MicroRNA 1207-3P in Prostate Cancer

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MICRORNA 1207-3P IN PROSTATE CANCER

by

DIBASH K. DAS

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2018
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Dibash K. Das

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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THE CITY UNIVERSITY OF NEW YORK
Abstract

MicroRNA 1207-3p in Prostate Cancer

by

Dibash K. Das

Advisor: Dr. Olorunseun O. Ogunwobi

Prostate cancer (PCa) is the most commonly diagnosed male cancer and the second leading cause of cancer-related death for men in the United States. Understanding the molecular mechanisms involved in progression from the asymptomatic androgen-dependent PCa to the lethal castration resistant prostate cancer (CRPC) is a major challenge. MicroRNAs (miRNAs), are known to be dysregulated in PCa. MicroRNA-1207-3p (miR-1207-3p) is encoded by the non-protein coding gene locus PVT1 on the 8q24 human chromosomal region, an established PCa susceptibility locus. However, the role of miR-1207-3p in PCa is unclear. We have discovered that miR-1207-3p is significantly underexpressed in PCa cell lines compared to normal prostate epithelial cells. Moreover, the increased expression of miR-1207-3p in PCa cells significantly inhibits proliferation, migration, and induces apoptosis via direct molecular targeting of Fibronectin type III domain containing 1 (FNDC1) and consequent loss of expression of fibronectin (FN1), and consequent loss of expression of the androgen receptor (AR). PCa cell lines and patient-derived tissues revealed significant overexpression of FNDC1, FN1 and AR which are factors that positively correlate with aggressive PCa. Also, metastatic PCa displayed concurrent overexpression of FNDC1, FN1 and AR. Taken together, this is the first description of a novel miR-1207-3p/FNDC1/FN1/AR regulatory pathway in PCa. For the unbiased discovery of the molecular targets of miR-1207-3p, we designed and synthesized a novel synthetic biotinylated
miR-1207-3p duplex (NB1207), and a novel synthetic biotinylated scramble duplex (NB1). We observed that NB1207, but not the scrambled duplex NB1, directly targets the 3’UTR of FNDC1 and more effectively inhibits proliferation, inhibits migration and increases apoptosis of PCa cells including those aggressively tumorigenic. Interestingly, the location of miR-1207-3p on the 8q24 human chromosomal region is downstream of the proto-oncogene, c-MYC. c-MYC has been linked to castration resistant prostate cancer (CRPC). However, the mechanisms regulating c-MYC remain unclear in CRPC. In this study, we discovered that c-MYC is regulated and therapeutically targetable via the miR-1207-3p/FNDC1/FN1/AR pathway in CRPC. miR-1207-3p negatively correlates with c-MYC in prostate tumors with Gleason score ≥8. Additionally, we discovered that overexpression of miR-1207-3p significantly inhibited proliferation and increased apoptosis in CRPC cells. We also compared the efficacy of NB1207 and NB5, two novel synthetic analogs of miR-1207-3p, with the currently used therapies against CRPC: abiraterone, enzalutamide, and apalutamide (phase 3 clinical trial). Treatment with NB1207 and NB5 resulted in increased inhibition of AR-V7 protein expression, and more significant inhibition of proliferation and increases in apoptosis of CRPC cells compared to abiraterone, enzalutamide and apalutamide. These results demonstrate that synthetic analogs of miR-1207-3p, such as NB5 and NB1207, may be a novel strategy for successful therapeutic targeting of c-MYC via the miR-1207-3p/FNDC1/FN1/AR pathway in CRPC. In summary, the present study indicates that miR-1207-3p may have potential diagnostic, prognostic, and therapeutic applications in PCa.
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Chapter 3 in this dissertation is a combination of works published in Experimental Cell Research, Translational Oncology and RNA & Disease. Chapter 4 is being submitted to Cancer Discovery.
# Table of Contents

Abstract .................................................................................................................................iv

Acknowledgments ................................................................................................................vi

Table of Contents ................................................................................................................viii

List of Abbreviations ..........................................................................................................xi

List of Tables .......................................................................................................................xii

List of Figures ....................................................................................................................xiii

Chapter 1: Introduction ......................................................................................................1

Prostate cancer .....................................................................................................................2

The role of the androgen receptor (AR) and AR-V7 in prostate cancer .........................3

Role of microRNAs in cancer ............................................................................................4

The role of microRNA-1207-3p in prostate cancer ..........................................................6

FNDC1 and FN1 regulation by the miR-1207-3p pathway in prostate cancer ...............6

c-MYC regulation by the miR-1207-3p pathway in prostate cancer ............................7

Clinical significance of miR-1207-3p in prostate cancer .............................................7

Racial disparity in prostate cancer ...................................................................................8

Chapter 2: Materials and Methods ................................................................................10

Cells and cell culture conditions ....................................................................................11

Transfection of oligonucleotide inhibitor and mimic of miR-1207-3p .........................12

Transfection of siRNA ......................................................................................................12

RNA isolation and quantitative real-time polymerase chain reaction (qPCR) analysis ...13

Click-iT EdU Alexa Fluor 488 Imaging Kit ....................................................................13
Chapter 3: miR-1207-3p regulates the androgen receptor in prostate cancer via FNDC1/FN1

Introduction

Results

miR-1207-3p is significantly underexpressed 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.

miR-1207-3p directly targets FNDC1.

miR-1207-3p regulates FN1 via FNDC1.

FNDC1 regulates proliferation, apoptosis, and migration of PCa cells.

FN1 regulates proliferation, apoptosis and migration of PCa cells.

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

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

miR-1207-3p is differentially expressed in PCa in men of African ancestry compared to Caucasian men.

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

Discussion

Author contributions
Acknowledgements........................................................................................................45

Human and animal rights.................................................................................................45

Chapter 4: c-MYC is regulated and therapeutically targetable via the miR-1207-3p/FNDC1/FN1/AR pathway in castration resistant prostate cancer.................................46

Introduction...................................................................................................................47

Results............................................................................................................................49

Underexpression of miR-1207-3p and the overexpression of FNDC1, FN1, AR and c-MYC are significantly associated with aggressive prostate cancer.................................49

miR-1207-3p negatively correlates with c-MYC in prostate tumors with Gleason score ≥8..............................................................................................................................53

c-MYC is downstream of the novel miR-1207-3p/FNDC1/FN1/AR pathway..............54

A novel synthetic analog of miR-1207-3p, NB1207, significantly inhibits c-MYC expression in CRPC cells.................................................................................................57

NB1207 significantly inhibits migration, inhibits proliferation and induces apoptosis in CRPC cells....................................................................................................................59

NB1207 and NB5 inhibits AR-V7 expression and is more effective in inhibiting proliferation and apoptosis in CRPC cells compared to enzalutamide, abiraterone and apalutamide..........................................................60

Discussion.....................................................................................................................65

Author contributions.....................................................................................................69

Acknowledgements.....................................................................................................69

Chapter 5: Conclusion, Significance and Future Directions.........................................70

Chapter 6: Bibliography...............................................................................................76
List of Abbreviations

ADT Androgen deprivation therapy
AR Androgen receptor
AR-V7 Androgen receptor variant 7
CM Caucasian men
CRPC Castration resistant prostate cancer
FN1 Fibronectin
FNDC1 Fibronectin type III domain containing 1
GWAS Genome-wide association studies
lncRNA long non-coding RNA
miRNA microRNA
miR-1207-3p microRNA-1207-3p
NB1 biotinylated scramble duplex
NB2 scramble duplex
NB3 biotinylated mutant duplex
NB4 mutant duplex
NB5 miR-1207-3p duplex
NB1207 biotinylated miR-1207-3p duplex
moAA men of African Ancestry
PCa Prostate Cancer
PSA prostate specific antigen
PVT1 plasmacytoma variant translocation 1 gene
qRT-PCR real-time quantitative reverse transcription polymerase chain reaction
UTR untranslated region
List of Tables

Table 3.1: Fibronectin expression in prostate cancer tissues of 377 patients.........................35
Table 3.2: miR-1207-3p is differentially expressed between moAA and CM......................37
List of Figures

Figure 3.1: 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

Figure 3.2: FNDC1 is a direct molecular target of miR-1207-3p

Figure 3.3: miR-1207-3p regulates FN1 expression

Figure 3.4: Effect of siRNA knockdown of FNDC1

Figure 3.5: Effect of siRNA knockdown of fibronectin

Figure 3.6: miR-1207-3p/FNDC1/FN1/AR is a regulatory pathway in prostate cancer

Figure 3.7: FNDC1, FN1, and AR expression is correlated in prostate cancers and upregulation is associated with aggressive disease

Figure 3.8: Loss of miR-1207-3p expression promotes migration, proliferation and inhibits apoptosis

Figure 3.9: A novel miR-1207-3p/FNDC1/FN1/AR regulatory pathway in prostate cancer

Figure 4.1: c-MYC has been linked to prostate cancer aggressiveness

Figure 4.2: Underexpression of miR-1207-3p is significantly associated with aggressive prostate cancer

Figure 4.3: Overexpression of FNDC1, FN1, and AR are significantly associated with aggressive prostate cancer

Figure 4.4: Overexpression of c-MYC are significantly associated with aggressive prostate cancer
Figure 4.5: miR-1207-3p negatively correlates with c-MYC in tumors with Gleason score ≥8.................................................................54

Figure 4.6: c-MYC is not a direct molecular target of miR-1207-3p but is regulated by the miR-1207-3p/FNDC1/FN1/AR pathway.................................................................56

Figure 4.7: A novel miR-1207-3p/FNDC1/FN1/AR/c-MYC pathway in CRPC in which NB1207 significantly inhibits c-MYC expression in CRPC cells.................................58

Figure 4.8: NB1207 significantly inhibits migration, inhibits proliferation and induces apoptosis in CRPC cells.................................................................60

Figure 4.9: NB1207 and NB5 inhibits AR-V7 expression and is more effective in inhibiting proliferation in CRPC cells compared to enzalutamide, abiraterone and apalutamide.................................................................63

Figure 4.10: NB1207 and NB5 is more effective in increasing apoptosis in CRPC cells compared to enzalutamide, abiraterone and apalutamide.................................64

Figure 5.1: miR-1207-3p/FNDC1/FN1/AR/c-MYC is a novel regulatory pathway in prostate cancer.................................................................75
Chapter 1:
Introduction
**Prostate cancer**

Prostate cancer (PCa) is the most commonly diagnosed cancer among males in the western world\(^1\-3\). Accounting for 180,890 new cases in the US in 2016, PCa is a major cause of cancer morbidity and mortality\(^4\-6,7\). The annual morbidity grows as the rate of PCa has increased by 14% over the last two decades\(^3,6,8,9\). One of the challenges faced in combating PCa is its considerable heterogeneity\(^1,9,10\). Typically, early PCa is asymptomatic, and advanced PCA is associated with the appearance of symptoms such as blood in the urine, burning/pain during urination and metastasis to the bones\(^11\-13\). Active research continues in the areas of early detection and treatment of PCa, but these efforts have met obstacles. The current screening tool for PCa, prostate specific antigen (PSA), is highly controversial as it is not PCa-specific and has a high false positive rate\(^14,15\). This poor correlation leads to adverse consequences associated with unnecessary diagnosis and overtreatment of non-aggressive PCa\(^14\-16\). Unfortunately, treatments are highly aggressive, invasive, costly and may impact a man’s long-term quality of life due to complications or severe side effects\(^12,17,18\).

