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MicroRNA-186 and metastatic prostate cancer.

Dominique Zilpha Jones

MICRORNA-186 AND METASTATIC PROSTATE CANCER

By

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B.A., Smith College, 2010
M.S., University of Louisville, 2014

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DEDICATION

This dissertation is dedicated to my husband, Joseph Lamar Reed, beloved mother, Annette Jones, beloved grandmother, Thelma Jones, godmother, Ernestine Hill, family and friends who are the source of inspiration for my interest in cancer research.

ACKNOWLEDGMENTS

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ABSTRACT

MICRORNA-186 AND METASTATIC PROSTATE CANCER

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MicroRNA (miR) dysregulation alters cancer-associated gene expression, which contributes to cancer pathogenesis. For example, miR-186 over expression lead to enhanced proliferation and migration in pancreatic cancer cell models. However, the role of miR-186 in prostate cancer (PCa) remains controversial. Previously, miR-186-5p was up-regulated in PCa patient serum (stage III/IV) compared to controls. Furthermore, miR-186-5p was up-regulated in metastatic PCa (PC-3, MDA PCa 2b, LNCaP) relative to normal prostate epithelial cells (RWPE1). We hypothesized miR-186 inhibition will reduce aggressive PCa using metastatic cell models. To test this, we evaluated whether miR-186-5p inhibition would reduce aggressive PCa behavior and overexpression induce malignant transformation in normal cells.

Cell proliferation (BrdU incorporation), invasion (reduced growth factor matrigel), colony formation (soft agar) and cell death (trypan blue exclusion) were examined in miR-186-5p inhibited PCa cells (PC-3, MDA PCa 2b) and overexpressed RWPE1 cells. Aberrant gene expression was evaluated in stable

PC-3 and RWPE1 cells to identify miR-186 candidate targets via microarray and qRT-PCR analyses using published reports, miR databases and statistical filtering (FDR p-value ≤ 0.05 and ±1.2 fold change).

MiR-186-5p inhibition reduced proliferation (27-46%) in PCa (PC-3, MDA PCa 2b), cellular invasion (66%) in PC-3 cells and anchorage independent growth (28-64%) in PC-3 cells and post-DHT (10 nM) treatment in MDA PCa 2b cells. Over 2,343 genes were differentially expressed in stably miR-186 inhibited PC-3 and overexpressed RWPE1 cells relative to controls. Out of 23 selected candidates, AKAP12 gene and protein was up-regulated in miR-186-5p inhibited PC-3 cells. MiR-186-5p overexpression decreased AKAP12 protein in HEK 293T cells. Additional *in vitro* as well as *in vivo* studies are needed to further elucidate the role of additional targets in PCa. Ultimately, the identification of novel biomarkers may improve detection and effective treatment of aggressive PCa.

TABLE OF CONTENTS

PAGE

LIST OF TABLES

TABLE PAGE

LIST OF FIGURES

FIGURE PAGE

CHAPTER I

INTRODUCTION

Prostate Cancer as a public health problem

Prostate cancer (PCa) is the leading cancer and the 2nd leading cause of cancer related deaths among men in the U.S. [1]. Ninety-one percent of PCa cases are treatable among men diagnosed with localized or regional disease as evidenced by 100% 5-year survival rates [1]. This 5-year survival rates drop drastically to 28% for metastatic PCa [2]. Once PCa spreads to the bone and other organs, it is not responsive to conventional treatment strategies. To compound the problem further, 25% of all cases diagnosed with stage I-III disease will experience disease recurrence within five years post treatment.

PCa Risk Factors

There are a number of well-established and probable risk factors that contribute to the development of PCa. These established risk factors include older age (age ≥ 50 years), family history among 1st or 2nd degree relatives, low socioeconomic status, sedentary lifestyle, poor diet, genetic predispositions, hormone imbalances (androgen, testosterone, androgen

sensitivity), chronic inflammation, and African heritage. Data shows elevated risks of PCa development are generally associated with the aforementioned risk factors and especially men of African descent. Based on 30 years of record keeping, there is significant a racial disparity associated with PCa.

PCa Health Disparities

African American men are 2.5 times more likely to die from PCa relative to American men of European descent [1, 3]. A number of studies have attempted to determine biological explanations for this disparity through the evaluation of clinical prognostic/diagnostic factors [e.g., prostate specific antigen (PSA), PSA density, tumor stage], hormone levels, and the expression of apoptotic and metastatic-related genes [4-11]. Unfortunately, no definitive molecular mechanisms have been identified contributing to this strong disparity between African and European American men. Thus, further investigation into the molecular mechanisms associated with racial disparity is warranted to increase our understanding of this disease and develop customized targeted therapies for diverse populations. Although, this dissertation does not focus on race/ethnic specific biomarkers, we sought to understand the biology of a specific genomic biomarker within PCa cell lines common to both African-American and European-American men diagnosed with metastatic and/or non-metastatic disease.

PCa Staging and Risk Stratification

PCa is a heterogeneous abnormal growth or neoplasm with distinct categories of pathologic stages. These varying pathological stages help inform

medical and scientific personnel of the extent to which the cancer progressed and guides treatment strategies. There are four tumor stages, three lymph nodal stages (N stages) and three metastatic stages (M stages). The first tumor stage (T1) of disease involves the tumor found in a surgical procedure called transurethral resection of the prostate (TURP) and less than 5% is cancer (T1a) or more than 5% is cancer (T1b) or tumor discovered during biopsy (T1c). In the second stage (T2), the tumor is located in half (or less) of one side of the prostate (T2a), one half (or more) on one side (T2b) or both sides (T2c) and has not extended beyond the prostate. For the third stage (T3) of PCa, tumor growth has extended outside of the prostate, but not to seminal vesicles (T3a) or has invaded seminal vesicles but not the lymph nodes (T3b). In stage four (T4), tumor growth is outside of the prostate and tumor cells have migrated to distant organs (i.e., lymph nodes, liver, bone), which is commonly referred to as metastasis. For lymph nodal staging, N_x means no testing has been performed to detect cancer in the lymph node. N_0 indicates the cancer has not reached any lymph node. N_1 means the cancer has spread to the lymph nodes. For the degree of metastasis, the first level, M_x, indicates no testing has been performed to detect cancer in distant lymph nodes. M_b indicates cancer has metastasized to the bone. The last level, M_c, means the cancer has spread to other areas (e.g., lungs, brain, liver). The most common biological patterns of PCa metastasis are metastasis of tumor cells to the bone (90%), liver (65%), lymph nodes (59%) and lung (38%), respectively [12].

There are common classification and risk stratification systems utilized for PCa. These classification and risk stratification systems were used to determine biospeciemens collected from patients in this study with aggressive and less aggressive disease. The first is the Gleason grade system, which aids in the evaluation of prostate biopsies and determine disease prognosis for men with PCa [13]. Gleason grades range from 1 (normal tissue) through 5 (abnormal tissue). Different areas of a tumor often have different grades and two grades that characterize majority of tumor. The sum of the two grades is called a Gleason score or sum. A Gleason score of ≤ 7 describes well to moderate differentiated tissue or low aggressive disease. A Gleason score range of 8 ≤ for poorly differentiated tissue or aggressive disease.

Two common PCa risk stratification systems are the D'Amico risk criteria and National Comprehensive Cancer Network (NCCN) guidelines. Both the D'Amico and NCCN use PSA levels and Gleason scores to classify disease aggressiveness. The D'Amico risk criteria was established in 1998 and later adopted by the American Urological Association and European Association of Urology. This system is used to determine low, intermediate and high risk individuals diagnosed with non-metastatic disease [14]. Patients diagnosed according to following criteria; low risk [tumor stage T1/T2a, and PSA ≤10 ng/ ml, and Gleason Score ≤ 6], intermediate risk [tumor stage T2b, and/or PSA 10–20 ng/mL and/or Gleason Score 7] and high-risk [tumor stage ≥T2c, PSA >20 ng/mL or Gleason Score 8–10].

In the NCCN guidelines, there is a further breakdown of low and high-risk individuals and includes metastatic patients [14]. Low risk is divided into not very low risk [T1-T2a and Gleason Score 2–6 and PSA ≤10] and very low risk [T1c and Gleason Score ≤ 6 and PSA <10 and 3 > biopsy cores positive and ≤ 50% cancer in each core]. Intermediate risk is classified as patients diagnosed with T2b or T2c and/or Gleason Score = 7 and/or PSA >10–20. Lastly, high risk is divided into not very high risk [T3a or PSA >20 or Gleason Score 8–10] and very high risk [T3b-4].

Pathogenesis of PCa

The prostate gland is oval in shape anatomically located interior to the bladder. The urethra tube runs directly through the middle of the gland. The gland is divided into three different zones, transition, central and peripheral. The peripheral zone is the largest of the three zones with contains 66% of the fluid producing glands. The gland is composed of three types of cells consisting of glandular, muscle, and fibrous cells. Gland cells produce the fluid component of semen. Muscle cells control urine flow and ejaculation. Fibrous cells provide structural support of the gland.

In the normal prostate epithelium, there are several major processes that lead to prostate carcinogenesis such as chronic inflammation, oxidative stress/DNA damage and genomic alterations [15, 16]. Oxidative stress and DNA damage contributes to the pathogenesis of PCa [16]. Oxidative stress results from an imbalance in reactive oxygen species (ROS) and detoxifying enzyme

levels via the accumulation of ROS. Furthermore, oxidative stress can induce damages in lipids, proteins and most importantly DNA via suppression of the expression of anti-oxidant enzymes and induction of DNA adducts in PCa. Also, genomic changes such as loss of tumor suppressors, NKX3.1 and PTEN, TMPRSS2-ERG translocations and up-regulation of oncogene, c-Myc, EZH2 and Akt/mTOR and MAPK signaling are commonly detected in PCa Lastly, chronic inflammation mainly attributed to bacterial or viral agents, dietary factors (i.e., heterocyclic amines), genetic variations, amyloids, physical trauma, hormonal changes, and urine reflux has been previously implicated in PCa [17-21]. Chronic inflammation can transition into proliferative inflammatory atrophy (PIA) characterized by focal atrophic lesions and influx of inflammatory cells and stromal fibrosis [18, 21-23]. PIA lesions frequently developed into prostatic intraepithelial neoplasia (PIN) evident by histopathological studies [21, 22]. High grade PIN is widely accepted as the precursor of prostate carcinoma evident by numerous reports that show high grade PIN is strongly associated with prostate carcinoma in prostatic tumor tissue [22, 24-27].

However recently, cancer research has implicated non-coding RNA species called miRNAs as massive regulators of gene expression in tumorigenesis. Dysregulation of these gene regulators could lead to the induction of oxidative stress, genomic changes and DNA damage in cancers such as prostate. Thus, this dissertation evaluates the biological and genetic contributions of a differentially expressed miRNA to the prostate tumor phenotype via changes

in tumor cellular behavior (i.e., growth, proliferation, invasion, anchorage independent growth) and gene expression profiles.

Metastatic Disease

Beyond tumorigenesis, metastasis is a complex process with that dramatically increases the risk of death for cancer patients and proposes a major issue in clinical management of PCa and all cancers. The metastatic process shown in Figure 1 initially involves the enhanced proliferation of primary tumor cells, invasion of tumor cells into adjacent surrounding tissues and basement membrane and then detachment from the primary tumor [28]. These cells are considered to have undergone epithelial mesenchymal transition (EMT). Next, tumor cell invasion of and survival in the circulation system and lymphatic channels also known as intravasation, arrest within a distant organ, extravasate into surrounding tissue and colonization at a secondary site [28]. Mesenchymal epithelial transition (MET) is required for tumor cells to establish a secondary tumor site.

There are several models of metastasis, which include progression, transient compartment, fusion, gene transfer, early oncogenesis, and genetic predisposition [28]. The progression model proposed by Peter Nowell (1976) has been the most widely accepted model and has been supported by a number of studies. This model suggests that a series of mutational events occurs in subpopulations of the primary tumor or disseminated cells and results in a small fraction of cells acquiring full metastatic potential [29]. The rationale behind the

progression model is that primary tumor cells have a low probability to acquire all of the multiple alterations required for successful metastasis [29]. So only a few cells will successful metastasis. Although the progression model is supported by some studies, none of the proposed models fully explain all of the different clinical cases observed in metastasis. Some research suggest that metastatic pattern may be due to secretion of chemokines from different tissues to attract tumor cells and the expression different chemokine receptors on specific organs also known as the “homing theory” [30]. Driving migration from the primary tumor site, tumor cells quickly exhaust local nutrient supply and are forced to relocate to another nutrient-rich environment. Chemokines may act as local chemoattractants to direct tumor cells to specific organs rich with their respective ligands during metastasis [12, 31, 32].

PCa Treatment Options

Treatment options are recommended based on patient age and disease aggressiveness. Initial and primary treatment for 52% of men 64 years old or younger with localized and regional disease is a surgical procedure called radical prostatectomy, which involves the removal of the prostate and surrounding tissue [2]. This same patient population also receives a treatment combination of radical prostatectomy and radiation therapy.

For men 65-74 years old, radiation therapy is commonly used to treat PCa due the detection of aggressive disease. In patients older, surgery and/or

radiation are not common treatment options. Active surveillance is most likely recommended for older men with less aggressive disease. However, hormone therapy is the first line treatment option for any patient with metastatic disease. Newer hormone therapies (abiraterone acetate, enzalutamide) have shown to be effective against metastatic disease [2]. Abiraterone acetate (brand name: Zytiga) is a steroidal androgen synthesis inhibitor. Abiraterone, the active form of Abiraterone acetate, decreases circulating levels of androgens through the inhibition of CYP17A1 activity. However, Enzalutamide (brand name: Xtandi) is a non-steroidal pure antiandrogen that reduces PSA levels by preventing the androgen receptor from binding to DNA and co-activator proteins. As previously mentioned, hormone-resistant and/or hormone refractory PCa is not responsive to androgen deprivation therapy. Available treatment options for early stages of disease include withdrawal of anti-androgen, estrogen compounds, adrenal suppressants, chemotherapy (i.e., docetaxel) , immunotherapy (i.e., sipuleucel-T) and radiotherapy (i.e., Radium-223) as well as combination treatments [33]. These treatment strategies are fairly effective for local/regional disease; however, once PCa spreads to the bone and other organs, it is not as responsive to these conventional treatment methods. Therefore, it is imperative to develop improved targeted treatment strategies against advanced PCa. Chapters 2-4 of this dissertation focus on the identification, validation and characterization of a biomarker related to the beginning stages of aggressive disease (i.e., invasion, anchorage independent growth) in androgen-sensitive and -insensitive PCa cell lines.

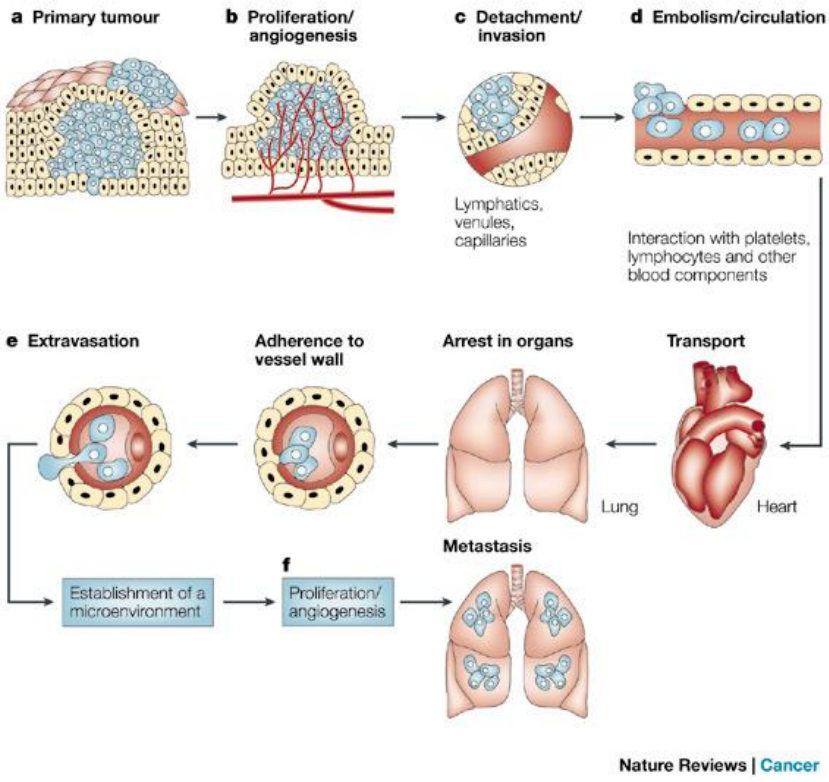

Figure 1. The Pathogenesis of Cancer Metastasis. This diagram was adapted from Fidler et al. 2003 Nature Reviews Cancer 3, 453-458 (June 2003) doi:10.1038/nrc1098. (http://www.nature.com.echo.louisville.edu/nrc/journal/v3/n6/fig_tab/nrc1098_F2.html)

Metastasis is a lethal disease in all malignancies and one canonical pathway of metastasis pathogenesis is displayed above. Tumor cells establish a primary tumor site and are provided nutrients to support growth and proliferation through angiogenesis via the integration of vascular network of blood vessels. Tumor growth leads the detachment and invasion of tumor cells into surrounding tissue and lymphatic system. Tumor cells escape the primary site into the blood circulation system where they interact with platelets, lymphocytes, other blood components and travel to distant organs. Tumor cells adhere to the vessel wall of distant organs and undergo extravasation to establish a microenvironment. Tumor cells proliferate via angiogenesis to establish a secondary tumor site.

PCa screening tools

The prostate specific antigen (PSA) test and digital rectal exam (DRE) are the primary screening tools used to detect PCa in the U.S. The PSA test measures the serum level (nanogram per milliliter) of the prostate specific antigen, a protein exclusively produced by cells in the prostate gland. PSA is a serine protease produced at high levels by normal prostate and malignant PCa cells. Historically, men with elevated PSA serum levels (PSA ≥ 4 ng/ml) were linked to a high risk of PCa development [6, 34]. The PSA test has a specificity range of 38.9-93.8% and sensitivity range of 20.5-83.4% dependent upon PSA cutoff values and type of assay standardization [35-37]. Furthermore, different variations of the PSA test such as PSA density, PSA velocity and percentage of free PSA were developed to aid in clinical decisions about PCa detection[36-38]. However, the appropriate thresholds for some of these tests remain to be determined for the detection of PCa. The wide spread use of PSA testing has led to a dramatic increase in PCa detection among U.S. men. Unfortunately, the frequent utilization of PSA testing has led to the over-diagnosis and overtreatment of localized disease, which is consistently observed in PCa screening studies [39-45].

Two major PCa screening trials, the European Randomized Study of Screening for PCa (ERSPC) and Prostate, Lung Colorectal and Ovarian (PLCO) Cancer Screening Trial, have influence the field's perspective on the effectiveness of PSA testing to detect cancer and reduce mortality [40, 44, 46]. In

the PLCO study, no significant difference was observed in the mortality rates of U.S. men receiving PSA/digital rectal exam (DRE) screenings relative to men with usual care after a 13 year-follow-up [44, 46]. In contrast, the ERSPC study observed that PSA-based screening was associated with a 20% reduction in PCa mortality after a 9-year follow-up among European men [40].

However, the use of the PSA test was strongly associated with a 50% rate of over-diagnosis of PCa in the screening group [40]. Additionally, PSA testing was associated with 12-13% false-positive results based upon data from the ERSPC and PLCO studies [40, 44, 46]. Unfortunately, it is well known now that the PSA test has a number of biological stimulants (e.g., bacterial infection, BPH, age) other than cancer that elevate PSA levels (PSA ≥ 4ng/ml) in men. Most PCa biopsies performed due to elevated PSA levels reveal BPH and inflammatory prostatitis. Although BPH does not cause PCa, its similar symptoms to PCa can sometimes lead to PCa misdiagnoses [47, 48]. The PLCO and ERSPC studies along with other reports failed to determine an overwhelming benefit for PSA testing in patients. It remains unclear whether PSA testing significantly lowers PCa mortality. This evidence has led the United States Preventive Services Task Force and American Urological Association to recommend against the use of the PSA test.

Future PCa biomarkers

Consequently, the PSA test is now considered an unreliable tool for PCa detection. Thus, novel biomarkers that are stably expressed in prostate tissue and biological fluids (e.g., serum, plasma) are desperately needed to clinical decisions about PCa detection and determining an individual's risk of developing non-metastatic versus metastatic disease. Additionally, these biomarkers will aid in the field of precision medicine to make inform clinical decisions about which patients will receive a biopsy and/or aggressive treatment. Single nucleotide polymorphisms (SNPs), known as genetic variations inherited in cancer-associated genes, were once viewed as potential tools to aid in clinical decisions. However, there is very limited evidence of the use of SNPs in a clinical setting and how they have improved clinical decisions. Recently, cancer researchers have turned their attention to more clinically relevant biomarkers (i.e., microRNAs, lncRNAs).

MicroRNAs regulate normal cellular growth and development processes as well as disease regulated processes (i.e., autoimmune disease, diabetes, Parkinson's disease, cancer). Relative to current PCa screening tools, microRNAs are ideal candidates to predict the presence of cancer and aid in the diagnosis of disease states and disease recurrence in patients due to their unique expression profiles that correspond to different tumor stages, Gleason scores, and metastatic status in cancer in tissue and biological fluids [49-52]. Numerous published reports show miRNAs are stably expressed in tumor

derived cells, plasma, serum and tissue, and consistently distinguish between different disease stages compared with non-cancerous biospecimens [53]. The non-invasive detection of highly sensitive non-coding RNA expression in biological fluids would greatly improve disease detection and prognosis of metastatic disease. MicroRNA expression profiles can provide more informative knowledge about the intrinsic signaling pathways that favor or suppress tumorigenesis [53, 54]. Ultimately, the utilization of miRNA expression profiles will aid in the pursuit of precision medicine and efforts to improve cancer care in patients [55, 56]. Thus, the focus of this dissertation is centered on discovering micro-RNA profiles of PCa and determining their functional role in the process of oncogenic transformation.

Biogenesis of microRNAs and their function

MicroRNAs are short endogenous RNA species stably expressed in plant/mammalian cells, biological fluids (e.g., serum, plasma, urine) and tissue. These RNA species are primarily transcribed from miRNA genes by RNA polymerase II as primary transcripts (pri-miRNAs) and subsequently cleaved by RNase III class enzyme, Drosha-DGCR8 (Pasha), in the cellular nucleus to produce ~70 nucleotide long hairpin structure also known as precursor miRNA (pre-miRNA) [57]. The Exportin-5-Ran-GTP complex transports the pre-miRNA to the cytoplasm and further cleaved by the Dicer-TRBP complex into a mature miRNA duplex. The functional strand of the mature miRNA duplex is incorporated into the RNA-induced silencing complex (RISC) with the Argonaute (AGO2)

protein. The mature miRNA transcript is 17-25 nucleotides in length. Normally, the passenger strand is marked for degradation of the miRNA transcript. Mature miRNA transcripts usually bind to the 3'UTR of their mRNA target with the assistance of AGO2 to negatively regulate mRNA translation. MiRNAs have thousands of mRNA targets, which contribute to their regulation of 60% of gene expression via translational repression of mRNA to protein, mRNA cleavage/degradation and de-adenylation [57].

