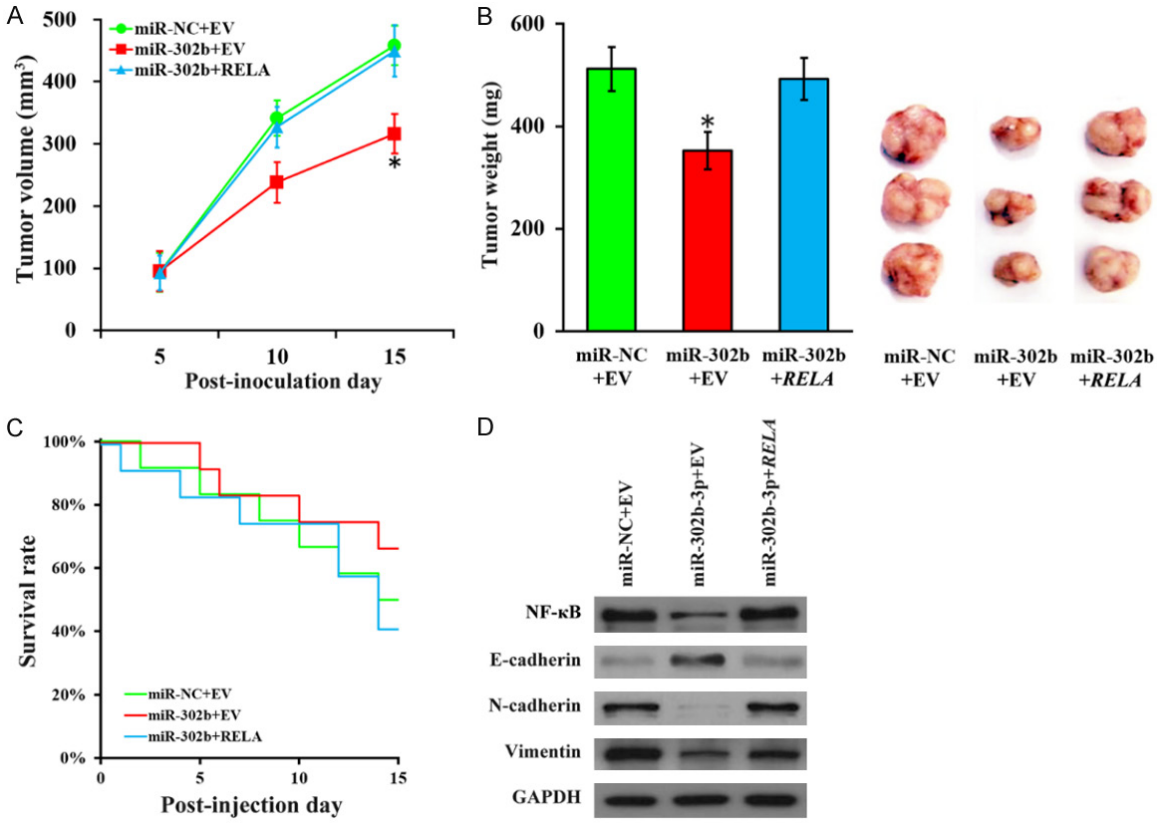

Figure 7. Re-introducing RELA plasmid abolishes miR-302b-3p effect on growth of xenograft tumor, survival of xenograft mice, and protein expressions of NF-κB and EMT markers in xenograft tumors. PC-3 cells stably expressing miR-302b-3p mimic (miR-302b) or negative control miR (miR-NC) were transfected with empty vector (+EV) or a plasmid containing the RELA open reading frame without its 3'UTR (+*RELA*), then inoculated into mice (n=12 each

group), and tumor size (A) was measured on indicated post-inoculation days. (B) On day 15, mice from all three groups were sacrificed to extract the xenograft and weigh the tumor, with representative tumor images shown on the left. Values were mean ± SD (n=12 each group). (C) Survival rates of mice from all three groups (n=12 each group) were monitored for up to 15 days. (D) On day 15, all mice were sacrificed to extract the xenograft, followed by Western blot analysis for NF-κB, epithelial marker E-cadherin, and mesenchymal markers N-cadherin and vimentin.

Co-transfection with miR-302b-3p drastically inhibited the luciferase activities with fusion with 3'UTR region of RELA. Consistently, the endogenous expression of RELA was greatly compromised at both transcript and protein levels by ectopic introduction of miR-302b-3p in PC-3 cells as well. In addition, we examined the potential influences of miR-302b-3p on tumor metastatis-related behaviors in prostate cancer cells. The remarkable decreases in respect to migration, invasion, attachment and detachment were observed in our system. Similarly, E-cadherin, anepithelial molecular marker, was greatly induced while N-cadherin and Vimentin, the mesenchymal markers, were tremendously repressed by miR-302b-3p in PC-3 cells. In vivo study using xenograft model mice further consolidated the anti-tumor activities of miR-302b-3p in prostate cancer, leading to more favorable overall survival in our animal experiment. To clarify the dependence of miR-302b-3p on RELA underlying its anti-tumor activity, we re-introduced RELA in the context of proficiency of miR-302b-3p in PC-3, which manifested the complete restoration in respect to cell migration, invasion, attachment, detachment and alterations on EMT molecular markers. In vivo study demonstrated that supplementation with RELA completely eliminated the tumor suppressive effects of miR-302b-3p, critically highlighting the importance of miR-302b-3p/RELA pathway in the tumor biology of prostate cancer.

