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miR-1207-3p regulates the androgen receptor in prostate cancer via FNDC1/fibronectin

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Abstract

Prostate cancer (PCa) is frequently diagnosed in men, and dysregulation of microRNAs is characteristic of many cancers. MicroRNA-1207-3p is encoded at the non-protein coding gene locus PVT1 on the 8q24 human chromosomal region, an established PCa susceptibility locus. However, the role of microRNA-1207-3p in PCa is unclear. We discovered that microRNA-1207-3p is significantly underexpressed in PCa cell lines in comparison to normal prostate epithelial cells. Increased expression of microRNA-1207-3p in PCa cells significantly

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Author contributions

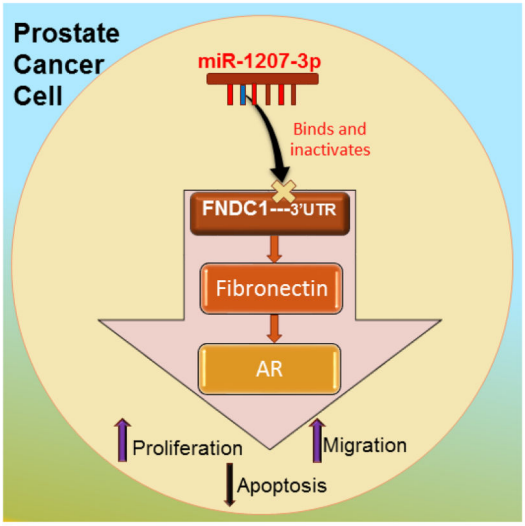
DD wrote the main manuscript and prepared figures 1-6. AI prepared figure 1b and reviewed the manuscript. JYP prepared table 1 and corresponding method section. TA and KK prepared supplementary figure S3 and corresponding method section. BDR prepared figure 5 and corresponding method section. JRO made intellectual contributions and reviewed the manuscript. OOO supervised all the work from conception to manuscript preparation and review. All authors reviewed the manuscript.

Disclosure of Potential Conflicts of Interest

The authors declare no potential conflicts of interests.

inhibits proliferation, migration, and induces apoptosis via direct molecular targeting of FNDC1, a protein which contains a conserved protein domain of fibronectin (FN1). FNDC1, FN1, and the androgen receptor (AR) are significantly overexpressed in PCa cell lines and human PCa, and positively correlate with aggressive PCa. Prostate tumor FN1 expression in patients that experienced PCa-specific death is significantly higher than in patients that remained alive. Furthermore, FNDC1, FN1 and AR are concomitantly overexpressed in metastatic PCa. Consequently, these studies have revealed a novel microRNA-1207-3p/FNDC1/FN1/AR regulatory pathway in PCa.

Graphical abstract



miR-1207-3p/FNDC1/FN1/AR is a novel regulatory pathway in prostate cancer.

Keywords

miR-1207-3p; fibronectin type III domain containing 1; fibronectin; androgen receptor; prostate cancer

Introduction

Prostate cancer (PCa) is the second most common cause of cancer-specific deaths in the U.S. accounting for approximately 13 percent of cancer-related deaths[1-3]. In 2014, it was estimated that the U.S. had 233,000 new cases of and 29,480 deaths from PCa [4,5]. Established risk factors include familial genetics, and African ancestry[4,6]. Thus, PCa is a multi-factorial, complex disease, with the exact mechanisms for its development and progression unclear. Understanding the molecular mechanisms underlying the development and progression of PCa is necessary. This will aid in the discovery of novel and efficacious biomarkers with applications in early PCa detection and molecular therapeutic targeting.

Genome-wide association studies (GWAS) have identified several loci and genetic variants that increase the risk of PCa. One of the most important susceptibility loci for PCa is the

8q24 human chromosomal region[7-9]. Recent studies demonstrated that PVT1, which is a 300kb long non-protein coding gene locus found at the 8q24 human chromosomal region, is dysregulated in PCa[10-14]. The PVT1 gene locus encodes six microRNAs (miRNAs). Interestingly, five of the six microRNAs are not associated with annotated PVT1 exons, including microRNA-1207-3p (miR-1207-3p)[10,15]. Furthermore, no known biological functions for this intron-derived miR-1207-3p has been previously reported.

Aberrant miRNA expression and function has potential applications in cancers. The objective of this study was to determine the expression, function, and molecular mechanisms of action of miR-1207-3p in PCa. Our study discovered a novel role of miR-1207-3p in the regulation of critical cellular functions in PCa via targeting of fibronectin type III domain containing 1 (FNDC1), leading to loss of fibronectin (FN1) expression and subsequent loss of androgen receptor (AR) expression. We also discovered that components of this novel molecular pathway, FNDC1, FN1, and AR are overexpressed in metastatic PCa. This discovery of a novel miR-1207-3p-dependent regulatory mechanism in PCa reveals the possibility of clinical applications for miR-1207-3p in PCa.

Materials and methods

Cell culture

Detailed description is contained in supplemental text. All cell lines have been tested and authenticated.

Transfection of oligonucleotide inhibitor and mimic of miR-1207-3p

Cells were seeded in 6-well plates. After reaching 60% - 70% confluence, media is replaced with Opti-MEM (Thermo Fisher Scientific Inc; Wilmington, DE, U.S.A) and cells are transfected with either a 50nM non-targeting negative control oligonucleotide (MISSION® Synthetic microRNA Negative Control, product# NCSTUD001), 50nM miR-1207-3p oligonucleotide mimic (MISSION® microRNA Mimic, product# HMI0066), or 50nM miR-1207-3p oligonucleotide inhibitor (MISSION® Synthetic microRNA Inhibitor, human, product# HSTUD0066) (Sigma-Aldrich, St. Louis, MO, USA) using Lipofectamine RNAiMAX (Thermo Fisher Scientific Inc; Wilmington, DE, USA) according to the manufacturer's instructions. Transfected cells are then incubated at 37°C for 24 hours after which media is replaced with cell line specific culture media.

Transfection of siRNAs

Transfection of siRNAs was performed as previously described [16].

RNA isolation and quantitative real-time polymerase chain reaction (qPCR) analysis

RNA isolation and qPCR were performed as previously described [17]. miR-1207-3p and U6 snRNA primers were purchased from Thermo Fisher Scientific Inc; Wilmington, DE, U.S.A. Primers for human fibronectin mRNA, and, human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were custom-designed using OligoPerfect™ Designer (Thermo Fisher Scientific Inc; Wilmington, DE, U.S.A).

