miR-23b Represses Proto-oncogene Src Kinase and Functions as Methylation-Silenced Tumor Suppressor with Diagnostic and Prognostic Significance in Prostate Cancer

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Abstract
The miRNAs have great potential as biomarkers and therapeutic agents owing to their ability to control multiple genes and potential to influence cellular behavior. Here, we identified that miR-23b is a methylation-silenced tumor suppressor in prostate cancer. We showed that miR-23b expression is controlled by promoter methylation and has great promise as a diagnostic and prognostic biomarker in prostate cancer. High levels of miR-23b expression are positively correlated with higher overall and recurrence-free survival in patients with prostate cancer. Furthermore, we elucidated the tumor suppressor role of miR-23b using in vitro and in vivo models. We showed that proto-oncogene Src kinase and Akt are direct targets of miR-23b. Increased expression of miR-23b inhibited proliferation, colony formation, migration/invasion, and triggered G0/G1 cell-cycle arrest and apoptosis in prostate cancer. Overexpression of miR-23b inhibited epithelial-to-mesenchymal transition (EMT) causing a decline in mesenchymal markers Vimentin and Snail and increasing the epithelial marker, E-cadherin. Depletion of Src by RNA interference conferred similar functional effects as that of miR-23b reconstitution. miR-23b expression caused a dramatic decrease in tumor growth in nude mice and attenuated Src expression in excised tumors compared with a control miR. These findings suggest that miR-23b is a methylation-silenced tumor suppressor that may be a useful biomarker in prostate cancer. Loss of miR-23b may confer proliferative advantage and promote prostate cancer migration and invasion, and reexpression of miR-23b may contribute to the epigenetic therapy for prostate cancer. Cancer Res. 72(24); 6435–46. ©2012 AACR.

Introduction
Prostate cancer is the most frequently diagnosed malignant tumor and second leading cause of cancer deaths in American men. It is estimated that 240, 890 newly diagnosed prostate cancer cases and 33,720 attributed deaths will occur in 2011 (1). Current prostate cancer treatments consisting of malignant prostate ablation by radical prostatectomy, radiotherapy, hormonal therapy, and/or neoadjuvant chemotherapy are generally curative for the majority of patients diagnosed with localized and androgen-dependent prostate cancer. However, progression to androgen-independent and metastatic disease states is often accompanied by a recurrence of prostate cancer and treatment remains a clinical challenge (2, 3).

The miRNAs are non–protein-coding sequences thought to regulate more than 90% of human genes (4). Growing evidence has strongly implicated the involvement of miRNAs in carcinogenesis (5, 6). From a clinical point of view, miRNAs have great potential as diagnostic and therapeutic agents. Microarray analysis has shown a general downregulation of miRNAs in tumors when compared with normal tissues (7). Owing to the tissue specificity of miRNAs, they have become a useful tool for defining the origin of tumors in poorly differentiated cancers (8). Prognosis and survival of patients depends on the cancer stage at diagnosis. For this reason, an important issue in clinical cancer research is to identify early biomarkers of the early tumorigenic process. miRNA signatures have been reported to be useful tools for early diagnosis of cancer (9, 10).

DNA hypermethylation of CpG sites within CpG islands is known to lead to the inactivation of many tumor-suppressive miRNAs (11). One of the most common causes of the loss for tumor suppressor miRNAs is silencing of their primary transcripts by CpG island hypermethylation (12–16). The DNA methylation profile of tumors is useful to define tumor type, clinical prognosis, and treatment response (17, 18). Epigenetic silencing of miRNAs is also involved in the acquisition of an invasive phenotype and the development of metastasis (19). Inactivation of oncogenic miRNAs (20, 21) or restoration of
tumor suppressor miRNAs (12–14) has great potential for cancer treatment. Here, we report that (i) miR-23b is frequently silenced through tumor-specific DNA methylation in prostate cancer, (ii) miR-23b acts as a tumor suppressor miRNA and has diagnostic/prognostic implication in prostate cancer, (iii) miR-23b directly targets proto-oncogene Src kinase and Akt, (iv) miR-23b has anti-proliferative/-migratory/-invasive effects by downregulating molecules involved in these pathways, (v) miR-23b inhibits epithelial-to-mesenchymal transition (EMT) markers, and (vi) miR-23b decreased in vivo tumor growth and Src kinase expression in nude mice xenografts.

