miRNA-34b Inhibits Prostate Cancer through Demethylation, Active Chromatin Modifications, and AKT Pathways

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Abstract

Purpose: miRNAs can act as oncomirs or tumor-suppressor miRs in cancer. This study was undertaken to investigate the status and role of miR-34b in prostate cancer.

Experimental Design: Profiling of miR-34b was carried out in human prostate cancer cell lines and clinical samples by quantitative real-time PCR and in situ hybridization. Statistical analyses were done to assess diagnostic/prognostic potential. Biological significance was elucidated by carrying out a series of experiments in vitro and in vivo.

Results: We report that miR-34b is silenced in human prostate cancer and the mechanism is through CpG hypermethylation. miR-34b directly targeted methyltransferases and deacetylases resulting in a positive feedback loop inducing partial demethylation and active chromatin modifications. miR-34b expression could predict overall and recurrence-free survival such that patients with high miR-34b levels had longer survival. Functionally, miR-34b inhibited cell proliferation, colony formation, migration/invasion, and triggered Go/G1 cell-cycle arrest and apoptosis by directly targeting the Akt and its downstream proliferative genes. miR-34b caused a decline in the mesenchymal markers vimentin, ZO1, N-cadherin, and Snail with an increase in E-cadherin expression, thus inhibiting epithelial-to-mesenchymal transition. Finally we showed the antitumor effect of miR-34b in vivo. MiR-34b caused a dramatic decrease in tumor growth in nude mice compared with cont-miR.

Conclusion: These findings offer new insight into the role of miR-34b in the inhibition of prostate cancer through demethylation, active chromatin modification, and Akt pathways and may provide a rationale for the development of new strategies targeting epigenetic regulation of miRNAs for the treatment of prostate cancer. Clin Cancer Res; 19(1); 73–84. ©2012 AACR.

Introduction

miRNAs are non–protein-coding RNAs thought to regulate the expression of up to 90% of human genes (1). Growing evidence has strongly implicated the involvement of miRNAs in carcinogenesis (2, 3). Dysregulated miRNAs may function as oncogenes (4), such as the miR-17–92 cluster (5), or tumor suppressor genes such as miR-205 (6–8). A single miRNA can have hundreds of target mRNAs, highlighting the importance of this gene regulation system in cellular functions (9). The study of miRNAs has become the subject of intense interest, especially after the discovery of their fundamental role in a myriad of cellular and biological processes ranging from development to disease (10). From a clinical point of view, miRNAs have great potential as diagnostic and therapeutic agents. Because microarray analysis shows a general downregulation of miRNAs in tumors when compared with normal tissues (11). As a result of the remarkable tissue specificity of miRNAs, they have become a very useful tool for defining the origin of tumors in poorly differentiated cancers (12). The prognosis and survival of patients depend on the stage of the cancer at diagnosis. For this reason, 1 of the most important issues in clinical cancer research is to find early biomarkers of the tumorigenic process. miRNA signatures have been reported to be powerful tools for early diagnosis in renal cancer and have been found to distinguish between metastatic and nonmetastatic tumors. The 5-year survival rate of patients with primary metastasis was reported to be 10% as compared with 70% to 90% in nonmetastatic patients (13).

Among various epigenetic mechanisms of cancer-related gene silencing, DNA hypermethylation of CpG sites within CpG islands is known to lead to the inactivation of many tumor suppressor miRNAs (14). Previous reports have
Translational Relevance

This study documents the pleiotropic role of miR-34b in prostate cancer. Our results provide evidence that miR-34b acts as a tumor suppressor with diagnostic and prognostic significance in prostate cancer. Reconstitution of miR-34b downregulated DNA methyltransferase (DNMT) and histone deacetylase (HDAC), resulting in partial demethylation and induction of active chromatin modifications. miR-34b had antiproliferative and anti-invasive effects by impairing the Akt proliferative pathway genes and the epithelial-to-mesenchymal transition markers. We also confirmed the growth-suppressive effect of miR-34b in in vivo xenografts. These findings offer novel insight into the association between dysregulation of miRNA and methylation/chromatin modification and provide a strong rationale for the development of new strategies targeting the epigenetic regulation of miRNAs for the treatment of prostate cancer.