For the human study at Moffitt Cancer Center, prostate cancer tissues were collected from 377 primary prostate cancers with macro-dissection. Total RNA of tumor tissues was extracted and RNA samples from the 377 patient cases were analyzed by qPCR to examine the mRNA expression levels of fibronectin. Ct-values were determined using the SDS ver2.3 software (Bio-Rad). Fibronectin expression was normalized with GAPDH expression within each sample. Relative quantification of target gene expression was evaluated using the comparative cycle threshold (Ct) method. The following primers were used: Fibronectin-F: TCG AGG AGG AAA TTC CAA TG; Fibronectin-R: ACA CAC GTG CAC CTC ATC AT; GAPDH-F: ACC ACA GTC CAT GCC ATC AC; GAPDH-R: TCC ACC ACC CTG TTG CTG TA.

Western blot analysis

Western blotting was performed as previously described[18]. Primary antibodies used were against fibronectin: ab2413 (ABcam plc, Cambridge, UK), human FNDC1 (Y-12): sc-107546 (Santa Cruz Biotechnology, TX, USA), human GAPDH: g9545 and beta-actin: a5441 (Sigma-Aldrich, St. Louis, MO, USA). Secondary antibodies used were either against mouse or rabbit (Sigma-Aldrich, St. Louis, MO, USA) or goat (Santa Cruz Biotechnology, TX, USA), as appropriate. In some cases, Li-COR Odyssey CLx with infrared fluorescence, IRDye secondary antibodies and imagers were also used to detect western blots without film or chemiluminescent substrates. Western blots were analyzed and quantified with Odyssey imager, Image Studio version 5.

Click-iT EdU Alexa Fluor 488 Imaging

Click-iT EdU Alexa Fluor 488 Imaging assay was performed as described[19]. Additional description of this method is contained in the supplemental text.

Wound Healing Assay

Wound healing assay was performed as previously described[17]. Images were taken using Motic Images Plus 2.0 Software (Motic; British Columbia, Canada).

Annexin V Assay

Annexin V staining was performed as described[20]. Additional description of this method is contained in the supplemental text.

Luciferase reporter assay

Luciferase reporter assay was performed as described. Additional description of this method is contained in the supplemental text.

RNA pulldown assay

RNA pulldown assay was performed with modifications of the protocol described by Wani *et al*[21]. Cells were seeded in 10cm plates. Cells were transfected with either a 1nM synthetic biotinylated scramble duplex (patent pending), or 1nM synthetic biotinylated miR-1207-3p duplex (patent pending) using Lipofectamine RNAiMAX (Thermo Fisher Scientific Inc; Wilmington, DE, USA) according to the manufacturer's instructions. Transfected cells were

then incubated at 37°C for 24 hours after which media was replaced with cell line specific culture media. Beads were washed, blocked and prepared as described[21]. Cells were harvested and lysed after 24 hours. Target mRNA was captured and post-capture bead washing was performed. Next, target mRNA and control lysate RNA were purified using Qiagen RNeasy kit according to manufacturer's RNA clean-up protocol. The target mRNA and the control lysate were then quantified by Nanodrop 1000 spectrophotometer. For the identification of direct targets of the mature miRNA, using the synthetic biotinylated miR-1207-3p duplex, samples were analyzed by qPCR to examine the mRNA expression levels and enrichment of FNDC1.

Statistical Analysis

Data was collected from at least three independent experiments. All results are presented as mean ± standard error of the mean (SEM). Unless otherwise indicated, analysis of statistical significance of differences between groups was performed using two-tailed Student's *t*-test, and only values with $P < 0.05$ were deemed significant.

For comparison of variables, a student's t test or analysis of variance (ANOVA) test were used for analysis of each set of continuous and categorical data. Statistical differences in the relative microRNA expression profiles were determined with one-way and two-way analysis of variance (ANOVA) using the SPSS Statistics software (http://www-01.ibm.com/software/analytics/spss/) on normalized data. P values <0.05 were considered significant. One-way ANOVA was used for Figures 1 B - E, Figure 3A and C. Two-way ANOVA was used for figures 6B, C, G-I.

Results

miR-1207-3p is significantly underexpressed in PCa cells

To investigate the role of miR-1207-3p in PCa, a panel of 8 prostate cell lines modeling different clinical characteristics of PCa was used. The panel included WPE1-NA22 (derived from RWPE-1, indolent, androgen-dependent, from Caucasian male), MDA PCa 2b (aggressive, androgen-dependent, from Black male), PC-3 (aggressive, androgen-independent, from Caucasian male), E006AA (indolent, androgen-independent, from Black male), E006AA-hT (derived from E006AA, aggressive, androgen-independent, from Black male), LNCaP (aggressive, androgen-dependent, from Caucasian male) and C4-2B (derived from LNCaP, aggressive, androgen-independent, from Caucasian male) and compared to a non-tumorigenic prostate epithelial cell line, RWPE-1 (from Caucasian male) (Figure 1a). As shown in figure 1b, miR-1207-3p expression is significantly underexpressed in all the human PCa cell lines in comparison to non-tumorigenic prostate epithelial cells, RWPE-1. We observed approximately a 50% decrease in androgen-dependent PCa cell lines (WPE1-NA22, MDA PCa 2b and LNCaP). Interestingly, miR-1207-3p was even further reduced, by approximately 80%, in androgen-independent cell lines (PC-3, E006AA, E006AA-hT and C4-2B). This indicates that loss of miR-1207-3p may be an early event in PCa development that contributes to PCa progression.

To study the role of miR-1207-3p expression in PCa cell function, a synthetic oligonucleotide mimic of miR-1207-3p (miR-1207-3p mimic), a synthetic oligonucleotide inhibitor of miR-1207-3p (miR-1207-3p inhibitor), or a synthetic non-targeting negative control oligonucleotide (negative control) were transfected into the cells using Lipofectamine® RNAiMAX. A dose-response experiment analyzed using qPCR demonstrated that the miR-1207-3p inhibitor inhibits endogenous expression of miR-1207-3p while the miR-1207-3p mimic imitates endogenous miR-1207-3p expression; both in a dose-dependent fashion (Figure 1c-e). The 50nM concentration of the miR-1207-3p inhibitor and the miR-1207-3p mimic that showed maximal specific effect on miR-1207-3p expression was used to determine the role of miR-1207-3p in regulating proliferation, migration and apoptosis in PCa cells.