Materials and Methods

Cell culture, plasmids, and probes/primers

Human prostate cancer cell lines PC3, DU145, LNCaP and a nonmalignant prostate cell line RWPE1 were obtained from the American Type Culture Collection (ATCC) and grown according to ATCC protocol. These human-derived cell lines were authenticated by DNA short-tandem repeat analysis by ATCC. The experiments with cell lines were carried out within 6 months of their procurement/resuscitation. Plasmids pEZX-MT01 miRNA 3′-untranslated region (UTR) target expression clones for Src (HmirT017696-MT01), Akt (HmirT004995-MT01) and miRNA Target clone control vector for pEZX-MT01 (CmiT000001-MT01) were purchased from Genecopoeia. Taq-Man probes for hsa-miR-23b (miR-23b) and negative control pre-miR were purchased from Applied Biosystems. siRNA duplexes (Src (Human)-3 unique 27mer siRNA duplexes [SR304574]) were purchased from Origene Technologies, Inc.

Quantitative real-time PCR

Tissue samples from radical prostatectomy were obtained from the Veterans Affairs Medical Center (San Francisco, CA). Total RNA was extracted and assayed for mature miRNAs and mRNAs using the TaqMan MicroRNA Assays and Gene Expression Assays, respectively, in accordance with the manufacturer’s instructions (Applied Biosystems). All real-time reactions were run in a 7500 Fast Real Time PCR system (Applied Biosystems). Relative expression was calculated using the comparative \( \Delta \Delta C_t \).

Methylation analysis of miR-23b by quantitative methylation-specific PCR

To investigate the mechanism involved in reduced levels of miR-23b in prostate cancer, we conducted methylation analysis in the 1.0-kb upstream sequence of miR-23b. Two CpG islands: CG-1 (−120 to −3 bp) and CG-2 (−820 to −650 bp) were located in 1.0 kb upstream sequence and further analyzed for methylation status in cell lines and tissue samples. DNA was available only for 38 (19 pairs) of laser-captured microdissected (LCM) tissue samples, and these samples were from the same cohort of 118 samples for which RNA expression was available. DNA was bisulfate-converted using EZ DNA Methylation-Gold Kit (Zymo Research) according to the manufacturer’s protocol. The converted DNA was amplified by PCR with 400 pmol/L of either primer set F1/R1, or F2/R2, and HotStar Taq Plus DNA Polymerase (Qiagen). PCR was carried out by denaturation at 95°C for 5 minutes, followed by 15 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds. Two microliters of the PCR product was added to 40 μL solution containing 20 μL TaqMan Fast Universal PCR Master Mix (2×; Applied Biosystems), 500 pmol/L primers F1/R1 or F2/R2. The mixed solution was aliquoted evenly into 2 tubes and was added 1 μL, 5 μmol/L probe for methylation reaction (PM) probe for methylation (M) reaction and 1 μL, 5 μmol/L probe for unmethylation reaction (PU) probe for unmethylation (U) reaction, respectively. Methylation in CGI-1 and CGI-2 was measured by quantitative real-time PCR (qRT-PCR) with an Applied Biosystems 7500 Fast Sequence Detection. For each sample, the percentage of methylation was calculated by the difference of \( C_t \) in M reaction (\( C_tM \)) and \( C_t \) in U reaction (\( C_tU \)).

In situ hybridization

In situ hybridization (ISH) was conducted as described previously (22). Briefly, cell lines and tissues were stained using DIG-labeled locked nucleic acid (LNA)-based probes specific for mir-23b and U6 following the manufacturer’s protocol (Exiqon, Inc.) and detected using anti-DIG-Fluorescein, Fab Fragments (Roche Applied Science for cell lines and BM Purple AP Substrate (Roche Applied Science) for tissues. ISH results for tissue array were graded according to quick score (percentage of cells stained × intensity of stain) and normalized to U6 levels.