shown that disruption of DNA methylation in cancer cell lines induces upregulation of substantial numbers of miRNAs (15, 16) and, thus, helps identify candidate tumor suppressor miRNAs that are silenced by methylation. For example, treatment of bladder cancer cells with histone deacetylase (HDAC) and DNA methyltransferase (DNMT) inhibitors induced demethylation and re-expression of miR-127 (17). Similarly, miR-34b/c was found to be methylated and downregulated in colorectal and gastric cancers (16, 18). The DNA methylation profile of tumors can be used as a signature to define tumor type, clinical prognosis, and treatment response (19, 20). MiRNAs transcribed from CpG islands undergo DNA methylation-associated repression with a similar chromatin context to protein-coding genes (15, 16, 21). Epigenetic regulation of miRNA genes is tightly linked to chromatin signatures and transcriptionally active miRNA genes are characterized by active chromatin marks, such as trimethylated histone H3 lysine 4 (H3K4; ref. 22). Epigenetic silencing of miRNAs is also involved in the acquisition of an invasive phenotype and the development of metastasis (23). Inactivation of tumor-promoting miRNAs (24, 25) or restoration of tumor-suppressor miRNAs (15, 17, 26) has great potential for use in cancer treatment.

Here we report that: (i) miR-34b is significantly downregulated in prostate cancer and the mechanism of silencing is through CpG hypermethylation; (ii) reconstitution of miR-34b induced partial demethylation and active chromatin modifications in upstream sequence of miR-34b gene; (iii) miR-34b acts as a tumor suppressor miRNA in prostate cancer; (iv) miR-34b has antiproliferative effects and targets Akt pathway genes; (v) miR-34b has antimigratory/invasive effects and downregulates markers of epithelial-to-mesenchymal transition (EMT); and (vi) miR-34b suppresses tumor growth in an in vivo xenograft nude mouse model.

Materials and Methods

Cell culture, plasmids, and probes

Human prostate cancer cell lines PC3, LNCaP, and a nonmalignant renal cell line RWPE1 were obtained from the American Type Culture Collection (ATCC; Manassas, Virginia) and grown according to the ATCC protocol and as described previously (7). These human-derived cell lines were authenticated by DNA short-tandem repeat analysis by the ATCC. The experiments with cell lines were carried out within 6 months of their procurement/resuscitation. Plasmids of 3'-UTR target expression clones were purchased from GeneCopoeia. TaqMan probes and miRNA precursors were purchased from Applied Biosystems. The probes for methylation-specific real-time PCR were synthesized by Applied Biosystems, then labeled with 6FAM reporter at the 5'-end and with MGB quencher at the 3'-end.

Quantitative real-time PCR and in situ hybridization

Tissue samples from radical prostatectomy were obtained from the Veterans Affairs Medical Center, San Francisco, California. Written informed consent was obtained from all patients and the study was approved by the UCSF Committee on Human Research (Approval number: H9058–35751-01). Laser capture microdissection was employed to get total RNA. All reactions were run in a 7500 Fast Real Time PCR System and miRNA assays were conducted in accordance with the manufacturer’s instructions (Applied Biosystems). Relative expression was calculated using comparative \(Ct\).

In situ hybridization (ISH) was carried out as described previously (7). The detailed method is described in Supplementary Materials and Methods. ISH results for tissue array were graded according to quick score (percentage of cells stained \(\times\) intensity of stain) and normalized to U6 levels.