Overexpression of miR-1207-3p suppresses endogenous expression of fibronectin type III domain containing 1 (FNDC1) and fibronectin (FN1) in PCa cells

To examine the molecular mechanisms through which miR-1207-3p exerts its PCa-inhibitory effects, the molecular targets of miR-1207-3p were investigated. The molecular targets of miR-1207-3p have never been studied in any system. Potential targets were initially screened using two different miRNA molecular target prediction algorithm tools (miRBase: http://www.mirbase.org/ and miRDB: http://mirdb.org/) which identified Fibronectin type III domain containing 1 (FNDC1) as a putative molecular target of miR-1207-3p. FNDCI contains the conserved ‘Fibronectin type III domain’ of Fibronectin (FN1).[22,23] FN1 is a glycoprotein consisting of three domains.[24-26] These FN1 domains (type I, type II and type III), have undergone exon shuffling resulting in many of them also being found in other molecules.[22,27,28] FN1 has been implicated in carcinogenesis and known to be a regulator of cell migration, proliferation and apoptosis.[29-32]

To initially determine if there is a relationship between FNDC1 expression, FN1 expression and miR-1207-3p expression, we analyzed protein expression of FNDC1 in the prostate epithelial cell lines described in figure 1a. We observed that FNDC1 protein expression was consistently higher in all the PCa cell lines compared to the non-tumorigenic prostate cell line, RWPE-1 (Figure 2a). In repeated experiments, RWPE-1 had very low FNDC1 protein expression. Further, overexpression of miR-1207-3p significantly inhibited the protein expression of FNDC1 by about 75% (Figure 2b).

miR-1207-3p directly targets FNDC1

To confirm that FNDC1 is a direct molecular target of miR-1207-3p, we performed a dual-luciferase reporter assay using the Luc-Pair™ Duo-Luciferase assay system to determine if miR-1207-3p binds to the 3’ untranslated region (UTR) of the FNDC1 mRNA. We used widely used PCa cell lines that model various characteristics of prostate cancer. Because of the significantly low level of endogenous expression of miR-1207-3p in the PC-3 and MDA PCa 2b PCa cell lines and their widespread use, we used them as cellular models for this assay. PC-3 and MDA PCa 2b cells were co-transfected with both the plasmid containing the sequence of the FNDC1 3’UTR and miR-1207-3p 50nM mimic. Cells were transfected with 3’UTR clones of FNDC1 with a synthetic non-targeting oligonucleotide negative control as the control. We observed a direct and specific interaction between exogenous miR-1207-3p

and the FNDC1 3'UTR. Overexpression of miR-1207-3p led to the suppression of activity of the luciferase reporter gene fused to the FNDC1 3'UTR by about 40% in PC-3 cells and about 60% in MDA PCa 2b cells (Figure 2c) compared to the cells transfected with the non-targeting 50nM oligonucleotide negative control.

As nearly 20% of miRNA mediated repression of target mRNAs occur without the canonical base pairing to the seed sequence, but rather by imperfect binding to the center of miRNA sequence, we further confirmed and validated that miR-1207-3p directly binds to FNDC1 by performing RNA pulldown using a synthetic biotinylated miR-1207-3p duplex.[21] This approach allows for the sensitive and specific detection of miRNA-mRNA interactions[21]. MDA PCa 2b cells was transfected with either 1nM synthetic biotinylated miR-1207-3p duplex or 1nM synthetic biotinylated scramble duplex as control. RNA was subsequently pulled down with streptavidin coated magnetic beads. The RNA was then analyzed for FNDC1 expression with qPCR. We observed that compared to the RNA pulled down by the synthetic biotinylated scramble duplex, the RNA pulled down by the synthetic biotinylated miR-1207-3p duplex was significantly enriched for FNDC1 by about 2,000-fold (Figure 2d). Therefore, FNDC1 is a direct molecular target of miR-1207-3p.

miR-1207-3p regulates FN1 via FNDC1

To examine the relationship between FN1 expression and miR-1207-3p, we analyzed mRNA and protein expression of FN1 in the previously described cell lines (Figure 1a). We observed that FN1 mRNA levels were significantly higher in all the PCa cells compared to the non-tumorigenic prostate cell line, RWPE-1. FN1 mRNA expression was relatively low in RWPE-1 cells (Figure 3a). Interestingly, FN1 expression was higher in all the PCa cell lines from Black men (MDA PCa 2b, E006AA and E006AA-hT) than in those from White men, suggesting that FN1 may play a role in the racial disparity in PCa (Figure 3a). Further validation by western blotting showed that increased FN1 protein expression in all the PCa cell lines as compared to the non-tumorigenic prostate epithelial cell line, RWPE-1. FN1 protein expression was very low in RWPE-1 (Figure 3b). Moreover, three of the five cell lines demonstrating the highest expression of FN1 were those derived from Black men. Furthermore, we assessed the effect of inhibition or overexpression of miR-1207-3p on FN1 expression in RWPE-1 cells. qPCR analysis showed that overexpression of miR-1207-3p induced nearly a 30% reduction of endogenous FN1 mRNA expression in RWPE-1 cells, while the inhibition of miR-1207-3p led to nearly a 4-fold increase in endogenous FN1 mRNA expression (Figure 3c). In addition, western blotting confirmed that overexpression of miR-1207-3p can significantly inhibit the protein level of FN1, with up to a 40% decrease in FN1 protein expression in three different PCa cell lines examined (Figure 3d). These findings demonstrate that FN1 is a component of the molecular pathway regulated by miR-1207-3p in PCa.

FNDC1 regulates proliferation, apoptosis, and migration of PCa cells

To determine if miR-1207-3p effects on PCa cellular function are due to its inhibitory effect on FNDC1 expression, a FNDC1 small-interfering RNA (siRNA) was designed and used to determine if the effects of loss of FNDC1 expression are identical to the effects of overexpression of miR-1207-3p on PCa cellular function. A dose-response experiment using

MDA PCa 2b cells was performed to determine the efficacy of our custom-designed FNDC1 siRNA in silencing FNDC1 protein expression (Supplementary Figure S1_a). FNDC1 siRNA downregulated the expression of FNDC1 protein expression in MDA PCa 2b cells after 24-hour transfection best at 100pM concentration. This 100pM concentration was used to determine the effect of silencing FNDC1 expression on apoptosis, proliferation and migration of PCa cells. To investigate whether FNDC1 regulates apoptosis, an Annexin V analysis was performed. Transient transfection with FNDC1 siRNA induced nearly 2-fold increase in apoptosis when compared to the scramble siRNA in C4-2b cells (Supplementary Figure S1_b). The EdU proliferation assay revealed that proliferation of MDA PCa 2b cells was inhibited by about 80% when compared to the scramble siRNA (Supplementary Figure S1_c). We observed that migration, assessed via wound healing, of C4-2b cells was reduced significantly by transfection with FNDC1 siRNA as compared to the scramble siRNA by about 50% (Supplementary Figure S1_d). These results demonstrate that knockdown of FNDC1 via siRNA suppresses cellular proliferation and migration while inducing apoptosis in a manner similar to that observed for overexpression of miR-1207-3p, thus indicating that the cellular effects of miR-1207-3p are due to its effect on FNDC1 expression.

FN1 regulates proliferation, apoptosis and migration of PCa cells

Initially, a dose-response experiment was used to determine the efficacy of the custom-designed FN1 siRNA and appropriate concentrations for silencing FN1 protein expression (Supplementary Figure S2_a). We observed that FN1 protein expression in PC-3 cells were reduced most effectively at a concentration of 25pM, with an inhibition by approximately 80%. Therefore, this concentration was chosen to determine if the knockdown of FN1 using siRNA affects cell proliferation, migration and apoptosis in PCa cells in a manner similar to the effect of overexpression of miR-1207-3p. FN1 protein expression decreased by up to 50% after transfection with FN1 siRNA compared to the scramble negative control siRNA in three different PCa cell lines tested (Supplementary Figure S2_b).