Since their discovery in the early 1990s by the laboratories of Drs. Ruvkun and Ambros through the present, these naturally occurring, short, non-coding RNA species have been demonstrated to be influential mediators of gene expression in multicellular organisms. Lin-4 was the first and origin miRNA discovered in 1993 through genetic testing in nematodes or microscopic worms. Further studies revealed a regulatory function for this small RNA. MiRNAs regulate cellular development, behavior and differentiation in the normal cells of multicellular organisms [58, 59]. However, these non-coding RNAs are mostly absent in unicellular organisms such as mouse embryonic stem cells. Majority of mammalian miRNA genes are located within the introns of host genes and co-expressed their host genes as well. Their primary function is the promotion of cellular differentiation in normal cells, which is essential for multicellular organisms. For instance, during early zebrafish development most miRNAs are not expressed, but in later development miRNAs have tissue-specific expression. MiRNAs are widely conserved in plant and mammalian cells with a high abundance per cell [59]. Furthermore, miRNAs have been implicated in diverse

biological functions such as programmed cell death, early and late vertebrate development. For instance, some miRNAs regulate the Notching signaling pathway, which is essential for proper patterning and development. Majority of mammalian miRNA genes are located within the introns of host genes and co-expressed their host genes as well. Yet within the last decade, numerous studies have shown the dysregulation of various microRNAs (miRNAs/miRs) within diseases such as PCa [53, 54, 60-65]. The differential expression of miRNAs in a variety of solid tumors demonstrate an opportunity for miRNAs to serve as promising biomarkers and/or therapeutic targets due to their detectability in human tissue and biological fluids and strongly influence of cancer hallmarks. MiRNAs may serve as promising non-invasive tools to aid in the improvement of cancer diagnosis, prognosis, clinical management, and prevention strategies.

MicroRNAs in PCa

A number of miRNAs are differentially expressed in PCa [53, 54, 60-65]. Non-coding RNAs such as miRNAs exert their effects through the modulation of gene expression to either promote or repress tumorigenesis and disease progression in various cancers, including prostate [66, 67]. To date, there are approximately 120 clinical trials evaluating miRNA expression profiles in relation to cancer [68]. However, less than five percent (~4.2%) of these clinical trials are investigating miRNA profiles associated with PCa development, progression and treatment.

Dysregulation of miRNA expression contributes to the pathogenesis of PCa through their regulation of target genes involved in cellular behaviors (e.g., proliferation, motility, invasion, metastasis, angiogenesis). Fifty percent or more of human miRNA genes are located in fragile sites, breakpoint regions and regions of loss of heterozygosity and/or amplification, which can lead to the dysregulation of miRNAs. Oncogenic miRNAs are commonly up-regulated in PCa in support of tumorigenesis. In contrast, tumor suppressor miRNAs are down-regulated to allow the tumor phenotype to persist and prosper. However, miRNAs do not always function the same way in every cancer type. Sometimes oncogenic miRNAs also play a dual role as a tumor-suppressor within the same cancer or other tumor types. Tumor suppression-related miRNAs can promote tumorigenesis in other cancer types. Recently, studies demonstrate some miRNAs function in a dual capacity as both oncogene and tumor-suppressor within PCa [51, 69, 70].

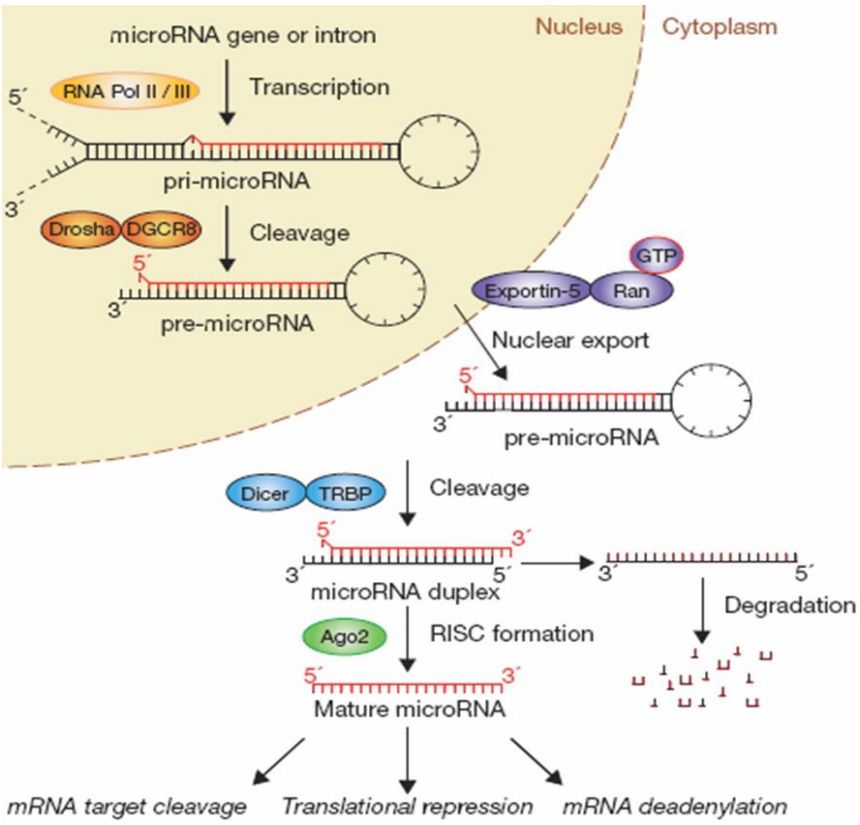

Figure 2. Intracellular Biogenesis of microRNA. This image was adapted from Winter et. al Nat Cell Biol. 2009 Mar;11(3):228-34. doi: 10.1038/ncb0309-228. The microRNA gene is transcribed by RNA polymerase II and/or III into the primary microRNA transcript (pri-miRNA) in the nucleus. Pri-miRNA transcript is cleaved by the microprocessor complex Drosha-DGCR8, into a precursor hairpin (~70nts) and exported into the cytoplasm by Exportn-5 Ran-GTP. RNase, Dicer, and TAR RNA binding protein (TRBP) complex with cleaves the precursor hairpin into a double stranded into a microRNA duplex. The leading miRNA strand is incorporated into the RISC complex and co-associated with Argonate 2 (Ago2) protein. Mature microRNA transcript commonly binds to the 3'UTR of target mRNA transcript and promote mRNA degradation, translational repression and/or deadenylation.

Oncogenic miRNAs

Some oncogenic miRNAs that promote tumorigenesis in PCa include miRs-18a, -21, -32, -96, -106b-25 cluster, -125b, -221 and -222 [53, 71]. For instance, overexpression of miR-221/222 was observed in androgen insensitive metastatic PC-3 cells [72]. Inhibition of these miRNAs resulted in the reduction of colony formation by 4.5-fold in metastatic PC-3 cells [72] . In contrast, overexpression of miRs-221and -222 enhanced cellular proliferation by 2-fold and colony formation by 3-fold in metastatic androgen sensitive LNCaP cells via down-regulation of $p27^{Kip1}$(CDKN1B), a major cyclin dependent kinase inhibitor. Mutation or loss expression of p27 can lead to uncontrolled cellular proliferation.

MiR-125b is another oncogenic miRNA in PCa. MiR-125b expression was up-regulated in androgen independent PCa LNCaP sublines, cds1 and cds2, and prostatic tumor tissue compared to PCa LNCaP cells and benign tissue, respectively [73]. Overexpression of miR-125b in cds1 cells significantly increased cellular proliferation via down-regulation of BAK1, a pro-apoptotic gene.

MiR-21 is a well-known oncogenic miRNA not only in PCa, but in other solid tumors as well. Inhibition of miR-21 expression in metastatic androgen-independent DU145 and PC-3 cells significantly reduced cell motility 72 and 48 hrs post-transfection, respectively [74]. MARCKS was identified as a direct target of miR-21 evident by the up-regulation of MARCKS protein expression in a dose-

dependent treatment with anti-sense miR-21 in both DU145 and PC-3 cells. Additionally, inhibition of miR-21 via the anti-sense miR-21 significantly reduced the cellular invasion of DU145 cells relative to cells transfected with the anti-sense control (p-value = 0.00079). This report suggests that cell motility reduction in DU145 and PC-3 cells is due to the up-regulation of MARCKS, a gene involved in the regulation of the actin cytoskeleton.

The miR-106b-25 cluster is a group of miRNAs including miRs-106b, -93 and -25. Several reports implicate that the miR-106b-25 cluster plays an oncogenic role in PCa. The miR-106b-25 cluster is up-regulated in metastatic PCa cell lines and malignant tissue relative to normal cells and tissue [75, 76]. For instance, Poliseno and associates (2010) observed that transient overexpression of the miR-106b-25 cluster down-regulated the protein expression of PTEN, a major tumor suppressor, by 40% in metastatic DU145 cells [76]. In the same report, inhibition of the mir-106b-25 cluster and other miRNAs (miRs-19a, -22) reduced cellular growth in DU145 cells after 6 and 7 days relative to controls. Also, stably miR-106b-25 cluster overexpressing DU145 cells increased tumor growth in nude mice relative to PIG retroviral vector as the empty vector control. Furthermore, MCM7, the host gene of the miR-106b-25 cluster, exhibited an oncogenic function via the increase of colony formation relative to empty vector and known oncogenes, c-MYC and RAS. Overexpression of MCM7 and miR-106-25 cluster members enhanced the transformation of wild type mouse embryonic fibroblasts (MEFs) relative to miR-

106-25 cluster alone. In another study, overexpression of the miR-106b-25 cluster in 22Rv1 cells significantly increased ECM binding and colony formation in soft agar [75]. Additionally, miR-106b, one of the miR-106b-25 cluster members, modestly reduced CASP7 protein levels in PCa xenograft 22Rv1, normal prostate epithelial RWPE1 and LNCaP cells. However, only the inhibition of miR-106b reduced ECM binding and colony formation in soft agar relative to the control and double inhibition of miR-106b and CASP7 in DU145 cells. This observation indicates that there are other miR-106b targets involved in altering ECM binding and anchorage independence in PCa. Further, in the same study, PCa patients who exhibited a high expression of miR-106b in their tumor tissue showed early biochemical recurrence compared to patients with a low expression (p-value = 0.014). Liang and co-workers (2014) observed that 24 hrs of hypoxia induced the expression of the miR-106b-25 cluster and reduced the gene and protein expression of REST (RE-1 silencing transcription factor), a tumor suppressor, in LNCaP cells [77]. Moreover, ectopic expression of miR-106b-25 cluster down-regulated REST protein expression in metastatic androgen-independent PC-3 cells as well.

In PCa, miR-96 also promotes the tumor phenotype via its effects on cellular proliferation and colony formation. In a report by Yu and colleagues (2014), transient overexpression of miR-96 significantly enhanced cellular proliferation and colony formation in LNCaP cells relative to negative control [78]. Inhibition of miR-96 in metastatic PC-3 cells reduced cell proliferation and colony

formation in the LNCaP cells. In both cell lines, FOXO1, a tumor suppressor gene was identified a target of miR-96. This report suggests that the suppression of cell proliferation and colony formation in PC-3 cells and the increased in these cellular behaviors in LNCaP cells were primarily due to the up-regulation and down-regulation of FOXO1 protein level, respectively. In a recent study, Siu and co-workers (2015) identified AKT1S1 as a target of miR-96 in established clonal PTEN/TP53 double knockout AC1and AC3 cell lines, which exhibited a high expression of miR-96. Additionally, the expression of miR-96 and AKT1S1 is mediated by TGFβ signaling. TGFβ treatment (10ng/ml) increased miR-96 expression and reduced AKT1S1 expression in PC-3 and relative to vehicle controls.

MiRs-32 and -148a promote prostate tumorigenesis through their regulation of cell cycle and cell growth. In a study by Jalava and colleagues (2012), overexpression of miR-32 and/or miR-148a significantly increased the growth of LNCaP cells [79]. Further analysis revealed that a significant population of LNCaP cells transfected with miR-148a were in the DNA synthesis (S) phase of the cell cycle. Moreover, overexpression of miR-32 was shown to down-regulate mRNA and protein expression of BTG2 in LNCaP cells. Whereas, PIK3IP1 was identified as a direct target of miR148a via its transcript and protein down-regulation in miR-148a overexpressing LNCaP cells. In the same study, BTG2 staining was lower in castration-resistant PCa (CRPC) (n = 94) tissue specimens relative to PCa specimens (n =170) and associated with advanced

disease (pT3 vs pT2) among cancer patients. In fact, the absence of BTG2 was strongly associated with a shorter progression-free survival of PCa relative to patients with BTG2 expression.

MiR-18a is another miRNA that plays an oncogenic role in PCa. It is up-regulated in metastatic PCa cell lines and tumor tissues relative to normal prostate epithelial cells and adjacent tissue [80]. Hsu and co-workers (2014) identified STK4, a tumor suppressor gene, as a putative target of miR-18a evident by the down-regulation of STK4 mRNA and protein expression in ectopic miR-18a expressing PC-3 and DU145 cells. Inhibition of miR-18a significantly reduced colony formation and cell proliferation in 22Rv1 cells. Tumor growth of anti-miR-18a 22Rv1 cells implanted in nude mice was reduced relative to the growth of anti-miR control cells.

Tumor suppressor miRNAs

In contrast, tumor suppressive miRNAs (e.g., miRs -29b, -34, -101, -126-3p, -145, 146a, -200 family, -675) play an important role in the repression of the tumor phenotype associated with PCa [53, 71]. For instance, Ru and associates (2012) observed miR-29b was down-regulated in androgen insensitive- and sensitive-PCa cell lines, PC-3 and LNCaP, and tumor tissue relative to immortalized normal prostate epithelial cells and non-tumor tissue [81]. Overexpression of miR-29b impairs wound healing and decreases cellular invasion in PC-3M cells, a highly metastatic PCa cell line derived from PC-3 cells. Ectopic miR-29b expression significantly reduces the number of

metastases *in vivo*. In the same report, Snail, a major EMT marker, was identified as a direct target of miR-29b due to a decrease in Snail protein expression in miR-29b overexpressing PC-3M cells. Furthermore, miR-29b inhibition resulted in an up-regulation of Snail protein expression in LNCaP cells.

MiR-34a is a mediator of p53 that acts as a tumor suppressor in PCa. It is significantly under-expressed in laser captured micro-dissected (LCM) malignant tissue (n = 10) and metastatic PC-3 cells compared with adjacent normal tissue and normal epithelial prostate cells, respectively [82]. Cellular invasion and colony formation in soft agar was suppressed in stably miR-34a transfected PC-3 cells. Moreover, the tumor growth of stably miR-34a transfected PC-3 cells was reduced in nude mice. PC-3 cells overexpressing miR-34a exhibited a higher apoptotic population relative to the negative control. Overexpression of miR-34a down-regulated the protein expressions of c-Myc oncogene and metastasis mediator RhoA. The report suggests that miR-34a-induced reduction of cellular invasion, colony formation and tumor growth via the induction of apoptosis and down-regulation of c-Myc.

In a recent report (2014), miR-146a was shown to function as a tumor suppressor in PCa [83]. Stable miR-146a overexpression significantly inhibits anchorage independent growth in the prostate epithelial non-metastatic P69 and highly metastatic M12 (a subline of P69) cell lines relative to vector control. In contrast, miR-146a inhibition in both cell lines increased colony formation in soft

agar. In M12 cells, miR-146a overexpression enhanced both cellular proliferation and invasion compared with the vector control. The inhibition of miR-146a produced the reverse effects on these cellular processes. Similar effects were observed in relation to cell proliferation in PC-3 and P69 cells relative to vector control. Additionally, Rac1 was identified as a direct target of miR-146a evident by the down-regulation of Rac1 protein level in both P69 and M12 cells. Moreover, the expression of miR-146a was down-regulated and Rac1 was up-regulated in tumor tissue (n = 28) relative normal tissue (n = 28), which exhibits an inverse relationship between their expression profiles. In an earlier report, miR-146a was significantly under-expressed in hormone-refractory PCa cell lines (LNCaP, C4-2B, PC-3) and tumor tissue relative to androgen-dependent cell lines (LNCaP, PC-3-AR9) and non-cancerous tissue [84]. Forced overexpression of miR-146a down-regulated ROCK1, an oncogene, protein expression in PC-3 cells.

MiR-145 is a severely under-expressed miRNA in PCa cells and functions as a tumor suppressor. For instance, one report observed that miR-145 expression was significantly down-regulated in metastatic PCa cells (i.e., DU145, LNCaP, PC-3) relative to normal epithelial PWR-1E [85]. Also, low expression of miR-145 was detected in prostate tumor tissue compared with normal adjacent tissue. Moreover, miR-145 overexpression reduced cell viability in PC-3 cells in a time dependent manner within 72 hrs.

The miR-200 family is a well-known tumor suppressor family in PCa. Forced expression of miR-200b in PC-3 cells significantly decreased the tumorigenic potential in an orthotropic PCa model relative to parental PC-3 and negative control cells [86]. Tumor tissue obtained from this model exhibited a reduction in cellular proliferation via staining for Ki-67, a proliferation marker, relative to controls. Furthermore, overexpression of miR-200b significantly decreased angiogenesis evident by a reduction in microvascular density and cell invasion of within miR-200b positive PC-3 tumors to negative control [86]. Also, overexpression of miR-200b negatively influences the EMT via down-regulation of mRNA and protein expression of ZEB1, a potent EMT mediator, in PC-3 cells. MiR-200c is low in LNCaP and DU145 cells [87]. A loss of miR-200c expression was observed in PC-3B1, derived from SCID mice bone marrow injected with PC-3 cells, and PC-3 due to DNA hyper methylation of miR-200c of CpG islands.

MiR-101 is another miRNA that functions exclusively as a tumor suppressor in PCa. In a report by Varambally and associates (2009), ectopic expression of miR-101 decreased cellular invasion and tumor growth of metastatic DU145 cells relative to control miR and empty vector, respectively [88]. Additionally, DU145 tumor growth reduction was accompanied by a decrease in EZH2 protein expression. EZH2, a histone methyltransferase, is commonly elevated in aggressive and localized PCa. In contrast, miR-101 is normally under-expressed in metastatic PCa (n = 32) relative to benign controls (n =26). This inverse relationship between miR-101 and EZH2 suggests that

EZH2 may be a potential target of miR-101. The report further suggests that the loss of miR-101 expression allows for the unrestricted up-regulation of EZH2 to maintain the tumor phenotype of DU145 cells.

MiR-675 also plays a tumor suppressor role in PCa. Derived from long non-coding RNA (lncRNA) H19, miR-675 is significantly under-expressed in highly metastatic M12 cells relative to non-metastatic prostate epithelial P69 cell line [89]. Inhibition of miR-675 expression in P69 cells resulted in a significant increase of cellular migration. In contrast, miR-675 overexpression dramatically reduced cellular migration of metastatic PC-3 cells, but not M12 cells. In P69 cells, miR-675 overexpression led to a decrease in the protein expression of TGFBI, an extracellular matrix protein. TGFBI also known as transforming growth factor β induced protein is highly expressed in metastatic PCa and has been previously implicated in the promotion of cell adhesion, motility, invasion and metastasis in other cancers [89].

Dual role miRNAs in PCa

Interestingly, there are only two miRNAs (miRs-133, -375) that have been identified to exhibit both an oncogenic and tumor suppressor role in PCa [51, 69, 70]. MiR-133b was significantly under-expressed in PCa tumor specimen (n = 69) compared normal adjacent tissue (n = 69) [69]. A low tumor to normal tissue miR-133b ratio was indicative of a shorter disease recurrence-free survival in patients compared with patients with a higher ratio. Moreover, a univariate

analysis revealed a marginal association between miR-133b and a decrease in risk of biochemical recurrence (HR = 0.16; 95% CI = 0.02-1.06; p-value = 0.06). Overexpression of miR-133b in androgen-independent PC-3 cells significantly reduced cellular proliferation 96 and 144 hrs post-transfection relative to miR-control. MiR-133b overexpressing PC-3 cells treated with recombinant human TRAIL (20ng/ml) showed a significant induction of apoptosis, which suggests miR-133b promotes cellular death. However in another study by Li and associates (2014), overexpression of miR-133b in androgen-dependent PCa cell lines (LNCaP, 22Rv1) significantly enhanced cell proliferation. Indeed, ectopic miR-133b expression decreased the apoptotic cell population in LNCaP cells relative to negative control. In contrast, forced miR-133b overexpression increased the apoptotic cell population in PC-3 cells. MiR-133b expression was the lowest in less aggressive LNCaP cells and highest in aggressive PC-3 cells. Additionally, DHT treatment (100nM) within 48 hrs induced an increase in miR-133b expression in LNCaP cells relative to vehicle control. Androgen-sensitivity mediates miR-133b expression levels and its effect on tumor phenotypes.

MiR-375 also has a dual function in PCa dependent on androgen sensitivity status. Costa and colleagues (2015) observed that miR-375 is significantly up-regulated in prostatic tumor tissue (n =114) relative to normal tissue (n =15) [70]. Moreover, higher levels of miR-375 were expressed in cases with higher Gleason scores (Gleason score ≥ 7) and advanced disease (pT3b) relative to patients with lower Gleason scores (Gleason score < 7) and tumor

stages (pT2 n = 58, pT3a n = 24). Ectopic miR-375 expression induced apoptosis and reduced cellular proliferation and invasion in metastatic PC-3 cells. In normal prostate epithelial RWPE1 cells, miR-375 overexpression decreased cell proliferation. However, this observation did not reach a level of significance. Mechanistically, overexpression of miR-375 down-regulated the CCND2 transcript, a regulator of the cell cycle, which is also normally under-expressed in PCa. This suggests that miR-375 expression promotes cell cycle arrest in both metastatic PCa cells and normal epithelial cells. The aforementioned studies on miR-133b and miR-375 support the concept that miRNAs can have a dual function within one disease type. These studies aid in the understanding of how to appropriately target miRNAs that play both an oncogenic and tumor suppressive roles to identify robust markers in PCa.

Clinical Applications of microRNAs

Clinical trials are critically evaluating the role of miRNA expression in relation to PCa and their therapeutic use against disease progression. Unfortunately, a small percentage (4.2%) of clinical trials is evaluating miRNA expression profiles in PCa. Currently, there are six clinical trials investigating miRNA expression in relation to high-risk PCa, androgen deprivation therapy, therapies (i.e., abiraterone acetate, enzalutamide, radiotherapy) against metastatic hormone-resistant disease and high-risk PCa [NCT01220427, NCT02366494, NCT01503229, NCT02471469, NCT01444820, NCT02391051]. Three of the six clinical trials are currently recruiting study participants and two

trials are ongoing. Currently, no data have been published in relation to any of the clinical trials. In a phase 2 clinical trial, the efficacy of abiraterone acetate treatment against metastatic and hormone-resistant PCa is being evaluated by the University of Washington [NCT01503229]. This study is utilizing miRNA expression profiles to monitor molecular changes in tumor metastases. In a phase 3 clinical trial, investigators are evaluating acute and delayed genito-urinary and gastrointestinal toxicity in high-risk prostate adenocarcinoma patients treated with hypofractionated and conventional radiation therapy [NCT01444820]. As secondary outcome of this study, the expression of X-chromosome-linked micro-RNAs will be evaluated in response to radiation.