Effective methods for early detection of clinically relevant PCa are crucial and should minimize unnecessary, aggressive treatments and cancer-related deaths. Currently, there are no satisfactory biomarkers available that are both effective for the early detection of PCa and sensitive enough to discriminate between indolent and aggressive PCa\(^1,13,19\). Understanding the molecular mechanisms underlying the development of PCa is necessary for the discovery of novel and efficacious biomarkers which offer specificity and improve risk stratification and treatment outcomes for patients.
The role of AR and AR-V7 in prostate cancer

The Androgen hormone stimulates and controls the development and maintenance of male characteristics by binding to AR\textsuperscript{20,21}. The AR, a member of the steroid hormone receptor superfamily, contains 8 canonical exons encoded in four functional domains\textsuperscript{17,22,23}. The domains include the NH2-terminal transactivation domain (NTD), the DNA-binding domain (DBD), the hinge region, and the ligand-binding domain (LBD)\textsuperscript{17,22,23}. The AR carries out its cellular functions as a ligand-activated transcription factor\textsuperscript{17,22,23}. However, PCa is an androgen-driven disease and the androgen receptor (AR) plays a critical role in the development and progression of PCa\textsuperscript{17,20,21}. PCa typically starts as androgen-dependent and most men are responsive to medical and surgical androgen deprivation therapies (ADT)\textsuperscript{12,17,21,24,25}. However, in spite of these efforts, the cancer inevitably reoccurs in up to 30% of men, who progress to androgen-independent PCa also known as castration-resistant PCa (CRPC)\textsuperscript{6,12,17,21}. It is this highly aggressive form of PCa that is lethal\textsuperscript{17,21,24}.

The therapeutic options for advanced PCa are limited, and minimally effective \textsuperscript{12,26,27}. Current CRPC anti-androgen therapies such as abiraterone, enzalutamide and apalutamide have focused on the androgen-dependent activation of AR through its ligand-binding domain (LBD)\textsuperscript{20,22,28}. Unfortunately, several studies have shown that androgen receptor variants (AR-Vs) which lack the LBD are resistant to these LBD-targeting AR antagonists or agents that repress androgen biosynthesis\textsuperscript{22,28-30}. AR-V7 is the most abundant and the most studied member because it is the only known variant of which the encoded endogenous protein is detectable in clinical specimens\textsuperscript{22,28,30,31}. AR-V7 contains the first three canonical exons of the AR, however AR-V7 is followed by variant-specific cryptic exon 3 (CE3) within intron 3 which prematurely truncates the
AR in a manner that deletes exons 4-8\textsuperscript{28,30,32}. These deleted exons normally code for the hinge region and LBD\textsuperscript{29-31}. Consequently, AR-V7 remains constitutively active as a transcription factor, independent of androgen signaling\textsuperscript{28-30}. Furthermore, studies have shown that the detection of AR-V7 in PCa has been correlated with CRPC and resistance against enzalutamide and abiraterone\textsuperscript{22,28,30}. Consequently, although the mechanism is not fully understood, the androgen receptor (AR) along with AR-V7 are considered important targets for CRPC therapy\textsuperscript{12,17,25}.

**Role of microRNAs in cancer**

MicroRNAs (miRNAs) are small (18-22 nucleotides) non-protein coding regulatory RNAs, which play an essential role in post-transcriptional gene regulation\textsuperscript{27,33-35}. The canonical biogenesis of miRNAs in vertebrate and invertebrate cells involves two key processing steps\textsuperscript{35-37}. In the nucleus, the RNase III enzyme complex Drosha, cleaves long primary miRNA transcripts (pri-miRNA) containing one or more hairpin structures to yield stem-loop precursor miRNAs (pre-miRNA) that are approximately 55-70 nucleotides in length\textsuperscript{35-37}. These pre-miRNAs are then exported to the cytoplasm where they are subsequently cleaved by another RNase III enzyme, Dicer, to remove the loop structure to produce a mature miRNA\textsuperscript{35-37}.

MicroRNAs are conserved, endogenously synthesized and have been shown to regulate the expression of nearly 60% of human genes by regulating many biological processes including proliferation, migration and apoptosis\textsuperscript{27,33-36,38}. Consequently, miRNAs possess great potential as ideal therapeutic tools for human cancers\textsuperscript{7,35,39}. The canonical mechanism of miRNA targeting involves an interaction between the 5’ end of the miRNA called the ‘seed region’ (from nucleotides 2 to 8) and the 3’ untranslated region (3’ UTR) of the mRNA\textsuperscript{35-37}. The seed region is the most
conserved region of miRNAs, and in many instances a seed match alone is sufficient to confer mRNA recognition, which results in the suppression of protein translation. In the case of perfect complementarity between the miRNA and the mRNA target site, the mRNA is degraded. However, because high complementarity is not required for miRNA regulation on an mRNA, a single miRNA can target multiple genes.

An abundant body of data has demonstrated that miRNA expression are found to be aberrant in many cancers including PCa. Many miRNAs’ expression reflect the tumor of origin, functioning as either oncogenes or tumor suppressors according to the roles of their target genes which strongly supports their use as therapeutic tools for cancer management. Since miRNA expression profiles may serve as “unique signature” in classifying human cancers, they have potential to be used for effective early detection and prognostic biomarkers in PCa as well. Several recently discovered miRNAs based on serum, tumor, blood, urine and tissue-derived samples obtained from human patients from various cancers have demonstrated the stability and robustness of miRNAs to serve as clinical biomarkers. A biomarker that reliably discriminates/distinguishes indolent from aggressive PCa is useful for treatment stratification. Aggressive treatment may be initiated early in rapidly lethal PCa while unnecessary for otherwise indolent PCa. Furthermore, it raises the potential for successful miRNA based therapy. Ultimately, few reports describe the role of miRNAs in PCa aggressiveness, highlighting the need for understanding of which specific miRNAs directly regulating PCa and by which mechanisms.
The role of miRNA-1207-3p in prostate cancer

It is now well-known that the 8q24 human chromosomal locus has profound genomic instability in PCa\(^2,47-50\). Several studies, including one published recently from our lab, have shown that the 300kb long non-protein coding gene locus, PVT1, located on the 8q24 chromosomal locus, is dysregulated in PCa\(^47\). PVT1 has at least 12 different exons and encodes six microRNAs, including microRNA-1207-3p (miR-1207-3p)\(^1,47,50,51\).

Five of the six miRNAs are not associated with annotated PVT1 exons, rather they are intronic, including miR-1207-3p\(^1,47\). However, no study has been performed on the status and biological functions of miR-1207-3p in PCa to date. Our study is the first to determine the role of miR-1207-3p in PCa\(^1\). To explore the molecular mechanisms through which miR-1207-3p exerts its PCa, the molecular targets of miR-1207-3p must be identified. No studies have ever shed light on the direct molecular targets of miR-1207-3p in any system. To investigate this, synthetic biotinylated analogs of miR-1207-3p were designed and synthesized\(^1\).

FNDC1 and FN1 regulation by the miR-1207-3p pathway in prostate cancer

We identified Fibronectin type III domain containing 1 (FNDC1) as a putative molecular target of miR-1207-3p. FNDC1 contains the conserved ‘Fibronectin type III domain’ of Fibronectin (FN1)\(^52-55\). FN1 is a glycoprotein consisting of three domains\(^56-58\). These FN1 domains (type I, type II and type III), have undergone exon shuffling resulting in their abundance in other molecules\(^54,59,60\). FN1 has been implicated in carcinogenesis and known to be a regulator of cell migration, proliferation and apoptosis\(^61-64\). So far, only two miRNAs have been shown to directly target a member of the FN1 family in PCa, miRs-143 and-145, which are both downregulated in
PCa cell lines and tissues\textsuperscript{65,66}. However, these studies did not reach a consensus on their effects on FN1\textsuperscript{34,65,67}.

The biological function of FNDC1 has not been well studied. The FNDC1 gene is located on the 6q25.3 human chromosomal region\textsuperscript{52,68}. The limited data available on FNDC1 reports that FNDC1 has also been associated with prostate leiomyosarcoma\textsuperscript{94,69}. Prostate leiomyosarcoma is a mesenchymal tumor that is particularly rare, highly aggressive, and accounts for less than 3.7% of primary prostate diseases\textsuperscript{69,70}. However, a functional role for FNDC1 in PCa has not been reported. Our study is the first to demonstrate a microRNA regulation of FNDC1.

c-MYC regulation by the miR-1207-3p pathway in prostate cancer
Studies have shown that PVT1/MYC cooperation is a fundamental feature in all cancers with 8q24 amplification and 98% of the 8q24 amplicons contained amplification of both the MYC and PVT1 loci\textsuperscript{71-74}. c-MYC is a proto-oncogene linked to PCa aggressiveness\textsuperscript{48,71,72,74-76}. Furthermore, c-MYC has been a consequence of antiandrogen treatment, and has been reported to be downstream of AR in some PCa\textsuperscript{71,74,75,77}. Interestingly, miR-1207-3p is encoded at the PVT1 gene locus, which is located downstream of c-MYC on the 8q24 human chromosomal region\textsuperscript{48,71,72,74,78}. Although miRNAs have also been implicated in the regulation of c-MYC, the mechanisms regulating c-MYC remains unclear in PCa\textsuperscript{79,80}. Consequently, we aimed to determine if c-MYC is regulated and therapeutically targetable via our novel miR-1207-3p pathway.

Clinical significance of miR-1207-3p in prostate cancer
Using our novel analogs, we sought to investigate if miR-1207-3p regulates AR as this may play a role in the progression from androgen-dependent to the life-threatening castration-resistant
prostate cancer (CPRC)\textsuperscript{5,17,34}. At diagnosis, clinical and pathologic features, such as prostate specific antigen (PSA) level, pathologic Gleason grade, and tumor stage, are used to inform clinical decision-making\textsuperscript{8,9,43}. The Gleason grading system is used to help evaluate the prognosis of men with PCa using samples from a prostate biopsy\textsuperscript{25}. To determine if miR-1207-3p can be a useful non-invasive biomarker in PCa clinical decision-making, we examined the expression levels of miR-1207-3p and its molecular targets in PCa patients by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR), and publicly available gene expression datasets in the Oncomine database. To assess miR-1207-3p’s ability to improve risk stratification in human clinical PCa, we analyzed correlation with Gleason scores. Additionally, because CRPC has been shown to be resistant to current anti-androgen therapies, preventing/treating antiandrogen resistance is a major clinical challenge. Therefore, we compared the efficacy of NB1207 and NB5, two novel synthetic analogs of miR-1207-3p, with current anti-AR treatments for CRPC (abiraterone, enzalutamide and apalutamide) \textit{in vitro}.