Flow cytometry, cell viability, migration, clonability, and invasion assays

Fluorescence-activated cell-sorting (FACS) analysis for cell cycle and apoptosis was done 72 hours posttransfection using nuclear stain, 4′,6-diamidino-2-phenylindole (DAPI), for cell-cycle analysis or Annexin V-FITC/7-AAD Kit (Beckman Coulter, Inc.) for apoptosis analysis according to the manufacturer’s protocol. Cell viability was determined at 24, 48, and 72 hours by using the CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit (Promega) according to the manufacturer’s protocol. For colony formation assay, cells were seeded at low density (1,000 or 200 cells per plate) and allowed to grow until visible colonies appeared. Then, cells were stained with Giemsa and colonies were counted. Cytoselect 24-well cell migration and invasion assay kit (Cell Biosabs., Inc.) was used for migration and invasion assays according to manufacturer’s protocol.

Immunoblotting and immunofluorescence

Immunoblotting was conducted as described previously (23). The antibodies used were specific for Src (2123; Cell Signaling), pSrc (2101; Cell Signaling), AKEK1/2 (4694; Cell Signaling), pMEK1/2 (Ser217/221; 9154; Cell Signaling), p44/42 MAPK (Erk1/2; 4695; Cell Signaling), p-p44/42 MAPK (Thr202/Tyr204; 4370; Cell Signaling), STAT3 (9132), p-STAT3 (Tyr705; 9145; Cell Signaling), Akt (4685; Cell Signaling), p-Akt (Ser473; 4060; Cell Signaling), Bad (9292; Cell Signaling), p-Bad (Ser136; 4366; Cell Signaling), FAK (3285; Cell Signaling), e-Jun (9165; Cell Signaling), and GAPDH (sc-32233; Santa Cruz Biotechnology, Inc.). Blots were visualized using Western blotting luminal reagent (sc-2048; Santa Cruz Biotechnology, Inc.).
For immunofluorescence, cells were transfected with precursors of miR-23b or control-miR for 72 hours, washed, and fixed with acetone-methanol (1:1) mixture and hybridized with the specific primary antibodies against EMT markers. Cells were washed and hybridized with fluorescein-conjugated secondary antibody (1:1,000) and mounted with ProLong Gold antifade reagent with DAPI (Invitrogen Life Technologies).

Luciferase assays

The complimentary sites in 3′UTR of Src and Akt for miR-23b and mutated sequences are given in Supplementary Table S7. The Src, Akt, and control vectors were purchased from Genetopeia and named Src-3′UTR and Akt 3′UTR. Mutated 3′UTR sequences of Src and Akt were cloned and named Mut Src3′UTR and Mut Akt3′UTR. For reporter assays, cells were transiently transfected with wild-type or mutated reporter plasmid and miR-23b or control-miR. Firefly luciferase activities were measured using the Dual Luciferase Assay (Promega) 18 hours after transfection, and the results were normalized with Renilla luciferase. Each reporter plasmid was transfected at least 3 times, and each sample was assayed in triplicate.

In vivo intratumoral delivery of miR-23b and genistein

The antitumor effect of miR-23b was determined by local administration of miR-23b precursor in established tumors. Each mouse was injected subcutaneously with 5.0 × 10^6 PC3 prostate cancer cells. Once palpable tumors developed (average volume = 55 mm^3), 6.25 μg of synthetic miRNA complexed with 1.6 μL siPORT Amine transfection reagent (Ambion) in 50 μL PBS was delivered 8 times intratumorally at 3-day intervals. Tumor growth was followed for 21 days from the first injection. All animal care was in accordance with the institutional guidelines.