Chemopreventive agents that alter miRNA expression offer other clinical applications for miRNAs as well [85, 90-98]. For instance, Geinstein (50 μM), a dietary isoflavone, up-regulates the gene and protein levels of ARHI, a tumor suppressor gene, via down-regulation of oncogenic miRs-221 and -222 in metastatic PC-3 cells [94]. Another chemopreventive agent, Resveratrol is a natural phytoalexin that down-regulates oncogenic miRNAs (miRs-7, -17, -18b -20a, -20b -106a, -106b, -129, -185, -764*) and up-regulates tumor suppressor miRNAs (miRs-149, -150, -575, -671-5p, -939, -1290) in metastatic LNCaP cells [93]. Epigallocatechin-3-gallate (EGCG), a major polyphenol in green tea, up-regulates tumor suppressor miR-330 expression in hormone-refractory PCa xenograft model relative to vehicle controls [95]. Chemopreventive agents that enhance the expression of tumor suppressor miRNAs and repress the

expression of oncomiRs may serve as excellent therapeutic agents against cancer to reduce disease burden in patients. Ultimately, miRNA profiles could be utilized to monitor physiological changes in response to disease progression and/or conventional treatments to evaluate the effectiveness of therapeutic agents and patient response to therapies [99-112].

This research project seeks to provide insight on the role of an understudied miRNA, miR-186, in prostate tumorigenesis. In this study, elevated expression levels of miR-186-5p were detected in PCa patients diagnosed with tumor stage I, III and IV relative to disease-free individuals. Furthermore, its expression was up-regulated in androgen sensitive and insensitive metastatic PCa cell lines relative normal epithelial cells. However, only two reports have evaluated miR-186 expression in PCa [63, 113]. The studies present conflicting data on the expression of miR-186-5p in prostate tumor tissue. Furthermore, the aforementioned studies do not provide a definitive function of miR-186-5p in PCa.

To address this gap research and define the true role of miR-186-5p in PCa, we characterized the expression of miR-186-5p *in vitro* and determine the impact of miR-186-5p expression on the prostate tumor phenotype and epithelial PCa cells. Moreover, we identified a potential mRNA target regulated by miR-186 in PCa. Characterization of miR-186 in vitro will serve as the foundation of future studies evaluating the role of miR-186 prostate tumorigenesis.

Our study findings suggest that miR-186-5p inhibition reduces, proliferation, anchorage independent growth and invasion in metastatic PCa (i.e.,

PC-3, MDA PCa 2b) and also miR-186 overexpression in normal epithelial cells (i.e., RWPE1) proliferation and increased cell death. These tumor specific effects reveal miR-186-5p inhibition reduces prostate tumor phenotype aggressiveness and miR-186-5p is potentially oncogenic and supports prostate tumorigenesis via its endogenous up-regulation. Furthermore, we showed miR-186-5p inhibition led to the up-regulation of tumor suppressor gene, AKAP12. Ultimately, the detection of elevated miR-186-5p serum levels in combination with current PCa screening tools may improve the detection of aggressive disease and identification of high-risk patients. Additionally, miR-186-5p and its regulated genes may serve as potential therapeutic targets for current therapeutic agents to modulate and improve treatment efficacy.

CHAPTER II

DIFFERENTIAL EXPRESSION OF MIRNAS IN THE SERUM OF PROSTATE CANCER PATIENTS

Introduction:

The prostate specific antigen (PSA) test and digital rectal exam are gold standards for PCa detection. However, these tools demonstrate a marginal or no impact on prostate mortality rates among men in the U.S. and Europe. In 2009, two major epidemiological studies, the Prostate Lung Colorectal and Ovarian (PLCO) Screening Trial and European Randomized Study of Screening for PCa (ERSPC), evaluated the effectiveness of PSA testing and DRE against PCa mortality [40, 44, 46]. Based upon the data collected from these and other studies, the use of PSA testing by physicians or urologists has been discouraged for patients due to the recommendations made by the American Urological Association and the U.S. Preventive Services Task Force. Therefore, additional biomarkers are needed to aid in the improvement of PCa detection.

As described in Chapter 1, miRNA dysregulation has been shown to play a pivotal role in the development and/or disease progression of PCa [71, 114, 115, 116]. These non-coding RNAs bind to the 3'UTR region of their mRNA targets and invoke post-transcriptional changes such as degradation,

repression of translation to protein, and deadenylation of mRNA targets. MicroRNAs target a vast number of mRNA transcripts. Consequently, microRNAs influence about 30-60% gene expression in tumor-associated processes (e.g., proliferation, motility, invasion, metastasis, angiogenesis) [53, 54, 60-65]. For instance, aberrant miRNA expression in PCa can distinguish between clinical features such as poorly and moderate differentiated tumor tissue, primary, metastatic disease, non-recurrent disease and biochemical recurrent patients in PCa [52, 117]. MiRNAs may serve as potential prognostic and diagnostic markers to aid in the detection and clinical management of PCa [62, 113]. Additionally, these short, non-coding RNAs can also be utilized as complementary tools for current screening procedures in PCa detection due to their ability to reflect the physiological changes in cancer, high stability and detectability in mammalian cells, tissue and different biological fluids [52, 65, 118].

MiRNAs generally participate in the regulation of cellular homeostasis, as previously discussed in Chapter 1. However in cancer, they can function as either oncogenes, tumor suppressors or both-depending on tumor type and hormonal sensitivity in some cancers such as PCa. MiRNAs are found in cells, tissues and biological fluids (saliva, urine, plasma, and serum). Changes in miRNA expression profiles have been linked to disease progression and different tumor stages of disease in PCa. These non-coding RNAs are highly stable and detectable in tissue, cell lines and biological fluids for hrs and/or days after

synthesis. The stability of miRNAs are attributed to the complex they form with a protein called Argonaute (AGO2), which prevents degradation of miRNAs [119]. During miRNA biosynthesis, miRNAs form a complex with AGO2 and this complex facilitates the degradation of target mRNA transcript and/or repression of translation to protein.

A population of miRNAs are secreted from various cell types (i.e., tumor cells) and released into the extracellular space [120]. Commonly referred to as circulating miRNAs, miRNAs in biological fluids are released via exosomes (50-100 nm), microvesicles (100-1000 nm), or are complexed to AGO2 and/or in high density lipoprotein particles shed from various cell types. Circulating miRNAs travel through the extracellular space and where they can be released into plasma, serum, saliva and/or urine [118]. These extracellular vesicles are mediate cell-to-cell communication and transport genetic information (i.e., miRNAs, mRNA) between different cells [118]. Reports suggest circulating miRNA expression profiles correspond to distinct stages in PCa [52, 117, 121]. Due to their high stability, detectability in biological fluids strong correlation to physiological changes, miRNAs are ideal candidates for disease detection and prognosis in PCa. However, there are major issues associated with the extraction of circulating miRNAs and the normalization methods required for their quantitative analyses in biological fluids, namely serum.

Challenges of miRNA detection

MiRNA expression profiles have the potential to serve as prognostic and diagnostic markers in PCa. However, the low abundance of miRNA in serum and use of different normalization techniques for miRNA expression across different solid tumor types plague miRNA detection. MiRNA extraction from serum yield low abundance of circulating miRNA and sometimes there are difficulties quantifying nucleic acid concentrations via spectrophotometry. Highly robust and sensitivity quantitation methods are needed to measure the concentration miRNA in serum. Currently, there are no endogenous controls for miRNA expression normalization in serum. In the literature, various normalization techniques for miRNA expression are used in different tumor types [118]. However, there is overlap between some cancer types. In breast, lymphoma, colon and oral cancer, miR-16 and 18s are used as controls to normalize miRNA expression in serum. In contrast, RNU6B is used as a control for miRNA expression in the serum of gastric and colon cancer patients. In PCa, reports by Brase et al. (2010) and Mitchell et al. (2008) spike denatured serum samples with cel-miRs-39, -54 or 238 to normalize miRNA profiles [52, 122]. No control has been determined as the best overall control for miRNA expression among cel-miRs-39, -54 or 238 in PCa. In other PCa studies, RNU6B, median Ct value and absolute quantification are normalization techniques for serum and/or plasma samples [60]. Ultimately, the utilization of various normalization procedures could lead to great inconsistencies among serum-based miRNA expression profiles in prostate and other cancers.

Circulating miRNAs as prognostic and diagnostic tools

Circulating miRNAs non-invasively discriminate between PCa and healthy individuals as well as localized versus metastatic disease. Several reports have demonstrated miRNA expression profiles correspond to specific disease stages and advanced disease [52, 65, 117, 123, 124]. MiRs-141 and -375 were significantly up-regulated in prostatic tumor tissue (n=36) relative to benign prostate tissue (n=36) (p-value < 0.001) [52]. These miRNAs were also elevated in the serum of PCa patients with lymph node metastasis score N1 (n = 43) compared to those without lymph node spread (n =23) (p-value < 0.05). In this study, miRNA expression was normalized using miRs-cel-39, cel-54, and cel-238 in serum to control for RNA extraction efficiency and cDNA synthesis. However, only miR-141 was significantly higher in patients with aggressive disease (Gleason Score > 8) relative to intermediate differentiated tumor tissue (Gleason Score = 7) (p-value < 0.05). Additionally, miR-375 expression was significantly higher in metastatic PCa serum samples compared with localized disease serum (FDR p-value = 0.036). In a recent report (2013), elevated serum levels of miR-141 corresponded to a greater number of bone metastases (metastases > 5) in hormone-naive PCa (p-value = 0.028), hormone-sensitive (p-value = 0.014) and hormone-refractory (p-value = 0.028) PCa patients relative to patients with lower metastases (3 > metastases) [123]. Serum-based miRNA expression profiles in the previous study were normalized to Sp6 and miR-cel-39. Circulating oncogenic miRs-195, -26a, and let7i were significantly up-regulated in the serum of PCa patients (n = 37) compared to BPH patients (n = 18) (p-value = 0.015-

0.048) [65]. The expression of all miRNA expression profiles in the serum was normalized to miR-cel-39. Furthermore, this study suggests that these miRNAs may serve as monitoring tools in disease management since the removal of the prostate dramatically reduced the expression these circulating miRNAs. Furthermore, miRNAs can distinguish between patients with advance disease and low-grade disease. In a report by Mihelich and associates (2015), 14 miRNAs (miRs-let-7a, -103, -107, -130b, -106a, -26b, -451, -223, -93, -24, 30c, -874, -100, -146a) predicted the absence of high-grade PCa (Gleason grade 4 and/or 5) in the plasma of BPH (n = 50) and low-grade PCa (Gleason grade =3) patients (n = 50) with a negative predictive value of 0.938 and positive predictive score of 0.366 (p-value < 0.05) [117]. MiRNA expression profiles analyzed in serum were normalized to Sp6 and miR-cel-39 expression. A negative predictive value is the probability that patients with a negative screening test truly have a negative disease diagnosis. However, a positive predictive score is the probability that patients truly have a positive diagnosis. In the same study, 8 miRNAs accurately predicted a subset of PCa patients at low risk of biochemical recurrence with a negative predictive value of 0.941. The data suggest that these 8 miRNAs may serve a pre-surgical predictor of disease recurrence. In metastatic castration resistant PCa patients (n = 25), the up-regulation of miR-423-3p in plasma was significantly associated with high Gleason score (Gleason Score ≥ 8), lymph nodal N1 status, and biochemical recurrence (PSA ≥ 0.2 ng/mL on two occasions after prostatectomy) relative to patients with localized disease (n = 25) (p-value < 0.004) [124]. Similarly to previous studies, circulating

miRNA expression profiles were normalized to miR-cel-39, Sp6, and Sp3, respectively.

In this study, we evaluated the expression of 377 mature microRNAs in the serum of European-American PCa patients diagnosed with tumor stage I, III and IV disease. The goal of this evaluation was to identify differentially expressed circulating miRNAs in the serum that influence PCa development and/or aggressive disease. Fortunately, we identified two up-regulated circulating miRNAs (miRs-106b-5p and -186-5p) in the serum of PCa patients relative to non-cancerous controls. Moreover, we evaluated the expression of a serum-based miRNA, miR-186-5p, in different prostate tumor cell lines. Circulating miRNAs reflect physiological changes associated with the disease states within PCa. The study findings identified an up-regulated circulating miRNA, miR-186-5p, in PCa patient serum, which was also up-regulated in three metastatic PCa cell lines (i.e., PC-3, LNCaP, MDA PCa 2b). This miRNA may play a role in aggressive tumor phenotype associated prostate tumorigenesis and serve as a potential prognostic marker for the clinical management of PCa.

Methods:

Human Serum Biospecimens

Serum (0.5-1 ml) from 15 men with PCa and 5 non-cancerous individuals was collected from patients prior to any therapy and obtained by BioServe Biotechnologies Biorepository (Beltsville, MD). Samples were divided into non-cancerous controls (n=5), patients diagnosed with PCa stage I (n=5), stage III (n=5), and stage IV (n=5). De-identified clinical characteristic data was provided for each patient by BioServe Biotechnologies (Beltsville, MD). Descriptive statistics were performed to determine patient median age, age range, median weight, weight range, body mass index (BMI) median, and BMI range. A high percentage of PCa patients had a smoking history (66.7%) and tumor classification of adenocarcinoma (60%). Statistical analyses for age, PSA levels and BMI were performed using the Wilcoxon Rank-Sum test and cut-off significance level of p-value ≤ 0.05.

microRNA Isolation from Serum

Total isolation of microRNA was performed using the miRVana microRNA Isolation kit (Catalog # AM 1561, Thermo Fisher Scientific, Waltham, MA). Serum (250 µl) samples from 20 patients were transferred to phase tubes. Trizol LS Reagent (1 ml) was added to each sample in 1.5 ml tubes in a chemical hood and pipetted up and down 30 times at room temperature. Each sample was spiked with 2ul of cel-miR-39 (1nM, internal control miRNA) and incubated at room temperature for 5 minutes (mins). Pure ACS grade 98% chloroform (200 µl)

was added to each sample and shaken up and down for 15 seconds (secs). Samples were immediately transferred to phase lock tubes and incubated for 5 mins at room temperature. Samples were centrifuged at 12,000xg at 4^{o}C for 15 mins. Aqueous phase (400 µl) of each sample was transferred to new 1.5ml tubes and 100% isopropanol (500 µl, 1.25x times aqueous phase volume) was added to each sample. Samples were pipetted up and down several times, each mixture (700 µl) was applied to a filter cartridge and centrifuged at 10,000 xg for 30 secs. This step was continued until each sample was completely filtered through at room temperature. Flow-through was discarded after each centrifugation. Sample filters were washed with wash solution 1 (700 µl) one time and wash solution 2/3 (500 µl) two times using centrifugation at 10,000xg for 30 secs at room temperature. Filters were centrifuged an additional time at 10,000xg for 1 minute to remove residual fluid and transferred to new 1.5ml tubes. Nuclease-free H_2O preheated at 95^{o}C was applied to each filter and incubated for 5 mins. Total RNA was eluted using centrifugation at 12,000 xg for 1 minute to room temperature. Serum samples were stored at -80^{o}C until further use. Statistical analysis for miRNA validation was performed using Unpaired Student T-test and cut-off significance level of p-value ≤ 0.05

miRNA Expression

Reverse transcription of total RNA (10 ng) was performed using TaqMan microRNA Reverse Transcription kit, miRNA specific RT primers and Peltier Thermal Cycler (PTC 200). Cycling cDNA was synthesized using the following

cycling conditions 16^{o}C for 30 mins, 42^{o}C for 30 mins, 85^{o}C for 5 mins and hold indefinitely at 4^{o}C. qRT-PCR was performed using miRNA specific PCR TaqMan Assays (Life Technologies) and the following cycling conditions: hold for 2 mins at 50^{o}C, 10 mins at 95^{o}C for 1 cycle, 15 secs at 95^{o}C and 1 minute at 60^{o}C for 40 cycles. Amplification of cDNA was performed using the Applied Biosystems Step One PCR system (Applied Biosystems, Carlsbad, CA). The relative expression of microRNA compared with U44 snRNA was calculated using $2^{-\Delta\Delta Ct}$ method.

Taqman Array Human MicroRNA

Expression profiling for miRNA was performed using the Taqman Array Human MicroRNA Pool A Cards v.2 (Thermo Fisher Scientific, Waltham, MA, Carlsbad, CA) according to the manufacturer's recommendations. The array card consisted of 377 miRNA assays, three endogenous controls (RNU6, RNU44 and RNU48) and one negative control unrelated to human (ath-miR-159a). Each total RNA sample was diluted to 2 ng/µl using nuclease-free water. Diluted RNA samples were reverse transcribed to produce cDNA in a 7.5 µl reaction using the TaqMan miRNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, Carlsbad, CA) in addition to Megaplex RT Primers Human Pool A 10X (Thermo Fisher Scientific, Waltham, MA, Carlsbad, CA). Samples were pre-amplified using TaqMan PreAmp Master Master Mix 2X and Megaplex PreAmp Primers Human Pool A (Thermo Fisher Scientific, Waltham, MA, Carlsbad, CA) in a 25 µl reaction. The pre-amplification conditions were initiated in PCR tubes

at were 95°C for 10 min, 55°C for 2 mins and 72°C for 2 mins followed by 12 cycles of 95°C for 15 secs and 60°C for 4 mins and followed by 99.9°C for 10 mins and held at 4°C. Pre-amplified products were diluted with 75 µl of TE (0.1X, pH 8.0) and stored at -15°C. Then samples were thawed and added into a PCR reaction mix and 100 µl of mix was dispensed into each port of the TaqMan MicroRNA array. Then array was centrifuged for 2 times at 1200 rpm for 1 minute, sealed and placed into the ABI 7900 Real Time PCR system.

Taqman Array Human MicroRNA Statistical Analysis

MicroRNA expression profiles of 377 targets (Pool A TLDA card) in total RNA derived from PCa serum (n = 15) and non-cancerous controls (n = 5) were evaluated using R-programming software. MicroRNA profiles were normalized to the global median Ct value for each array (global Ct median value - target Ct value). After global normalization, missing Ct values associated targets were imputed using k nearest neighbor (kNN) imputation. miRNA expression profiles with too many missing values were screened off and not included in final analysis. Differentially up-regulated and down-regulated miRNAs for each each PCa stage vs. controls were determined using moderated t-tests and ANOVA in the R package *limma* [125-127]. Statistical analyses on miRNA expression profiles were adjusted for multiple hypothesis testing using the False Discovery Rate (FDR) method. MicroRNA profiles that reached statistical significance (FDR p-value ≤ 0.05) were further analyzed individually.

Cell Line	Disease/Site	Patient Age (yrs)/ Ethnicity	Tumor Stage	AR-sensitivity	Tumorigenic (nude mice)
DU 145	Human Prostate adenocarcinoma Brain metastasis	69 /European-American	Grade IV	No	Yes
PC-3	Human Prostate adenocarcinoma Bone metastasis	62 /European-American	Grade IV	No	Yes
LNCaP	Human Prostate adenocarcinoma Left supraclavicular lymph node metastasis	50 /European-American	Grade IV	Yes	Yes
C4-2B	Human LNCaP tumor derived adenocarcinoma in mouse Bone metastasis			No	Yes
22Rv1	Human castration resistant carcinoma in mouse CWR22 xenograft			Yes, weak	Yes
E006AA	Human Prostate adenocarcinoma Left middle lobe of prostate	50/ African-American	Grade I or II	Yes	No
MDA-PCa-2b	Human Prostate adenocarcinoma Bone metastasis	63/ African American		Yes	Yes
RWPE-1	Human normal prostate epithelium	54/European-American		Yes	No
RWPE-2	v-Ki-Ras transformation of RWPE1	54/ European-American		Yes	Yes
HEK 293T	Adenovirus 5 transformation of human embryonic kidney epithelium	Fetus			Yes

Table 1. Characteristics of Human Cell lines. Cell lines utilized in the current study are described in the table above. Tumor grade was not available for 22Rv1, C4-2B and MDA PCa 2b PCa cell lines.

Cell Culture

Immortalized European American metastatic (PC-3, DU145) and normal prostatic epithelial cell lines (RWPE1, RWPE2) were obtained from American Type Culture Collection (ATCC) (Manassas, VA). E006AA and MDA-PCa-2b cells were obtained from Drs. Shahriar Koochekpour and Deepak Kumar. All cell lines were grown and maintained in a humidified incubator at 37^{o}C with 5% CO_2.

E006AA is an immortalized androgen-dependent human primary PCa epithelial cell line derived from a 50 year old African American male diagnosed with stage 2 (T2aN0M0) localized PCa. This cell line was obtained from Dr. Shahriar Koochekpour at Roswell Park Cancer Institute (Buffalo, NY). The E006AA cell line was sub-cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium supplemented with 10% of fetal bovine serum (FBS), 1% of 200 nM L-glutamine [100x] (Catalog# 25030-081, Thermo Fisher Scientific, Waltham, MA) and 1% of antibiotic [10,000 I.U./ml of penicillin, 10,000 µg/ml of Streptomycin, 25 µg/ml Amphoterricin B] (Catalog # 30-004-Cl, Mediatech Inc., Manassas, VA).

MDA-PCa-2b is an androgen dependent human immortalized epithelial cell line derived from a 63-year old African American male diagnosed with lung and bone metastasis. The cell line was obtained from Dr. Deepak Kumar at the University of the District of Columbia (Washington, D.C.). MDA-PCa-2b cells were sub-cultured in Kaighn's modified Ham's F-12K medium (Catalog #21127-022, Thermo Fisher Scientific, Waltham, MA) supplemented with hydrocortisone

(100 pg/ml) (Catalog# H0396-100MG, Sigma) and human recombinant epidermal growth factor (EGF) (10 ng/ml), FBS (20%) and antibiotic [10,000 I.U./ml of penicillin, 10,000ug/ml of Streptomycin, 25 µg/ml Amphoterricin B] (Catalog # 30-004-Cl, Mediatech Inc., Manassas, VA).

PC-3 is an androgen-independent immortalized human epithelial cell line derived from a 62-year old European American male with prostatic adenocarcinoma grade IV and bone metastasis. PC-3 cells were sub-cultured in Kaighn's modified Ham's F-12K medium (Catalog #21127-022, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% of FBS, 1% of 200nM L-glutamine [100x] (Catalog# 25030-081, Thermo Fisher Scientific, Waltham, MA) and 1% of antibiotic [10,000 I.U./ml of penicillin, 10,000 µg/ml of Streptomycin, 25 µg/ml Amphoterricin B] (Catalog # 30-004-Cl, Mediatech Inc., Manassas, VA).

LNCaP clone is an androgen dependent human immortalized epithelial cell line derived from a 50-year old European American male diagnosed with left supraclavicular lymph node metastasis. LNCaP cells were sub-cultured in RPMI 1640 (Catalog # 11875-093, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% of FBS, 1% of 200 nM L-glutamine [100x] (Catalog# 25030-081, Thermo Fisher Scientific, Waltham, MA) and 1% of antibiotic [10,000 I.U./ml of penicillin, 10,000 µg/ml of Streptomycin, 25 µg/ml Amphoterricin B] (Catalog # 30-004-Cl, Mediatech Inc., Manassas, VA).

22Rv1 is an immortalized human prostate carcinoma epithelial cell line derived from a CWR22 xenograft. After castration –induced regression and relapse of parental androgen-dependent CWR22 xenograft, cells were serially propagated in mice. The cell line was obtained from Dr. Deepak Kumar at the University of the District of Columbia (Washington, D.C.). 22Rv1 cells were sub-cultured in RPMI 1640 (Catalog # 11875-093, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% of FBS, 1% of 200 nM L-glutamine [100x] (Catalog# 25030-081, Thermo Fisher Scientific, Waltham, MA) and 1% of antibiotic [10,000 I.U./ml of penicillin, 10,000ug/ml of Streptomycin, 25 µg/ml Amphoterricin B] (Catalog # 30-004-Cl, Mediatech Inc., Manassas, VA).