A direct analysis of cell proliferation was performed using EdU labeling on MDA PCa 2b cells. After 24 hours of transfection with either a scramble siRNA or 25pM FN1 siRNA, treatment with FN1 siRNA significantly inhibited cell proliferation by about 70% (Supplementary Figure S2_c). An analysis of the effects of FN1 siRNA on apoptosis using PC-3 cells was done by Annexin V analysis. We observed about 50% increase in apoptosis with FN1 silencing (Supplementary Figure S2_d). To assess the effect on cell migration, wound healing assays were performed using WPE1-NA22 cells. Inhibition of FN1 expression using the 25pm siRNA significantly inhibited migration by about 80% in WPE1-NA22 compared to the scramble (Supplementary Figure S2_e). These results indicate that FN1 regulates apoptosis, proliferation and migration of PCa cells. The silencing of FN1 induced apoptosis, and inhibited proliferation and migration in a manner similar to that of silencing of FNDC1, and overexpression of miR-1207-3p.

miR-1207-3p regulates the androgen receptor via FNDC1/FN1

Prostate cancer (PCa) is an androgen-driven disease and the androgen receptor (AR) plays a critical role in the development and progression of PCa. We initially assessed AR protein expression in the non-tumorigenic prostate epithelial cell line and PCa cell lines using

western blotting. We observed that AR protein expression, similar to FNDC1 and FN1 protein expression, is overexpressed in the PCa cell lines compared to the non-tumorigenic prostate epithelial cell line, RWPE-1. Moreover, AR protein expression, similar to FNDC1 and FN1 protein expression, is very low in RWPE-1 (Figure 4a). In addition, FNDC1, FN1 and AR protein are overexpressed in the androgen-independent C4-2B PCa cell line compared to the androgen-dependent LNCaP PCa cell line (Figure 4b). Thus, the data show that FNDC1 and FN1 expression positively correlate to AR expression in PCa cell lines. It is interesting to note that AR expression is negatively correlated with miR-1207-3p expression in PCa cell lines but is independent of the aggressiveness of the PCa cell line (Figure 1b). To determine if miR-1207-3p regulation of FNDC1/FN1 regulates AR expression, we examined the effect of silencing FNDC1 or FN1 expression on AR expression in PCa. FN1 siRNA was transfected into MDA PCa 2b cells and we observed that compared to the scramble siRNA, FN1 siRNA inhibits AR expression (Figure 4c.) Therefore, AR expression is dependent on FN1 expression.

As we have already confirmed that miR-1207-3p directly targets the 3'UTR of FNDC1 leading to loss of its expression, we wanted to determine if loss of FNDC1 expression leads to the loss of FN1 and subsequent AR expression. Therefore, FNDC1 siRNA was transfected into MDA PCa 2b cells. To further clarify the role of miR-1207-3p in regulating FNDC1, and downstream molecular mechanisms, we also compared the effect of overexpression of miR-1207-3p to the effect of a negative control oligonucleotide. The western blot data clearly show that the overexpression of miR-1207-3p inhibits the protein expression of FNDC1 (>60%), FN1 (~40%), and AR expression (~20%). The data positively correlates to that of the effect of silencing FNDC1, with protein expression inhibitions of >70% for FNDC1, >50% for FN1. Remarkably, AR has the most striking inhibition at nearly 85% (Figure 4d). Taken together, the data indicates that miR-1207-3p binds to the 3'UTR of FNDC1 leading to loss of expression of FNDC1, consequent loss of expression of FN1, and consequent loss of expression of AR. Thus, we have demonstrated, for the first time a miR-1207-3p/FNDC1/FN1/AR novel regulatory pathway.

FNDC1, FN1, and AR expression are positively correlated in human prostate cancers and upregulation is associated with aggressive disease

In view of the novel role we discovered of FNDC1, FN1 and AR as downstream molecular mechanisms of action of miR-1207-3p in PCa, we wanted to determine if they are relevant to clinically significant disease in human PCa patients. We independently analyzed data from four separate studies: Moffitt Cancer Center-based study, analysis of a prostate cancer RNA-seq dataset deposited at the Array Express archive of the European Bioinformatics Institute (EBI) (http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-567/), and analysis of two publicly available gene expression datasets in the Oncomine database (www.oncomine.org).

In an independent study based at the Moffitt Cancer Center, we discovered that FN1 expression in PCa cases that resulted in overall death (3.01±1.92 versus 2.18±1.67, P=0.001) or PCa-specific death (2.88±2.11 versus 2.18±1.68, P=0.001) is significantly higher than PCa cases that remained alive. These results are based on analysis of FN1 expression in 377 cases (Table 1).

For analysis of the dataset deposited with the EBI, we specifically designed a novel automated RNA-seq pipeline. The data was from 14 primary prostate cancers and their paired adjacent normal prostate tissue from a Chinese population[13]. The analysis revealed that 11 of the patients had reliably analyzable ($P<0.05$) expression of FN1 and AR (Supplementary Figure S3_a.) Data from the samples of patient 7 were not included because it lacked reliably analyzable FN1 or AR expression. Of the 11 patients whose data could be reliably analyzed, a subset of this group (patients 2, 5, and 13), showed concurrent increased FN1 and AR expression in prostate cancer samples as compared to normal prostate samples (Supplementary Figure S3_b). The boxplots show similar overexpression of FN1 and AR, thus demonstrating that prostate cancer tissue has higher FN1 and AR gene expression compared to normal prostate tissue for patients 2, 5 and 13 (Supplementary Figure S3_c).

Further, we independently investigated two additional publicly available gene expression datasets in the Oncomine database (www.oncomine.org) for expression of FNDC1, FN1, and AR. Analysis of the study conducted by Lapointe et al [33] revealed similar levels of upregulation of all three genes in localized prostate cancers as compared to benign prostate tissues (FNDC1: 1.17 fold, p=0.002; FN1: 1.15 fold, p=0.005; AR: 1.12 fold, p=0.117) (Figure 5a). Furthermore, metastatic prostatic cancers showed an even higher level of upregulation as compared to the localized prostate cancers (FNDC1: 1.80 fold, $p=5.96e^{-4}$; FN1: 2.65 fold, p=0.063; AR: 2.87 fold, p=0.001) (Figure 5b). Similarly, in the dataset from the study by Grasso et al. [34], metastatic prostate cancers showed a significant upregulation of all three genes when compared to localized primary prostate cancers (FNDC1: 3.87 fold, $p=6.49e^{-9}$; FN1: 3.36 fold, $p=1.01e^{-7}$; AR: 3.34 fold, $p=6.27e^{-4}$) (Figure 5c). Together, these findings indicate a positive correlation of FNDC1, FN1, and AR expression in prostate cancers similar to that seen in prostate cancer cell lines, and their upregulation in metastatic prostate cancers supports a role for FNDC1, FN1, and AR in aggressive prostate cancers.