\textbf{Racial disparity in prostate cancer}

For reasons still unclear, men of African ancestry (moAA) have the worlds’ highest incidence of PCa \textsuperscript{47,81-84}. MoAA exhibit 19\% increased incidence and 37\% increased mortality rate from PCa compared to Caucasian men (CM) and disparities in tumor aggressiveness remain after controlling for social determinants\textsuperscript{82-86}. Consequently, aggressiveness of PCa may be due to specific biological factors\textsuperscript{83,87}. To address this high mortality, effective early detection and therapeutic strategies are needed\textsuperscript{82,83,87,88}. Undoubtedly, miRNAs are dysregulated in PCa\textsuperscript{85,89}. However, miR-1207-3p expression in PCa in moAA is unknown. Therefore, we investigated the expression of miR-1207-3p and its molecular targets in PCa cells of moAA by evaluating the expression profile of miR-
1207-3p, FNDC1, FN1, AR and c-MYC in the prostate tissues in a cohort of West African Black males. Our study seeks to determine if miR-1207-3p and its molecular targets may be useful in increasing our understanding of the molecular mechanisms underlying PCa in moAA. Moreover, such strategies have the prospective to ultimately result in novel diagnostic, prognostic biomarkers as well as specific approaches for treatment that will help reduce PCa disparities in moAA.
Chapter 2:  
Materials and Methods
Cells and cell culture conditions

Androgen-dependent RWPE-1 cells were cultured in Keratinocyte-Serum free medium (SFM) supplemented with 0.05 mg/ml bovine pituitary extract (BPE), 5 ng/ml epidermal growth factor (EGF) and 1% Penicillin-Streptomycin. Androgen-dependent WPE1-NA22 cells were cultured in Keratinocyte-SFM medium supplemented with 0.05 mg/ml BPE, 5 ng/ml EGF and 1% Penicillin/Streptomycin. Androgen-dependent MDA PCA 2b cells were cultured in F-12K medium supplemented with 20% fetal bovine serum, 25 ng/ml cholera toxin, 10 ng/ml mouse Epidermal Growth Factor, 0.005 mM phosphoethanolamine, 100 pg/ml hydrocortisone, 45 nM selenious acid, 0.005 mg/ml bovine insulin. Androgen-independent PC-3 cells were cultured in F-12K medium supplemented with 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin. Androgen-independent 22Rv1 cells were cultured in RPMI 1640 supplemented with 10% heat inactivated FBS, and 1% Penicillin/Streptomycin. RWPE-1, WPE1-NA22, PC-3, MDA PCA 2b and 22RV1 cell lines were purchased from the American Type Culture Collection. Androgen-dependent LNCaP cells were cultured in RPMI 1640 supplemented with 10% heat inactivated FBS, 1% Penicillin/Streptomycin and 10nM testosterone. Androgen-independent C4-2B cells were cultured in DMEM supplemented with 200ml Ham’s F12, 10% heat-inactivated FBS, 1% Penicillin/Streptomycin, insulin (5µg/ml), triiodothyronine (13.65 pg/ml), human apo-transferrin (4.4 µg/ml), d-Biotin (0.244 µg/ml), and Adenine (12.5 µg/ml). The C4-2B cell line was obtained from MD Anderson Cancer Center under a materials transfer agreement with Hunter College of The City University of New York. Androgen-independent E006AA and E006AA-hT were cultured in DMEM supplemented with 1% fetal bovine serum and 1% Penicillin/Streptomycin. E006AA and E006AA-hT were obtained from Dr. Shahriar Koochekpour under a materials transfer agreement between Roswell Park Cancer Institute and Hunter College of The City.
University of New York\textsuperscript{1}. These cells were cultured at 37C/5\% CO2, under humidified atmosphere.

**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), 50nM miR-1207-3p oligonucleotide inhibitor (MISSION® Synthetic microRNA Inhibitor, human, product# HSTUD0066) (Sigma-Aldrich, St. Louis, MO, USA), or novel synthetic miRNA-1207-3p duplexes (biotinylated scramble duplex (NB1), scramble duplex (NB2), biotinylated mutant duplex (NB3), mutant duplex (NB4), miR-1207-3p duplex (NB5), or biotinylated miR-1207-3p duplex (NB1207), (patent pending)), using Lipofectamine RNAiMAX (Thermo Fisher Scientific Inc; Wilmington, DE, U.S.A) according to the manufacturer’s instructions. Transfected cells are then incubated at 37°C for a total duration of 24 hours before cells are lysated\textsuperscript{1}.

**Transfection of siRNA**

Cells were transfected with fibronectin, FNDC1, AR or c-MYC siRNAs. Negative control (scramble) siRNAs which does not lead to specific degradation of any known mRNA was used as the negative control\textsuperscript{1}.
RNA isolation and quantitative real-time polymerase chain reaction (qPCR) analysis

RNA isolation and qPCR were performed as previously described. miR-1207-3p and RNU6_B12 snRNA primers were purchased from (Qiagen, Hilden, Germany). 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) and purchased through Millipore Sigma (St Louis, U.S.A).

Click-iT EdU Alexa Fluor 488 Imaging Kit

Cells were transfected with either oligonucleotides of miR-1207-3p, negative controls, siRNAs against FNCD1, FN1, AR or c-MYC as well as synthetic analogs and mutants of miR-1207-3p. Assay was performed according to manufacturer’s instructions: (Click-iT™ EdU Alexa Fluor™ 488 Imaging Kit, Cat. No.: C10337, ThermoFisher Scientific Inc; Wilmington, DE, U.S.A). EdU is a nucleoside analog of thymidine and is incorporated into DNA during active DNA synthesis. Detection is based on a click reaction, 2-3 a copper-catalyzed covalent reaction between an azide and an alkyne. EdU contains the alkyne and Alexa Fluor dye contains the azide. Cells were imaged and analyzed using the Motic Images Plus 2.0 Software (Motic; British Columbia, Canada) and positive EdU signal is indicative of S-phase cell population.

Annexin V Assay

Cells were transfected with either oligonucleotides of miR-1207-3p, negative controls, siRNAs against FNCD1, FN1, AR or c-MYC as well as synthetic analogs and mutants of miR-1207-3p for 24 hours prior to Annexin V staining. Assay was performed according to manufacturer’s
instructions: (ApoScreen® Annexin V Apoptosis Kit-FITC, Cat. No. 10010-02, SouthernBiotech Birmingham, U.S.A). Annexin V and PI staining were analyzed using flow cytometry\(^1\).

**Luciferase reporter assay**

Human FNDC1 and miRNA target clone control vector for pEZX-MT06 were purchased from GeneCopoeia, Inc, Rockville, MD, USA. Cells were seeded co-transfected with luciferase-expressing 3’ UTR clones of FNDC1 along with a 50nM miR-1207-3p oligonucleotide mimic. As the control, cells were also co-transfected with 3’UTR clones of FNDC1 with miR-1207-3p synthetic non-targeting oligonucleotide negative control. The lysate was analyzed by SpectraMax i3x multi-mode detection platform\(^1\).

**Western blot analysis**

Western blotting was performed as previously described\(^9\). Primary antibodies used were against human fibronectin: ab2413 (Abcam plc, Cambridge, UK), human FNDC1 (Y-12): sc-107546 (Santa Cruz Biotechnology, TX, U.S.A), human AR: sc-7305 (Santa Cruz Biotechnology, TX, U.S.A), human c-MYC: ab32072 (Abcam plc, Cambridge, UK), human AR-V7-specific: ab198394 (Abcam plc, Cambridge, UK), human GAPDH: g9545 and beta-actin: a5441 (Sigma-Aldrich, St. Louis, MO, U.S.A). Secondary antibodies used were either against mouse or rabbit (Sigma-Aldrich, St. Louis, MO, U.S.A) or goat (Santa Cruz Biotechnology, TX, U.S.A), as appropriate. In some cases, LI-COR with infrared fluorescence, IRDye secondary antibodies and LI-COR Odyssey CLx imaging system were also used to detect western blots without film or chemiluminescent substrates. Western blots were analyzed and quantified using Odyssey CLx imaging system, Image Studio version 5\(^1\).
RNA pulldown assay

RNA pulldown assay was performed with modifications of the protocol described by Wani et al\textsuperscript{92}. Cells were seeded in 10 cm plates. Cells were transfected with either 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, U.S.A) for 24 hours, then lysed, according to the manufacturer’s instructions. Beads were washed, blocked and prepared as described\textsuperscript{92}. Target mRNA was captured and post-capture bead washing was performed. Next, target mRNA and control lysate RNA were purified using Qiagen RNeasy kit (Cat no: 74104, Qiagen, Hilden, Germany) 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 qRT-PCR to examine the mRNA expression levels and enrichment of FNDC1 and c-MYC\textsuperscript{1}.

Patients and Samples

For the human study at Moffitt Cancer Center, prostate cancer tissues were collected from 377 primary prostate cancers with macro-dissection (Table 3.1). Total RNA of tumor tissues was extracted and RNA samples from the 377 patient cases were analyzed by qRT-PCR 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 $2^{\Delta\Delta Ct}$ method. The following primers were used: Fibronectin-F: TCG AGG AGG AAA TTC CAA
To evaluate miR-1207-3p expression in clinical samples, we used tissue specimens from 404 patients (389 CM, and 15 moAA) with primary adenocarcinoma of the prostate from 1988 to 2003 at the Moffitt Cancer Center, Tampa, Florida, USA. After histopathologic confirmation, PCa tissues were obtained from unstained slides with macro-dissection. Total RNA was extracted from PCa tissues using superscript IV first-strand Synthesis system (Life technologies No. 18091200) and quantified using UV spectrophotometer NanoDrop 2000 (Thermo Scientific, USA). The cDNA templates were synthesized from RNA samples by SuperScript using random hexamers. RNA samples from the 404 patient samples were analyzed by qRT-PCR to examine the expression of miR-1207-3p.

Gene expression was determined using power SYBR Green Real time PCR Master Mix (Life technologies No. 4367649) and 2.0 µl of cDNA template. qRT-PCR was performed on a ABI7900 machine using the following amplification conditions: 10 min at 95°C; followed by 45 cycles of 15 seconds at 95°C; 30 seconds at 55°C; and 30 seconds at 72°C. All assays were carried out in triplicate to control for technical variance. Ct values were determined using the SDS ver2.3 software (Bio-Rad). miR 1207-3p expression was normalized with RNUB6_12 expressions within each sample. Relative quantification of target gene expression was evaluated using the comparative $2^{\Delta\Delta Ct}$. 
Institutional Review Board approvals were obtained for the study protocol at the University of South Florida and the Moffitt Cancer Center, Tampa, Florida (Tables 3.1 & 3.2). Signed informed consent was obtained from all study participants. Clinicopathological and demographic information of the patients are summarized in Table 3.1 and Table 3.2.\textsuperscript{1,2,93}

In a separate study, we evaluated the expression profile of miR-1207-3p, FNDC1, FN1, AR and c-MYC in histologically confirmed normal prostate tissue (n=24), benign prostate tissue (n=44) and malignant prostate tissue (n=29) from prostatectomy or transrectal ultrasound-guided biopsies in patients recruited at the University College Hospital, Ibadan, Nigeria. Analysis of miR-1207-3p, FNDC1, FN1, AR, and c-MYC expression in malignant prostate tissue with Gleason scores ≥8 versus Gleason scores <8 was performed. This study was performed following protocols approved by both City University of New York Institutional Review Board and the University of Ibadan Institutional Ethics Board. RNA extraction, cDNA synthesis and qRT-PCR were performed to analyze mRNA expression of miR-1207-3p, FNDC1, FN1, AR and c-MYC.

**Statistical Analysis**

Differences in two and three clinical outcome groups were compared using the two-tailed Student’s $t$-test and ANOVA test, respectively. For evaluating the association of miR-1207-3p expression with PCa death and PCa recurrence in CM, a competing risk approach was applied. miR-1207-3p expression was categorized to three groups: low (<3), median (3-6) and high (≥6) based on the tertile cut-points. Factors associated with PCa death were evaluated using competing-risk regression.\textsuperscript{94} The cumulative incidence curves of PCa death were generated by the subgroups of miR-1207-3p expression. For evaluating miR-1207-3p expression associated with PCa survival,
PCa death was the primary interest and non-PCa death was treated as a competing risk. Survival time was defined as the time from date of PCa diagnosis to date of death. For evaluating association of miR-1207-3p expression with PCa recurrence, PCa recurrence was the primary interest and non-recurrence death was treated as a competing risk. Time to event was defined as time from the date of PCa diagnosis to PCa recurrence or non-recurrence death, whichever comes first. Data were censored at the last follow-up date. The same approach was applied to evaluate the association of miR-1207-3p expression with PCa recurrence. The candidate factors taken into consideration in modeling included age at diagnosis, Gleason score, and tumor stage. PSA was excluded from the models because the PSA values were missing in 150 CM. The level of significance was established using a P value less than 0.05\(^2\).