C4-2B is a bone metastatic subline derived from C4-2 cells orthotopically injected into castrated male mice. C4-2B cells were extracted from large ossesus tumors in mice. C4-2B cells were sub-cultured in T-medium supplemented with 10% of FBS, 1% of 200 nM L-glutamine [100x] (Catalog# 25030-081, Thermo Fisher Scientific, Waltham, MA) and 1% of antibiotic [10,000 I.U./ml of penicillin, 10,000 µg/ml of Streptomycin, 25ug/ml Amphoterricin B] (Catalog # 30-004-Cl, Mediatech Inc., Manassas, VA). T-medium is composed of the following: Kaighn's modified Ham's F-12K medium, Dulbecco's Modified Eagle's medium, 200 µg/ml of Biotin (Catalog# 84639, Sigma Aldrich, St. Louis, MO), 200 ng/ml 3,3,5-triloda-l-thyronine sodium salt (Catalog# 16397, Sigma Aldrich, St. Louis, MO), 5mg/ml of transferrin (Catalog# T8158, Sigma Aldrich, St. Louis, MO), 10 mg/ml of adenine hydrochloride (Catalog# A9795, Sigma Aldrich, St. Louis, MO)

and 2.5ml of ITS Liquid Media Supplement (100X) (Catalog# 13146, Sigma Aldrich, St. Louis, MO).

DU145 is an androgen-independent immortalized human epithelial cell line derived from a 69 year old European American male with prostate carcinoma and brain metastasis. DU145 cells were sub-cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium supplemented with 10% of fetal bovine serum (FBS), 1% of 200 nM L-glutamine [100x] (Catalog# 25030-081, Thermo Fisher Scientific, Waltham, MA) and 1% of antibiotic [10,000 I.U./ml of penicillin, 10,000 µg/ml of Streptomycin, 25 µg/ml Amphoterricin B] (Catalog # 30-004-Cl, Mediatech Inc., Manassas, VA).

RWPE-1 is an immortalized normal human prostate epithelial cell line obtained from a European American donor and immortalized with human papillomavirus 18. RWPE-2 is an immortalized normal prostate epithelial cell line derived from RWPE1 cells and transformed by v-ki-Ras. Both RWPE1 and RWPE2 were sub- cultured in Keratinocyte-SFM (Catalog # 17005-042, Thermo Fisher Scientific, Waltham, MA) supplemented with bovine pituitary extract (BPE) (50 µg/ml), human recombinant epidermal growth factor (EGF) (5 ng/ml), and 1% of antibiotic [10,000 I.U./ml of penicillin, 10,000ug/ml of Streptomycin, 25 µg/ml Amphoterricin B] (Catalog # 30-004-Cl, Mediatech Inc., Manassas, VA).

microRNA Isolation from cell lines

Total isolation of mRNA and microRNA was performed using the miRVana microRNA Isolation kit (Catalog# AM 1561, Thermo Fisher Scientific, Waltham, MA). Cells were seeded at an optimal cell density into 60 mm plates in growth medium. Cells were washed once with 1X PBS (2 ml), lysed in dishes with mirVana lysis buffer (600 µl), harvested with cell scraper and pipetted into 1.5ml tubes. Cells were vortexed for 30 secs to completely lyse cells. Homogenate additive (1/10 of cell lysate volume) was added to cell lysates and vortexed for 30 secs. Samples were incubated on ice for 10 mins and equal volume of acid/phenol chloroform mixture was added to each tube. Samples were vortexed for 60 secs and separate into aqueous and organic phases through centrifugation at 10,000xg for 5 mins at 4^{o}C using Eppendorf Centrifuge 5810R. Aqueous phase (≥ 400 µl) was pipetted into new 1.5ml tubes and 100% EtOH (1.5 x aqueous layer volume) was added to each tube. Samples were added to filters in new collection tubes, centrifuged at 10,000 xg for 30 secs. Samples were processed at room temperature and flow-through was discarded after centrifugation. Filters were washed once with 700 µl of wash 1 solution and centrifuged at 10,000 xg for 30 secs. Filters were washed twice with 500µl of wash 2/3 solution and centrifuged at 10,000 xg for 30 secs. Filters were centrifuged at 10,000 xg for 1 minute to remove residual fluid and transferred to new collection tubes. Elution buffer (60 µl) was added to the center of each filter and incubated for 5 mins. miRNA was eluted through centrifugation at 10,000 xg

for 1 minute at room temperature. Samples were placed on ice and RNA concentrations were determined using a Nanodrop Spectrophotometer.

Results:

Population Description

Serum was collected from PCa patients diagnosed with tumor stage I, III, IV and disease free patients that self-identified as men with European ancestry as reported in Table 2. Controls were received a diagnosis of a normal prostate at the time of serum collection. PCa cases (n = 15) had an age range of 47-72 and a median age of 69. Non-cancerous controls had an age range of 56-71 and a median age of 69. No significance difference was observed between the median age of cases and controls (p-value = 0.726). The prostate serum antigen (PSA) median among cases was significantly higher relative to non-cancerous controls (p-value = 0.048). Sixty percent (n = 9) of the cases were identified as adenocarcinoma and 40% (n = 6) of the cases were not reported or classified. Sixty-seven percent of the PCa patients (n =10) had a history of smoking; however the smoking status among the controls was not available for this study population. Relative to the controls, no difference was detected between PCa patient BMI median relative to controls. Majority of the cancer patients received at least 2 types of therapy (73.3%) against PCa, which include chemotherapy (n = 2, 13.3%), radiation (n = 8, 53.3%), surgery (n = 7, 46.7%), and hormonal therapy (n =14, 93.3%).

Characteristics	Cases	Controls	p-value
# of Serum samples	15	5	
Age			0.726
Range	47-72	56-71	
Median	69	68	
PSA, n (%)			0.048
Range	5-57	4-13	
Median	11	6	
<4 ng/ml	0 (0%)	0 (0%)	
≥4 ng/ml	11 (73.3%)	5 (100%)	
Missing values	4 (26.7%)	0 (0%)	
Body Mass Index (BMI), n=20			0.225
Range	22.1-40.5	23-30.4	
Median	28.1	24.93	
Smoking History, n (%)			
Non-smoker	5 (33.3%)	n/a	
Smoker	10 (66.7%)	n/a	
Tumor classification, n (%)			
Adenocarcinoma	9 (60%)	0 (0%)	
Unknown	6 (40%)	0 (0%)	
Gleason Score (GS), n (%)			
GS < 7	4 (27%)		
GS ≥ 7	9 (60%)		
Missing values	2 (13%)		
Treatment, n (%)			
Chemotherapy	2 (13.3%)		
Radiation	8 (53.3%)		
Surgery	7 (46.7%)		
Hormonal therapy	14 (93.3%)		
2 ≤ treatments	11 (73.3%)		
3 ≤ treatments	4 (26.7%)		
4 ≤ treatments	1 (6.7%)		

Table 2. Population Description of PCa Patients. Clinical data for patients from Bioserve is displayed in the above table. Patients (n=20) self-identified as European American males. Majority of PCa cases were diagnosed with adenocarcinoma (60%), had a smoking history (66.7%) and received 2 or more therapies. Relative to controls, cases had higher median serum PSA level, weight and BMI value relative to controls.

Differentially expressed serum-based miRNAs in PCa

MicroRNA profiling was performed using total RNA from collected from serum of PCa patients diagnosed with stages I (n = 5), III (n = 5) and IV (n = 5) relative to non-cancerous controls (n = 5), as described in Chapter 2 methods section. MicroRNA array profiling measured the expression of 377 miRNAs in the serum of PCa patient diagnosed with localized disease (stage I), pre-metastatic and bone metastatic disease (Stage IV) relative to non-cancerous controls (n = 5), as summarized in Figure 3. After global normalization of miRNA profiles, array analysis revealed 26 miRNAs were differentially expressed in PCa patients. Relative to non-cancerous controls, 17 miRNAs (miRs-18a, -122, -133a, -145, -150, -186, -191, -197, -296, -342-3p, -374, -454, -484, -518d, -744, -885-5p, -let-7b) were down-regulated in serum from PCa patients, shown in Table 3. In contrast, nine miRNAs (miRs-21, -92a, -99b -106b, -142-3p, -185, -302b, -320, -520e) were up-regulated in PCa serum relative to controls (Table 2). However, only 6 miRNAs (miRs-106b-5p, -186-5p, -302b-3p, -342-3p, -520e, -885-5p) were adherent to the following selection criteria; FDR p-value ≤ 0.05 and fold change (0.5 > FC, FC ≥ 2). These miRNAs were selected for further validation via qRT-PCR using singleplex Taqman miRNA assays.

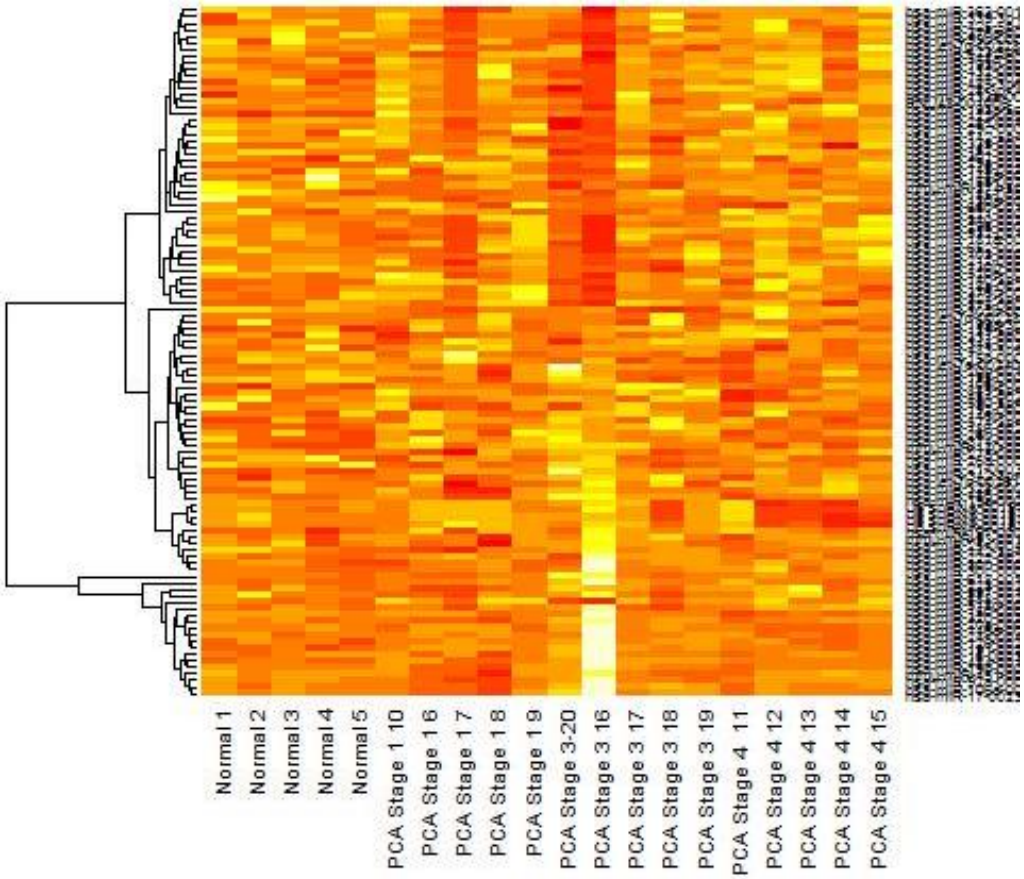

Figure 3. Heatmap of Differentially expressed miRNAs in PCa serum. Color scheme for heatmap: orange (no change), red (high expression) and yellow (low expression). The expression of 377 miRNAs in the serum of patients with diagnosed with tumor stage I, III and IV PCa and non-cancerous controls.

	Stage I (FC)	Stage I (p-value)	Stage III (FC)	Stage III (p-value)	Stage IV (FC)	Stage IV (p-value)
hsa-miR-106b-5p	4.68	**0.038**	6.66	**0.016**	4.89	**0.044**
hsa-miR-142-3p	2.83	0.167				
hsa-miR-185-5p	5.14	0.147				
hsa-miR-21-5p	4.06	0.065	4.67	0.062	2.98	0.311
hsa-miR-302b-3p	73.45	**0.012**	512.83	**0.001**		
hsa-miR-320a					1.95	0.412
hsa-miR-520e	7.66	0.147	30.16	**0.018**		
hsa-miR-92a-3p	2.38	0.147				
hsa-miR-99b-5p	2.59	0.205				
hsa-miR-122-5p	0.08	0.065	0.08	0.099	0.06	0.053
hsa-miR-133a-5p			0.15	0.199		
hsa-miR-145-5p	0.33	0.186	0.26	0.187	0.30	0.311
hsa-miR-150-5p					0.21	0.241
hsa-miR-18a-5p	0.17	0.167				
hsa-miR-186-5p	0.34	0.147	0.11	**0.005**		
hsa-miR-191-5p	0.55	0.242	0.41	0.144		
hsa-miR-197-3p	0.42	0.240				
hsa-miR-296-5p					0.29	0.328
hsa-miR-342-3p	0.27	**0.026**	0.25	**0.018**	0.25	**0.028**
hsa-miR-374a-5p	0.32	0.167				
hsa-miR-454-3p			0.27	0.199		
hsa-miR-484			0.46	0.187		
hsa-miR-518d-3p	0.31	0.186				
hsa-miR-885-5p			0.04	**0.011**		
hsa-let-7b-5p			0.25	0.242		
hsa-miR-744-5p					0.32	0.412

Table 3. Differentially expressed miRNAs in PCa serum**.** After global normalization, 26 miRNAs were differentially expressed in PCa serum relative to non-cancerous controls. Relative to non-cancerous controls, only 9 miRNAs were up-regulated in PCa stage I, III and IV disease. The remaining 17 miRNAs were down-regulated in PCa serum. After adjusting for multiple hypothesis testing (false discovery rate [FDR] p-value ≤ 0.05), only 6 miRNAs were determined to be differentially expressed in prostate serum. Significant FDR p-values are bolded in the above table.

Validation of miRNAs

The expression of miRs-106b-5p, -186-5p, -302b-3p, -342-3p, -520e, and -885-5p was measured in two independent total RNA isolations from serum (Table 2). All miRNA expression was normalized to a spiked-in external control, miR-cel-39 and analyzed using specific miRNA Taqman qRT-PCR assays. No differences were detected in the expression of cel-miR-39 in serum between non-cancerous and PCa cases (Figure 5). Among the selected miRNAs, the expression of miRs-520e and 302b were below the detectable level (Ct value < 37 or undetermined) in PCa patient and non-cancerous control serum. The expression of miR-885-5p was inconsistent between total RNA isolations for patients and controls (Figure 8). Therefore, this miRNA was not further validated *in vitro*. MiR-342-5p was up-regulated in PCa serum relative to non-cancerous controls. Unfortunately, this up-regulation was not statistically significant. However, miRs106b-5p and -186-5p were significantly up-regulated in PCa serum relative to non-cancerous controls in both independent isolations (Figures 6-7). MiR-186-5p was selected for further validation and characterization *in vitro* to determine its role in PCa due to the limited evidence available on this miRNA in PCa.

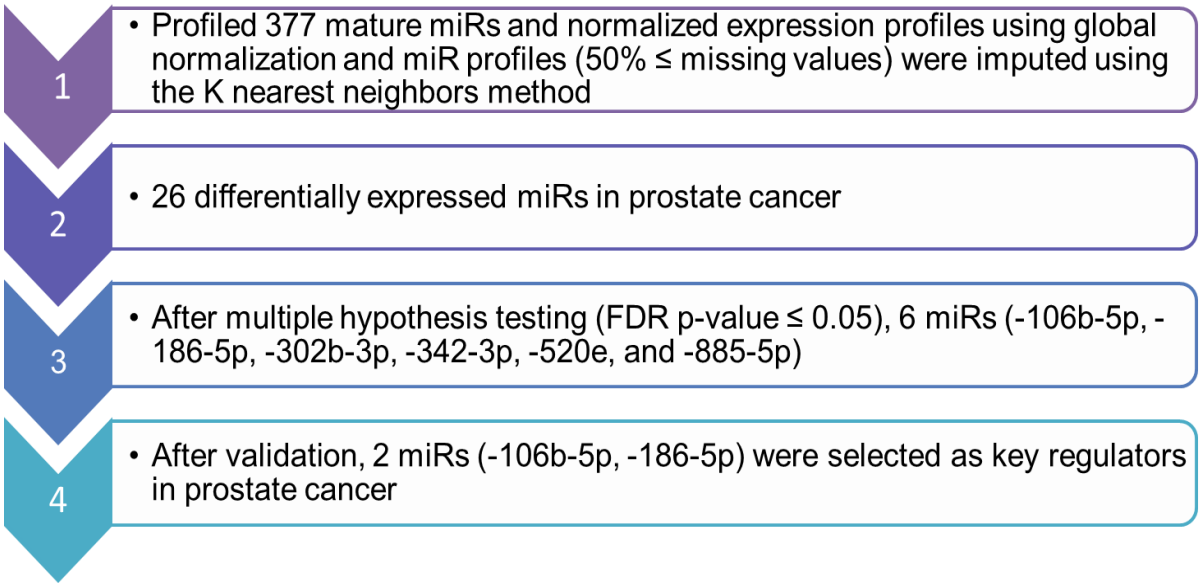

Figure 4. Flowchart of miRNA profiling for serum-based miRNAs. The expression of 377 mature miRNA profiles was evaluated in the serum of PCa patients (n = 15) and non-cancerous controls (n= 5). MiRNA profiles were normalized to global median Ct value among all the miRNA profiles. Next, miRNA profiles associated with missing values (≥ 50) were imputed using K nearest neighbor imputation program. After global normalization, the array determined that 26 miRNAs were differentially expressed in PCa serum relative to non-cancerous controls. After adjusting for multiple hypothesis testing using the false discovery rate (FDR) (FDR p-value ≤ 0.05), 6 miRNAs were determined to be differentially expressed serum-based miRNAs. Selected miRNAs were individually validated in the serum of PCa and controls. After validation, the expression of only two miRNAs (-106b-5p, -186-5p) remained consistent.

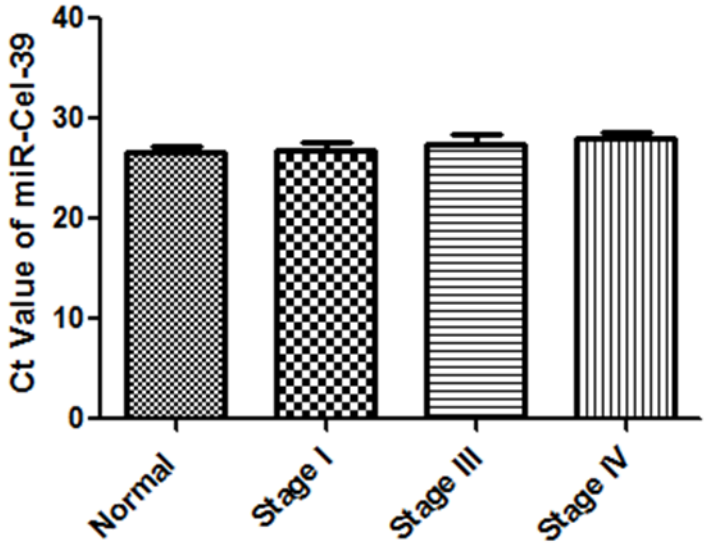

Figure 5. Ct values of MiR-Cel-39 (cel-39) in PCa serum and non-cancerous controls. MiR-Cel-39 is a non-human synthetic miRNA was used as an external control for miRNA isolation from serum. Cel-39 was spiked into denatured serum to control for the normalization of miRNA expression in non-cancerous controls and PCa patients. No significance differences were determined in the Ct values between controls and cases (p-value = 0.116). Statistical analysis was performed using a non-parametric one way ANOVA test and Dunn's multiple hypothesis test and significance cut-off p-value ≤ 0.05.

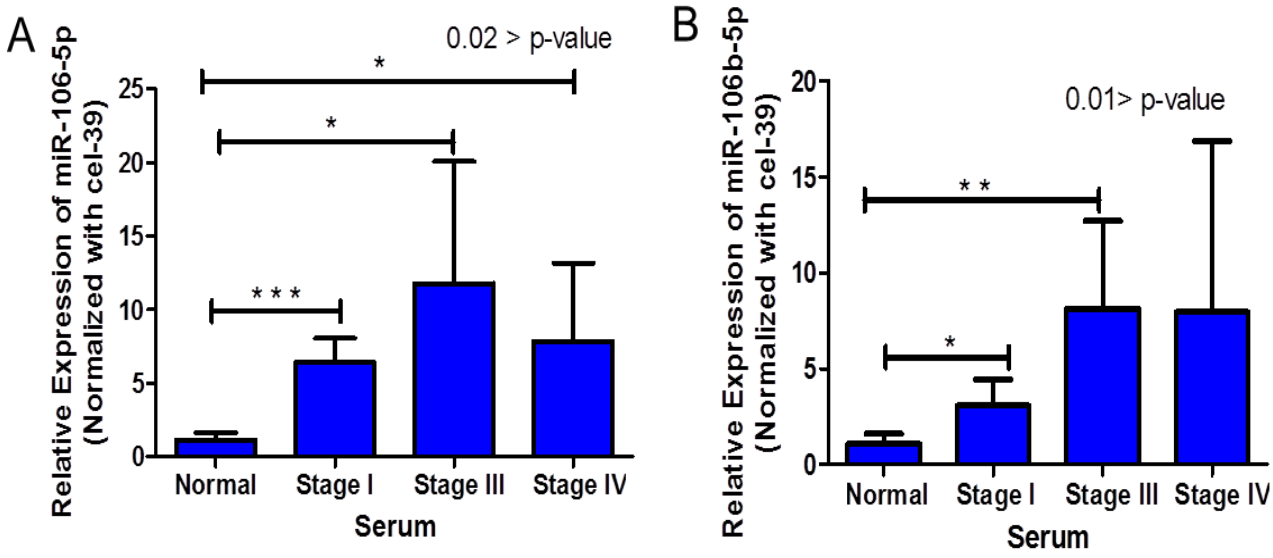

Figure 6. MiR-106b-5p expression in PCa serum. Expression of miR-106b-5p and -186 was validated in two independent isolations of miRNA from serum. MiRNA expression was measured in total RNA derived from PCa serum (n = 15) relative to non-cancerous controls (n = 5). A) MiR-106b-5p was up-regulated by 3.1-11.8-fold in patients diagnosed with tumor stage I, II, and III disease relative to non-cancerous controls in the 1st isolation (p-value > 0.02). B) MiR-106b-5p was up-regulated in only tumor stage I and III disease relative to controls. Statistical analysis was performed using Unpaired t-test and significance level of 0.05 (*p-value < 0.05, **p-value < 0.01, ***p-value < 0.0002).