Methylation analysis of miR-34b by sequencing and quantitative methylation-specific PCR

DNA was available for 32 pairs of laser-capture microdissected samples. Out of 32, 19 pairs are from the same cohort for which miRNA expression was available. Methylation status of tissue samples was analyzed by quantitative methylation-specific PCR (qMSP) within the 1.0-kb region upstream of the miR-34b gene. For prostate cancer cell lines, methylation was determined by sequencing and percentage of methylation was calculated. The sequences are given in Supplementary Table S2.

Immunoblotting and immunofluorescence

Immunoblotting was carried out as described previously (7) and is also described in Supplementary Materials. For immunofluorescence, cells were transfected with precursors of miR-34b or cont-miR for 72 hours, washed, and fixed with acetone–methanol (1:1) mixture. Cells were then blocked with 10% normal goat serum blocking solution (Zymed Laboratories) and hybridized with the specific primary antibodies against EMT markers. Cells were washed and hybridized with fluorescein conjugated secondary
antibody (1:1,000), then washed and mounted with Pro-Long Gold antifade reagent with DAPI (Invitrogen-Life Technologies).

Luciferase assays
For reporter assays, cells were transiently transfected with wild-type or mutant reporter plasmid and miR-34b 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. Complementary sequences are given in Supplementary Table S2.

Chromatin immunoprecipitation assay
Chromatin immunoprecipitation (ChIP) assays were conducted using the EZ-ChIP Kit (Upstate Biotechnology) as described previously (7). Immunoprecipitation was carried out using antibodies purchased from Upstate Biotechnology. Power SYBR Green PCR Mastermix was used to carry out real-time PCR with a 7500 Fast Real-Time PCR System (both from Applied Biosystems). Signals were also confirmed by conventional PCR and gel analyses. Primer sequences are given in Supplementary Table S2.

Flow cytometry, cell viability, migration, clonability, and invasion assays
Fluorescence-activated cell sorting (FACS) analysis for cell cycle and apoptosis was done 72 hours after transfection using nuclear-stain DAPI for cell-cycle analysis or Annexin V-FITC/7-AAD KIT (Beckman Coulter) 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 instructions. For colony-formation assay, cells were seeded at low density (1,000 cells/plate) and allowed to grow until visible colonies appeared. Then, cells were stained with Giemsa and the colonies were counted. The Cytoselect 24-well cell migration and invasion assay kit (Cell Biolabs) was used for migration and invasion assays according to the manufacturer’s instructions.

In vivo intratumoral delivery of miR-34b
The antitumor effect of miR-34b was determined by local administration of miR-34b precursor in established tumors. Each mouse was injected with 5.0 x 10^6 PC3 prostate cancer cells subcutaneously, and there were 5 mice in each group. After palpable tumors developed (average volume, 50 mm^3), 6.25 µg of synthetic miRNA-34b and/or control 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 institutional guidelines.

Statistical analysis
Statistical analyses were conducted with StatView version 5 for Windows, GraphPad Prism 5, and MedCalc version 10.3.2. All quantified data represent an average of at least triplicate samples or as indicated. Error bars represent standard deviation of the mean. All tests carried out were 2-tailed, and P values 0.05 or less were considered statistically significant. Receiver operating curves (ROC) were calculated to determine the potential of miR-34b or its methylation to discriminate between malignant and nonmalignant samples. Chi-square tests were conducted to determine the correlation among miR-34b expression, clinicopathologic characteristics, and disease-progression analysis. Kaplan–Meier (log-rank test) analysis was also carried out for survival analysis.