For functional experiments (proliferation, migration and apoptosis), results were 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 or Tukey post-hoc test were used for analysis of each set of continuous and categorical data. Statistical differences in the relative miRNA expression profiles were determined with one-way, two-way analysis of variance (ANOVA) and Tukey post-hoc test using the SPSS Statistics software (http://www-01.ibm.com/software/analytics/spss/) on normalized data. P values <0.05 were considered significant\(^1,2\).
Chapter 3:

miR-1207-3p regulates the androgen receptor in prostate cancer via FNDC1/FN1
Introduction

PCa is a multi-factorial, complex disease, with the exact mechanisms for its development and progression unclear\textsuperscript{10,12,95,96}. 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 the 8q24 human chromosomal region as one of the most important susceptibility loci for PCa\textsuperscript{13,48,51,86,97,98}. 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\textsuperscript{51,97,99-101}. The PVT1 gene locus encodes a cluster of six microRNAs (miRNAs), including microRNA-1207-3p (miR-1207-3p)\textsuperscript{47,50,93}. 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\textsuperscript{7,27,41}. 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 direct 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.
Results

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

To investigate the expression profile, the functional role, and the molecular mechanisms of miR-1207-3p in PCa, a panel of 8 prostate cell lines modeling different clinical characteristics of PCa was used because of the widely known heterogeneity of PCa. In detail, the panel included the RWPE-1 (non-tumorigenic prostate epithelial cell line, Caucasian male (CM)), WPE1-NA22 (derived from RWPE-1, indolent, androgen-dependent, CM), MDA PCa 2b (aggressive, androgen dependent, from a male of African ancestry (moAA)), PC-3 (aggressive, androgen-independent, CM), E006AA (indolent, androgen-independent, moAA), E006AA-hT (derived from E006AA, aggressive, androgen-independent, moAA), LNCaP (aggressive, androgen-dependent, CM) and C4-2B (derived from LNCaP, aggressive, androgen-independent, CM) (Figure 3.1A). As shown in figure 3.1B, 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 nearly 50% less miR-1207-3p expression in androgen-dependent PCa cell lines (WPE1-NA22, MDA PCa 2b and LNCaP). Interestingly, the expression level of miR-1207-3p was even further decreased, by approximately 80%, in androgen-independent cell lines (PC-3, E006AA, E006AA-hT and C4-2B). The data suggest that miR-1207-3p underexpression may be associated with the onset and progression of PCa and could potentially be used as a new predictive biomarker for monitoring the susceptibility of development of androgen-independent PCa.

To elucidate the role of miR-1207-3p expression in PCa cell function, commercially available 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 qRT-PCR demonstrated that the miR-1207-3p inhibitor suppresses 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 3.1C-E). A 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.

Figure 3.1: 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; moAA, men of African Ancestry; AR, androgen receptor. (B) Three separate qPCR experiments were performed. Each experiment was done in quadruplicates. The data showed that miR-1207-3p expression was decreased in all the tumorigenic cell lines. (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. As a control, RWPE-1 cells were treated with 50nM non-targeting negative control oligonucleotide. (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. As a control, WPE1-NA22 cells were treated with 50nM non-targeting negative control oligonucleotide. Dose-dependent inhibition and dose dependent overexpression of miR-1207-3p expression by the oligonucleotide inhibitor and mimic of miR-1207-3p was observed. Results are 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 $P<0.05$. (Originally published in Experimental Cell Research).

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

To explore the molecular mechanisms through which miR-1207-3p exerts its inhibitory effects in PCa, the potential molecular targets of miR-1207-3p were investigated. The molecular targets of miR-1207-3p have never been studied in any system. To identify potential targets, two different miRNA molecular target prediction algorithm tools, miRBase and miRDB, we used. Fibronectin type III domain containing 1 (FNDC1) was identified as a putative molecular target of miR-1207-3p. FNDC1 contains the conserved ‘Fibronectin type III domain’ of Fibronectin (FN1)\textsuperscript{52,55}. However, a functional role for FNDC1 in PCa has not been reported.

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 3.1A. 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 3.2A). 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 3.2B).
**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. Due to the heterogeneity of PCa, we utilized widely used PCa cell lines that model various characteristics of PCa. 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 3.2C) 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 (Patent application publication number, US20170121711 A1). This approach allows for the sensitive and specific detection of miRNA-mRNA interactions. MDA PCa 2b cells were transfected with either 1nM synthetic biotinylated miR-1207-3p duplex or 1nM synthetic biotinylated scramble duplex as a control.
RNA was subsequently pulled down with streptavidin coated magnetic beads. The RNA was then analyzed for FNDC1 expression with qRT-PCR. 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 approximately 2,000-fold (Figure 3.2D). Therefore, FNDC1 is a direct molecular target of miR-1207-3p.

Figure 3.2: 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) Biotinylated microRNA pulldown was performed for the isolation of direct targets of a mature miRNA, using synthetic biotinylated miRNA duplexes. Biotinylated scramble duplex and biotinylated microRNA-1207-3p duplex were transfected into MDA PCa 2b cells. Pull-down miRNA enrichments were assayed by qRT-PCR. Activity is normalized to the biotinylated scramble duplex set to 1.0. Bars represent SD. *P<0.05. (Originally published in Experimental Cell Research1).
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 3.1). The data revealed higher FN1 mRNA expression in all the PCa cell lines compared to non-tumorigenic RWPE-1 cell line\(^1\) (Figure 3.3A). Interestingly, PCa cell lines from moAA (MDA PCa 2b, E006AA and E006AA-hT) showed higher FN1 expression compared with cells from Caucasian men, suggesting that FN1 may play a role in the racial disparity in PCa (Figure 3.3A). Additional validation by western blotting confirmed increased FN1 protein expression in all the PCa cell lines compared to the non-tumorigenic prostate epithelial cell line, RWPE-1 (Figure 3.3B). Further work was performed, to assess the effect of inhibition or overexpression of miR-1207-3p on FN1 expression in RWPE-1 cells. qRT-PCR 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 3.3C). 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 3.3D). These findings demonstrate that FN1 is a component of the molecular pathway regulated by miR-1207-3p in PCa.
Figure 3.3: miR-1207-3p regulates FN1 expression. (A & B) Two separate qRT-PCR and western blotting experiments were performed. Each qRT-PCR experiment was performed in quadruplicates. (C) Effect of overexpression and inhibition of miR-1207-3p on fibronectin expression in RWPE-1 cells. qRT-PCR experiments were performed in quadruplicates, and three separate times. Statistical differences for A & C were determined with one-way ANOVA. All the criterions for significance was set at \( P < 0.05 \). (D) Effect of overexpression of miR-1207-3p on fibronectin expression in PC-3, E006AA cells, and E006AA-hT cells. Western blot experiments were performed two separate times. (Originally published in Experimental Cell Research).

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 (Figure 3.4A). 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 (Figure 3.4B). The EdU proliferation assay revealed that proliferation of MDA PCa 2b cells was inhibited by approximately 80% when compared to the scramble siRNA (Figure 3.4C). We observed that migration, assessed via wound healing, of C4-2B cells was reduced significantly by approximately 55% by transfection with FNDC1 siRNA as compared to the scramble siRNA (Figure 3.4D). 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.
Figure 3.4: Effect of siRNA knockdown of FNDC1. (A) A dose response test of the effect of siRNA knockdown of FNDC1 was performed on MDA-PCa 2b using western blotting. Cells were treated with a non-targeting scramble siRNA and increasing concentrations of siRNA against FNDC1. Inhibition of FNDC1 expression by the FNDC1 siRNA was dose-dependent. (B) Effect of 100pM siRNA against FNDC1 on apoptosis of C4-2B cells via Annexin V staining. Inhibition of FNDC1 expression by the siRNA increases apoptosis. (C) Effect of 100pM siRNA against FNDC1 on proliferation in MDA PCa 2b cells using the Click-iT Edu proliferation assay (10X magnification). Inhibition of FNDC1 expression by the siRNA inhibits proliferation. (D) Effect of 100pM siRNA against FNDC1 on C4-2b cells. Inhibition of FNDC1 expression by the siRNA inhibits migration. (Originally published in Experimental Cell Research^1).
FN1 regulates proliferation, apoptosis and migration of PCa cells

Next, a dose-response experiment was used to determine the efficacy of the custom-designed FN1 siRNA and appropriate concentrations for silencing FN1 protein expression (Figure 3.5A). 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 (Figure 3.5B).

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% (Figure 3.5C). 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 after FN1 silencing (Figure 3.5D). 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 (Figure 3.5E). 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.
Figure 3.5: Effect of siRNA knockdown of fibronectin. (A) A dose response test of the effect of siRNA knockdown of fibronectin was performed on PC-3 cells using western blotting. Cells were transfected with 10pM non-targeting scramble siRNA and increasing concentrations of siRNA against fibronectin. Inhibition of fibronectin expression by our fibronectin siRNA was dose-dependent. (B) Effect of 25pM siRNA against fibronectin in PC-3 cells, E006AA cells, and E006AA-hT cells, respectively. Western blot experiments were performed three separate times. (C) Effect of a 25pM siRNA against fibronectin on proliferation in MDA PCa 2b cells was assessed using the Click-iT Edu proliferation assay (10X magnification). Inhibition of fibronectin expression by the fibronectin siRNA inhibits proliferation. (D) Effect of 25pM siRNA against fibronectin on PC-3. Inhibition of fibronectin expression by the fibronectin siRNA increases apoptosis. (E) Effect of 25pM siRNA against fibronectin on WPE1-NA22. Inhibition of fibronectin expression by the fibronectin siRNA inhibits migration. (Originally published in Experimental Cell Research.)
miR-1207-3p regulates the androgen receptor via FNDC1/FN1

It is well established that aberrant AR expression and activity play a critical role in the development and progression of PCa. Thus, we wanted to investigate if miR-1207-3p regulates AR in our novel pathway as this may play a determinant role in the progression from androgen-dependent PCa to the incurable androgen-independent PCa (CRPC). 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 3.6A). 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 3.6B). Thus, the result shows 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 3.1B). 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 3.6C). 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
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 result clearly shows that the overexpression of miR-1207-3p inhibits the protein expression of FNDC1 (>60%), FN1 (~40%), and AR expression (~20%) (Figure 3.6D). The result positively correlates to that of the effect of silencing FNDC1, with protein expression inhibition of >70% for FNDC1, >50% for FN1. Remarkably, AR exhibits the most striking inhibition at nearly 85% (Figure 3.6D). Taken together, the results indicate 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.

**Figure 3.6:** 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, Fibronectin, and AR expression in LNCaP and C4-2b cells, respectively. (C) Effect of silencing fibronectin 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. (Originally published in Experimental Cell Research).
FNDC1, FN1, and AR expression are positively correlated in human prostate cancers and upregulation is associated with aggressive disease

In view of this novel discovery 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 three separate studies: Moffitt Cancer Center-based study, 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 patients that remained alive. These results are based on analysis of FN1 expression in 377 cases (Table 3.1).
Table 3.1. Fibronectin expression in prostate cancer tissues of 377 patients. List and characteristics of human prostate cancer samples. Variables: number, mean; SD, standard deviation; P-value. (Originally published in Experimental Cell Research)1).

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.96 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 3.7A). 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 3.7B). Similarly, in the dataset from the study by Grasso et al.105, 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 3.7C). Together, these findings indicate a positive correlation between 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.

**Figure 3.7:** FNDC1, FN1, and AR expression is correlated in prostate cancers and upregulation is associated with aggressive disease. (A) Expression of FNDC1, FN1 and AR genes in benign prostate tissues compared to localized prostate cancer. (B) Expression of FNDC1, FN1 and AR genes in localized prostate cancer compared to metastatic prostate cancer. (C) Expression of FNDC1, FN1 and AR genes in localized primary prostate cancer compared to metastatic prostate cancer. Results demonstrate upregulation of FNDC1, fibronectin and AR in aggressive disease. (Originally published in Experimental Cell Research).

miR-1207-3p is differentially expressed in PCa in men of African ancestry compared to Caucasian men

For reasons still unclear, men of African ancestry (moAA) have the highest incidence of PCa globally^47,81-83^. MoAA have approximately two-thirds higher incidence and two times the
increased mortality rate compared to Caucasian men (CM)\textsuperscript{47,83,85,106}. Additionally, moAA are more frequently diagnosed with worse clinicopathological features (PSA levels, Gleason grades, advanced tumor stage, etc.) than CM\textsuperscript{81,83,88,107}.