Statistical analysis

Statistical analyses were conducted with GraphPad-Prism 5 and MedCalc. All quantified data represent average of at least triplicate samples or as indicated. Error bars represent SD of mean. All tests conducted were 2-tailed, and P < 0.05 was considered statistically significant. Receiver-operating curves (ROC) were calculated to determine potential of miR-23b or its methylation to discriminate between malignant and nonmalignant samples and hence can be used as a diagnostic marker for prostate cancer. To determine whether miR-23b has any prognostic significance, we divided 151 cases into low miR-23b expression tumor (T)/normal (N) < 0.8-fold and high miR-23b (expression T/N > 0.8-fold) groups and conducted Kaplan–Meier survival analysis and multiple regression analysis. In Kaplan–Meier analysis, the miR-23b high group displayed significantly higher overall survival (OS) probability than the miR-23b low group (log-rank test: P < 0.0001; HR, 3.3; 95% CI, 3–13; Fig. 2B). Kaplan–Meier survival analysis for recurrence-free survival (RFS) was conducted using 105 cases. Cases with high miR-23b expression had better RFS than with low miR-23b expression cases (log-rank test: P < 0.002; HR, 6; 95% CI, 3–13; Fig. 2C). We conducted multiple regression analysis for the same set of patients using entry, forward, backward, and stepwise methods one by one (Supplementary Tables S2–S5). Multiple regression analysis revealed that miR-23b expression is an independent predictor of biochemical recurrence (P < 0.02) as determined in entry, forward, backward and stepwise methods (Supplementary Table S2). We also determined the correlation of miR-23b expression with clinicopathologic variables such as Gleason grade, pathologic stage (pT), and biochemical recurrence (Fig. 2D) and details are described in detail in Supplementary Results. Correlation tests revealed that cases with low miR-23b expression increased from low-grade, low pathologic stage to high-grade and high pathologic stage (Fig. 2D). Patients who had prostate-specific antigen (PSA) recurrence also had significantly low miR-23b expression. These findings suggest that miR-23b has a potential to be a diagnostic and prognostic marker for predicting the biochemical recurrence of patients with prostate cancer, although addition of more samples may strengthen these results.

miR-23b is significantly downregulated in prostate cancer

To identify silenced miRNAs in prostate cancer, we conducted microarray screening using cancer cell lines and a nonmalignant cell line. Mir-23b was found to be a significantly downregulated miRNA in cancer compared with the nonmalignant cell line. We validated the microarray data by miRNA qRT-PCR analysis. The results confirmed that miR-23b was downregulated in cancer cell lines (Fig. 1A). In situ hybridization also confirmed the presence of miR-23b expression (green signal) in RWPE1 cells compared with cancer cell lines (Fig. 1B). DU145 cells express higher levels of miR-23b but the green signal is absent because we have to keep the exposure time same across the cell lines. To examine the biologic significance of miR-23b, its expression was analyzed by qRT-PCR in 236 (118 pairs) LCM matched tissue samples (Fig. 1C; Supplementary Fig. S1) and an unmatched group of 27 benign prostatic hyperplasia (BPH) and 20 tumor samples (Fig. 1D) along with another cohort of 48 samples where expression was analyzed by ISH (Fig. 1E). Among all samples, miR-23b expression was significantly downregulated in cancer samples compared with normal or BPH (Fig. 1C–E). These results indicate a putative tumor suppressor role of miR-23b in prostate cancer.

Diagnostic and prognostic significance of miR-23b in prostate cancer

Clinical demographics of the study cohort are summarized in Supplementary Table S1. ROC analyses were conducted to evaluate the ability of miR-23b expression to discriminate between normal and tumor cases using 151 pairs of tissue samples. An area under the ROC curve (AUC) of 0.975 [95% confidence interval (CI), 0.950–0.999; Fig. 2A] was obtained, suggesting that miR-23b expression can discriminate between malignant and nonmalignant samples and hence can be used as a diagnostic marker for prostate cancer. To determine whether miR-23b has any prognostic significance, we divided 151 cases into low miR-23b (expression T/N < 0.8-fold) and high miR-23b (expression T/N > 0.8-fold) groups and conducted Kaplan–Meier survival analysis and multiple regression analysis. In Kaplan–Meier analysis, the miR-23b high group displayed significantly higher overall survival (OS) probability than the miR-23b low group (log-rank test: P < 0.0001; HR, 3.3; 95% CI, 3–13; Fig. 2B). Kaplan–Meier survival analysis for recurrence-free survival (RFS) was conducted using 105 cases. Cases with high miR-23b expression had better RFS than with low miR-23b expression cases (log-rank test: P < 0.002; HR, 6; 95% CI, 3–13; Fig. 2C). We conducted multiple regression analysis for the same set of patients using entry, forward, backward, and stepwise methods one by one (Supplementary Tables S2–S5). Multiple regression analysis revealed that miR-23b expression is an independent predictor of biochemical recurrence (P < 0.02) as determined in entry, forward, backward and stepwise methods (Supplementary Table S2). We also determined the correlation of miR-23b expression with clinicopathologic variables such as Gleason grade, pathologic stage (pT), and biochemical recurrence (Fig. 2D) and details are described in detail in Supplementary Results. Correlation tests revealed that cases with low miR-23b expression increased from low-grade, low pathologic stage to high-grade and high pathologic stage (Fig. 2D). Patients who had prostate-specific antigen (PSA) recurrence also had significantly low miR-23b expression. These findings suggest that miR-23b has a potential to be a diagnostic and prognostic marker for predicting the biochemical recurrence of patients with prostate cancer, although addition of more samples may strengthen these results.
Mechanism of silencing of miR-23b in prostate cancer is through CpG hypermethylation: correlation between miR-23b methylation status with expression and use as diagnostic marker