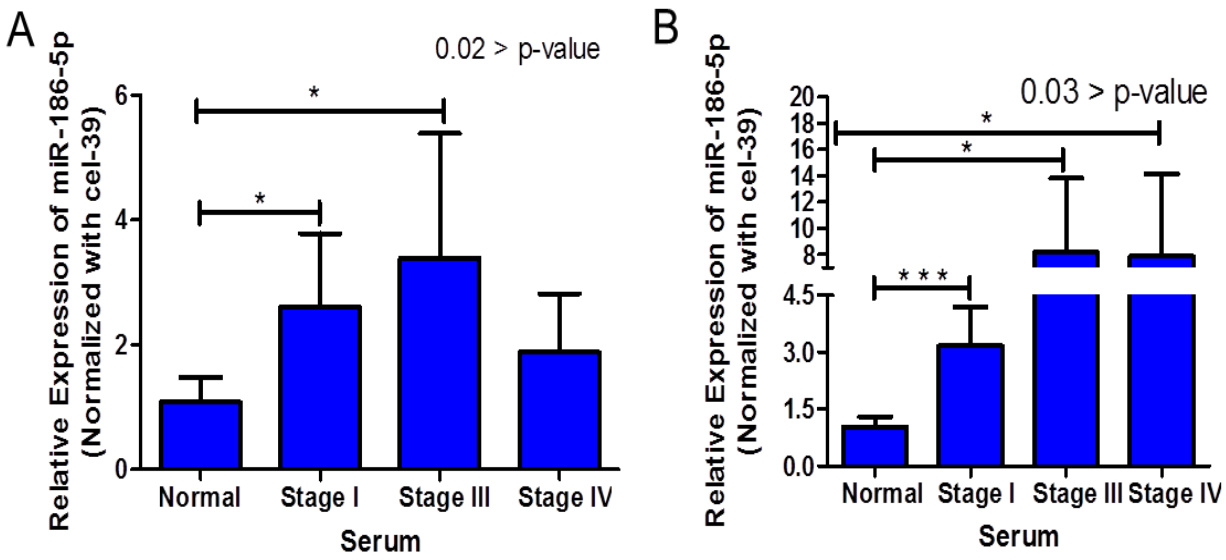

Figure 7. MiR-186-5p expression in PCa serum. Expression of miRs-186-5p was validated in two independent isolations of miRNA from serum. MiRNA expression was measured in total RNA derived from PCa serum (n = 15) relative to non-cancerous controls (n = 5). A) MiR-186-5p was significantly up-regulated by 2.6-8.1-fold in tumor stage I and II serum from both isolations relative to non-cancerous controls (p-value < 0.03). B) However, miR-186 expression was only significantly elevated by 7.9-fold in stage IV disease in the second isolation relative to non-cancerous controls (p-value = 0.02). Statistical analysis was performed using Unpaired t-test and significance level of 0.05 (*p-value < 0.05, ***p-value < 0.002).

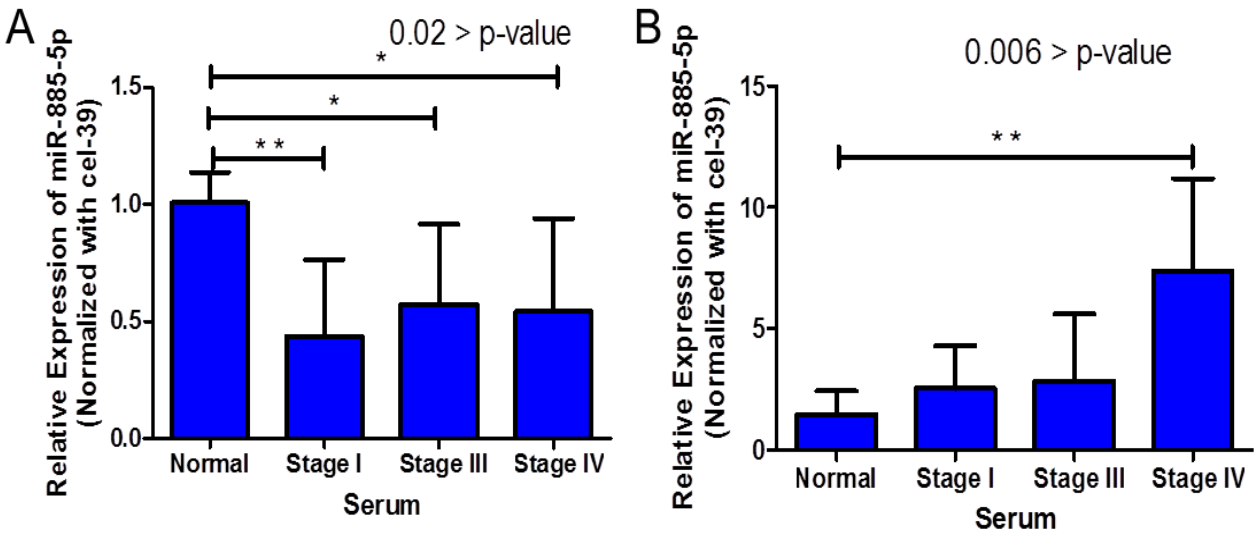

Figure 8. MiR-885-5p expression in PCa serum. Expression of miR-885-5p was validated in two independent isolations of miRNA from serum. MiRNA expression was measured in total RNA derived from PCa serum (n = 15) relative to non-cancerous controls (n = 5). A) MiR-885-5p was significantly down-regulated by 46-57% in patients diagnosed with stage I and II disease relative to non-cancerous controls (p-value = 0.02-0.0034). B) However, in the second isolation miR-885-5p expression was up-regulated by 2.5-7.4 fold in stage III disease relative to non-cancerous patients (p-value = 0.005). Statistical analysis was performed using Unpaired t-test and significance level of 0.05 (*p-value < 0.02, ** p-value < 0.004).

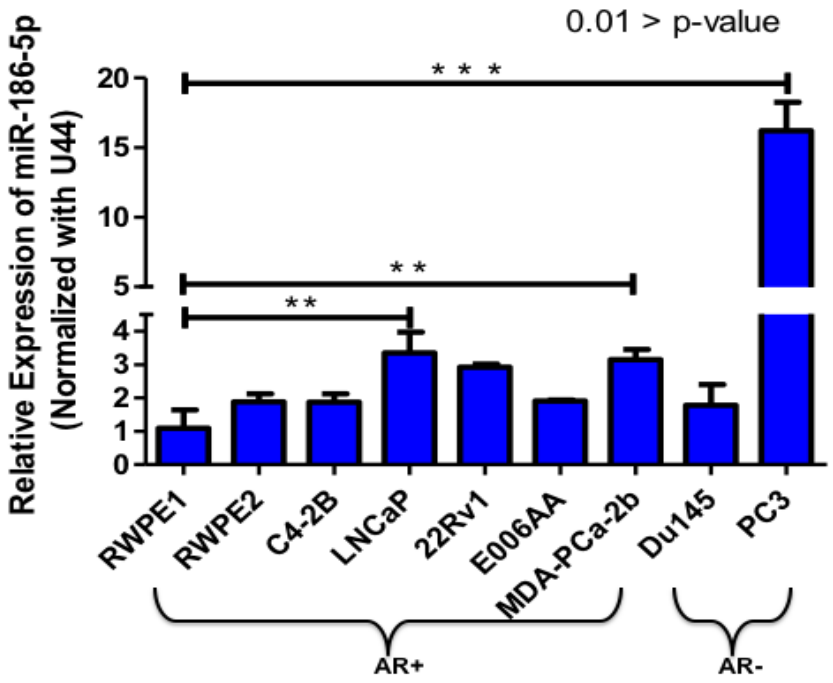

Figure 9. Mature miR-186-5p expression in androgen dependent and independent PCa cell lines. The relative expression of miR-186-5p was measured in normal prostate epithelial cells (RWPE1, RWPE2), non-metastatic (E006AA), prostate carcinoma xenograft (22Rv1), bone (PC-3), lymph node (LNCaP), brain (DU145) metastatic PCa cells using specific Taqman primers and qRT-PCR. MiR-186 expression was not significantly higher in RWPE2, E006AA, 22Rv1, and DU145 cell lines relative RWPE1 cells. However, miR-186-5pwas up-regulated by 3.2-16.2-fold in three metastatic PCa cell lines (LNCaP, MDA PCa-2b, PC-3) relative to normal prostate epithelial RWPE1 cells. Metastatic PC-3 cells exhibited a 16-fold increase, the highest expression, in the expression of miR-186-5p relative to normal prostate epithelial cells (p-value = 0.0002). MiRNA expression was normalized to endogenous control miR, RNU44, and measured in three independent experiments. Statistical analysis of experiments was performed using one way ANOVA (** p-value < 0.01, *** p-value < 0.0003).

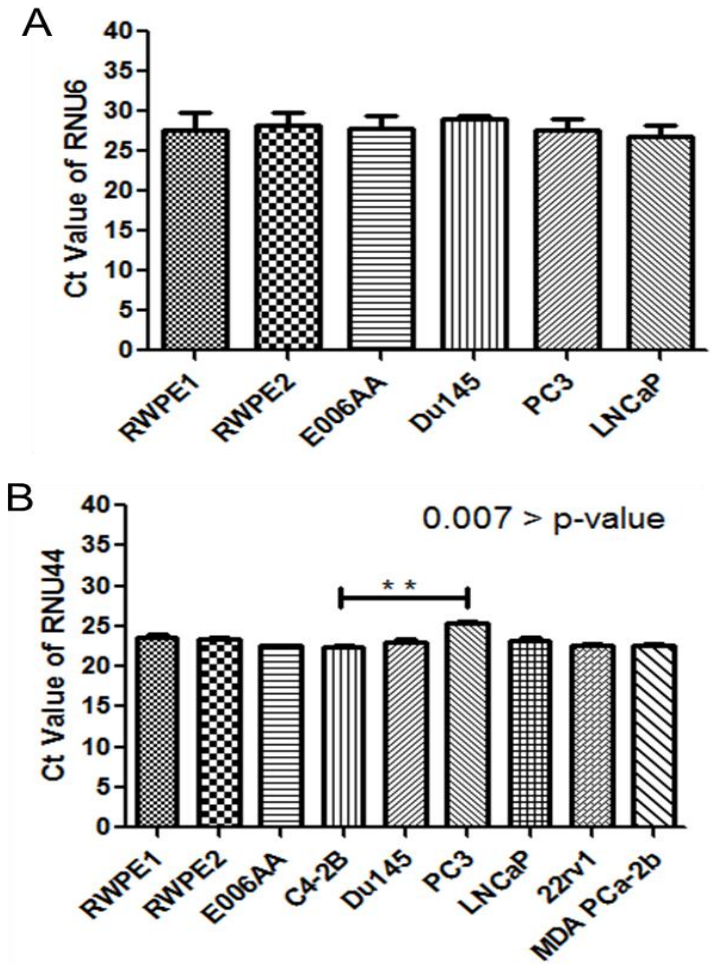

Figure 10. Ct values of RNU6 and RNU44 in normal prostate epithelial and PCa cell lines. A) Higher Ct values were detected for RNUB6 in normal epithelial and PCa cell lines (p-value = 0.571). B) However, lower Ct values (higher expression) were detected for RNU44 in normal epithelial and PCa cell lines relative to RNU6. The Ct value of RNU44 in PC-3 cells was significantly higher relative to C4-2B (p-value = 0.0061). Statistical analysis was performed using a non-parametric one way non-parametric ANOVA test and Dunn's multiple hypothesis test and significance cut-off p-value ≤ 0.05 (** p-value < 0.007).

Expression profile of miR-186-5p in metastatic and non-metastatic PCa cell lines

The expression of miR-186-5p was measured in androgen sensitive metastatic (i.e., LNCaP, C4-2B, 22Rv1, MDA-PCa-2b), androgen sensitive non-metastatic (i.e., E006AA) and insensitive metastatic (DU145, PC-3) PCa cell lines relative to normal epithelial prostate cells (RWPE1) and k-Ras transformed normal epithelial cells (RWPE2) using qRT-PCR. These cell lines are also described in Table 1. MiRNA expression was normalized to the expression of endogenous control miR RNU44 in PCa cell lines. RNU44 was selected as an endogenous control miR as supposed to RNU6 due to lower Ct values, which are indicative of higher expression in normal epithelial and PCa cell lines used in this study (Figure 10). Expression of miR-186-5p was not significantly up-regulated in transformed normal epithelial (RWPE2), prostate carcinoma xenograft (22RV1), metastatic PCa (DU145) and non-metastatic PCa (E006AA) cells relative to RWPE1 cells. However, miR-186-5p was significantly up-regulated by 3.2-16.2-fold in metastatic PCa cells (LNCaP, MDA-PCa-2b and PC-3) relative to the control cell line (RWPE1), as shown in Figure 9 (p-value < 0.01). In LNCaP cells, miR-186-5p expression was up-regulated by 3.4-fold relative to normal epithelial cells (p-value $= 0.009$). Additionally, the miR-186-5p was up-regulated by 3.2-fold in MDA PCa 2b cells relative to RWPE1 cells (p-value $= 0.005$). Interestingly, miR-186-5p expression was most pronounced in androgen insensitive metastatic PC-3 cells with a 16.2-fold increase relative to RWPE1 cells (p-value $= 0.0002$). However, miR-186-5p expression in PCa cell lines did not exhibit a dependence on androgen sensitivity status.

Discussion:

Circulating non-coding RNAs (e.g., mRNAs) in serum play a pivotal role in many cancers, including PCa. MiRNAs are essential regulators of genes involved in various hallmarks of cancer. At the beginning of this study, there were limited reports on the expression of circulating miRNAs in PCa. To address this research gap, we initiated a pilot study to evaluate whether circulating miRNAs were differentially expressed in the serum collected from PCa patients diagnosed with non-metastatic and metastatic disease relative to disease-free men. In the current study, six circulating miRNAs (-106b-5p, -186-5p, -302b-3p, -342-3p, -520e, -885-5p) were differentially expressed in the serum of PCa patients using a intermediate high-throughput Taqman array cards. Two circulating miRNAs (-106b-5p, 186-5p) were up-regulated in the serum of PCa patients when compared to controls, following qRT-PCR. Since miR-106b-5p was characterized as an oncogenic miRNA with several direct gene targets using PCa cell models, further analysis was focused on miR-186 [75, 76, 96, 128, 129]. In addition to the up-regulation of miR-186 in the serum of PCa patients, we also demonstrated miR-186 was up-related in a few metastatic PCa cell models (PC-3, MDA-PCA-2b, LNCaP).

Some discrepancies were detected in the expression of circulating miRNAs between the microarray and individual qRT-PCR analyses. For instance, miR-186-5p was down-regulated in the serum of PCa patients using the microarray data but up-regulated following qRT-PCR. Discrepancies between the micro-array and qRT-PCR were presumably attributed to the difference in normalization techniques (i.e., global normalization and miR-cel-39 external

control) used for these two genomic approaches. In particular, analysis of miRNA using Taqman array requires global normalization; whereas, qRT-PCR required the use of an external control (i.e. miR-39) that was spiked in the serum samples. However, qRT-PCR findings that used an external control were considered more reliable than Taqman Array data. Global normalization of Taqman array data was heavily reliant upon median Ct values of all miRNAs analyzed, which may lead to false positive results or mis-interpretation of the directionality of the expression of circulating miRNAs. Normalization of qRT-PCR data of circulating miRNAs using miR-cel-39 as an external control helped to control for sample losses during the total RNA isolation, cDNA generation, miRNA isolation steps. Since there is no appropriate endogenous control for circulating miRNAs, previously published reports suggest the use of synthetic miRNA Caenorhabditis elegans 39 to normalize circulating miRNA profiles among PCa patients [52, 117, 123, 124]. Consequently, miR-cel-39 was used in the current study to normalize miRNA expression in serum due to its common use in literature in relation to PCa.

Although there are no published reports on the expression of miR-186 in the serum from PCa patients and appropriate controls, miR-186-5p expression has been evaluated in two independent prostate tumor tissue-based studies with mixed results [63, 113]. In one study, miR-186-5p was significantly down-regulated in fresh frozen tumor tissue (n = 50) relative to benign hyperplasia (n = 30) and matched non-malignant tissue (n = 50) among German men [113]. This

study failed to remove surrounding normal tissue from cancerous tissue using micro-dissection, which may have skewed miR-186-5p expression within a non-pure population of tumor tissue.

Commensurate with our serum based findings, Ambs and colleagues (2008) observed an up-regulation of miR-186-5p expression in laser microdissected tumor tissue of prostate patients diagnosed with extraprostatic disease (i.e., extension of the tumor outside or independent of the prostate) relative to patients with no extraprostatic disease among European (n = 30) and African American (n = 30) men [63]. Unlike the previous report among German PCa patients, this 2008 study evaluated miR-186-5p expression using micro-dissected tissue, which provides a pure population of tumor cells in which the miRNA profiles are not contaminated, skewed or diluted by normal surrounding tissue (personal communication with Dr. Lacey McNally, University of Louisville). Therefore, miR-186-5p expression in the Ambs study rather than the German study was given more consideration in relation to our study findings.

Previously, miR-186-5p expression has been reported as up-regulated in several different malignancies [99-104, 130]. Furthermore, miR-186 has demonstrated an oncogenic function via its down-regulation of anti-tumor associated genes in cancer. For instance, miR-186 overexpression led to the down-regulation of tumor suppressors, FOXO1 and NR5A2, *in vitro* in endometrial and pancreatic cancer, respectively [100, 103]. Additionally in cervical cancer cells, miR-186 overexpression down-regulated the gene

expression of pro-apoptotic gene, P2X7 [101]. However, the role of miR-186 in cancer may be tumor specific because it appears to have a tumor suppressor role in other cancers [106-112]. In the next chapter of this dissertation, we demonstrate inhibition of miR-186 corresponds with a suppression of cellular proliferation, anchorage independent growth and cellular invasion suggest miR-186 exhibits an oncogenic role in PCa within our metastatic PCa cell models.

We have considered study limitations, strengths and future directions. The analysis of circulating miRNA expression was restricted by a small sample size of PCa patient serum. To our knowledge, our lab is the first to demonstrate the up-regulation of miRNA-186-5p in the serum of PCa patients as well as metastatic cell models. Given the discrepancy in the directionality of the Taqman array and qRT-PCR of serum-based miRNAs, we speculate global normalization may not be the best normalization technique for circulating miRNAs in PCa. We propose normalization of circulating miRNA profiles using an external control like miR-cel-39 may be more appropriate than a global normalization used for the Taqman array analysis. To prevent future issues in miRNA profiling during high-throughput analysis, we will include miR-cel-39 and other synthetic Caenorhabditis elegans miRNAs within customized microarrays to normalize the expression of serum-based miRNAs. In future studies, we will confirm the up-regulation of miR-186-5p and other oncomiRs in serum and matched micro-dissected PCa tumor samples relative to age-matched controls using ethnically/racially diverse sub-groups. Additionally, we will evaluate miR-186-3p,

the passenger strand of miR-186, expression to determine whether only miR-186-5p is uniquely up-regulated in PCa serum and cell lines. The up-regulation of miR-186 based on disease-state or disease aggressiveness within independent studies may support it's utility as a prognostic tool.

Circulating non-coding RNAs (i.e., miRNAs) have the great potential to serve as clinical biomarkers capable of distinguishing between lethal and non-lethal disease. In addition, miRNA's have the capacity to monitor physiological changes and evaluate the effectiveness of different therapies against PCa in patients. For instance, after prostate patients underwent a radical prostatectomy the serum-based expression of oncogenic miRs-195, -26a and -let-7i dramatically reduced in patients. Moreover, several studies demonstrate that miRNA expression profiles relate to early stage, advanced, and recurrent disease [52, 65, 117, 121, 123, 124]. MiRNA expression profiles that correspond to specific disease stages provide an opportunity to improve current prognostication and clinical management strategies. Furthermore, miRNA expression may help enhance the capacity of current PCa tools, namely PSA test and digital rectal examination to identify individuals with a high-risk of developing aggressive or recurrent disease as well as those who may benefit from more aggressive treatment or follow-up care. Ultimately, miRNAs may serve as non-invasive biomarkers with the capacity to improve PCa prognostication, treatment and clinical management strategies.

CHAPTER III

THE IMPACT OF MIR-186 ON THE PROSTATE CANCER PHENOTYPE *IN VITRO*

Introduction:

MiRNAs are differentially expressed in tumor cells and greatly influence several cellular hallmarks of cancer (i.e., proliferation, migration, anchorage independent growth and invasion) via their regulation of target mRNAs [73, 75, 79, 81, 89]. Specifically, miR-186 expression alters tumor cellular behavior *in vitro* in several malignancies including bladder, pancreatic, ovarian and lung [103, 106, 107, 112, 130, 131]. MiR-186 is an intronic miRNA located on chromosome 1 at 1p31.1 within the ZRANB2 gene locus. This miRNA is differentially expressed in various tumor types including PCa [63, 99-104, 106-113]. In some cancers, miR-186 plays an oncogenic role due its overexpression, but in other neoplasms miR-186 functions as a tumor suppressor. Previously, we demonstrated an up-regulation of miR-186-5p expression levels in the serum of PCa patients diagnosed with stage I, III and IV disease relative to non-cancerous controls. Additionally, we also observed a significant up-regulation of miR-186-5p in metastatic PCa cell lines (LNCaP, MDA PCa-2b, PC-3), but highest in PC-3 relative to normal prostate epithelial RWPE1 cells. However, miR-186-5p has never been characterized as an oncogenic miRNA in PCa.

Several reports demonstrate miR-186 expression is down-regulated in cancer-associated fibroblasts, oral squamous, non-small cell lung, colorectal, bladder and ovarian cancer [106-112, 130, 131]. In two reports, miR-186 was under-expressed in non-small lung cancer (NSCLC) tumor tissue and cell lines (A549, NCI-H358, H157, H1299) relative to adjacent normal tissue and normal bronchial epithelial cells (16HBE) [106, 107]. In the same studies, overexpression of miR-186-5p inhibited cellular migration and invasion in NSCLC cell lines [106, 107]. Overexpression of miR-186 in bladder cancer cell lines (i.e., HT-1346, RT4) lead to a decrease in cellular proliferation, and invasion [130]. Furthermore in a recent report (2015), miR-186 expression was significantly low in cisplatin-resistant epithelial ovarian cancer (EOC) cell lines (i.e., ACRP, C13*, OVCAR3/DDP) relative to cisplatin-sensitive ovarian cancer cell lines (i.e., A2780, OV2008, OVCAR3) [112]. Ectopic expression of miR-186 reduced cell motility via reduction of vimentin and increase of E-cadherin protein expressions in cisplatin-resistant EOC cell line, ACRP. Additionally, higher miR-186 expression was exhibited in platinum sensitive ovarian tumor tissue from patients with progression-free survival greater than 6 months. In another study, miR-186 overexpression reduced proliferation and invasion of bladder cancer cells [131]. Collectively, these aforementioned reports suggest that miR-186 plays a tumor suppressive role in NSCLC, and ovarian cancer.