Consequently, we investigated the clinical significance of miR-1207-3p in PCa and to determine if it contributes to the disproportionate aggressiveness of PCa in moAA. We observed that miR-1207-3p expression was significantly less in PCa tissues of moAA in comparison to the PCa tissues of CM (3.00±2.65 versus 5.36±3.76, \(P = 0.016\)) (Table 3.2)\textsuperscript{2}. This suggests that miR-1207-3p may play a role in explaining the disproportionately increased aggressiveness of PCa in moAA\textsuperscript{2}.

<table>
<thead>
<tr>
<th>Variables</th>
<th>N</th>
<th>Mean ±SD</th>
<th>(P)</th>
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</thead>
<tbody>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>moAA</td>
<td>15</td>
<td>3.00±2.65</td>
<td></td>
</tr>
<tr>
<td>CM</td>
<td>389</td>
<td>5.36±3.76</td>
<td>0.016</td>
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Table 3.2: miR-1207-3p is differentially expressed between moAA and CM. Description and characteristics of human prostate cancer samples from 404 patients. N, Number; Avg, Average; SD, Standard Deviation; \(P\), \(P\)-value. (Originally published in Translational Oncology\textsuperscript{2}).

**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 evaluated its role in cell migration. Wound healing assays were performed using RWPE-1 (Figure 3.8A), WPE1-NA22 (Figure 3.8B) and PC-3 cells (Figure 3.8C). As shown in figure 3.8A-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 inhibited migration by 50% in both PCa cell lines, WPE1-NA22 (Figure 3.8B) and PC-3 (Figure 3.8C), thus 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 3.8D). 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 3.8E). 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 3.8F). These results indicate that miR-1207-3p induces apoptosis in PCa cells.

We next investigated whether miR-1207-3p regulates proliferation, by performing 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. As shown in figure 3.8G-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 3.8G). 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 3.8H & 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. The results demonstrate that increased expression of miR-1207-3p significantly inhibits cellular proliferation in PCa.

**Figure 3.8: 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 (10X magnification) (G-I). Results 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 $P<0.05$. (Originally published in Experimental Cell Research1).
Prostate cancer (PCa) is the most frequently diagnosed solid organ cancer in males in the developed world. Despite the high prevalence, 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. These small non-coding RNAs are attractive for clinical applications for many reasons. One compelling reason is that miRNAs direct targeting of many mRNAs that are translated to proteins explains miRNAs differential expression between non-cancer and cancer cells. Understanding the regulatory pathways controlled by miRNAs 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 a definitive therapeutic strategy in PCa.

miRNA-1207-3p is encoded at the non-protein coding gene locus PVT1 on the 8q24 human chromosomal region, an established PCa susceptibility locus. However, miRNA-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 central cellular functions associated with 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, based on the
observation that miR-1207-3p expression was further reduced in PCa cell lines derived from castration-resistant PCa (CRPC) versus castration-sensitive PCa, this study suggests that loss of miR-1207-3p may potentially be an early PCa biomarker of progression to CRPC.

It is well known that being of African ancestry is a non-modifiable risk factor for PCa.\cite{82,83} 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.\cite{83,109} Although the importance of miRNAs in PCa in moAA is still unclear, our results show that miR-1207-3p expression is particularly low in PCa cell lines derived from moAA (MDA PCa 2b, E006AA and E006AA-hT). More importantly, results from human studies suggests that miR-1207-3p may contribute to the disproportionate aggressiveness of PCa in moAA. In this regard, miR-1207-3p is differentially expressed in primary tumors of moAA tissues compared to primary tumors of CM. To our knowledge, this is the first report of a microRNA that directly correlates with aggressive PCa, yet shows divergent expression between CM and moAA\cite{2}. These data suggest that it may be possible to predict clinical behavior of PCa based upon miRNA-1207-3p expression in primary PCa. While the overall sample size of the present study is relatively large, a limitation is the small sample size of the moAA sub-group. Although challenging, due to issues related to recruitment, a larger study enriched with a substantial proportion of moAA is warranted. In addition, the present study did not examine miR-1207-3p expression in non-tumor versus tumor tissues. Therefore, the current data do not provide information regarding whether miR-1207-3p is differentially expressed in normal prostate versus PCa tissue\cite{2}. Further studies evaluating the role of miR-1207-3p in PCa racial disparity are required.
Since the seven tumorigenic PCa cell lines studied showed consistent and significant underexpression of miR-1207-3p 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. Whereas inhibition of miR-1207-3p expression leads to inhibition of apoptosis and increases in cellular proliferation of PCa cells, overexpression of miR-1207-3p significantly induced apoptosis and inhibited proliferation of PCa cells. Strikingly, inhibition of miR-1207-3p led to an approximately four-fold increase in the migratory capacity of both non-tumorigenic and tumorigenic prostate epithelial cells. These data demonstrate that miR-1207-3p regulates key cellular processes dysregulated in the development and progression of PCa. 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, independently identify FNDC1 as a putative molecular target of miR-1207-3p. As demonstrated, 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, we are first to demonstrate FNDC1 as a direct molecular target of miR-1207-3p. FNDC1 contains a major component of the structural domain of FN1. FN1 has been implicated in many cancers and known to be a major regulator of migration of cancer cells. Overexpression of miR-1207-3p downregulates FN1, and specific silencing of FNDC1 also downregulates FN1. Thus, miR-1207-3p effects in PCa are due to binding to and loss of FNDC1, and subsequent loss of FN1.
The biological function of FNDC1 has not been well studied. To our knowledge, this study is the first to demonstrate microRNA regulation of FNDC1. So far, only two miRNAs have been shown to directly target a member of the FN1 family in PCa, miRs-143 and-145 which are both downregulated in PCa cell lines and tissues\textsuperscript{65,66}. However, these studies did not reach a consensus on their effects on FN1\textsuperscript{34,65,67}. Nevertheless, FN1 is upregulated in PCa as we observed from three independently analyzed human studies\textsuperscript{64,112}. By identifying miR-1207-3p as a regulator of FN1, and its subsequent role in the regulation of proliferation, apoptosis, and migration, FNDC1 is shown to have a clear and prominent role in PCa.

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\textsuperscript{104}. Although AR signaling has been studied for decades, the molecular mechanisms behind its dysfunction remain unclear\textsuperscript{21,104,113}. Undoubtedly, AR dysfunction precedes the progression CRPC\textsuperscript{21,103}. We demonstrated that AR protein expression, in positive correlation with FN1 and FNDC1 protein expression, is significantly more elevated in the CRPC cellular model, C4-2B, in comparison to its castration-responsive counterpart, LNCaP. In summary, we have demonstrated the molecular effects of miR-1207-3p: loss of FNDC1 expression and consequent loss of FN1 expression lead to loss of AR expression. Thus, this is the first description of a novel miR-1207-3p/FNDC1/FN1/AR regulatory pathway in PCa (Figure 3.9)\textsuperscript{1,93}. 
Figure 3.9: A novel miR-1207-3p/FNDC1/FN1/AR regulatory pathway in prostate cancer. MicroRNA-1207-3p inhibits proliferation and migration and induces apoptosis of prostate cancer cells by the direct molecular targeting of the 3’UTR of FNDC1. miR-1207-3p induced loss of FNDC1 expression leads to the sequential loss of FN1 and AR expression. (Originally published in RNA & Disease93).

The clinical relevance of this novel regulatory pathway was investigated in several independently analyzed human prostate studies: Moffitt Cancer Center-based study, 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 PCa. Our Moffitt 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. Importantly, 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. This underscores the importance of further studying miR-1207-3p for potential therapeutic applications especially for metastatic PCa.
Author contributions

Dibash K. Das wrote the published articles and prepared figures 3.1-3.9 and tables 3.1-3.2\textsuperscript{1,2,93}. Michelle Naidoo helped in data acquisition for figure 3.6\textsuperscript{1}. Adeodat Ilboudo helped prepared figure 3.1B\textsuperscript{1}. Brian D. Robinson led data acquisition and prepared figure 3.8 and corresponding method section\textsuperscript{1}. Jong Y. Park led data acquisition and manuscript writing for tables 3.1 & 3.2 and corresponding method section\textsuperscript{2}. Hui-Yi Lin contributed to biostatistical analysis and writing for tables 3.1 & 3.2\textsuperscript{2}. Dibash K. Das, Jong Y. Park and Olorunseun O. Ogunwobi contributed to data analysis of tables 3.1 & 3.2\textsuperscript{2}. Joseph R. Osborne made intellectual contributions and reviewed the published work in Experimental Cell Research and Translational Oncology\textsuperscript{1,2}. Olorunseun O. Ogunwobi supervised all the work from conception to publication preparation for all 3 articles\textsuperscript{1,2,93}.

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Human and animal rights

Signed informed consent was obtained from all study participants.
Chapter 4:
c-MYC is regulated and therapeutically targetable via the miR-1207-3p/FNDC1/FN1/AR pathway in castration resistant prostate cancer
Introduction

Although PCa is second leading cause of cancer-related death for men in the US, men with PCa do not die from localized prostate cancer\textsuperscript{9,12,11,25,103,105}. Consequently, the major scientific challenge lies in understanding the molecular mechanisms by which prostate cancer progresses from the castration-responsive subtype to the lethal castration-resistant PCa (CRPC)\textsuperscript{17,25,103}.

In the US, the average 5-year survival rate for localized PCa is 100\%, but for CRPC it is less than 30\%\textsuperscript{17,18,114}. CRPC is resistant to androgen deprivation therapies (ADT), and is currently incurable\textsuperscript{17,18,25,103}. Despite CRPCs resistance to ADT, it is now well established that a central mechanism driving CRPC progression is sustained androgen receptor (AR) signaling despite castration levels of testosterone which can be attributed to variable combinations of AR specific gene-amplification, mutations, increased AR sensitivity, and AR gene rearrangement promoting synthesis of constitutively-active truncated AR splice variants\textsuperscript{32,115-118}. Given the failure of ADT to completely abrogate AR-mediated functions in CRPC patients, there remains a significant demand for novel, effective and safe therapeutic strategies for CRPC\textsuperscript{115,118}. Furthermore, the current tool for screening for PCa, prostate specific antigen (PSA), is highly controversial as it is not PCa-specific and has a high false positive rate\textsuperscript{14,15}. Consequently, new biomarkers are urgently needed for the early detection of PCa, and it is crucial that these novel biomarkers are sensitive enough to discriminate between indolent PCa and CRPC.

Because miRNAs are found to be dysregulated in many cancers, they have the potential to be used as potent tools to support the diagnosis and early detection of cancers\textsuperscript{7,27,43}. Of note, miR-1207-3p is located downstream of the well-established proto-oncogene c-MYC on the 8q24 human
chromosomal region (Figure 4.1)\textsuperscript{72,97}. c-MYC overexpression at the RNA and protein levels has subsequently been linked to a wide variety of human cancers\textsuperscript{72,75,76}. Additionally, c-MYC is commonly found to be amplified in up to 72\% of CRPCs\textsuperscript{72,75,76}. Furthermore, significant amplification of c-MYC has been a consequence of antiandrogen treatment, and c-MYC is downstream of AR in some PCa\textsuperscript{75,76}. Also, miRNAs have been implicated in the regulation of c-MYC\textsuperscript{79,80,119}. Nonetheless, the mechanisms regulating c-MYC remain unclear in PCa.

**Figure 4.1: c-MYC has been linked to prostate cancer aggressiveness.** The chromosomal region 8q24 is associated with aggressive PCa. miR-1207-3p is encoded at the *PVT1* gene locus, which is located downstream of c-MYC.