miR-1207-3p regulates migration, apoptosis and proliferation of PCa cells

To investigate the function of miR-1207-3p in prostate epithelial cells, we first investigated if it plays a role in cell migration. Therefore, wound healing assays were performed using RWPE-1 (Figure 6a), WPE1-NA22 (Figure 6b) and PC-3 cells (Figure 6c). As shown in figure 6a-c, inhibition of miR-1207-3p expression with the miR-1207-3p inhibitor led to a four-fold increase in the migratory capacity of all three prostate epithelial cell lines. Conversely, overexpression of miR-1207-3p using the mimic of miR-1207-3p significantly inhibited migration by 50% in both PCa cell lines, WPE1-NA22 (Figure 6b) and PC-3 (Figure 6c), thus strongly showing that miR-1207-3p suppresses PCa cell migration.

To investigate whether miR-1207-3p affects cell apoptosis, we performed Annexin V staining using flow cytometry on RWPE-1, MDA PCa 2b and C4-2b cells. We observed that for the normal prostate epithelial cell line, RWPE-1, the overexpression of miR-1207-3p mimic only slightly increased apoptotic activity, however the miR-1207-3p inhibitor decreased apoptosis by nearly 2-fold (Figure 6d). miR-1207-3p inhibitor decreased apoptosis, but overexpression of the miR-1207-3p mimic resulted in increased apoptotic activity when compared to the negative control in MDA PCa 2b cells (Figure 6e). Similarly, the overexpression of miR-1207-3p mimic resulted in increased apoptosis in C4-2b cells, by

nearly 2-fold, when compared to the negative control (Figure 6f). These results indicate that miR-1207-3p induces apoptosis in PCa cells.

We next investigated whether miR-1207-3p regulates proliferation, we performed the Click-iT EdU Alexa Flour 488 imaging assay. This sensitive method measures a cell's ability to proliferate by assessing the cells in S-phase. This is accomplished by incorporating EdU (5-ethynyl-2'-deoxyuridine), a thymidine analog into DNA during active DNA synthesis [35]. As indicated in figure 6 g-i, the effect of miR-1207-3p mimic and the miR-1207-3p inhibitor were compared to the negative control in RWPE-1, MDA PCa 2b and WPE1-NA22 cells. For the normal prostate epithelial cell line, RWPE-1, we observed that overexpression of miR-1207-3p reduced EdU positive cells by approximately 30% compared to the negative control, while the inhibition of miR-1207-3p led to a nearly 2.5-fold increase in proliferation (Figure 6g). For MDA PCa 2b and WPE1-NA22 PCa cell lines, we observed that EdU positive cells were reduced by nearly 70% by the overexpression of miR-1207-3p compared to the negative control, while the inhibition of miR-1207-3p led to an approximate 40% increase in proliferation (Figure 6 h & i). The diminished percentage of cells in S-phase caused by the overexpression of miR-1207-3p indicates that cells with overexpression of miR-1207-3p suspend the cell cycle as they are unsuccessful to progress through the S-phase. Therefore, the data demonstrate that increased expression of miR-1207-3p significantly inhibited cellular proliferation in PCa.

Discussion

Prostate cancer (PCa) is the most frequently diagnosed solid organ cancer in males in the developed world [36]. Even though PCa is so prevalent, the molecular mechanisms underlying its development and progression remain largely unclear. Increasing evidence has demonstrated that many miRNAs are aberrantly expressed in cancers, suggesting that variations in miRNA expression are common events in tumorigenesis[37-39]. These small non-coding RNAs are attractive for clinical applications for many reasons. One compelling reason is that their direct targeting of many mRNAs that are translated to proteins explains their differential expression between non-cancer and cancer cells. Understanding the regulatory pathways controlled by microRNAs is crucial as they can have significant impact on the regulation of various genes. The current study provides novel insight into the underlying molecular mechanisms for a potential tumor suppressive role for miR-1207-3p in PCa. The data suggest that it may be possible to exploit miR-1207-3p as part of a therapeutic strategy in PCa.

MicroRNA-1207-3p is encoded at the non-protein coding gene locus PVT1 on the 8q24 human chromosomal region, an established PCa susceptibility locus[10,15]. However, microRNA-1207-3p is not associated with any annotated PVT1 exons and no known functions have previously been described for miR-1207-3p. Thus, the function of miR-1207-3p may be different from that of PVT1 or any of its annotated exons. As the expression of miR-1207-3p in PCa has not previously been reported, our study is the first to demonstrate that miR-1207-3p regulates molecular mechanisms and key cellular functions implicated in PCa development and progression. An important discovery is that miR-1207-3p is significantly underexpressed in human PCa cell lines as compared to normal

prostate epithelial cells (by more than two-fold). Furthermore, we observed that it is underexpressed in both indolent and aggressive human PCa cell lines. This suggests that an early event in PCa development could be the loss of miR-1207-3p.

It is well known that being of African ancestry is a non-modifiable risk factor for PCa.[40] These men are more than twice as likely to be diagnosed with PCa and more likely to have aggressive subtypes which increases their risk of mortality.[40,41] Interestingly, from our data, we observed that miR-1207-3p expression is particularly low in PCa cell lines derived from Black men (MDA PCa 2b, E006AA and E006AA-hT). We also observed that FN1 is overall significantly more expressed in PCa cell lines derived from Black men as compared to those derived from Caucasian men. Further studies into the role of miR-1207-3p in the racial disparity that is notable in PCa are required.

Further, we observed that miR-1207-3p expression was further reduced in PCa cell lines derived from castration-resistant PCa (CRPC) as compared to cell lines derived from castration-sensitive PCa. This suggests that loss of miR-1207-3p may be able to be an early PCa biomarker of potential for progression to CRPC.

Because miR-1207-3p is consistently and significantly underexpressed in the seven tumorigenic PCa cell lines we examined as compared to the non-tumorigenic prostate epithelial cell line RWPE-1, we investigated the role of miR-1207-3p in regulating key cellular processes (proliferation, apoptosis, and migration) that are dysregulated during the development and progression of PCa. We made the novel discovery that inhibition of miR-1207-3p expression leads to significant inhibition of apoptosis, and significant increases in cellular proliferation of PCa cells. Conversely, overexpression of miR-1207-3p significantly induced apoptosis, and significantly inhibited proliferation of PCa cells. Strikingly, inhibition of miR-1207-3p led to an approximately 4-fold increase in the migratory capacity of both non-tumorigenic and tumorigenic prostate epithelial cells. And overexpression of miR-1207-3p significantly inhibits migration of PCa cells. These data demonstrate that miR-1207-3p regulates key cellular processes dysregulated in the development and progression of PCa.[42] Consequently, it is possible that miR-1207-3p could be applied to modulate these processes for therapeutic effects in PCa. Therefore, strategies to increase expression of miR-1207-3p in prostate epithelial cells deserve thorough investigation.