Two CpG islands, CG-1 and CG-2, were located in 1.0 kb upstream sequence (Fig. 3A). CG-1 was the methylation "hot spot" that was methylated in cancer cell lines and in tumors. Our results showed a hypermethylated sequence in matched tumor tissue samples compared with their normal counterparts (Fig. 3B). Similarly, prostate cancer cell lines were also hypermethylated compared with the nonmalignant RWPE1 cell line (Fig. 3C). These results indicate that miR-23b is silenced by hypermethylation in prostate cancer. To investigate whether miR-23b methylation status affects its expression in prostate cancer cell lines, we treated cells with the demethylating agent 5-aza-deoxycytidine (5-Aza, 1 μmol/L) for 1 week and then determined methylation status and miR-23b expression by methylation-specific qRT-PCR and qRT-PCR, respectively. The methylation percentage in 5-Aza–treated cells was significantly decreased compared with untreated controls along with a concomitant increase in miR-23b expression (Fig. 3C and D). To determine the correlation between percent methylation of miR-23b and expression levels of its target Src kinase, we analyzed the expression of miR-23b, Src kinase, and the methylation of miR-23b in a different set of matched patient samples (12 pairs). A negative correlation was observed between miR-23b and Src expression (P < 0.01), whereas a positive correlation was found between percentage of methylation of miR-23b and Src expression (P < 0.03; Fig. 3H) in the same patient samples.

To confirm that promoter methylation is an important mechanism of miR-23b suppression in patients with prostate cancer, we investigated the correlation between methylation status of miR-23b and its expression in the same samples. There was a significant inverse correlation between percentage of methylation and miR-23b expression such that cases with higher miR-23b expression had a lower percentage of methylation (P < 0.01; Fig. 3B and E; Supplementary Table S6). Sequences of primers and probes are given in Supplementary Table S7. We also conducted ROC analysis to evaluate the percentage of miR-23b methylation to distinguish malignant from normal cases in matched tissue samples and cell lines. For tissues, the AUC was 0.742 (95% CI, 0.575–0.870; Fig. 3G), whereas for cell lines (PC3 and DU145 vs. RWPE1), the AUC was a perfect 1.00 (95% CI, 0.664–1.000; Fig. 3H), indicating that miR-23b methylation can distinguish between disease and normal cases.

miR-23b overexpression suppresses prostate cancer cell proliferation, migration/invasion and colony formation and induces G0–G1 cell-cycle arrest and apoptosis

We determined the functional significance of miR-23b–overexpressing prostate cancer. A significant decrease in cell proliferation was observed over time in miR-23b–transfected
miR-23b in Prostate Cancer

miR-23b directly represses proto-oncogene Src kinase and its downstream genes involved in proliferation/survival/invasion and migration pathways in prostate carcinoma

We investigated whether Src kinase is a direct functional target of miR-23b in prostate cancer. Complimentary sites in 3’UTR of Src kinase for miR-23b are given in Supplementary Table S7. We chose Src kinase because it has been reported to be a center stage molecule in cancer with pleiotropic effects due to the multiple signaling pathways engaged by Src kinase (Fig. 5A). In prostate cancer, Src kinase has been reported to be involved in proliferation, migration, and invasion. Our results showed that Src kinase is overexpressed in prostate cancer cell lines compared with RWPE1 cells (Fig. 5B). Transient transfection of human prostate cancer cells with Src 3’UTR plasmids along with miR-23b led to a significant decrease in relative luciferase units when compared with Src Mut3’UTR vector and cont-miR or Src Mut3’UTR vector and miR-23b (Fig. 5C). These results indicate that Src kinase is a direct target of miR-23b in prostate cancer.