Results
Downregulation of miR-34b expression and its correlation with clinicopathologic characteristics of patients
Expression of miR-34b was examined in prostate cell lines and tissue samples by miRNA-quantitative real-time PCR (qRT-PCR). The expression of miR-34b was downregulated in cancer cell lines PC3 and LNCaP compared with the nonmalignant cell line RWPE1 (Fig. 1A). To examine the biological significance of miR-34b, its expression was analyzed in 148 laser-captured microdissected (LCM) matched human tissue samples (Fig. 1A) together with an unmatched group of 27 benign prostatic hyperplasia (BPH) and 20 tumor samples (Supplementary Fig. S1A). To validate the reproducibility of the data, we also carried out in situ hybridization on commercially available tissue arrays (Cat. No. PR956; US Biomax) to determine miR-34b expression in an independent cohort of tissues samples (Supplementary Fig. S1B). Almost all carcinoma samples showed significant downregulation of miR-34b expression with respect to either normal or BPH samples (Fig. 1B, Supplementary Fig. S1A and S1B). Among the 74 pairs of matched samples, 63 pairs had either significantly lower or no expression of miR-34b (<0.8-fold) in tumors versus normal samples (P < 0.0001). 3 pairs had equal expression (0.8- to 1.2-fold), whereas 8 pairs had higher miR-23b expression (>1.2-fold) in tumors compared with their matched normal samples. These results suggest that miR-34b is significantly downregulated and might be a putative tumor suppressor in prostate cancer.

Clinical demographics of the study cohorts are summarized in Supplementary Table S1. ON the basis of the relative expression of miR-34b, we grouped the samples into low and high miR-34b expression groups and assessed the correlation with clinicopathologic variables like Gleason grade, pathologic stage (pT), and biochemical recurrence (Supplementary Fig. S1C). The time of recurrence was defined as the first postoperative PSA value greater than 0.1 ng/mL, confirmed by at least 1 undetectable PSA level (detection limit <0.04 ng/mL) after surgery. Seventeen patients experienced a biochemical relapse according to this criterion. A significant correlation was observed between miR-34b expression and clinical variables. Decreased miR-34b expression was observed in 96% of cases with higher
pathologic stage (pT3-pT4) compared with 80% cases of low pathologic stage (pT2; \( P < 0.001 \)). To analyze the correlation with Gleason grade, we divided the samples into low (4–6), medium (7), and high (8–10) Gleason grade groups. Low expression of miR-34b was found in 73% cases of low, 90% cases of medium, and 100% cases of high Gleason grade groups (\( P < 0.0001 \)). In PSA recurrence patients, 82% of cases had decreased miR-34b expression compared with 18% cases with high miR-34b expression (\( P < 0.0001 \)). These results reveal that the number of cases with low miR-34b expression increases from low-grade, low pathologic stage to high-grade and high pathologic stage. Furthermore, PSA recurrence patients also had significantly low miR-34b expression, suggesting that miR-34b might have prognostic value in prostate cancer (Supplementary Fig. S1C).

ROC analyses were conducted to evaluate the capability of miR-34b to discriminate between normal and tumor tissues using 148 LCM tissue samples. An area under the ROC curve (AUC) of 0.973 (\( P < 0.0001; 95\% \) confidence interval [95\% CI], 0.930–0.993; Fig. 1C) was obtained suggesting that miR-34b expression can discriminate between malignant and nonmalignant samples and might be useful as a diagnostic marker for prostate cancer. To determine whether miR-34b expression has prognostic significance, we divided the matched samples into low (T/N < 0.8-fold) and high (T/N > 0.8-fold) miR-34b expression groups and conducted Kaplan–Meier survival analysis. In Kaplan-Meier analysis, the miR-34b high expression group displayed significantly higher overall survival probability compared with the low expression group (log-rank test, \( P < 0.02 \); Fig. 1C). We also analyzed recurrence-free survival and found that the miR-34b high expression group had significantly higher recurrence-free survival probability compared with the low expression group (log-rank test, \( P = 0.02 \); Fig. 1C).
survival and risk of biochemical recurrence. Results indicated that cases that had low miR-34b expression had poor recurrence-free survival (log-rank test, $P < 0.02$) and a higher risk of biochemical recurrence (hazard ratio, 3.3; 95% CI, 1.3–8.7; Fig. 1C). These results suggest that miR-34b can predict the biochemical recurrence of prostate cancer and has potential to be a diagnostic/prognostic biomarker, although a larger sample size is needed to confirm these results.