The effect of miR-1207-3p on cellular migration is especially remarkable. Two miRNA molecular target algorithm tools, miRBase and miRDB, were used independently to identify FNDC1 as a putative molecular target of miR-1207-3p. Further, overexpression of miR-1207-3p inhibited expression of FNDC1 in multiple PCa cell lines. In addition, luciferase reporter assay and RNA pulldown assay confirmed that miR-1207-3p directly binds to FNDC1. Thus, for the first time, we have confirmed FNDC1 as a direct molecular target of miR-1207-3p. FNDC1 contains a major component of the structural domain of FN1 [22,23,25,27,43]. FN1 has been implicated in many cancers and known to be a major regulator of migration of cancer cells [29-32,44]. Overexpression of miR-1207-3p downregulates FN1, and specific silencing of FNDC1 also downregulates FN1. Thus,

miR-1207-3p effects in PCa (including effects on migration) are due to binding to and loss of FNDC1, and subsequent loss of FN1.

Only limited data exist about the function of FNDC1. The very few reports about FNDC1 have shown that it is also expressed in the kidney, may regulate G protein signaling and play some role in hypoxia-induced apoptosis of cardiomyocytes.[45-47] FNDC1 has also been associated with prostate leiomyosarcoma.[48 4343] Prostate leiomyosarcoma is a mesenchymal tumor that is particularly rare, highly aggressive, and accounts for less than 3.7% of primary prostate diseases.[48,49] To our knowledge, this study is the first to demonstrate microRNA regulation of FNDC1. Also, to our knowledge, only two miRNAs has been shown to directly target a member of the FN1 family in PCa, miRs-143 and-145.[50,51] miR-143 and miR-145 are downregulated in PCa cell lines and tissues.[51] However, their true effects on FN1 are unknown at this time as data are conflicting.[38,50,52] Nevertheless, FN1 is upregulated in PCa [32,53] as we observed from four independently analyzed human studies. Consequently, we have identified miR-1207-3p as a miRNA that regulates proliferation, apoptosis, and migration in PCa by regulating FN1 via directly targeting FNDC1. Moreover, for the first time, FNDC1 is shown to have a clear and prominent role in the regulation of proliferation, apoptosis, and migration in PCa.[54]

We have also demonstrated that miR-1207-3p regulates AR activity via FNDC1 and FN1. AR signaling is not only crucial for normal prostate growth and development but also for the onset and progression of prostate carcinogenesis.[6] Although AR signaling has been studied for decades, the molecular mechanisms behind its dysfunction remain unclear.[6,55,56] Furthermore, AR dysfunction is usually associated with the progression to castration-resistant PCa.[56,57] We observed that AR protein expression, in positive correlation with FN1 and FNDC1 protein expression, is significantly more elevated in the castration-resistant cellular model, C4-2B, in comparison to its castration-responsive counterpart, LNCaP. In addition, we demonstrate that the molecular effects of miR-1207-3p (loss of FNDC1 expression and consequent loss of FN1 expression) lead to loss of AR expression: silencing of FN1 protein expression leads to loss of AR protein expression. Thus, this is the first description of the miR-1207-3p/FNDC1/FN1/AR regulatory pathway in PCa.

The clinical relevance of this novel regulatory pathway was investigated in four independently analyzed human prostate studies: Moffitt Cancer Center-based study, analysis of a prostate cancer RNA-seq dataset deposited at the Array Express archive of the European Bioinformatics Institute (EBI) (http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-567/), and analysis of two publicly available gene expression datasets in the Oncomine database (www.oncomine.org). To our knowledge, these are the first concurrent analyses of FNDC1, FN1, and AR in human prostate cancer. Analysis of the RNA-seq dataset from the EBI revealed positive correlation of FN1 and AR significant overexpression in a subset of the prostate cancer patients. This result suggests a very important role for precision medicine in the management of PCa as only a subset of patients may benefit from certain approaches.[58] Our Moffit Cancer Center-based study strongly showed that FN1 overexpression positively correlated with overall death and PCa-specific death in PCa. To our knowledge, this is the first description of FN1 as a prognostic biomarker in human clinical PCa. Interestingly, analysis of the two publicly available gene expression datasets in

the Oncomine database very strongly showed concurrent overexpression of FNDC1, FN1, and AR in metastatic PCa in comparison to primary tumors in both datasets. This is very clear evidence of an important role of this novel FNDC1/FN1/AR molecular pathway regulated by miR-1207-3p in the most clinically significant PCa, metastatic PCa. And this underscores the importance of further studying miR-1207-3p for potential therapeutic applications especially for metastatic PCa.

Conclusions

In conclusion, we identified loss of miR-1207-3p expression in PCa cells, and demonstrate that miR-1207-3p is an important regulator of proliferation, apoptosis, and migration of PCa cells. Importantly, we describe for the first time the miR-1207-3p/FNDC1/FN1/AR novel regulatory pathway. And we report, for the first time, clinical data indicating that this novel molecular pathway may be important in metastatic PCa. Consequently, this novel miR-1207-3p-dependent pathway may be actionable for clinical applications in PCa.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations

PCa	prostate cancer
miR-1207-3p	microRNA-1207-3p
FNDC1	fibronectin type III domain containing 1
FN1	fibronectin
AR	androgen receptor
GWAS	Genome-wide association studies
UTR	untranslated region

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Highlights

- Expression of microRNA-1207-3p is significantly lost in prostate cancer (PCa) cells.
- MicroRNA-1207-3p regulates proliferation, apoptosis, and migration of PCa cells via direct molecular targeting of the 3'UTR of FNDC1.
- MicroRNA-1207-3p-induced loss of FNDC1 expression leads to the loss of FN1 and subsequently AR expression.
- FNDC1, FN1, and AR are concurrently overexpressed in metastatic PCa.