In contrast, miR-186 expression is up-regulated in endometrial, pancreatic, esophageal, cervical, head/neck, and non-melanoma skin cancer [99-104, 130, 132]. For instance, Zhou and co-workers (2008) observed that miR-186

expression was up-regulated in HeLa cells, a cervical adenocarcinoma cell line, relative to human ecto-cervical-vaginal epithelial cells (hEVEC) [101]. MiR-186 expression was also shown to be up-regulated in endometrial tumor tissue and an endometrial cell line, Ishikawa, relative to controls [100]. In pancreatic cancer, miR-186 is up-regulated in tumor tissue (surgical specimens T2, T13, T29, T33, T34, T36-38, T40) and cell lines (BxPC-3, Panc-1, MIA, Hs766T, HPAFII, ASPC-1) relative to normal pancreatic tissue and human pancreatic ductal epithelial (HPDE) cells [102]. In another report, overexpression of miR-186 in pancreatic cell lines (Panc-1, BxPC-3, and MiaPaca-2) induced cellular proliferation and migration relative to negative controls [103]. These effects were reversed after inhibition of miR-186 in pancreatic cell lines. Moreover in the same report, miR-186 was significantly up-regulated in the tumor tissue of pancreatic cancer patients with poor survival (median survival of 13.8 months) relative to patients with low miR-186 expression with a longer survival time [103]. Additionally, overexpression of miR-186-5p led to enhanced cell proliferation, anchorage independent growth and colony formation of J82 bladder cancer cells [130]. Together these reports suggest an oncogenic function for miR-186 demonstrated via its overexpression in various cancers. However, whether miR-186 plays an oncogenic or tumor suppressor role in PCa is unknown.

To date, only two reports have evaluated the expression profile of miR-186 in PCa tumor tissue [63, 113]. However, these reports conflict with one another and do not reveal a definitive role of miR-186 in PCa. In a report by Erdmann and associates (2014), miR-186 expression was significantly down-

regulated by 2.7-3.4-fold in tumor tissue (n = 50) relative to tumor-free (n = 46) and benign hyperplasia (n = 30) tissue [113]. Furthermore, AMACR and PSMA were identified as putative targets of miR-186 in PCa through qRT-PCR and exemplary scatter plots; however, the study did not confirm the impact of miR-186 expression on these targets via western blot or luciferase reporter assays [113]. In another report, miR-186 expression was up-regulated by 1.3-fold in tumor tissue with extraprostatic disease (n = 17) relative to tumor tissue without extraprostatic disease (n = 35) [63]. Due to these conflicting reports, a definitive role for miR-186 remains to be identified in PCa.

Therefore, we addressed this research gap through the characterization of the effects miR-186 overexpression in normal cells and inhibition in PCa cell lines. The different hallmarks of cancer such as enhanced cellular proliferation, invasion and anchorage independence were evaluated in relation to miR-186-5p expression in metastatic PCa and normal prostate epithelial cells. We hypothesized the miR-186-5p inhibition will impede proliferation and invasion to reduce the aggressiveness of tumor phenotype associated with metastatic PCa. Furthermore, we hypothesized miR-186-5p overexpression in normal prostate epithelial cells would lead to the malignant transformation.

Methods:

Cell Culture

Cell lines (i.e. PC-3, MDA PC 2b, E006AA, RWPE1) previously described in Chapter 2 were used to perform characterization experiments.

DNA Isolation

Cells were plated in a T75 flask and grown to 80% confluency. Cells were washed once in 1X PBS (2 ml), trypsinized with 0.05% Trypsin for 5 mins and trypsin was neutralized with growth medium containing FBS. Cells were transferred to 15ml tubes and pelleted through centrifugation at 1200 rpm for 5 mins. Cell pellets were washed in 1X PBS (1 ml) and centrifuged at 1200 rpm for 5 mins. Genomic DNA was isolated from cell pellets using the DNeasy Blood and Tissue kit (Catalog# 69504, Qiagen, Valencia, CA). In brief, ATL buffer (180 µl) and proteinase K (20 µl) were added to each cell pellet and mixed thoroughly. Samples were transferred into 1.5ml tubes. Samples were incubated at 56^{o}C and constantly tapped until cells lysed completely. AL buffer (200 µl) was added to each sample and vortexed briefly. Samples were incubated at 56^{o}C for 10 mins and 100% EtOH (200 µl) was added to each tube and vortexed for 10 secs. Samples were applied to DNeasy mini-spin columns in collection tubes and centrifuged at 6,000xg for 1 minute at room temperature. Mini-spin columns were transferred to new collection tubes, washed with AW1 buffer (500ul) and centrifuged at 6,000xg for 1 minute. Samples were transferred to new collections, washed with AW2 buffer (500 µl) and centrifuged at 16,100xg for 4 mins. Mini-

spin columns were transferred to new collection tubes and heated AE elution buffer (100 µl) was added to each column. Samples incubated at room temperature for 1 mins and DNA was eluted through centrifugation at 6,000 xg for 1 minute. DNA concentrations were measured using a Nano Dropper Spectrophotometer.

RNA Isolation

Cells were plated at an optimal cell density in 60mm dishes and incubated at 37^{o}C for a given time frame. Cells were trypsinized with Trypsin 0.05% for 5 mins, neutralized with growth medium containing FBS and transferred to 15ml tubes. Cells were centrifuged at 1200rpm for 5 mins. Cell pellet was washed in 1X PBS (1 ml) once and centrifuged at 1200rpm for 5 mins. PBS was removed and 1 ml of Trizol Reagent (Invitrogen) was added to cell pellets, thoroughly mixed into solution and transferred to 1.5 ml tubes. Mixture incubated at room temperature for 5 mins. Chloroform (200 µl/1ml of Trizol) was added to mixture and shaken for 15 secs and incubated at room temperature for 3 mins. Samples were centrifuged at 12,000 xg for 15 mins at 4^{o}C. Aqueous phase (≥ 400ul) was transferred to new 1.5ml tubes and 100% isopropanol (500ul/ 1ml of Trizol) was added to each tube. Samples were vortexed for 10 secs and incubated at room temperature for 10 mins. Samples were centrifuged at 12,000xg for 10 mins at 4^{o}C. Supernatant was discarded and RNA pellets were washed in 80% EtOH (1ml per 1ml of Trizol). Samples were vortexed briefly (10 secs) and centrifuged at 7500 xg for 5 mins at 4^{o}C. RNA pellets air dried for 5-30 mins and

resuspended in molecular grade H_2O. Samples were incubated at 60^oC for 15 mins and RNA concentrations using a Nano drop Spectrophotometer.

microRNA Isolation from cell lines

Total isolation of mRNA and microRNA was performed using the miRVana microRNA Isolation kit (Catalog# AM 1561, Thermo Fisher Scientific, Waltham, MA). Cells were seeded at an optimal cell density into 60 mm plates in growth medium. Cells were washed once with 1X PBS (2 ml), lysed in dishes with mirVana lysis buffer (600 µl), harvested with cell scraper and pipetted into 1.5ml tubes. Cells were vortexed for 30 secs to completely lyse cells. Homogenate additive (1/10 of cell lysate volume) was added to cell lysates and vortexed for 30 secs. Samples were incubated on ice for 10 mins and equal volume of acid/phenol chloroform mixture was added to each tube. Samples were vortexed for 60 secs and separate into aqueous and organic phases through centrifugation at 10,000 xg for 5 mins at 4^oC using Eppendorf Centrifuge 5810R. Aqueous phase (≥ 400 µl) was pipetted into new 1.5ml tubes and 100% EtOH (1.5 x aqueous layer volume) was added to each tube. Samples were added to filters in new collection tubes, centrifuged at 10,000 xg for 30 secs. Samples were processed at room temperature and flow-through was discarded after centrifugation. Filters were washed once with 700 µl of wash 1 solution and centrifuged at 10,000 xg for 30 secs. Filters were washed twice with 500 µl of wash 2/3 solution and centrifuged at 10,000 xg for 30 secs. Filters were centrifuged at 10,000 xg for 1 minute to remove residual fluid and transferred to

new collection tubes. Elution buffer (60 µl) was added to the center of each filter and incubated for 5 mins. miRNA was eluted through centrifugation at 10,000 xg for 1 min at room temperature. Samples were placed on ice and RNA concentrations were determined using a Nanodrop Spectrophotometer.

Transient Transfection

RWPE-1 and RWPE-2 cells were seeded at a cell density of $8x10^5$ into 60 mm dishes and incubated 37^oC overnight. Cells were washed twice in PBS before transfection. Cells were transfected using mirVana miR-186 mimic (19 nM) (Assay# MC11753, Catalog# 4464066, Thermo Fisher Scientific, Waltham, MA, Carlsbad, CA), mirVana mimic scramble control (19 nM) (Catalog# 4464059, Thermo Fisher Scientific, Waltham, MA, Carlsbad, CA) and Superfect transfection reagent (Catalog# 301305 Qiagen, Valencia, CA) according to manufacturer's instructions. The miR-186 mimic is a small, chemically modified double-stranded RNA molecule that mimic the endogenous miRNA and enable miRNA functional analysis by up-regulation of miRNA activity. The mimic is the same length as the mature miRNA transcript. In brief, a 10 µM stock solution was made for the mirVana mimic scramble control (10 pmoles/µl) and mirVana miR-186 mimic (10 pmoles/µl). Cells were transfected in 60mm dishes with 50pmoles of mirVana mimic scramble control and mirVana miR-186 mimic in Superfect reagent (10 µl), growth medium (600 µl) and 1ml of Opti-MEM Reduced Serum Media (Catalog# 11058921, Thermo Fisher Scientific, Waltham, MA). After a 2hr incubation at 37^oC, 1ml of growth medium was added each dish. After 24hrs,

cells were used cellular behavior assays (e.g., cellular proliferation, migration, invasion, and colonogenic assay).

PC-3 cells were seeded at a cell density of $6x10^5$ and MDA-PCa-2b cells were seeded at a cell density of $8x10^5$ into 60mm dishes and incubated at 37^oC overnight. Cells were transfected using miR-186-5p mirVana inhibitor (33 nM) (Assay# MH11753, Catalog# 4464084, Thermo Fisher Scientific, Waltham, MA, Carlsbad, CA), mirVana inhibitor scramble control (33 nM) (Catalog# 4464078, Thermo Fisher Scientific, Waltham, MA, Carlsbad, CA), JetPrime reagent and buffer (Catalog# 114-01, Polyplus Transfection, New York, NY) according to manufacturer's instructions. MiR-186-5p is a small, chemically modified single-stranded RNA molecule designed to specifically bind to and inhibit endogenous miRNA molecules and enable miRNA functional analysis by down-regulation of miRNA activity. The miRNA inhibitor is the reverse complement and same length as the mature miRNA transcript. In brief, a 10 µM stock solution was made for the mirVana inhibitor scramble control (10 pmoles/µl) and mirVana miR-186 inhibitor (10 pmoles/µl). Cells were transfected in 60 mm dishes with 72.6pmoles of mirVana inhibitor scramble control and mirVana miR-186 inhibitor in JetPrime reagent (2 µl), JetPrime buffer (200 µl) and 1ml of Opti-MEM Reduced Serum Media (Catalog# 11058921, Thermo Fisher Scientific, Waltham, MA). After a 2-hr incubation at 37^oC, 1ml of growth medium was added each dish. After 24hrs, cells were used cellular behavior assays (e.g., cellular proliferation, migration, invasion, and colonogenic assay). Post-transfection 24hrs, microRNA was

isolated from cell lines to confirm overexpression and inhibition of miR-186 via qRT-PCR.

HEK-293T cells were at a cell density of $8x10^5$ into 60 mm dishes and incubated 37^{o}C overnight. Cells were washed twice in PBS before transfection. Cells were transfected using mirVana miR-186 mimic (19 nM), mirVana mimic scramble control (19 nM) (Thermo Fisher Scientific, Waltham, MA, Carlsbad, CA) according to manufacturer's instructions. In brief, a 10 µM stock solution was made for the mirVana mimic scramble control (10 pmoles/µl) and mirVana miR-186 mimic (10pmoles/µl). Cells were transfected in 60mm dishes with 50pmoles of mirVana mimic scramble control and mirVana miR-186 mimic in 1ml of Opti-MEM Reduced Serum Media (Catalog# 11058921, Thermo Fisher Scientific, Waltham, MA), JetPrime reagent and buffer (Catalog# 114-01, Polyplus Transfection, New York, NY) according to manufacturer's instructions. After a 2hr incubation at 37^{o}C, 1 ml of growth medium was added each dish. After 24 hrs, cells were used western blot analyses.

Mir-186 precursor and anti-miR constructs

Genomic DNA from RWPE1 cells was used to clone a 579 bp BamHI/NotI fragment consisting of the miR-186 precursor using forward primer (GCGGATCCGAGCCATGCTTATGCTACTG), and reverse forward (GCGCGG CCGCCCAGGTATATGGCACAG) via PCR with the following cycling conditions: 95^{o}C for 5 mins for 1 cycle, 95^{o}C for 15 secs, 57.5^{o}C for 30 secs, 72^{o}C for 30 secs followed by 35 cycles and held 4^{o}C. Size of PCR product was confirmed via

gel electrophoresis and eluted from gel using Gen Elute Agarose Spin Column kit (Catalog# 56500, Sigma Aldrich, St. Louis, MO). PCR product was cloned into the pENTR/D-Topo vector (Thermo Fisher Scientific, Waltham, MA) using Gateway system and spread on kanamycin (50 mg/ml) agar plates. Plasmid DNA was isolated from bacterial colonies using the QIAprep Spin Miniprep Kit (Catalog # 27104, Qiagen, Valencia, CA) and sequenced by Eurofins Genomics (Louisville, KY) to verify correct cloning.

Designed forward and reverse anti-miR-186 oligos from sequence provided from Thermo Fisher Scientific, Waltham, MA and ordered from Eurofins Genomics (Louisville, KY). Anti-miR-186 forward (5'CACCgcGGATCC TGCTTGTAACTTTCCAAAGAATTCTCTCCTTTTGGGCTTTCTGGTTTTATTTTA AGCCCAAAGGTGAATTTTTTGGGAAGTTTGAGCT 3') (1mM) and anti-miR-186 reverse (5'GCGGCCgcAGCTCAAACTTCCCAAAAAATTCACCTTTGGGCTTAA AATAAAACCAGAAAGCCCAAAAGGAGAGAATTCTTTGGAAAGTTACAAGCA 3') (1mM) oligos were annealed together with 10X Annealing Buffer (100mM Tris-HCl pH 7.5, 1M NaCl, 10mM EDTA pH 7.5) according to cycling conditions: 87^{o}C (10^{o} degrees above Tm) for 10 mins, 87-25^{o}C at a rate of 0.1^{o}C/sec, 25^{o}C for 30 mins and hold at 4^{o}C. The annealed anti-miR-186 oligo was run on 0.1% TAE agarose gel Mir-186 precursor (579bp) and anti-miR-186 (100bp) was shuttled into pcDNA-DEST47 expression vector and sequenced by Eurofins Genomics (Louisville, KY) to verify correct cloning. The pcDNA-DEST47-miR-186 mimic construct was used to stably overexpress miR-186 in RWPE1 and RWPE2 cells

and the pcDNA-DEST47-Anti-miR-186 construct was used to stably inhibit miR-186 in PC-3 cells.

Stable Transfection

RWPE-1 cells were seeded at a cell density of $8x10^5$ into 60 mm dishes and incubated 37^oC overnight. Cells were washed twice in PBS before transfection. Cells were transfected using pcDNA-DEST47-miR-186 overexpression construct (1 µg), pcDNA-DEST47 as the negative control (1 µg) (Thermo Fisher Scientific, Waltham, MA) and 10 µl of Superfect reagent (Qiagen, Valencia, CA) according to manufacturer's instructions. PC-3 cells were seeded at a cell density of $6x10^5$ into 60mm dishes and incubated 37^oC overnight. Cells were transfected using pcDNA-DEST47-anti-miR-186 construct (1 µg), pcDNA-DEST47 as the negative control (1 µg) (Thermo Fisher Scientific, Waltham, MA) and JetPrime reagent (Polyplus Transfection, New York, NY) according to manufacturer's instructions. Forty-eight hours post-transfection, growth medium (3 ml) was replaced and cells were treated with G418 Sulfate (800 µg/ml) (Catalog# 30-234-CR, MEDIATECH INC) for 10-14 days and then cultured in G418 Sulfate (400 µg/ml) for expansion.

miRNA Expression

Reverse transcription of total RNA (10ng) was performed using TaqMan microRNA Reverse Transcription kit, miRNA specific RT primers and Peltier Thermal Cycler (PTC 200). cDNA was synthesized using the following cycling

conditions 16^{o}C for 30 mins, 42^{o}C for 30 mins, 85^{o}C for 5 mins and hold indefinitely at 4^{o}C. qRT-PCR was performed using miRNA specific PCR TaqMan Assays (Life Technologies) and the following cycling conditions: hold for 2 mins at 50^{o}C, 10 mins at 95^{o}C for 1 cycle, 15 secs at 95^{o}C and 1 minute at 60^{o}C for 40 cycles . Amplification of cDNA was performed using the Applied Biosystems Step One PCR system (Applied Biosystems, Carlsbad, CA). The relative expression of microRNA compared with U44 snRNA was calculated using $2^{-\Delta\Delta Ct}$ method.

Cell Proliferation Assays

Trypan Blue Exclusion Assay

Transiently and stably miR-186 transfected cells were seeded at a cell density of 10x10^{3} or 50x10^{3} into 6-well plates and incubated at 37^{o}C over the time course of 5-6 days. Cells was washed in PBS (1X) twice, cleaved with TrypLE Select (Catalog # 12563-029, Thermo Fisher Scientific, Waltham, MA) and placed in the incubator for 5 mins. Trypsin was neutralized with growth medium containing FBS and cells were transferred to 15 ml tubes. Cells were mixed thoroughly and used to make a 1:2 dilution of cells with 0.4 w/v Trypan Blue solution in PBS (Catalog# 25-900-C1, Mediatech, Manassas, VA). Cell dilution (10µl) was counted in triplicate at each time interval using a Hemacytometer.

5-bromo-2'-deoxyuridine (BrdU) Incorporation

Transiently transfected PC-3 ($2x10^3$), MDA PCa 2b ($2x10^3$) and RWPE1 ($5x10^3$) cells (100 µl/well) were seeded into 96-well black plates and incubated for 24 hrs at 37^oC overnight. Cellular proliferation was measured using the Cell Proliferation ELISA BrdU colorimetric kit (Catalog # 11647229001, Sigma Aldrich, St. Louis, MO). The 5-bromo-2'-deoxyuridine (BrdU) labeling solution (1:100, 10 µl/well) in growth medium was added to cells to monitor the incorporation of BrdU into newly synthesized DNA. Cells were incubated at 37^oC in BrdU labelling solution for 24hrs. For each experiment, experimental controls consisted of blanks (medium/BrdU/ BrdU anti-body) and background controls (cells/anti-body). After each time intervals, BrdU label was removed from adherent cells. Cells were incubated in FixDenat solution (200 µl/well) at room temperature for 30 mins and removed thoroughly. Anti-BrdU-POD working solution (1:100) was added to cells for 90 mins at room temperature and then removed. Cells were washed three times in PBS 1X solution. Substrate solution was added to each well and incubated at room temperature for 15 mins. Cellular proliferation was quantified using the difference in the absorption of BrdU incorporated in the DNA of newly proliferating cells at the testing wavelength, 370 nm, and the reference wavelength, 492 nm, using a Biotek Synergy HT plate reader.

ATP Lite Assay

Transiently miR-186 transfected PC-3 cells were seeded at a cell density of $2x10^3$ (100 µl/well) into 96-well black plates and incubated for 24 hrs at 37^oC. One vial of lyophilized substrate solution was reconstituted in 5 ml of substrate

buffer solution and gently agitated until solution was homogenous. Mammalian cell lysis buffer (50 µl) was added to each well of 96-well plates. Plates were shaken for 5 mins at 700 rpm to lyse cells and stabilize ATP. Immediately before measurement, substrate solution (50 µl) was added to each well. Cellular viability and proliferation was measured via luminescence intensity after 72, 96 and 120 hrs using ATPlite Luminescence Assay System Kit (Catalog# 6016941, Perkin Elmer, Waltham, MA) and Advanced Medical Imaging (AMI) instrument. ATPlite is an Adenosine TriPhosphate (ATP) monitoring system based on firefly (*Photinus pyralis*) luciferase and uses ATP as a marker of cell viability. Luminescence is emitted from the chemical of ATP with luciferase and D-luciferin. This luminescence assay is the alternative to colorimetric, fluorometric and radioisotopic assays for the quantitative evaluation of proliferation and cytotoxicity of cultured mammalian cells.

Anchorage Independent Growth Assay

Sterile 3.5% agar solution in 1X PBS was used to make a 0.7% agar solution with growth medium. The 0.7% agar-medium solution was used to make a 0.35% agar-medium solution. The 0.35% agar-medium solution (3 ml) was added to 6-well plates to form base layer and plates were incubated at 4^{o}C for 60 mins to set agar base. Transiently transfected and/or stable cells ($10x10^{3}$ cell density) in a final volume of 3 ml were gently mixed with 0.7% agar-medium solution (3 ml) to make a 0.35% agar-medium solution. The cells in the 0.35% agar-medium solution were added onto the agar base layer in the 6-well plates. Cells were allowed to incubate at 37^{o}C and form colonies in soft agar for 2-3

weeks. Colonies were counted at 4X magnification in bright field setting using bright field microscopy.

5-α-DHT Treatment

5 -alpha Dihydrotestosterone (DHT) (Catalog# D-073-1ML, Lot# FE02101503, Sigma Aldrich, St. Louis, MO) in methanol was purchased at 1 mg/ml (1mM). DHT (100ul) was diluted 1:10 in PBS 1X (900ul) to make a solution of 100 µM. Next, DHT solution (100 µM) was diluted 1:100 in 5ml of PBS 1X to make s solution of 1uM. DHT solution (1 µM) was diluted 1:10 in 5ml of PBS 1X. Androgen sensitive MDA PCa 2b cells were transfected with miR-186-5p inhibitor (33 nM) and scramble control (33 nM), serum starved for in reduced Opti-MEM I Reduced serum media (Catalog# 31985070, ThermoFisher Scientific, Waltham, MA) for 24 hrs and treated with DHT (10 nM) for 24 hrs in growth medium with FBS. Treated cells were plated in 0.35% soft agar with DHT (10 nM).

Cellular Invasion Assay

Polyethylene Terephthalate hanging inserts ($1.1cm^2$) with pore size of 8.0µm and placed in a 12-well plate. Reduced growth matrigel was thawed and 100 µl was added on top of inserts in a 12-well plate. Matrigel was incubated at 37^oC for 1hr to form a thin gel layer and cells ($25x10^3$) in serum-free medium were seeded on top of matrigel. A solution of growth medium (0.6 ml) containing FBS and FBS (0.6 ml) was pipetted into the lower chambers of a 12-well plate. Growth medium and FBS in the lower chamber was used to make contact with

insert membrane and create a chemoattractant gradient for cells. After 48hrs, inserts were washed in 1X PBS with sterile q-tip and cells that migrated through the insert into the lower chamber were washed with 1X PBS. Migrate cells in the lower chamber were stained with 0.4% crystal violet in ethanol and quantified using a 10X magnification and bright field microscopy.