This study investigated if c-MYC is regulated by the novel miR-1207-3p/FNDC1/FN1/AR pathway in CRPC. We also describe for the first time miR-1207-3p’s ability to potentially improve risk stratification in human clinical PCa. Additionally, using *in vitro* CRPC cell models, the efficacy of NB1207 and NB5 was compared with second generation anti-androgen drugs to evaluate their therapeutic potential in CRPC treatment.
Results

Underexpression of miR-1207-3p and the overexpression of FNDC1, FN1, AR and c-MYC are significantly associated with aggressive prostate cancer

We assessed the expression profile of miRNA-1207-3p, FNDC1, FN1, AR and c-MYC in histologically confirmed normal prostate (n=24), benign prostatic hyperplasia tissue (n=44) and malignant prostate tumor tissue (n=29) from prostatectomy or transrectal ultrasound-guided biopsies in patients recruited at the University College Hospital, Ibadan, Nigeria, following an Institutional Ethics Board approved protocol. Analysis revealed that miR-1207-3p is significantly underexpressed in prostate tumor tissues (0.10±0.02, 95% CI [0.05, 0.149], P=0.000) in comparison to normal prostate tissue (1.02±0.17, 95% CI [0.67, 1.365], P=0.000) (Figure 4.2). Furthermore, there was significant differential miR-1207-3p expression in benign prostatic hyperplasia tissues (0.743±.15, 95% CI [.43, 1.05] P=0.004) in comparison to prostate tumor tissues (0.10±0.02, 95% CI [0.05, 0.149], P=0.004) (Figure 4.2). This is the first description of miR-1207-3p differential expression in human clinical PCa.

Conversely, FNDC1 was significantly overexpressed in prostate tumor tissues (21.93±7.60, 95% CI [6.30, 37.54], p=0.006) in comparison to normal prostate tissues (1.62±0.41, 95% CI [0.756, 2.48], p=0.006) (Figure 4.3A). Additionally, we discovered that FNDC1 was significantly overexpressed in prostate tumor tissues (21.93±7.60, 95% CI [6.30, 37.54], p=0.001) in comparison to benign prostatic hyperplasia tissues (1.22±.17, 95% CI [.86, 1.57] P=0.001) (Figure 4.3A). We observed that similar positive correlation with advanced disease held true for FN1 (tumor: 13.71 ± 3.31, 95% CI [6.93, 20.50], P=0.000; normal: 1.11 ± 0.22, 95% CI [0.66, 1.57],
P=0.000), (tumor: 13.71 ± 3.31, 95% CI [6.93, 20.50], P=0.000; benign prostatic hyperplasia tissues: 1.12 ± 0.20, 95% CI [0.72, 1.53], P=0.000), AR (tumor: 20.33 ± 6.20, 95% CI [7.54, 33.13], P=0.001; normal: 1.06 ± 0.22, 95% CI [0.61, 1.52], P=0.001), (tumor: 20.33 ± 6.20, 95% CI [7.54, 33.13], P=0.000; benign: 1.69 ± 0.23, 95% CI [1.21, 2.16], P=0.000) (Figure 4.3A-C), and c-MYC (tumor: 34.76 ± 8.11, 95% CI [18.09, 51.43], p=0.000; normal: 1.99 ± 0.34, 95% CI [1.27, 2.70]), (tumor: 34.76 ± 8.11, 95% CI [18.09, 51.43], p=0.000; benign: 1.01 ± 0.21, 95% CI [0.59, 1.43]), p=0.000) (Figure 4.4). The significantly increased mean expression for FNDC1, FN1, AR and c-MYC in prostate tumor tissues in comparison to normal prostate tissues demonstrate that their overexpression is associated with increased risk of cancer progression. Of note, this is the first description of miR-1207-3p serving as a candidate biomarker which can differentiate between benign prostatic hyperplasia and aggressive PCa.
Figure 4.2: Underexpression of miR-1207-3p is significantly associated with aggressive PCa. miR-1207-3p, in histologically confirmed normal prostate (n=21), benign prostatic hyperplasia (n=41) and malignant prostate tissue (n=26) from prostatectomy or transrectal ultrasound-guided biopsies in patients recruited at the University College Hospital, Ibadan, Nigeria. Tissues were collected in compliance with Institutional Ethics Board approved protocols. All data were analyzed by the one-way ANOVA test & Tukey post-hoc test. P < 0.05 were considered significant.
Figure 4.3. Overexpression of FNDC1, FN1, and AR are significantly associated with aggressive PCa. (A-C) FNDC1, FN1, and AR in histologically confirmed normal prostate, benign prostatic hyperplasia, and malignant prostate tissue from prostatectomy or transrectal ultrasound-guided biopsies in patients recruited at the University College Hospital, Ibadan, Nigeria. Tissues were collected in compliance with Institutional Ethics Board approved protocols. All data were analyzed by the one-way ANOVA test & Tukey post-hoc test. P < 0.05 were considered significant.
Figure 4.4: Overexpression of c-MYC is significantly associated with aggressive PCa. c-MYC, in histologically confirmed normal prostate (n=21), benign prostatic hyperplasia (n=43) and malignant prostate tissue (n=27) from prostatectomy or transrectal ultrasound-guided biopsies in patients recruited at the University College Hospital, Ibadan, Nigeria. Tissues were collected in compliance with Institutional Ethics Board approved protocols. All data were analyzed by the one-way ANOVA test & Tukey post-hoc test. P < 0.05 were considered significant.

miR-1207-3p negatively correlates with c-MYC in prostate tumors with Gleason score ≥8. Next, we wanted to access miR-1207-3p’s ability to improve risk stratification in human clinical PCa. We analyzed miR-1207-3p in histologically confirmed malignant prostate tumor tissues (n=23). We observed that miR-1207-3p was underexpressed by nearly 3-fold in patients with a Gleason score ≥8 (n=10) versus those with a Gleason score <8 (n=13) (Figure 4.5A). In contrast, we observed that c-MYC was overexpressed by nearly 5-fold in patients with a Gleason score ≥8 (n=13) versus those with a Gleason score <8 (n=14) (Figure 4.5B). Therefore, miR-1207-3p
negatively correlates with c-MYC in tumors with Gleason score ≥8. This is the first description of miR-1207-3p’s potential role in risk stratification in human clinical PCa.

**Figure 4.5: miR-1207-3p negatively correlates with c-MYC in tumors with Gleason score ≥8.** Expression profile of miR-1207-3p and c-MYC in histologically confirmed malignant prostate tissue (n=27) from prostatectomy or transrectal ultrasound-guided biopsies in patients recruited at the University College Hospital, Ibadan, Nigeria. Tissues were collected in compliance with Institutional Ethics Board approved protocols. All data were analyzed by the one-way ANOVA test & Tukey post-hoc test. P < 0.05 were considered significant.

**c-MYC is downstream of the novel miR-1207-3p/FNDC1/FN1/AR pathway**

In light of our results demonstrating an inverse correlation between miR-1207-3p and c-MYC, we next investigated if c-MYC may possibly be a direct molecular target of miR-1207-3p. An RNA pulldown assay was performed using our custom-designed biotinylated miR-1207-3p duplex (NB1207) and our custom-designed biotinylated scramble oligonucleotide duplex (NB1), as a control. This technique allows for the definitive detection of miRNA-mRNA interactions\(^1,93\). C4-
2B cells were transfected with either NB1207 or NB1, as a control. RNA pulled down was then analyzed for c-MYC expression with qRT-PCR. We observed that compared to the RNA pulled down by our custom-designed biotinylated scramble oligonucleotide sequence, the RNA pulled down by our custom-designed biotinylated miR-1207-3p duplex was not enriched for c-MYC expression (Figure 4.6A). Therefore, c-MYC is not a direct molecular target of miR-1207-3p.

Although c-MYC is not a direct molecular target of miR-1207-3p, we observed an inverse association with miR-1207-3p and positive association with FNDC1, FN1 and AR in human PCa. Given that c-MYC has been reported to be downstream of AR in some PCa, we wanted to determine if c-MYC is downstream of the novel miR-1207-3p/FNDC1/FN1/AR pathway. Consequently, we assessed the effect of siRNAs against FNDC1, FN1 and AR on c-MYC mRNA and protein expression in the E006AA and the E006AA-hT PCa cell lines. Compared to the control scramble siRNA, siRNAs against FNDC1, FN1 and AR revealed significant suppression of c-MYC expression by >90% in the E006AA PCa cell line and >75% in the E006AA-hT PCa cell line, validating that c-MYC is downstream of the novel miR-1207-3p/FNDC1/FN1/AR pathway and that miR-1207-3p regulates c-MYC expression via the miR-1207-3p/FNDC1/FN1/AR pathway (Figure 4.6B-G).
Figure 4.6: c-MYC is not a direct molecular target of miR-1207-3p but is regulated by the miR-1207-3p/FNDC1/FN1/AR pathway. (A) Biotinylated miRNA pulldown was performed for the isolation of direct targets of a mature miRNA, using synthetic biotinylated miRNA duplexes. NB1 and NB1207 were transfected into C4-2B cells. Pull-down miRNA enrichments were assayed by RT-qPCR. (B-D) Effect of siRNAs against FNDC1, FN1 and AR on c-MYC mRNA expression in the indolent PCa cell line, E006AA, respectively. (E-G) Effect of siRNAs against FNDC1, FN1 and AR on c-MYC mRNA expression in the castration-resistant PCa cell line, E006AA-hT, respectively. Results are presented as mean ± SEM. *P<0.05 compared to the control.
A novel synthetic analog of miR-1207-3p, NB1207, significantly inhibits c-MYC expression in CRPC cells.

To further determine if miR-1207-3p regulates c-MYC, we assessed the effect of overexpression of miR-1207-3p on c-MYC protein expression, using NB1207, our synthetic analog of miR-1207-3p. Using our panel of PCa cell lines, we discovered that c-MYC expression is higher in aggressive CRPC cell lines compared to non-aggressive CRPC and androgen-dependent PCa cell lines (Figure 4.7A). Of note, our results reveal that c-MYC expression is higher in the aggressive C4-2B and E006AA-hT PCa cell lines when compared to their indolent counterparts, LNCaP and E006AA PCa cell lines, respectively (Figure 4.7A). This suggests that c-MYC is associated with aggressive PCa.

To determine if NB1207 inhibits c-MYC expression, we explored the effect of overexpression of NB1207 on c-MYC mRNA and protein expression. We observed that NB1207 significantly inhibited c-MYC mRNA expression when compared with a control synthetic sequence in the CRPC cell lines, PC-3 (~60%), E006AA-hT (~35%) and C4-2B (~50%) (Figure 4.7B). Moreover, we observed that NB1207 significantly inhibited c-MYC protein expression when compared with the control synthetic sequence in the CRPC cell lines PC-3, E006AA-hT and C4-2B either as effectively or more effectively than the commercially available miR-1207-3p mimic (Sigma-Aldrich MISSION® microRNA Mimic) (Figure 4.7C). We observed that in the CRPC cell lines, PC-3, E006AA-hT and C4-2B, miR-1207-3p regulates c-MYC expression via the miR-1207-3p/FNDC1/FN1/AR pathway (Figure 4.7D-F). Compared to the controls (no transfection, control synthetic sequence, and scramble siRNA), c-MYC protein expression is inhibited in the CRPC cell
lines C4-2B (>75%), PC-3 (>80%) and E006AA-hT (>40%) by overexpression of miR-1207-3p, and loss of expression of FNDC1, FN1, and AR (Figure 4.7D-F).

Figure 4.7: A novel miR-1207-3p/FNDC1/FN1/AR/c-MYC pathway in CRPC in which NB1207 significantly inhibits c-MYC expression in CRPC cells. (A) Relative protein expression of c-MYC in PCa cell lines. (B) Effect of transfection of NB1207 on c-MYC mRNA expression in CRPC cell lines. (C) Comparison of effect of transfection NB1 or NB1207 on c-MYC protein expression compared to negative control or commercial miR-1207-3p mimic in CRPC cell lines. (D-F) Effect of novel miR-1207-3p/FNDC1/FN1/AR/c-MYC pathway on c-MYC protein expression in CRPC cell lines. All results are presented as mean ± standard error of the mean (SEM). *P<0.05 compared to the control.
NB1207 significantly inhibits migration, inhibits proliferation and induces apoptosis in CRPC cells.