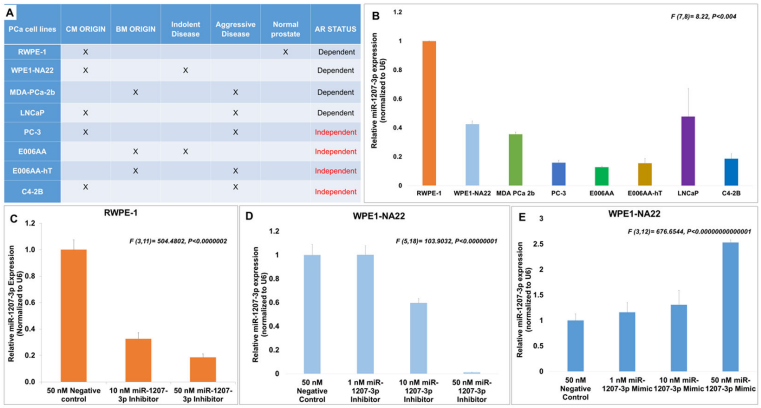

Figure 1. miR-1207-3p expression is lost in prostate cancer cells, and effect of an oligonucleotide inhibitor and oligonucleotide mimic of miR-1207-3p on miR-1207-3p expression

(A) Characterization of non-tumorigenic and tumorigenic prostate epithelial cells. List and characteristics of human prostate cell lines used. CM, Caucasian Male; BM, Black Male; AR, androgen receptor. (B) Three separate qPCR experiments were performed. Each experiment was done in quadruplicates. (C) A dose-response test of the effect of an oligonucleotide inhibitor of miR-1207-3p on miR-1207-3p expression was performed on RWPE-1 cells; 50 nM non-targeting negative control oligonucleotide was used as a control. (D & E) A dose-response test of the effect of an oligonucleotide inhibitor and an oligonucleotide mimic of miR-1207-3p on miR-1207-3p expression was performed on WPE1-NA22 cells, respectively. 50nM non-targeting negative control oligonucleotide was used a control. Data is presented as mean ± standard error of the mean (SEM). Statistical differences were determined with one-way ANOVA. All the criterions for significance was set at $P<0.05$.

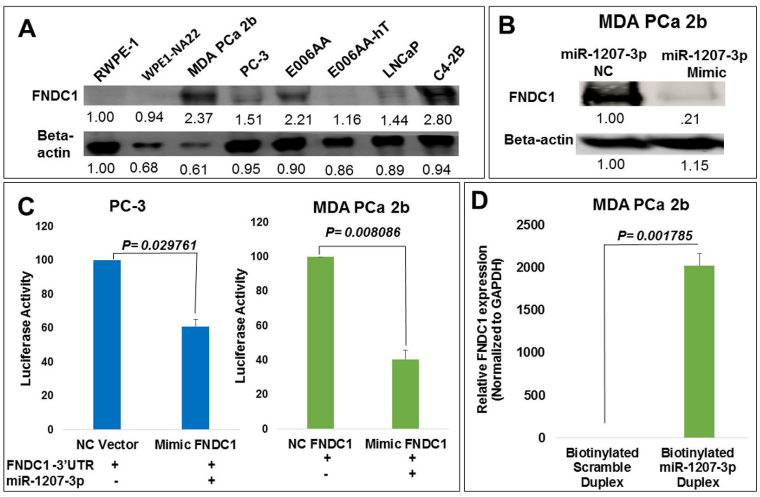

Figure 2. FNDC1 is a direct molecular target of miR-1207-3p

(A) Western blotting experiments were performed. FNDC1 is overexpressed in PCa cell lines when compared to the non-tumorigenic prostate epithelial cell line, RWPE-1. (B) Effect of overexpression of miR-1207-3p on FNDC1 in MDA PCa 2b cells. (C) Luc-Pair™ Duo-Luciferase Assay was used to determine if miR-1207-3p binds to the 3'UTR of FNDC1. PC-3 and MDA PCa 2b cells were co-transfected with GeneCopoeia pEZX-MT06 miRNA reporter empty vector or FNDC1 3'UTR-containing plasmid with a 50nM non-targeting negative control or miR-1207-3p 50nM mimic for 24 hours. FLuc and RLuc activity were measured. Activity is normalized to the negative control luciferase activity set to 1.0. Bars represent SD. **P*<0.05. Data show that microRNA-1207-3p directly targets the 3'UTR of FNDC1. (D) RNA pulldown assay was performed for the identification of direct binding of miR-1207-3p to FNDC1 mRNA, using a synthetic biotinylated miR-1207-3p duplex and a synthetic biotinylated scramble duplex as a control. The synthetic biotinylated scramble duplex and the synthetic biotinylated microRNA-1207-3p duplex were transfected into MDA PCa 2b cells. Enrichment for FNDC1 mRNA in pulled down RNA was analyzed by qPCR. Data represent relative expression. Bars represent SD. **P*<0.05. See also Figure S1.

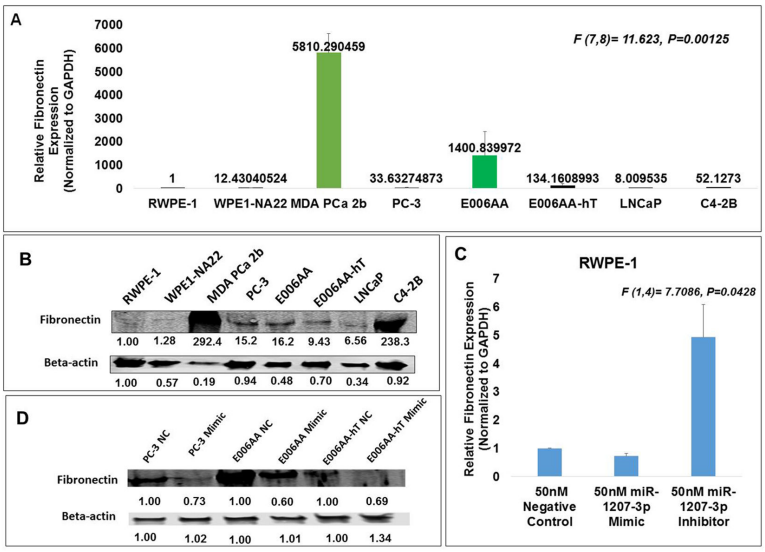

Figure 3. miR-1207-3p regulates FN1 expression

(A & B) FN1 is overexpressed in PCa cell lines when compared to the non-tumorigenic prostate epithelial cell line, RWPE-1. (C) Effect of overexpression and inhibition of miR-1207-3p on FN1 expression in RWPE-1 cells. (D) Effect of overexpression of miR-1207-3p on FN1 expression in PC-3, E006AA cells, and E006AA-hT cells. Statistical differences for A & C were determined with one-way ANOVA. All the criterions for significance was set at $P<0.05$. Western blot experiments were performed two separate times. qPCR experiments were performed in quadruplicates, and three separate times. See also Figure S2.