Results:

Mir-186 inhibition induces cellular death in both metastatic PCa and normal prostate epithelial cells

To determine whether alteration of miR-186 expression modified cellular death in PCa, we inhibited the expression of miR-186-5p in metastatic PCa cells (Figure 12A). We also evaluated the effect of cellular behavior in relation to miR-186-5p overexpressed in normal prostate epithelial cells (Figure 15A). We selected RNU44 as a endogenous control miR to normalize miRNA expression due to its expression being more stable and higher amplification in prostate cells than RNU6, a commonly used endogenous control miR in other cancers (Figure 10). First, we evaluated the duration of a transient transfection in PC-3 and RWPE1 cells. We determined that transient transfections last up to 144 hrs post transfection, which is beyond the time frame for the *in vitro* studies in this study (Figures 11, 14). A small chemically modified single-stranded RNA molecule inhibitor was used to inhibit miR-186-5p expression. The inhibitor is designed to specifically bind to and inhibit endogenous miRNA molecules and enable miRNA functional analysis by the down-regulation of miRNA activity. The miRNA inhibitor is the reverse complement and same length as the mature miRNA transcript. A small, chemically modified double-stranded RNA molecule was used to overexpress miR-186-5p. The mimic is designed to mimic the endogenous miRNA and enable miRNA functional analysis by the up-regulation of miRNA activity. The mimic is the same length as the mature miRNA transcript. MiR-186-5p expression was transiently inhibited in androgen insensitive PC-3 cells and androgen sensitive MDA PCa 2b cells as shown in Figure 12A. The death rate of

transfected PC-3 and MDA PCa 2b cells were evaluated for up to 120 hrs using trypan blue exclusion or cellular death assays as described in Chapter 2 methods. After 5 days, the cellular death of PC-3 and MDA PCa 2b cells treated with the miR-186-5p inhibitor (33nM) was increased by 20-31% relative to scramble control (33nM). Cellular death was increased by 28-31% in PC-3 treated with the miR-186 inhibitor relative to scramble control as shown in Figure 12B (p-value ≤ 0.055). In Figure 12B, miR-186 inhibition in MDA-PCa-2b cells induced cellular death by 20-31% relative to scramble control (p-value = 0.334). However, cellular death was not significantly reduced in PC-3 and MDA PCa 2b cells. Next, PC-3 cells were stably transfected with pcDNA-DEST47-anti-miR186 and pcDNA-DEST47 constructs. Stable anti-miR-186 PC-3 cells exhibited a 30% reduction in miR-186-5p expression relative to empty vector shown in Figure 13A (p-value = 0.0022). Stable miR-186-5p inhibition in metastatic PC-3 cells resulted in a 32-51% increased of cell death after 5 days relative to empty vector control as shown in Figure 13B (p-value ≤ 0.02).

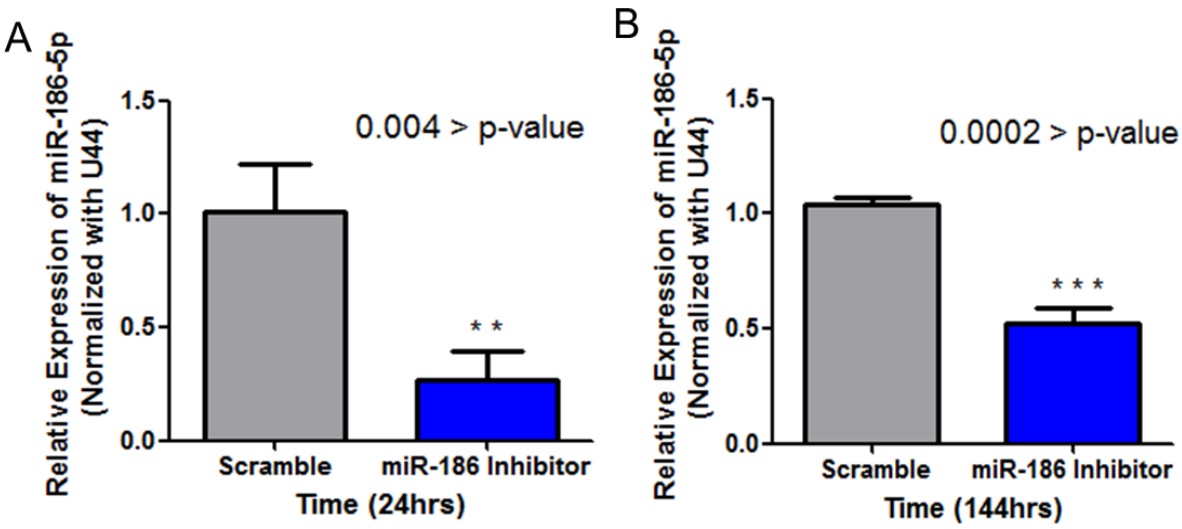

Figure 11. Stability of transient miR-186 inhibition in PCa cells. PC-3 cells were transiently transfected with scramble negative control and miR-186 inhibitor for 24hrs. Expression of miR-186-5p was measured A) 24 and B) 144 hrs post-transfection via qRT-PCR. Post-transfection, miR-186-5p expression was decreased by 73% (p-value = 0.003) and 50% (p-value = 0.0001), after 24 and 144 hrs, respectively (p-value < 0.004). We set scramble control to 1 fold change. Data was quantitated from at least three independent experiments and error bars are the standard deviation between experiments. Statistical analysis was performed using Unpaired t-test and significance level of p-value ≤ 0.05 (**p-value < 0.004, ***p-value < 0.0002).

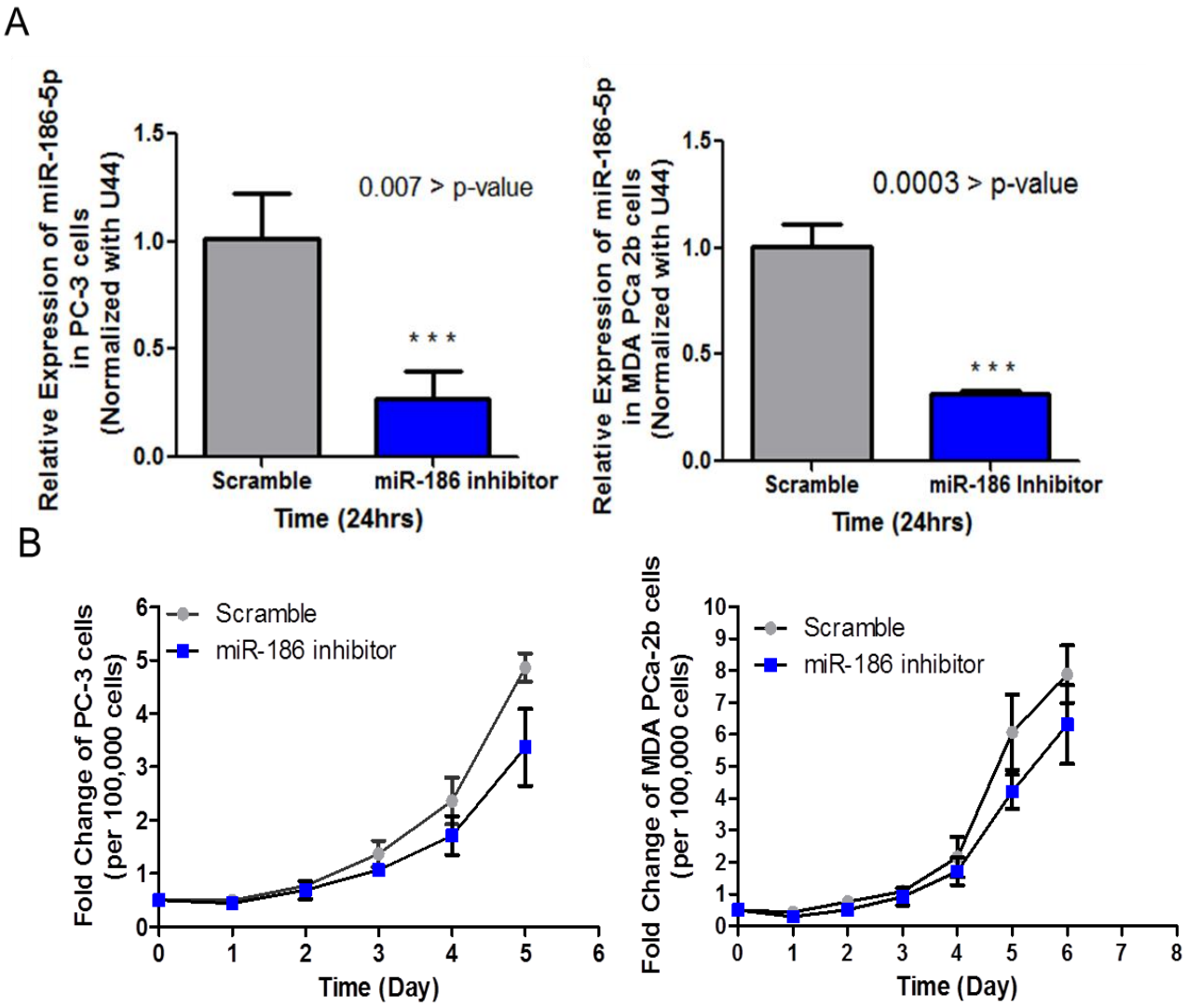

Figure 12. The effect of transient miR-186-5p inhibition on cell death in PCa. A) PC-3 and MDA PCa-2b cells were transiently transfected with miR-186-5p inhibitor (33nM) and significantly reduced miR-186-5p expression by 66-73% relative to scramble control as examined by qRT-PCR (p-value = 0.0061, 0.0002). B) Post-transfection, cellular death of PC-3 and MDA PCa-2b cells was monitored every 24 hrs up using trypan blue exclusion assay. MiR-186 inhibition induced cellular death by 31% (Day5) in PC-3 and by 20-31% (Day5, 6) in MDA PCa-2b cells relative to scramble control (p-value = 0.055, 0.334). Data was quantitated from at least two separate independent experiments and error bars are the standard deviation between experiments. Statistical analysis was performed using Unpaired t-test and significance level of 0.05 (***p-value < 0.007).

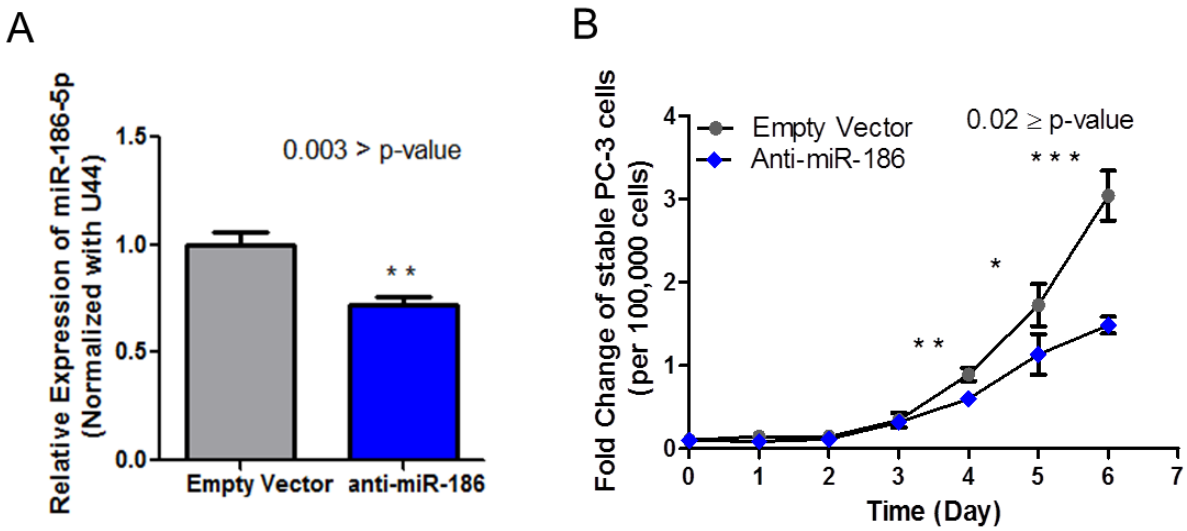

Figure 13. The effect of stable miR-186 inhibition on cellular death in PC-3 cells. A) PC-3 cells were stably transfected with pcDNA-DEST47-anti-miR-186-5p and pcDNA-DEST47 constructs using G418 treatment. MiR-186-5p expression was stably reduced by 30% in PC-3 cells relative to empty vector as revealed by qRT-PCR (p-value = 0.0022). B) Cellular death of stable anti-miR-186 PC-3 cells were monitored every 24hrs. Cell death was induced by 33-51% in stable anti-miR-186 PC-3 cells relative to empty vector as examined by trypan blue exclusion assay (p-value ≤ 0.02). Data was quantitated from one experiment and error bars correspond to between three separate cell counts standard deviation. Statistical analysis was performed using Unpaired t-test and significance level of p-value ≤ 0.05 (*p-value < 0.03, **p-value < 0.003, *** p-value < 0.0006)

Overexpression of miR-186-5p suppresses cellular death in normal prostate epithelial cells

To determine the impact of miR-186-5p expression on cellular death in normal prostate cells, miR-186-5p was transiently overexpressed in normal epithelial RWPE1 cells to evaluate the transformation capacity of potential oncogenic miR-186-5p alone. The stability of transient miR-186-5p overexpression was evaluated in normal prostate epithelial cells. Ectopic expression was observed to last 144 hrs post-transfection, shown in Figure 14. MiR-186-5p was transiently overexpressed by 761-fold increase in RWPE1 cells 24 hrs post-transfection with miR-186-5p mimic (19nM) relative to scramble control (19nM) as shown in Figure 15A. The death rate of transfected RWPE1 cells was monitored up to 120 hrs using trypan blue exclusion assays as described in Chapter 3 methods. Overexpression of miR-186 in normal epithelial RWPE1 cells significantly induced cellular death by 27% after 5 days relative to scramble control shown in Figure 15B (p-value = 0.0008). Next, in a more physiological model miR-186-5p was stably overexpressed in RWPE1 cells by 2.6 fold increase relative to empty vector (Figure 16A). Interestingly, stable overexpression of miR-186-5p resulted in a more pronounced increase of 50-76% in cellular death relative to empty vector control, shown in Figure 16B. After day 5, the RWPE1 cells expressing the miR-186 mimic construct displayed the greatest decrease of 76% in cellular death.

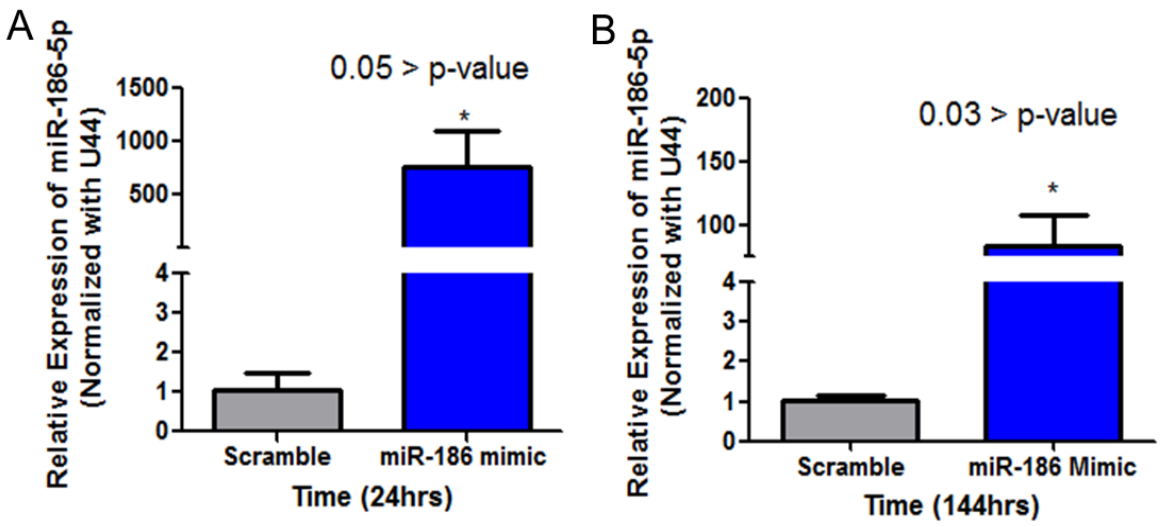

Figure 14. Stability of transient miR-186 overexpression in normal epithelial cells. RWPE1 cells were transiently transfected with scramble negative control and miR-186 mimic for 24hrs. Expression of miR-186-5p was measured A) 24 and B) 144 hrs post-transfection via qRT-PCR. Post-transfection, miR-186 was up-regulated by 761–fold (p-value = 0.0426) and 83-fold (p-value = 0.0213) relative to scramble control after 24hrs and 144hrs, respectively. Data was quantitated from at least three independent experiments and error bars correspond to standard deviation. Statistical analysis was performed using Unpaired t-test and significance level of p-value ≤ 0.05 (*p-value < 0.05).

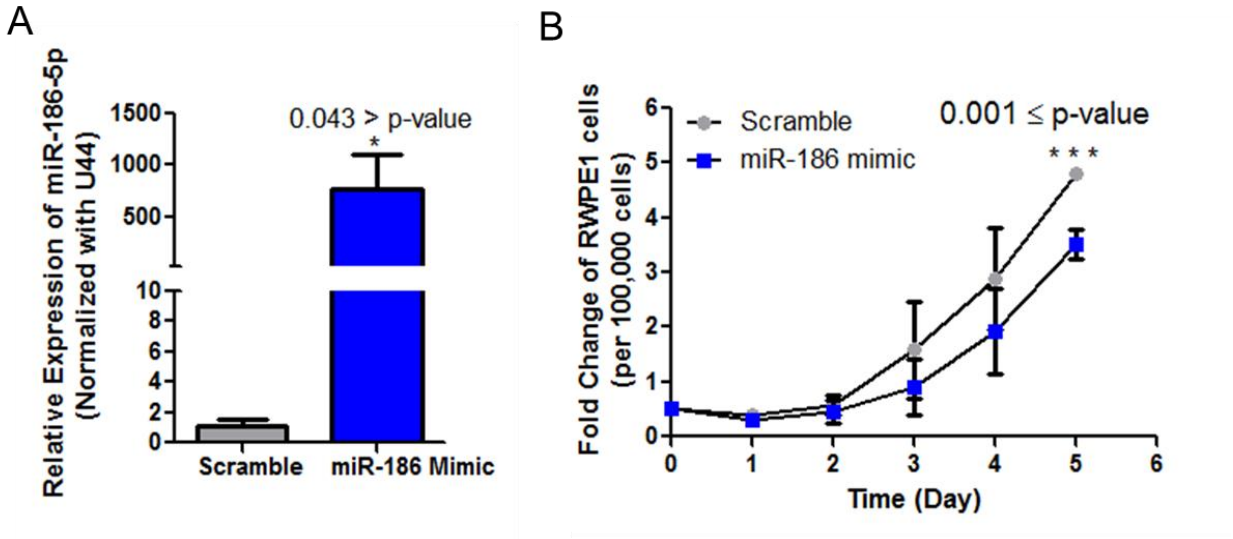

Figure 15. The effect of transient miR-186-5p overexpression on cellular death in normal epithelial cells. A) RWPE1 cells were transiently transfected with miR-186-5p mimic and scramble negative control for 24 hrs. MiR-186-5p expression was up-regulated in RWPE1 cells relative to negative control as examined by qRT-PCR. B) Post transfection, cellular death of RWPE1 cells were monitored every 24hrs up to 120hrs using trypan blue exclusion assay. Overexpression of miR-186 induced cellular death by 27% in RWPE1 cells relative to negative control (p-value = 0.0008). Data was quantitated from at least three separate independent experiments and error bars calculated with standard deviation. Statistical analysis was performed using Unpaired t-test and significance level of p-value ≤ 0.05 (***p-value < 0.001).

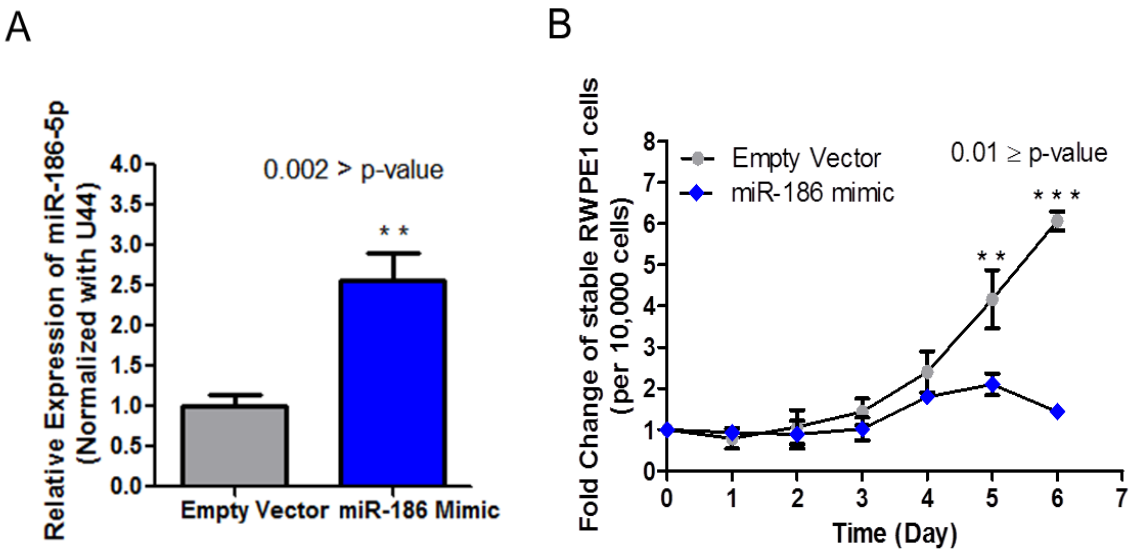

Figure 16. The effect of stable miR-186-5p overexpression on cell death in normal prostate epithelial cells. A) RWPE1 cells were stably transfected with pcDNA-DEST47-miR-186-5p mimic and pcDNA-DEST47 constructs using G418 treatment. MiR-186 was stably overexpressed by 2.6-fold in RWPE1 cells relative to empty vector as examined by qRT-PCR (p-value = 0.0019). B) Cellular death of stable miR-186 mimic RWPE1 cells every 24hrs up to 120hrs examined by trypan blue exclusion assay. Cellular death was increased by 50% on Day 5 (p-value < 0.0001) and 76% on Day 6 (p-value =0.009) in stable miR-186 mimic RWPE1 cells relative to empty vector. Data was quantitated from one experiment and error bars correspond to the standard deviation between three separate cell counts. Statistical analysis was performed using Unpaired t-test and significance level of p-value ≤ 0.05 (**p-value < 0.01, ***p-value < 0.0001).

MiR-186 alters the capacity of metastatic PCa cells to proliferate *in vitro*

Self-sustained cellular proliferation is an essential hallmark of cancer. Malignant cells produce their own growth signals [i.e., tumor growth factor-β (TGF-β), platelet-derived growth factor (PDGF), epidermal growth factor (EGF)] to stimulate uncontrolled proliferation in tumorigenesis [133]. To determine the effect of miR-186-5p expression on proliferation, we evaluated the cellular proliferation of transiently miR-186-5p inhibited metastatic PCa cells, PC-3 and MDA PCa 2b, and miR-186-5p overexpressing RWPE1 cells using BrdU assays as described in Chapter 2 methods. Cellular proliferation in both metastatic PC-3 and MDA PCa 2b cells was reduced significantly shown in Figure 17. Newly proliferating cells was decreased by 46% in PC-3 cells and 27% in MDA-PCa-2b cells relative to scramble controls shown in Figures 17A and 17B, respectively. Additionally, the effect of miR-186-5p on cell viability and proliferation was also detected in metastatic PC-3 cells using the ATP Lite Luminescence detection kit as described in the Chapter 2 methods. In Figure 18, a 48-58% reduction in cell viability and proliferation was detected in metastatic PC-3 cells after 72-120 hrs relative to scramble control. In contrast, transient miR-186 overexpression slightly reduced cellular proliferation by 10% in RWPE1 cells relative to scramble control (Figure 19A). Post-transfection, cellular proliferation was decreased by 24% in E006AA cells treated with miR-186-5p mimic relative to negative control as shown in Figure 19B (p-value = 0.0055).