We next compared the effects of NB1207 to that of a commercially available miR-1207-3p mimic and scramble control on PCa cell proliferation, migration and apoptosis. We investigated whether NB1207 regulates migration similar to the miR-1207-3p mimic. Wound healing assays were performed to assess effect on migration of the E006AA-hT PCa cell line. We observed that migration of E006AA-hT cells was inhibited by nearly 50% by NB1207 compared to a scramble control sequence (NB1), whereas the commercially available miR-1207-3p mimic had no effect on E006AA-hT (Figure 4.8A). Using the MTT cell proliferation assay, we observed that proliferation of E006AA-hT CRPC cells was inhibited by nearly 40% by NB1207 compared to NB1, while the commercially available miR-1207-3p mimic had no effect on E006AA-hT (Figure 4.8B). Therefore, the results demonstrate that NB1207 significantly inhibited cellular proliferation in CRPC. Lastly, using Annexin V assay to assess effect on apoptosis in E006AA-hT CRPC cells, we observed that apoptosis was increased by 2-fold in E006AA-hT cells by NB1207 compared to NB1, while the commercially available miR-1207-3p mimic had no effect (Figure 4.8C). In conclusion, we discovered that NB1207 more effectively inhibited migration, inhibited proliferation and increased apoptosis in the CRPC cell line E006AA-hT PCa with increased c-MYC expression when compared to the commercially available miR-1207-3p mimic. These data also demonstrate that the miR-1207-3p/FNDC1/FN1/AR pathway regulates c-MYC in CRPC.
Figure 4.8: NB1207 significantly inhibits migration, inhibits proliferation and induces apoptosis in CRPC cells. Transfection of NB1 or NB1207 for 24 hours in the CRPC cell line E006AA-hT. (A) Wound healing assay was performed to assess effect on migration. (B) MTT assay was performed to assess effect on proliferation. (C) Annexin V assay was performed to assess effect on apoptosis. Results are presented as mean ± standard error of the mean (SEM). *P<0.05 compared to the control.

NB1207 and NB5 are more effective in inhibiting proliferation and inducing apoptosis in CRPC cells compared to enzalutamide, abiraterone and apalutamide.

Based on consistent and highly reproducible data demonstrating underexpression of miR-1207-3p in CRPC cells, we hypothesized that strategies to provide exogenous miR-1207-3p may have therapeutic effect in CRPC. Current anti-androgen therapies have concentrated on the androgen-dependent activation of AR through its ligand-binding domain (LBD)\textsuperscript{20,115,118}. However, recent studies have reported that AR-Vs which lack the LBD are resistant to these anti-androgen therapies. Collectively, both ligand-dependent full-length AR (AR-FL) and AR-Vs regulate distinctive transcriptional strategies in CRPC\textsuperscript{20,115,118}. Therefore, preventing/treating antiandrogen resistance is a major clinical challenge. It is already known that the detection of
AR-V7 in PCa CTCs has been correlated with CRPC and resistance against enzalutamide and abiraterone\textsuperscript{20,115,118}. Consequently, we assessed the effects of miR-1207-3p on both the ligand-induced activities of full-length AR and a constitutively active truncated AR lacking the LBD, AR-V7 in the CRPC cell line 22Rv1. To determine whether differences in AR-V7 protein levels was a result of miR-1207-3p expression and to compare the effect NB1207 and NB5 to abiraterone, enzalutamide and apalutamide, we examined protein levels of endogenous AR-V7 in the CRPC cell lines, C4-2B and 22Rv1. As indicated by Figure 4.7A, treatment of C4-2B and 22Rv1 with NB1207 and NB5 dramatically reduced AR-V7 protein expression levels by more than 50\% compared to the negative controls. Furthermore, in the aggressive CRPC cell line C4-2B, NB1207 and NB5 more effectively inhibited AR-V7 compared to abiraterone, enzalutamide and apalutamide by \sim 40\%, \sim 35\% and \sim 25\%, respectively (Figure 4.9A). Similarly, in 22Rv1, NB1207 and NB5 more effectively inhibited AR-V7 compared to abiraterone, enzalutamide and apalutamide by \sim 50\%, \sim 60\% and \sim 30\%, respectively (Figure 4.9A). Taken together, these results demonstrate the specific reduction of AR-V7 levels by synthetic analogs of miR-1207-3p is evident in CRPC models which express AR-V7.

Next, we compared the efficacy of NB1207 and another novel synthetic miR-1207-3p analog (NB5, patent pending) with the efficacy of current anti-AR therapies abiraterone, enzalutamide and apalutamide on proliferation and apoptosis in CRPC cells which express both AR and AR-Vs. We first compared the efficacy in the androgen-sensitive LNCaP and E006AA PCa cell lines. Abiraterone, enzalutamide and apalutamide blocked cell proliferation as effectively as NB1207 and NB5 in the non-CRPC LNCaP (\sim 40\%) and E006AA (\sim 30\%) PCa cell lines assessed by MTT assay (Figure 4.9B-C). Next, we again compared the effectiveness of NB1207 and NB5 to
enzalutamide, abiraterone and apalutamide on proliferation of the CRPC cell lines, C4-2B, E006AA-hT, PC-3 and 22Rv1 by MTT assay. We observed that in the CRPC cell lines, enzalutamide, abiraterone and apalutamide failed to significantly inhibit cell proliferation (Figure 4.9D-G). However, both NB1207 and NB5 significantly inhibited the proliferative effect in C4-2B (~50%), E006AA-hT (~50%), PC-3 (~40%), and in 22Rv1 (~50%) (Figure 4.9D-G).

To confirm the observations observed by MTT assay in the CRPC cell lines, we performed the Click-iT EdU Alexa Flour 488 imaging assay. For the C4-2B and 22Rv1 CRPC cell lines, we observed that EdU positive cells were reduced by nearly 70% by both NB1207 and NB5 compared to the negative controls, while enzalutamide, abiraterone and apalutamide led to a 20%-30% decrease in proliferation (Figure 4.9H-I). As indicated in Figure 4.7H-I, NB1207 and NB5 demonstrated more than 2-fold decrease in cellular proliferation in CRPC cells compared to enzalutamide, abiraterone and apalutamide.

To examine whether NB1207 and NB5 overcomes the resistance to androgen antagonist therapy in CRPC cells in part by inducing cellular apoptosis, C4-2B and 22Rv1 cells were tested using Annexin V assay. We observed that NB1207 and NB5 increased apoptotic activity by more than 7-fold in both CRPC cell lines when compared to the negative controls (Figure 4.10A-B). More importantly, NB 1207 and NB5 were >20% more effective in increasing apoptotic activity compared to enzalutamide, abiraterone and apalutamide in C4-2B cells (Figure 4.10A). NB1207 and NB5 demonstrated even further superiority in 22Rv1 cells, as they demonstrated approximately 2-fold increase in apoptotic activity compared to enzalutamide, abiraterone and apalutamide (Figure 4.10B). Taken together, both NB1207 and NB5 inhibited proliferation and
increased apoptosis in CRPC cell lines which express both the androgen inducible AR-FL and the constitutively active truncated AR lacking the LBD (AR-V7).
Figure 4.9. NB1207 and NB5 inhibits AR-V7 expression and is more effective in inhibiting proliferation in CRPC cells compared to abiraterone, enzalutamide, and apalutamide. (A) Comparison of effect of transfection NB1207 and NB5 on AR-V7 protein expression compared to negative controls or abiraterone, enzalutamide and apalutamide in CRPC cell lines. MTT assay was performed to assess effect on cell viability. (B-G) Transfection of no transfection mix, DMSO, NB1, NB2, NB3, NB4, NB5, NB1207, enzalutamide, abiraterone or apalutamide for 24 hours in the non-aggressive cell lines LNCaP and E006AA (B-C) and the CRPC cell lines C4-2B, E006AA-hT, PC-3 and 22RV1 (D-G). EdU assay was performed to assess effect on cell proliferation. (H-I) Transfection of no transfection mix, DMSO, NB1, NB2, NB3, NB4, NB5, NB1207, enzalutamide, abiraterone or apalutamide for 24 hours in the CRPC cell lines C4-2B and 22Rv1.

Figure 4.10. NB1207 and NB5 are more effective in inducing apoptosis in CRPC cells compared to enzalutamide, abiraterone and apalutamide. Annexin V assay was performed to assess effect on cell apoptosis. (A-B) Transfection of no transfection mix, DMSO, NB1, NB2, NB3, NB4, NB5, NB1207, enzalutamide, abiraterone or apalutamide for 24 hours in the CRPC cell lines C4-2B and 22Rv1. Results are presented as mean ± standard error of the mean (SEM). *P<0.05 compared to the control.
Discussion

Men with advanced disease typically receive androgen deprivation therapy (ADT) with new second-generation therapeutic agents such as enzalutamide or abiraterone\textsuperscript{17,120}. Yet, it is well established that these patients who receive ADT inescapably succumb to their relapsed tumor after a period where the disease is in remission\textsuperscript{11,17,103}. As PCa is a heterogeneous disease, the chief obstacle is understanding the molecular mechanisms involved in this progression from the asymptomatic androgen-dependent PCa to the lethal CRPC. Consequently, new biomarkers and treatment strategies are urgently required to combat CRPC.

This is the first report to show that miR-1207-3p regulates c-MYC expression via the miR-1207-3p/FNDC1/FN1/AR pathway in aggressive PCa. c-MYC is a vital transcription factor and a crucial protein, and direct targeting may have severe consequences on patients\textsuperscript{72-75,79,121}. Because c-MYC targeting has proved difficult, our data demonstrating the regulation of c-MYC expression by the miR-1207-3p/FNDC1/FN1/AR pathway bears significant implications for treatment of c-MYC-driven cancers. Excitingly, targeting miR1207-3p may be less challenging and the effects may be less deleterious than targeting c-MYC itself for inhibiting c-MYC driven cancers\textsuperscript{72-75,79,121}. Our findings have illuminated the molecular details of miR-1207-3p/c-MYC cooperation and the synthesis of the novel NB1207 and NB5 as candidate therapeutics specifically targeting the miR-1207-3p/FNDC/FN1/AR/c-MYC pathway in CRPC.

We have also demonstrated the potential diagnostic and prognostic value of miR-1207-3p in CRPC. Currently, the Gleason grading system represents an invasive technique which evaluates
the prognosis of men with PCa using samples from a prostate biopsy. From the perspective of personalized medicine, an improved strategy for better patient stratification and treatment response should include the monitoring of specific miRNAs. Various studies have established the stability of miRNAs in serum, plasma and urine as well as the differential expression of miRNAs in normal and tumor tissue which demonstrates the potential of miRNAs as potential PCa biomarkers and predictors of therapy response. Accordingly, our prostate tissue analysis revealed that underexpression of miR-1207-3p and the overexpression of FNDC1, FN1, AR and c-MYC are significantly associated with aggressive PCa. Furthermore, we observed that miR-1207-3p, FNDC1, FN1, AR and c-MYC were significantly differentially expressed between benign prostatic hyperplasia and PCa. Also, miR-1207-3p was underexpressed while c-MYC was overexpressed in tumors with Gleason score $\geq 8$ in comparison to those with Gleason score <8. These findings demonstrate the potential usefulness of miR-1207-3p and c-MYC for PCa risk stratification.

The observation of decreased miR-1207-3p expression during PCa progression indicates that strategies to enhance miR-1207-3p in PCa may have therapeutic value in CRPC. miRNAs can simultaneously modulate several cancer-relevant gene pathways and can be exploited to increase the sensitivity of tumor cells to conventional anticancer agents. Because of this potential to be one-hit multi-target therapeutic agents against PCa, miRNAs are interesting therapeutic candidates. We observed that the novel candidate therapeutic, NB1207, significantly inhibited c-MYC protein expression in the CRPC cell lines. Furthermore, NB1207 significantly inhibited migration, inhibited proliferation and induced apoptosis in CRPC cell lines.
Therefore, these \textit{in vitro} studies demonstrate the potential of miR-1207-3p analogs, such as NB1207 and NB5 serving as therapeutics for CRPC.