We next determined whether overexpression of miR-23b regulates Src kinase at translational level and alters downstream signaling events. Transient transfection of prostate cancer cells with miR-23b significantly downregulated total Src and active Src (pSrcY416) protein expression (Fig. 5D). Western blot analysis showed reduced levels of downstream molecules that are involved in proliferation, migration, and invasion (Fig. 5E) in cells with suppressed Src expression following miR-23b overexpression. Akt is an important downstream gene of Src. We found complimentary miR-23b binding sequence in its 3’UTR (Supplementary Table S7). Our results showed that miR-23b can also directly target Akt (Supplementary Fig. S4). This further compliments our results that miR-23b exerts its effects in prostate cancer, at least partly, through Src–Akt pathway axis. We do not rule out the involvement of other target genes of miR-23b action in prostate cancer, although our results do indicate that Src–Akt axis pathway is partly involved.

Figure 2. Diagnostic and prognostic significance of miR-23b in prostate cancer. A, ROC analysis showing performance of miR-23b expression to discriminate between malignant and nonmalignant tissue samples. B and C, Kaplan–Meier analysis for OS and RFS based on miR-23b expression. D, χ² test showing correlation of clinicopathologic characteristics with miR-23b expression. Group H L, miR-23b high or low.

PC3 and DU145 cells (Fig. 4A) as compared with cells expressing cont-miR. miR-23b–transfected cells also had low colony formation ability, as the number of foci in miR-23b–expressing cells was decreased when compared with cont-miR–transfected cells (Fig. 4B; Supplementary Fig. S2). Less absorbance was observed at 560 nm with miR-23b–transfected cells than cont-miR in the migration assay (Fig. 4C), and miR-23b overexpression also significantly reduced the invasiveness of cancer cells (Fig. 4D). FACS analysis revealed that reexpression of miR-23b leads to a significant increase (22%–27%) in the number of cells in the G0–G1 phase of the cell cycle, whereas the S-phase population decreased from 19% to 20% to 4%, suggesting that miR-23b causes a G0–G1 arrest in miR-23b–transfected cells compared with controls (cont-miR; Fig. 4E). FACS analysis for apoptosis was conducted using Annexin-V/FTC/7-aminoactinomycin D (7-AAD) dye. The percentage of total apoptotic cells (early apoptotic + apoptotic) was significantly increased (13%–22%) in response to miR-23b overexpression compared with cont-miR (3%–4%) with a corresponding 13% to 20% decrease in the viable cell population (Fig. 4F). Functional assays were also conducted in androgen-dependent LNCaP cells, and the results were consistent (Supplementary Fig. S3). These results indicate a tumor suppressor role for miR-23b in prostate cancer.
Figure 3. Methylation status of miR-23b in prostate cancer. A, miR-23b gene and CpG islands within the 1 kb region upstream of miR-23b gene. CG1 and 2, CpG islands 1 and 2; F1 and 2, forward primers; R1 and 2, reverse primers; M1-2/U1-2 methylation- and unmethylation-specific probes. Sequences of primers and probes are given in Supplementary Table S7. B, miR-23b methylation percentage in matched tissue samples. C, miR-23b methylation percentage and demethylation by 5-Aza treatment in cell lines. D, induction of miR-23b expression by 5-Aza treatment in prostate cell lines. E, expression of miR-23b in the same samples in which methylation was analyzed to show that miR-23b expression is inversely correlated to percent methylation. F and G, ROC for percentage of methylation in tissues and cell lines.
miR-23b downregulates EMT markers and suppresses cell migration and invasion independent of proliferation

To further show that the effect of miR-23b on cell migration and invasion is an event independent of cell proliferation, we examined the effect of overexpression of miR-23b on EMT markers. A decrease in Vimentin and Slug (mesenchymal markers) and an increase in E-cadherin (epithelial marker) was observed in miR-23b-transfected cells compared with cont-miR (Fig. 5F and G). The process of EMT is involved in epithelial-derived tumors causing them to become invasive and metastatic. These results show that miR-23b suppressed EMT markers along with the other migratory/invasive genes such as Src and focal adhesion kinase (FAK) and thus confirm that effect of miR-23b on invasion/migration is independent of proliferation.