Mechanism of miR-34b silencing in prostate cancer is through CpG hypermethylation

To investigate the mechanism involved in the depleted levels of miR-34b in prostate cancer, we carried out methylation analysis on the 1.0-kb upstream sequence of miR-34b. Primers are shown in Supplementary Table S2. CpG rich regions were found (Supplementary Fig. S1D) and further analyzed for methylation status in prostate cancer cell lines and tissues. Our results show that this sequence is hypermethylated in prostate cancer cell lines, with an average methylation percentage of 94% (Fig. 1D). We also investigated the methylation status of miR-34b in 32 pairs of matched LCM tissue samples by methylation-specific qRT-PCR. Our results indicated that the tumor samples had a higher methylation percentage compared with their normal counterparts (Fig. 1D). For the 19 pairs of samples where DNA and miRNA was available, we found a significant inverse correlation between miR-34b expression and methylation. Samples with low miR-34b expression had higher average percentage of methylation and vice versa ($P < 0.0001$; Supplementary Fig. S1E). We also conducted ROC analysis to evaluate the ability of miR-34b methylation percentage to distinguish malignant from normal tissues. The AUC observed was 0.814 ($P < 0.0001$; 95% CI, 0.697–0.990; Fig. 1D), showing that the percentage of miR-34b methylation is help distinguish malignant from normal tissues.

Reconstitution of miR-34b suppresses DNMTs and HDACs

As miR-34b is epigenetically silenced in prostate cancer and DNA methylation is linked to histone modifications, we asked whether reconstitution of miR-34b in cancer cells could downregulate DNMTs or HDACs. We overexpressed miR-34b in PC3 and LNCaP cells (Fig. 2A). Our results show downregulation of DNMT1, DNMT3b, HDAC1, HDAC2, HDAC3, and HDAC4 protein expression in miR-34b-overexpressing cells compared with negative control (Fig. 2B). Complimentary sequences for miR-34b in the 5′-UTR of these genes is given in Supplementary Table S2. To determine whether miR-34b targets these genes directly, we carried out luciferase reporter activity assays. The results show that DNMT1, HDAC1, HDAC2, and HDAC4 are direct targets as the relative luciferase activity was significantly decreased with cotransfection of the wild-type 3′-UTRs of these genes together with miR-34b (Fig. 2C). A decrease in luciferase activity was also observed in the case of DNMT3b and HDAC3, although it was not significant. Further, we attenuated miR-34b expression by using antimiR-34b in LNCaP cells because these cells had higher expression than PC3. Depletion of miR-34b in LNCaP cells rescued the protein expression of these genes (Fig. 2D), which again confirms that miR-34b targets these genes in prostate cancer.

Ectopic expression of miR-34b induces partial demethylation and active chromatin modifications in 5′-upstream sequence of the miR-34b gene

Because miR-34b suppressed DNMTs, we looked to see if its reconstitution could also induce demethylation in prostate cancer cells. Our results show that overexpression of miR-34b caused partial demethylation of CpG sites in the 5′-upstream sequence of the miR-34b gene as methylation decreased from 96 ± 3% in controls to 84 ± 2% in miR-34b-transfected cells (Fig. 3A). Epigenetic regulation of miRNA genes is tightly linked to chromatin signatures and transcriptionally active miRNA genes are characterized by active chromatin marks such as trimethylated histone H3 lysine 4 (3H3K4; ref. 22). To investigate whether these active chromatin modifications are induced in epigenetically regulated miR-34b gene, we conducted ChiP assay by PCR and qRT-PCR in the same sequence where we determined methylation. We found enrichment of active chromatin modifications and a modest decrease in repressive ones (Fig. 3B). These changes are indicative of active genes and suggest that overexpression of the miR-34b gene downregulates DNMTs and HDACs, which in turn causes a positive feedback loop to induce demethylation and active modifications.