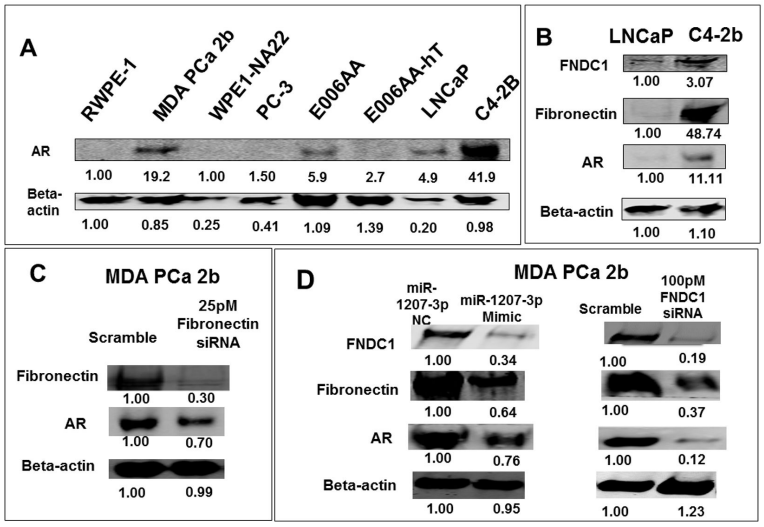

Figure 4. miR-1207-3p/FNDC1/FN1/AR is a regulatory pathway in prostate cancer

(A) AR is overexpressed in PCa cell lines when compared to the non-tumorigenic prostate epithelial cell line, RWPE-1. (B) Relative protein expression of FNDC1, FN1, and AR expression in LNCaP and C4-2b cells, respectively. (C) Effect of silencing FN1 expression on AR expression in MDA PCa 2b cells. (D) Effect of overexpression of miR-1207-3p and silencing of FNDC1 on FNDC1, FN1 and AR expression in MDA PCa 2b cells. Western blotting experiments were performed three separate times.

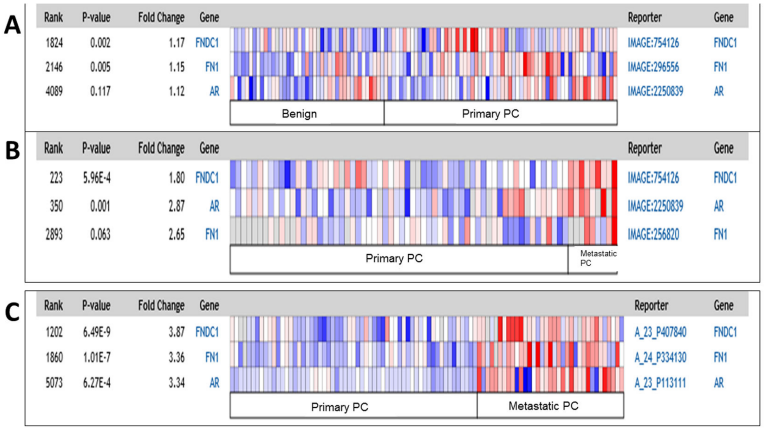

Figure 5. FNDC1, FN1, and AR are similarly significantly overexpressed in metastatic human prostate cancer

(A) Gene expression of FNDC1, FN1 and AR in benign prostate tissues as compared to localized prostate cancer. (B) Gene expression of FNDC1, FN1 and AR in primary prostate cancer as compared to metastatic prostate cancer. Data is from analysis of RNA-sequencing data from the study by Lapointe and colleagues. (C) Gene expression of FNDC1, FN1 and AR in primary prostate cancer as compared to metastatic prostate cancer. Data is from analysis of RNA-sequencing data from the study by Grasso and colleagues. See also Figure S3.

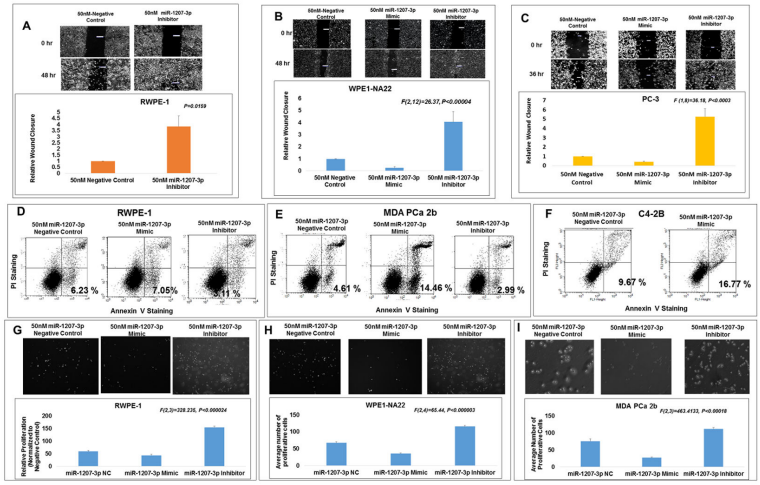

Figure 6. Loss of miR-1207-3p expression promotes migration, proliferation and inhibits apoptosis

Transfection of a synthetic oligonucleotide mimic or inhibitor of miR-1207-3p was performed. (A) Effect of inhibition of miR-1207-3p expression on migration of RWPE-1 cells. (B & C) Effect of overexpression of miR-1207-3p and inhibition of miR-1207-3p on migration of WPE1-NA22 and PC-3 cells, respectively. Data indicate that loss of miR-1207-3p promotes migration. (D) Effect of overexpression of miR-1207-3p and inhibition of miR-1207-3p expression on apoptosis of RWPE-1 and MDA PCa 2b was assessed using Annexin V staining, respectively (D & E). Effect of overexpression of miR-1207-3p on apoptosis of C4-2b cells assessed using Annexin V staining. Data indicate that overexpression of miR-1207-3p expression induces apoptosis (F). Effect of overexpression of miR-1207-3p and inhibition of miR-1207-3p expression on proliferation of RWPE-1, MDA PCa 2b and WPE1-NA22 cells assessed using the Click-iT EdU proliferation assay (10× magnification) (G-I). Data indicate that loss of miR-1207-3p increases proliferation. Data is presented as mean + standard error of the mean (SEM). Statistical differences were determined with two-way ANOVA for B-C, G-I. All the criterions for significance was set at $P<0.05$.

Table 1

FN1 expression in prostate cancer tissues of 377 patients

List and characteristics of human prostate cancer samples. Variables: number, mean; SD, standard deviation; P-value.

Variables		Number	Mean ±SD	P-value
Recurrence	Yes	136	2.56±2.00	
	No	241	2.38±1.66	.36
Death	Yes	120	3.01±1.92	
	No	257	2.18±1.67	.001
Death Specific	Alive	255	2.18±1.68	
	Dead	74	3.09±1.80	
	PCA specific	46	2.88±2.11	0.001
Race	Black	17	2.17±1.80	
	White	362	2.45±1.79	.55
Gleason Score	≤6	159	2.65±1.73	
	7	124	2.17±2.01	
	≥8	27	2.40±1.79	.099
Stage	1-2	281	2.43±1.83	
	3-4	89	2.50±1.74	.766
PSA	<4	27	1.95±2.21	
	4-10	156	2.44±1.83	
	>10	49	2.36±2.24	.499
Marital Status	Divorced	22	2.30±1.37	
	Married	321	2.51±1.74	
	Single	22	1.85±2.84	.227