Consequently, we investigated if NB1207 and NB5 could have efficacy against CRPC cells that have proven to be resistant to both first generation and second generation ADTs. The notion that those PCas continuing to progress during ADT are androgen independent has been reconsidered in the last few years and it is now accepted that AR signaling plays a significant role in CRPC despite castrate-levels of testosterone\textsuperscript{17,103,105,122}. Important advances in the understanding of the mechanism involved in CRPC pathogenesis have led to welcome breakthrough of several AR-targeting drugs such as abiraterone, enzalutamide and apalutamide\textsuperscript{17,125-129}. However, despite their promising effects in some patients with CRPC, approximately 20-40\% of patients do not benefit from these agents and eventually all the initial responders to these drugs acquire secondary drug resistance leading to death\textsuperscript{115,118}. Recent studies have reported that AR-Vs signal in the absence of the ligand binding domain (LBD) which are a crucial contributing factor for resistance to LBD-targeting AR antagonists or agents that repress androgen biosynthesis such as abiraterone, enzalutamide and apalutamide\textsuperscript{20,115,118}. Among the variants, AR-V7 is the most studied member because it is the only known variant of which the encoded endogenous protein is detectable in clinical specimens\textsuperscript{20,28,115}. Studies have shown a strong association between the presence and overexpression of AR-V7 in CRPC and resistance to the current drug regimen\textsuperscript{20,28,115,116}. This resistance is a major clinical challenge in the field and a clearer understanding of the mechanisms underlying resistance to these drugs would facilitate selection of alternative novel therapeutic strategies\textsuperscript{20,28,115}. Consequently, we have demonstrated that NB1207 and NB5 significantly inhibited AR-V7 protein expression in the CRPC models, C4-2B and 22Rv1.
In that regard, the efficacy of our novel candidate therapeutics, NB1207 and NB5, against CRPC cells is of tremendous interest. We discovered that NB1207 and NB5 is as effective as abiraterone, enzalutamide and apalutamide in significantly preventing proliferation and increasing apoptotic activity in non-CRPC cell lines. Strikingly, our results demonstrate significant anti-proliferative and pro-apoptotic effects of NB5 and NB1207 in CRPC cell lines whereas abiraterone, enzalutamide and apalutamide were less effective. Our novel miR-1207-3p/FNDC1/FN1/AR/c-MYC pathway suggests that the role of AR-V7 on CRPC development and PCa progression may be mediated in part via c-MYC. Moreover, NB5 and NB1207 may be promising therapeutic agents against AR-V7 expressed CRPC by serving as c-MYC inhibitors. Based on our data, it is likely this may be less susceptible than all current ADTs to the development of resistance in advanced PCa\textsuperscript{28,115}.

In conclusion, this is the first report to show the potential diagnostic and prognostic value of miR-1207-3p as it has been shown to be a candidate biomarker for risk stratification. NB1207 and NB5 are efficacious in inhibition of proliferation of CRPC and increasing the apoptotic activity of CRPC via targeting c-MYC. Overall, our results demonstrate a preclinical proof-of-principle that NB5 and NB1207, our novel analogs of miR-1207-3p, may be promising therapeutics for the treatment of CRPC.
Author contributions

Dibash K. Das wrote the manuscript and prepared all the figures. E. O. Olapade-Olaopa, Akintunde T. Orumuyi, Gabriel O. Ogun, S. A. Adebayo acquired, managed patients, provided the human tissue samples and helped prepare the corresponding method section for figures 4.2-4.5. Akintunde T. Orumuyi and Adeodat Ilboudo helped perform RNA extractions for figures 4.2-4.5 Cuong Bach helped prepare figures and perform the statistical analysis for figures 4.2-4.5. Anna Galuza helped in data acquisition for figure 4.7. J. David Warren assisted in synthesizing our novel synthetic duplexes using the Weill Cornell Medicine core facilities. Olorunseun O. Ogunwobi supervised all the work from conception to manuscript preparation and review. All authors reviewed the manuscript.

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Human and animal rights

Signed informed consent was obtained from all study participants.
Chapter 5:
Conclusion, Significance and Future Directions
In Chapter 1 we presented the rationale for investigating the role of miR-1207-3p in PCa. Understanding the molecular mechanisms of PCa progression is essential for the discovery of robust prognostic markers which we believe are capable of helping to identify those patients that are at the greatest risk of relapse\textsuperscript{17,20,25}. This will, in turn, enable us to optimize management strategies to control PCa progression. In that light, the importance of miRNAs in cancer biology has become apparent for the discovery of miRNA-based diagnostic, prognostic and theranostic biomarkers\textsuperscript{7,39,43,124}. Moreover, their relative stability in tissues and a variety of biofluids including blood and urine makes repeated measurements of miRNA levels by qRT-PCR assays very feasible\textsuperscript{8,39,124,130,131}. This can provide critical information on a disease’s molecular signature status over time through the course of treatment and recurrence without the need for a biopsy\textsuperscript{5,43}.

We made several novel findings. We discovered that expression of miR-1207-3p is significantly diminished in PCa cells\textsuperscript{1}. We also demonstrated that increased expression of miR-1207-3p in PCa cells significantly inhibits migration and proliferation, and induces apoptosis via direct molecular targeting of FNDC1\textsuperscript{1}. These functional experiments clearly demonstrate a mechanism by which miR-1207-3p regulates key cellular processes dysregulated in the development and progression of PCa. Importantly, we describe for the first time the miR-1207-3p/FNDC1/FN1/AR novel regulatory pathway in PCa: miR-1207-3p-induced loss of FNDC1 expression leads to the loss of FN1 and subsequently AR expression\textsuperscript{1}. Our novel pathway has important clinical implications as miRNAs possess several key features that make them attractive PCa biomarkers\textsuperscript{33,34,43}. Our clinical results indicate that FNDC1, FN1, and AR are concurrently overexpressed in metastatic PCa\textsuperscript{1}. Also, FN1 overexpression in prostate tumors positively correlated with overall death and PCa-
specific death\(^1\). To our knowledge this is the first description of FN1 as a potential prognostic biomarker in human clinical PCa.

To identify the target genes of miR-1207-3p in PCa and to investigate the involvement of miR-1207-3p target genes in PCa, we designed and synthesized a novel synthetic biotinylated miR-1207-3p duplex (NB1207) and scramble duplex (NB1) (Patent application publication number, US20170121711 A1). Prior to using the NB1207 for target discovery, we compared their effects to that of the previously used commercially available miR-1207-3p mimic on the 3’UTR of FNDC1, and PCa cell proliferation, migration and apoptosis. Of significance, we observed that NB1207 maybe be more efficient than the commercially available mimic as NB1207 significantly inhibited migration, inhibited proliferation and induced apoptosis in several CRPC cell lines while the mimic had little effect. We have already used these novel tools (NB1207 and NB1) to perform a RNA pull down assay that demonstrated that FNDC1 is a direct molecular target of miR-1207-3p. This gives us the confidence and the ability to identify all the molecular targets of miR-1207-3p. Future work will employ these tools in a RNA pulldown approach followed up with RNA sequencing of the enriched RNA pulled down to identify all directly bound RNA molecular targets of miR-1207-3p.

As a sub-study, we investigated the impact of racial disparity in PCa because moAA have a 2-fold higher chance of getting aggressive PCa. Despite progress being made in the field, significant PCa racial disparity persists\(^82,83,109\). In this regard, we discovered that miR-1207-3p is differentially expressed in primary tumors from moAA tissues compared to primary tumors of CM. To our knowledge, this is the first report of a microRNA that directly correlates with aggressive PCa and
has divergent expression between CM and moAA. The discovery of a miRNA biomarker that is capable of displaying differential expression in men of different ancestry is of great significance for the development and optimization of miR-1207-3p-based therapeutic strategies for personalized treatment approaches. Further studies are required to establish this.

In Chapter 4, we investigated the relationship of miR-1207-3p with the proto-oncogene, c-MYC. Our results demonstrated that it may be possible to predict clinical behavior of PCa based upon miRNA-1207-3p expression. Approximately 30% of PCa patients develop clinical recurrence, and the survival period of this phase of PCa is limited and extremely variable, the challenge lies in the identification of those patients most at risk for relapse\textsuperscript{6,12,17,25}. Because c-MYC is commonly found to be amplified in up to 72\% of CRPCs, targeting c-MYC could be a potential approach against CRPC. Yet, the mechanisms regulating c-MYC have not been fully delineated in PCa.

We discovered the underexpression of miR-1207-3p and the overexpression of FNDC1, FN1, AR and c-MYC is associated with increased risk of cancer progression and aggressive PCa. Investigations into miR-1207-3p ability to improve risk stratification in human clinical PCa by determining if there are any correlation with Gleason scores demonstrated that miR-1207-3p negatively correlates with c-MYC in tumors with Gleason score $\geq 8$. This is of clinical significance for several reasons. Currently, factors such as serum PSA level, Gleason score, and tumor stage are used for diagnosis, prognostication and treatment decision-making\textsuperscript{8,132}. However, none of these alone or in combination are adequate indicators for accurate clinical decision-making\textsuperscript{6,105,133}. It is likely that heterogeneity of PCa at the molecular level may account for the fact that even those patients displaying similar PSA levels, tumor stage, Gleason score can still have different clinical
outcomes 6,105,133. Furthermore, factors to detect recurrence such as increased serum PSA levels may actually lead to false positive diagnosis124. Moreover, increased PSA following radical prostatectomy can be caused by the presence of residual benign prostate tissue14,15,134. Considering these current limitations, our discovery adds to the “tool-box” to improve the clinical decision-making process2. To our knowledge, this is the first study to evaluate the expression of miR-1207-3p in any human disease and the first description of miR-1207-3p differential expression in human clinical PCa1. Importantly, this is the first report to show the potential diagnostic value of miR-1207-3p as it has been shown to be a candidate biomarker for risk stratification in human clinical PCa.

In Chapter 4, we also confirmed that c-MYC is not a direct molecular target of miR-1207-3p yet is regulated by our pathway. Consequently, we have discovered a novel miR-1207-3p/FNDC1/FN1/AR/c-MYC pathway in PCa. Additionally, using miR-1207-3p analogs (NB1207 and NB5), we discovered that overexpression of miR-1207-3p more effectively inhibited proliferation and increased apoptosis in the CRPC cell lines. Our reproducible results had prompted us to envision miR-1207-3p as a therapeutic to help health care professionals combat CRPC in patients. Current anti-androgen therapies including enzalutamide, abiraterone and apalutamide are unable to inhibit proliferation and increase apoptosis in CRPC cell lines such as C4-2B. Thus the question which remained is can our novel miR-1207-3p analogs demonstrate efficacy in CRPC where current CRPC treatments have failed? We observed that treatment with NB1207 and NB5 inhibited AR-V7 protein expression in CRPC cells and more significantly inhibited proliferation and increased apoptosis of CRPC cells compared to abiraterone, enzalutamide and apalutamide. These results demonstrate that use of synthetic analogs of miR-
1207-3p such as NB5 and NB1207 may be a novel strategy for successful therapeutic targeting of c-MYC in CRPC.

In summary, we present mechanistic and clinical data characterizing the novel miR-1207-3p/FNDC1/FN1/AR/c-MYC molecular pathway suggesting its role in PCa (Figure 5.1). We conclude that the present study signifies that miR-1207-3p may have potential diagnostic, prognostic, and therapeutic applications in PCa. Furthermore, our results demonstrate proof-of-principle that NB1207 and NB5 may be promising therapeutics for the treatment of CRPC.

**Figure 5.1:** miR-1207-3p/FNDC1/FN1/AR/c-MYC is a novel regulatory pathway in PCa.
Chapter 6:

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