Functional significance of miR-34b in prostate cancer

Next, we determined the functional significance of miR-34b in prostate cancer by carrying out cell cycle, proliferation, clonability, migration and invasion assays. A significant decrease in cell proliferation was observed over time in PC3 and LNCaP cells overexpressing miR-34b (Fig. 4A) as compared with controls. The miR-34b-transfected cells also had low colony-forming ability, as both size and number of foci in miR-34b-overexpressing cells was reduced when compared with controls (Fig. 4A). FACS analysis revealed that re-expression of miR-34b leads to a significant increase (12–17%) in the number of cells in the G0–G1 phase of the cell cycle, whereas the S-phase population decreased from 20% to 5% to 8% suggesting that miR-34b causes a G0–G1 arrest in miR-34b-transfected cells (Fig. 4B). FACS analysis for apoptosis was carried out using Annexin-V-FITC-7-AAD dye. The percentage of total apoptotic cells (early apoptotic + apoptotic) was significantly increased (12–15%) in response to miR-34b transfection compared with cont-miR (1%), with a corresponding 16% decrease in the viable cell population (Fig. 4C). Collectively, these results indicate a tumor suppressor role of miR-34b in prostate cancer. To determine the pathway involved in the antiproliferative effect of miR-34b, we analyzed the protein expression of Akt proliferative pathway genes after miR-34b overexpression (Fig. 4D). miR-34b significantly suppressed phosphorylation of AKT(Ser473) and its downstream target GS3β.
Figure 2. Ectopic expression of miR-34b reduced expression of methyltransferases (DNMT) and deacetylases (HDAC) in prostate cancer cells. A, overexpression of miR-34b in PC3 and LNCaP cells. B, ectopic expression of miR-34b inhibits DNMTs and HDACs at the protein level. C, luciferase assays showing decreased reporter activity after cotransfection of wild-type 3′-UTR with miR-34b in PC3 prostate cancer cells. The scrambled 3′-UTR sequence had no effect on reporter activity. D, inhibition of miR-34b by antagoMirs rescued the expression of DNMTs and HDACs.
ly higher in tumors receiving miR-34b compared with tumors and found that miR-34b expression was significant.

We measured the expression of miR-34b in harvested miR-34b by local administration into established tumors. Quantification of the immunoprecipitated upstream region of miR-34b was determined by qRT-PCR and normalized to input DNA. Signals were also confirmed by conventional PCR, and PCR product for each sample was resolved on 2% agarose gel and visualized by staining with ethidium bromide.

In vivo tumorigenicity

Intratumoral delivery of miR-34b suppresses EMT markers and suppresses cell migration and invasion independent of proliferation

Cell migration and invasion assays revealed that miR-34b-overexpressing cells were less proficient than controls in migrating or invading a barrier membrane, with less absorbance observed at 560 nm (Fig. 5A and B). We examined the effect of overexpression of miR-34b on EMT markers and observed a decrease in vimentin, ZO1, N-cadherin, and Snail (mesenchymal markers) and an increase in E-cadherin (epithelial marker) (Fig. 5C–D). The process of EMT is involved in epithelial-derived tumors, causing them to become invasive and metastatic, and these results show that miR-34b exerts antiproliferative effects in prostate cancer at least partly through the Akt pathway.

miR-34b downregulates EMT markers and suppresses cell migration and invasion independent of proliferation

One of the most common causes of the loss of tumor suppressor miRNAs in human cancer is the silencing of their primary transcripts by CpG island promoter hypermethylation. MiR-34b downregulates DNMTs, HDACs, and induces partial demethylation and active chromatin modifications in prostate cancer.

Discussion

In this study, we provide evidence that miR-34b is silenced in prostate cancer and the mechanism is mediated through CpG hypermethylation. MiR-34b downregulates DNMTs, HDACs, and induces partial demethylation and active chromatin modifications in prostate cancer. Low expression of miR-34b can predict overall and recurrence-free survival and can distinguish normal from malignant tissues. We also elucidated the tumor suppressor role of miR-34b in prostate cancer and determined that the antiproliferative and antimigratory/invasive effects of miR-34b is partly through downregulation of the Akt pathway and EMT markers. We also show that miR-34b inhibits tumorigenicity both in vitro and in vivo, this being the first report documenting the detailed role of miR-34b in prostate cancer.