Depletion of Src by RNA interference mimics miR-23b reconstitution in prostate cancer

Phenocopy experiments were also carried out by siRNA inhibition of Src (Fig. 6; Supplementary Fig. S5). We used a validated siRNA that resulted in 80% to 90% Src gene depletion of Src by RNA interference mimics miR-23b reconstitution in prostate cancer.

Figure 4. Transient transfection of miR-23b inhibits prostate cancer cell proliferation, colony formation, migration, invasion, and induces apoptosis and cell-cycle arrest. A, proliferation of PC3 and DU145 cells after miR-23b transfection was significantly reduced compared with cont-miR. B, miR-23b overexpression significantly inhibits colony-forming ability of prostate cancer cells. C, migration assays of PC3 and DU145 cells transfected with miR-23b. D, invasion assay shows a significant decrease in the number of invading PC3 and DU145 cells transfected with miR-23b. E, cell-cycle analysis showing an increase in the G0–G1 phase of PC3 and DU145 cells overexpressing miR-23b. F, apoptosis assay showing induction of apoptosis by miR-23b. \( P < 0.05, \pm SD.\)
knockdown at the mRNA and protein levels (Fig. 6A) for further experiments. Our results showed that siRNA inhibition of Src increased G0–G1 cell-cycle population (16%–21%), whereas there was a decrease of 11% to 12% in S-phase cell population (Fig. 6B and C). Approximately 15% to 18% of the cells were in the apoptotic fraction in Src siRNA–transfected cells compared with 4% in nonspecific control (Fig. 6D and E). These results suggest that inhibition of Src (that in turn depletes the downstream Akt axis) by miR-23b overexpression is partly responsible for the observed phenotype in prostate cancer cells, and siRNA depletion of Src mimics the effect of miR-23b Overexpression. We further carried out experiments with antimiR-23b and anti-miR-Neg-control to determine whether the protein expressions of all the downregulated genes (by miR-23b) are rescued. For this experiment, we chose DU145 cell line as it expresses higher levels of miR-23b than PC3. Indeed the protein expression of all genes was rescued by anti-miR-23b (Fig. 6F).

**Intratumoral delivery of miR-23b suppresses tumorigenicity in vivo**

We also conducted in vivo growth suppression experiments to determine the tumor-suppressive effect of miR-23b after local administration in established tumors. Tumor growth was...
significantly suppressed by miR-23b over the course of experiment compared with the cont-miR. Average tumor volume in cont-miR was 850 mm$^3$ compared with the average tumor volume of 38 mm$^3$ in mice that received miR-23b (Fig. 7A) at the termination of the experiment. We also checked the expression of miR-23b or Src kinase in harvested tumors. Our results showed that miR-23b expression was significantly high with a corresponding significant decrease in the target Src kinase gene expression in miR-23b–treated tumors compared with controls (Fig. 7B and C). These results confirm the tumor suppressor effect of miR-23b in a prostate xenograft model.

Discussion

In this study, we found miR-23b to be significantly silenced/downregulated in human prostate tumor samples when compared with adjacent normal samples. The downregulation of miR-23b expression was also observed in prostate cancer cell lines when compared with a nonmalignant cell line. This is consistent with a previous miRNA profiling analysis of 9 prostate carcinoma samples and various cell lines that showed downregulation of miR-23b (24). The suppression of miR-23b expression in tumors and cancer cell lines suggests a tumor suppressor role in prostate cancer. However, neither the functional role nor the diagnostic or prognostic implications of miR-23b in prostate cancer have been previously defined.

DNA methylation–mediated downregulation of miRNAs by proximal CpG islands has been described by a number of groups (15, 25), and identification of other targets for methylation may clarify the specific molecular events involved in prostate cancer progression, enabling the prevention,
diagnosis, and treatment of prostate cancer to be approached at a molecular level. Here, we show that miR-23b is frequently silenced through tumor-specific DNA methylation in prostate cancer tissues and cell lines and the methylation can be used as a diagnostic marker to distinguish malignant from normal cases. miRNAs possess several features that make them attractive candidates as new prognostic biomarkers and powerful tools for the early diagnosis of cancer (26). In this study, we found that miR-23b was predictive of OS and RFS. Multiple regression analysis also showed that miR-23b is an independent predictor of biochemical recurrence. miR-23b expression also distinguished malignant from normal tissues, indicating the diagnostic significance of miR-23b in prostate cancer.