Multiple genes targeted by miR-302b-3p have been so far identified. Wang et al. showed that deficient miR-302b alleviated LPS-elicited damages through affecting Smad3 in C28/I2 chondrocytic cells [20]. Guo et al. demonstrated that miR-302b-3p blockaded cell proliferation via AKT pathway through IGF-1R in human stomach cancer [21]. In esophageal cancer, Zhang et al. suggested that miR-302b alleviated cancer-related inflammation through targeting ERBB4, IRF2 and CXCR4 [22]. Lee et al. provided evidence showing that miR-32binhibited the signaling of insulin-like growth factor-binding protein 2 pathway and promoted apoptosis of glioma cells via modulating nuclear factor 1A [23]. Cataldo et al. demonstrated miR-302b improved cisplatin sensitivity of breast cancer cells by targeting E2F1 and the cellular DNA repair process [24]. Here, for the first time, we identified and validated that RELA was directly targeted by miR-302b-3p in prostate cancer, which broadened our cognition on kaleidoscopic biological roles of miR-302b-3p.

NF-κB was a ubiquitous transcription factor involving in multiple essential biological processes: immunity, inflammation, cell growth, differentiation, apoptosis, and tumorigenesis, etc. [25]. Dysregulated NF-κB signaling has been intimately linked to incidence and development of human malignancies, which was summarized in the comprehensive review by Perkins ND [26]. Range of microRNAs has been uncovered to involve in NF-κB signaling modulation. For instance, Qi et al. found that miR-224-5p modulated TNFα-induced adipocyte apoptosis via NF-κB activation [27]. Zheng et al. demonstrated that miR-135a was induced and aggravated myocardial depression in sepsis through the p38/MAPK/NF-κB axis [28]. It was also suggested that miR-29a contributed to lipopolysaccharide (LPS)-stimulated inflammatory responses via the Akt1/NF-κB signaling pathway in murine macrophages [29]. Wang et al. proved that miR-302d and miR-16 functioned as tumor suppressor and inhibited human glioblastoma multiforme via FGF2/NF-κB [30]. Intriguingly, Wang et al. demonstrated that miR-302b suppressed metastasis and cell invasion in human hepatocellular carcinoma cells through direct interaction with AKT2 [31] Here we for the first time report that a potential genetic interaction between RELA/NF-κB and miR-302b-3p might be implicated in the tumorigenesis of prostate cancer both *in vitro* and *in vivo*.

Noteworthily, the molecular mechanisms underlying aberrant down-regulated miR-302b-3p in prostate cancer was still to be defined so far. For example, miR-302b-3p did not behave similarly in androgen positive prostate cancer cells as in androgen negative cells (data not shown),

suggesting that androgen is also potentially associated with the suppressive role of miR-302b in prostate cancer. The epigenetic programming and genomic copy number variation might operate in this context, which is definitely worthy of further investigations in the future.

Conclusion

In summary, our data uncovered the role of miR-302b-3p in prostate cancer, whose down-regulation relieved the negatively regulatory actions on RELA expression, and thereby contributed to the metastatic phenotype in prostate cancer.

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Disclosure of conflict of interest

None.

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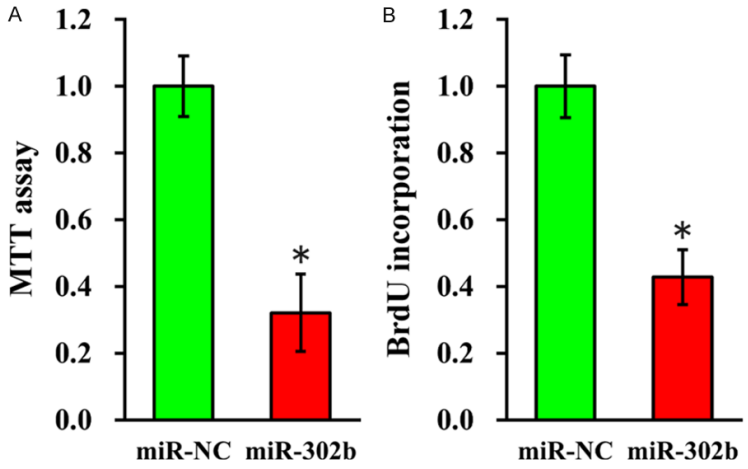

Figure S1. MiR-302b-3p inhibits proliferation of prostate cancer cells *in vitro*. PC-3 cells stably expressing negative control miR (miR-NC) or miR-302b-3p mimic (miR-302b) were subjected to (A) MTT assay and (B) BrdU incorporation assay to analyze their proliferation. Values were mean ± SD from at least three independent experiments. $^{*}P<0.05$, compared to miR-NC.