One of the most common causes of the loss of tumor suppressor miRNAs in human cancer is the silencing of their primary transcripts by CpG island promoter hypermethylation (17, 27). In fact, DNA methylation–mediated downregulation of miRNAs by proximal CpG islands has been described by a number of groups (16, 17, 28). Further identification of additional methylation targets may clarify the specific molecular events involved in prostate cancer progression, which hopefully will lead to prevention, diagnosis, and treatment of prostate cancer at the molecular level.

miR-34b in Prostate Cancer

Figure 3. Induction of partial demethylation and active chromatin modifications. A, ectopic expression of miR-34b induces partial demethylation of CpG islands. B, enrichment of active chromatin modifications by overexpression of miR-34b. ChIP assay in PC3 cells transfected with miR-34b or control miR was carried out. Quantification of the immunoprecipitated upstream region of miR-34b was determined by qRT-PCR and normalized to input DNA. Signals were also confirmed by conventional PCR, and PCR product for each sample was resolved on 2% agarose gel and visualized by staining with ethidium bromide.
level. Here, we identified miR-34b is frequently silenced in tumors compared with normal samples and the mechanism of silencing is through DNA methylation in prostate cancer tissues. Previous studies have reported CpG methylation of miR34b/c in colorectal cancer (16), oral squamous cell carcinoma (28), malignant melanoma where it correlated...
Figure 5. miR-34b impairs prostate cancer cell migration and invasion independent of antiproliferative effect. A and B, migration and invasion assays of PC3 and LNCaP cells transfected with miR-34b or a cont-miR. C and D, downregulation of EMT markers at the protein level determined by Western blot analysis and immunofluorescence in PC3 prostate cancer cells transfected with miR-34b or cont-miR.
with metastatic potential (26), and various other cancers (29). Our study indicates that miR-34b methylation status can discriminate between normal prostate tissue and cancer samples making it a potential diagnostic marker for prostate cancer. miRNAs possess several features that make them attractive candidates as new prognostic biomarkers and tools for the early diagnosis of cancer (13, 30, 31). In this study, we show that miR-34b expression could predict overall survival and recurrence-free survival such that patients with high miR-34b levels had longer survival. Similarly, promoter hypermethylation of miR-34b/c has been reported as a common event in non–small cell lung cancer (NSCLC) and a potential prognostic factor for stage 1 NSCLC. Thus, aberrant DNA methylation of miR-34b/c was correlated with a high probability of recurrence and associated with poor overall and disease-free survival of patients with stage 1 NSCLC (32).

To link altered miR-34b expression with aberrant CpG methylation, we looked at the ectopic effect of miR-34b on DNMTs and HDACs because their levels are reported to be elevated in prostate cancer (33, 34). DNMTs catalyze the methylation of genomic DNA resulting in transcriptional repression. DNMTs are also known to recruit HDACs, leading to histone deacetylation and transcriptional repression (35, 36). DNMTs and HDACs are expressed at higher levels in prostate cancer than the nonmalignant prostate cells although the degree of expression varies among the cell lines on the basis of the cell context (33, 37, 38). Androgen-independent cell lines such as Du145 and PC3 express HDACs at higher levels than androgen-dependent cell lines such as LNCaP (37). Various reports have shown that miRNAs downregulate DNMTs (39) and HDACs (40). The miRNA-29 family directly targets DNMTs and restored normal patterns of DNA methylation in lung cancer cell lines (39). Similarly, restoration of miR-97 levels in Waldenstrom macroglobulinemia (WM) cells downregulated HDACs with an upregulation of histone-H3 and –H4 (40). Further epigenetic regulation of miRNA genes has been found to be tightly linked to chromatin signatures, and transcriptionally active miRNA genes are characterized by