An obstacle to understanding miRNA function has been the relative lack of experimentally validated targets. We validated Src kinase to be a direct target of miR-23b in prostate cancer. Src kinase requires phosphorylation within a segment of the kinase domain termed the activation loop for full catalytic activity and this auto-phosphorylation site is tyrosine416 (27). Src kinase has been reported to be activated in prostate cancer (28, 29, 30). We showed that miR-23b inhibits Src kinase at protein levels by directly binding to its 3’UTR complimentary sequences. Src is involved in multiple signaling pathways including Ras/Raf/ERK1/2, PI3K/AKT, β-catenin/c-Myc/cyclin D1, and FAK/p130CAS/MMP-9, and increased Src activity correlates with more aggressive phenotypes (28, 29, 31, 32). We observed that inhibition of Src by miR-23b overexpression reduced signaling via the MEK/ERK pathway. A previous study by Chang and colleagues (28) has shown that Src inhibition by small-molecule inhibitors induced apoptosis and cell-cycle arrest at the G0–G1 phase of the cell cycle in prostate cancer cell lines. Our results revealed that inhibition of Src by miR-23b overexpression induced apoptosis and G0–G1 arrest in prostate cancer cells. This effect on the cell cycle and apoptosis prompted us to study the effect on Src downstream target genes AKT, BAD, and STAT3, a Src target and key transcriptional factor for c-Myc and cyclin D1 (33) that are involved in the G0–G1 phase of cell cycle. We found that all these genes were downregulated at the protein level. Inhibition of Src has been found to decrease the invasion and migration of prostate cancer cells (29) through selective inhibition of Src substrates, such as FAK. Our results indicate that miR-23b inhibited prostate cell migration and invasion and also downregulated c-Jun and FAK. Overexpression of miR-23b caused a decline in the mesenchymal markers Vimentin and Snail, whereas there was an increase in the expression of E-cadherin, an epithelial marker. These results suggest that miR-23b suppresses migration and invasion independent of cell proliferation and that it has an anti-metastatic effect on prostate cancer cells. To determine whether Src inhibition is, at least partly, responsible for the changes observed after miR-23b overexpression, we carried out phenocopy experiments after inhibiting Src by siRNA. Our results showed that inhibition of Src was responsible for G0–G1 cell-cycle arrest, whereas there was a decrease in S-phase cell population. Induction of apoptosis was also observed in Src siRNA–transfected cells compared with non-control. These results were similar to that of miR-23b overexpression assays. We further determined that the protein expressions of the genes that were downregulated by miR-23b were rescued by anti-miR-23b. These results strongly indicate that the tumor-suppressive effect of miR-23b is, at least partly, mediated by Src–Akt axis inhibition in prostate cancer.

The antiproliferative effects of miR-23b observed in this study were confirmed in prostate tumor xenograft models. In conclusion, our study shows that miR-23b has an important tumor suppressor role both in vitro and in vivo. Accumulating evidence indicates that modulation of miRNA also represents an attractive strategy for therapeutic gene

Figure 7. miR-23b inhibit tumor growth in vivo. *, *P < 0.05. A, tumor volume following intratumoral injection of control or miR-23b precursor into established tumors. #, P < 0.05. B, average expression of miR-23b in excised tumors. D, depletion of Src kinase by miR-23b in excised tumors. Protein was extracted from 2 tumors that received control or miR-23b precursor (23B-1-2).
silencing, miRNAs potentially influence cellular behavior through the regulation of extensive gene expression networks. Therapeutic modulation of a single miRNA may therefore affect many pathways simultaneously to achieve clinical benefit. Our study is the first report showing that miR-23b has diagnostic/prognostic significance and inhibits the Src–Akt axis genes, a center stage pathway that regulates proliferation, survival, and migration/invasion in cancer. It also highlights the therapeutic potential of miR-23b in the treatment of prostate cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
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