Figure 6. Antitumorigenic effect of miR-34b in vivo. A, average expression of miR-34b in excised tumors. B, tumor volume following intratumoral injection of Cont-miR or miR-34b precursor into established tumors. P < 0.05. C, schematic representation of pleiotropic tumor suppressor effect of miR-34b in prostate cancer. miR-34b is silenced through hypermethylation in prostate cancer. Reconstitution of miR-34b results in downregulation of DNMTs and HDACs. This provides a positive feedback to further demethylate hypermethylated miR-34b and induce active chromatin modifications that are signatures of active genes. miR-34b overexpression also inhibits the Akt pathway and EMT markers resulting in antiproliferative, antimigratory/anti-invasive effects in prostate cancer.
Our study also elucidated the tumor suppressor role and functional significance of miR-34b in prostate cancer. Results showed that miR-34b has an antiproliferative effect and induced $G_{0}$/$G_{1}$ cell-cycle arrest and apoptosis. To determine the antiproliferative and apoptotic effects of miR-34b, we focused on Akt-GSK-betacatenin-cMyc-Cyclin D1 proliferative pathway genes. Signaling through PI3K/Akt pathway controls cell proliferation and apoptosis (41, 42). Akt mediates these effects by modulating cell-cycle and apoptosis regulatory proteins (43), and changes in expression of these genes have been linked to prostate cancer (44). Other studies have reported that advanced human prostate cancer was accompanied by the expression of phosphorylated Akt (45) and alterations in the serine/threonine kinase Akt/PKB pathway have been detected in a number of human malignancies (46). Akt has a wide range of downstream targets that regulate tumor-associated cell processes such as cell growth, cell-cycle progression, survival, migration, EMT, and angiogenesis (46). Our results confirmed that miR-34b directly targeted Akt and caused downregulation of downstream proteins pGSK, total β-catenin, c-Myc, and cyclin D1 that are involved in cell proliferation/survival. These results suggest that the antiproliferative effect of miR-34b is partly mediated through the Akt pathway. We also observed a decrease in the migratory and invasive capabilities of prostate cancer cells overexpressing miR-34b and found that it effected EMT markers. EMT is a multifaceted transdifferentiation program that enables tumor cells to acquire malignancy-associated phenotypes (47). This initial step of local invasion may be triggered by signals that carcinoma cells receive from the nearby stroma, causing them to undergo an EMT and subsequently metastasize to distant tissue sites (48). Considerable research has been focused on identifying the critical regulators of this metastatic process including both proteins and miRNAs (48). Our results show that overexpression of miR-34b caused a decline in the mesenchymal markers vimentin, ZO1, N-cadherin, and Snail whereas there was an increase in the expression of E-cadherin, an epithelial marker. These results suggest that miR-34b suppresses migration and invasion independent of cell proliferation and that it has an anti-metastatic effect on prostate cancer cells.

The antitumorigenic effects of miR-34b observed in this study were also confirmed in a prostate cancer xenograft nude mouse model. In vivo experiments showed a striking suppression in subcutaneous tumor growth in nude mice where miR-34b was directly administered to tumors compared with controls. Therefore, our study shows that miR-34b has an important tumor suppressor role both in vitro and in vivo.

In summary, our study shows that miR-34b is an important tumor suppressor that is epigenetically silenced in prostate cancer. Overexpression of miR-34b reduced DNMTs and HDACs, inducing partial demethylation and active chromatin modifications. MiR-34b expression can also predict overall and recurrence-free survival of patients with prostate cancer. MiR-34b induces antiproliferative and anti-invasive effects partly by inhibiting the Akt pathway and EMT markers. This study documents the pleiotropic role of miR-34b in prostate cancer. These findings offer novel insight into the association between dysregulation of miRNA and methylation/chromatin modification and provide a strong rationale for the development of new strategies targeting the epigenetic regulation of miRNAs for the treatment of prostate cancer.

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

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