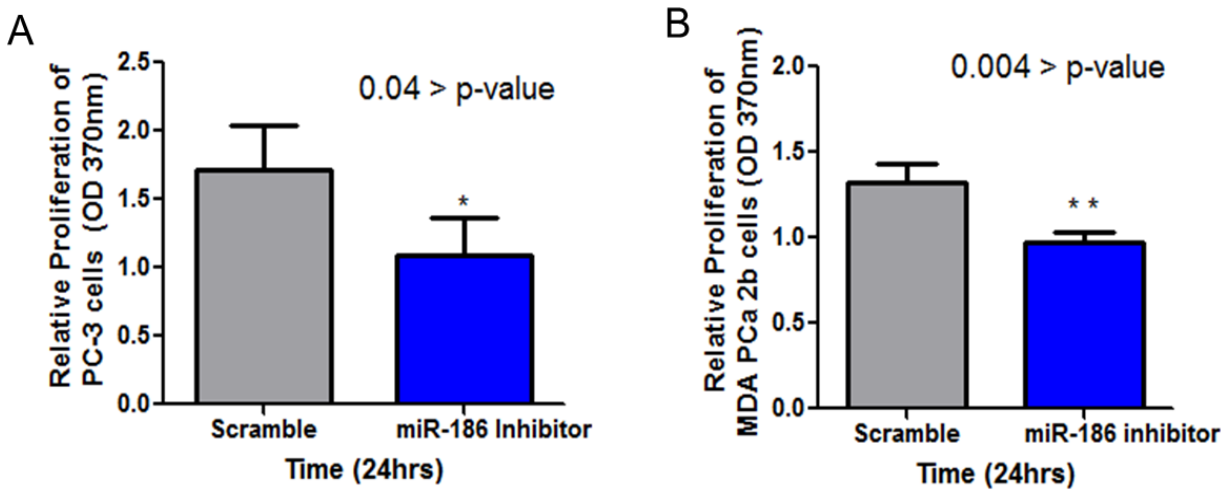

Figure 17. The effect of miR-186 inhibition on cellular proliferation in metastatic PCa. PC-3 and MDA PCa-2b cells were transiently transfected for 24 hrs with miR-186-5p inhibitor and scramble negative control. Cellular proliferation of PC-3 and MDA PCa-2b cells was examined after 24hrs by BrdU colorimetric assays. A) Post-transfection, cellular proliferation was decreased by 46% in PC-3 cells treated with miR-186-5p inhibitor relative to scramble control (p-value = 0.032). B) Post-transfection, cellular proliferation was decreased by 27% in MDA PCa-2b cells treated with miR-186-5p inhibitor relative to scramble control (p-value = 0.0039). Statistical analysis was performed using an Unpaired Student's T test and significance cut-off p-value ≤ 0.05. Data was quantitated from at least three separate independent experiments and error bars calculated with standard deviation. Statistical analysis was performed using Unpaired t-test and significance level of p-value ≤ 0.05 (*p-value, 0.04, **p-value < 0.004).

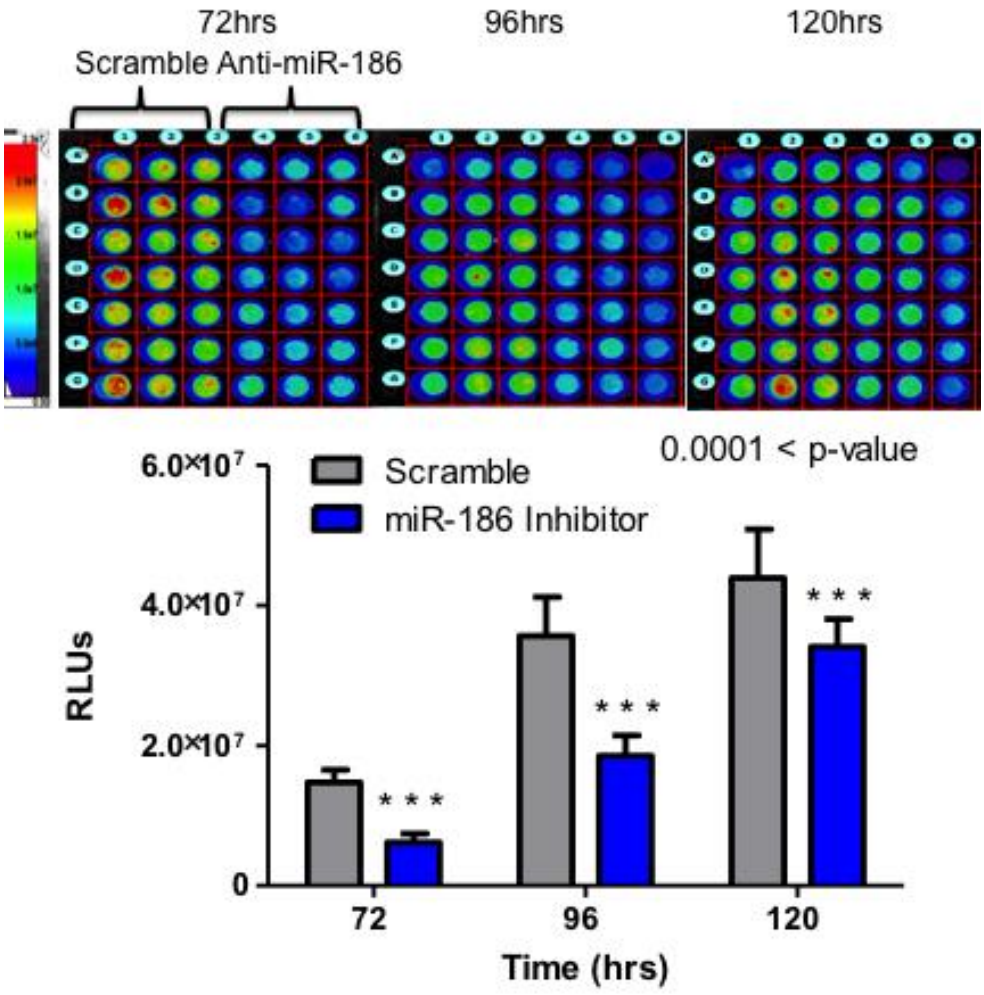

Figure 18. Proliferation of miR-186-5p inhibited PC-3 cells. PC-3 cells were transiently transfected with miR-186-5p inhibitor and scramble control for 24 hrs. Cellular proliferation of PC-3 cells 72, 96 and 120hrs post transfection evaluated by ATP Lite assay. MiR-186-5p inhibition reduced proliferation by 22-58% in PC-3 cells relative to scramble controls (p-value < 0.0001). Cell proliferation was decreased by 58% after 72hrs, 48% after 96hrs and 22% after 120hrs. Data was quantitated from one experiment and error bars were calculated from the standard deviation of 6 replicates using the ATPLite assay Statistical analysis was performed using Unpaired t-test and significance level of p-value ≤ 0.05 (***p-value < 0.0001).

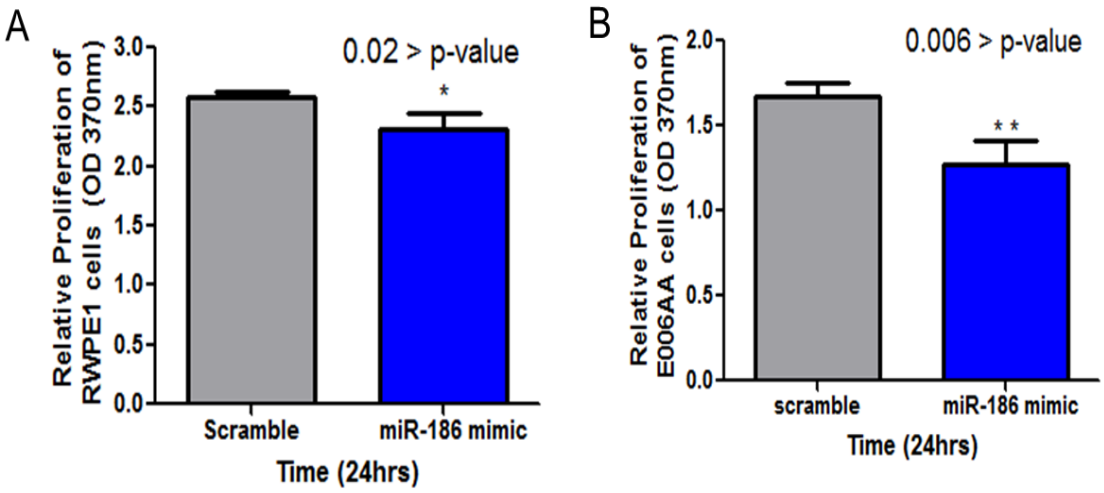

Figure 19. The effect of miR-186-5p overexpression on proliferation in normal prostate epithelial and non-metastatic PCa cells. RWPE1 and E006AA cells were transiently transfected for 24hrs with miR-186-5p mimic and scramble negative control. Cellular proliferation of RWPE1 and E006AA cells was examined after 24 hrs by BrdU colorimetric assays. A) Post-transfection, cellular proliferation was decreased by 10% in RWPE1 cells treated with miR-186-5p mimic relative to negative control (p-value = 0.013). B) Post-transfection, cellular proliferation was decreased by 24% in E006AA cells treated with miR-186-5p mimic relative to negative control (p-value = 0.0055). Data was quantitated from three independent experiments and error bars calculated with standard deviation. Statistical analysis was performed using Unpaired t-test and significance level of p-value ≤ 0.05 (*p-value < 0.02, **p-value < 0.006).

MiR-186-5p inhibition alters anchorage independence in metastatic PCa

To evaluate the effect of miR-186 expression on anchorage independent growth, we seeded transfected normal epithelial (RWPE1), non-metastatic (E006AA) and metastatic (PC-3, MDA PCa-2b) cells into 0.35% soft agar and their growth was monitored at 37^{o}C for 2-3 weeks. RWPE1 and E006AA cells exhibited low miR-186 expression. MiR-186 was transiently overexpressed in both cell lines and plated into 0.35% soft agar. Over the course of 2-3 weeks, both RWPE1 and E006AA treated with scramble control and miR-186-5p mimic exhibited no colony formation (Data not shown).

MiR-186 was up-regulated in metastatic PCa cell lines, PC-3 and MDA PCa-2b, relative to normal prostate epithelial RWPE1 cells. We transiently inhibited the expression of miR-186 in PC-3 and MDA PCa 2b cells and seeded cells into 0.35% soft agar. In Figure 20A, a reduction of 64% in colony formation of metastatic PC-3 cells treated with the miR-186 inhibitor relative to scramble control (p-value = 0.0022). MiR-186-5p inhibition did not significantly impede colony formation in androgen sensitive metastatic MDA PCa 2b cells shown in Figure 20 B (p-value = 0.335). However, after stimulation of the androgen receptor with 5-alpha-DHT treatment (10 nM) a modest 28% decrease in colony formation was observed in MDA PCa 2b cells treated with the miR-186-6p inhibitor relative to scramble control (p-value = 0.034).

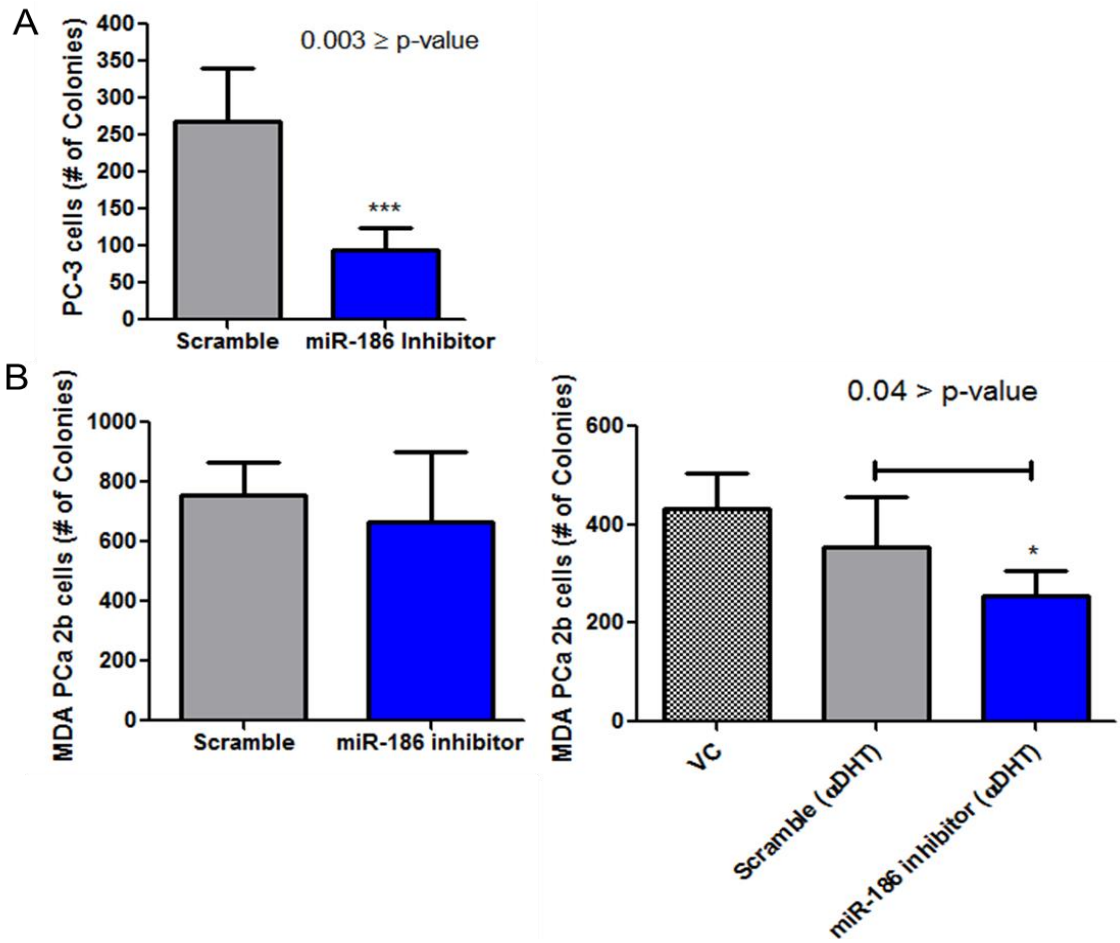

Figure 20. The effect of miR-186-5p inhibition on anchorage independence in metastatic PCa. Metastatic PC-3 and MDA PCa 2b cells were transiently transfected with scramble negative control and miR-186 inhibitor for 24hrs. Post-transfection, cells were grown in 0.35% soft agar for 2-3 weeks at 37^{o}C. Colony formation of PC-3 cells treated with miR-186 inhibitor was significantly decreased by 64% relative to scramble control (p-value = 0.0022). In MDA PCa 2b cells, colony formation was slightly reduced by reduced by 12% relative to scramble control (p-value = 0.335). However, stimulation of the androgen receptor with α-DHT treatment (10nM) after miR-186-5p inhibition did significantly decrease colony formation in by 28% in MDA PCa 2b cells (p-value= 0.034). Data was quantitated from at least three independent experiments and error bars calculated with standard deviation. Statistical analysis was performed using Unpaired t-test and significance level of p-value ≤ 0.05 (*p-value < 0.04, ***p-value < 0.003).

Suppression of cellular invasion in metastatic PCa

To determine whether inhibition of miR-186-5p impacted cellular invasion of metastatic PCa cells, metastatic PC-3 and MDA PCa 2b cells were transiently transfected with the miR-186-5p inhibitor and scramble for 24hrs. Post-transfection, PC-3 and MDA PCa 2b cells were seeded on top of reduced growth factor matrigel in 12-well plates. A 1:1 solution of growth medium and FBS was pipetted into the lower chamber of each well to serve as a chemoattractant for PCa cells. Reduced growth factor matrigel was used to reduce the influence growth factors on the cellular invasion of PCa cells. After 48 hrs, the cells were fixed in 100% methanol and stained in 0.4% crystal violet overnight. In Figure 21A, a significant reduction in cell invasion (66%) was observed in metastatic PC-3 cells transfected with the miR-186-5p inhibitor relative to scramble control (p-value = 0.0024). In metastatic MDA PCa 2b cells, miR-186 inhibition reduced cellular invasion by 38% relative scramble control as shown in Figure 21B (p-value = 0.0892). Unfortunately, this reduction was not statistically significant between treatment groups.

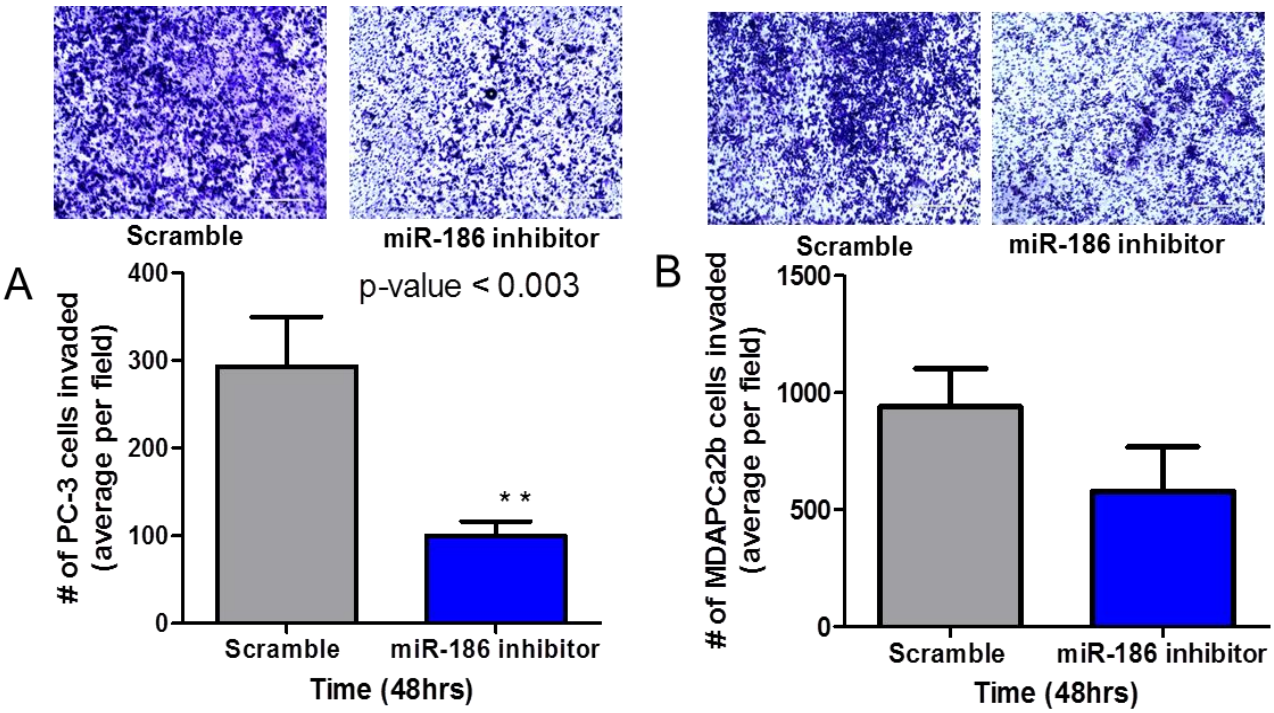

Figure 21. Cellular invasion of miR-186-5p inhibited metastatic PCa cells. PC-3 and MDA PCa 2b cells were transiently transfected with scramble control and miR-186-5p inhibitor for 24 hrs. Post-transfection, cells were seeded on top of reduced growth factor matrigel. After 48 hrs, cells were fixed in methanol and stained with 0.4% crystal violet. A) Cell invasion was reduced by 66% in metastatic PC-3 cells treated with the miR-186-5p inhibitor relative to the scramble control (p-value = 0.0024). B) However, miR-186-5p inhibition did not significantly reduced cell invasion in MDA PCa 2b cells (p-value = 0.0892). Data was quantitated from at least three independent experiments and using an average of 3 different fields of view at 10X magnification. Statistical analysis was performed using an Unpaired Student's T test and significance level of p-value ≤ 0.05 (**p-value < 0.003).

Discussion:

Aberrant expression of intronic miR-186 has been implicated in various solid tumor malignancies [99-104, 106-112, 130, 131]. The influence of miR-186-5p expression on tumor cellular behavior has undergone characterization in a limited number of cancers [103, 106, 107, 112, 130, 131]. For instance, miR-186-5p overexpression enhanced the cellular proliferation, migration, colony formation and anchorage independent growth of pancreatic and bladder cancer cells [103, 130]. However, there are no published reports on the role of miR-186-5p in relation to different cancer hallmarks using PCa cell models (i.e., proliferation, invasion, anchorage independent growth). We hypothesized miR-186-5p inhibition would impede the tumor phenotype in metastatic cells and miR-186-5p overexpression would increase the aggressiveness of non-metastatic cells and induce malignant transformation of normal epithelial cells. In the current study, inhibition of miR-186-5p led to a reduction in cell proliferation, invasion, and anchorage independent growth in metastatic PCa cells but not non-metastatic cell models. Moreover, miR-186 overexpression increased cellular death and proliferation in normal prostate epithelial cells. These findings implicate an oncogenic role for miR-186-5p in both metastatic PCa and normal epithelial cells.

In vitro characterization of miR-186-5p revealed pertinent information related to its effects on PCa and normal cell behavior. For instance, cell death was not significantly affected by transient miR-186-5p inhibition in metastatic PC-3 and MDA PCa 2b PCa cell lines. However, stable miR-186 inhibition significantly increased cell death in PC-3 cells. This data suggests permanent

rather than transient knockdown of miR-186 in the cellular genome in PCa cells may lead to a significant increase in cell death metastatic PC-3 PCa cells. Additional studies are needed to assess whether permanent miR-186 inhibition will influence cellular death in other metastatic cell lines (e.g., MDA PCa 2b). Moreover, transient inhibition of miR-186-5p significantly reduced the proliferative capacity of metastatic PC-3 and MDA PCa 2b PCa cells. In agreement with our *in vitro* data, miR-186-5p inhibition was also associated with a decrease in cellular proliferation of pancreatic and bladder cancer cell lines [103, 130].

Initially, miR-186-5p overexpression was expected to increase the cellular death and proliferation of normal prostate epithelial RWPE1 cells. However, in the current study, transient overexpression of miR-186-5p led to a decrease in proliferation and increase in cell death in RWPE1 cells. Similar to our findings, overexpression of another oncogenic miRNA, miR-375 in RWPE1 cells resulted in a reduction of cell proliferation, presumably due to an increase in cellular death [70]. However in the current study, cellular death has not been identified as the biological explanation for reduced proliferation yet. We speculate reduction in cellular proliferation in miR-186 overexpressed RWPE1 cells may be attributed to increased cellular death; however, subsequent studies are needed to test this hypothesis. In addition, we cannot rule out the possibility that miR-186-5p overexpression in the normal epithelial cell lines may lead to necrosis or autophagy. Ultimately, miR-186-5p overexpression alone was not sufficient for malignant transformation in the normal epithelial cells. In order for malignant

transformation to take place in the normal epithelium, multiple genomic changes and insults induced by oxidative stress, and mutations in major tumor suppressors (i.e., PTEN, p53, NKX3.1) and oncogenes (i.e. c-Myc) must occur based upon Knudson hypothesis or multiple hits hypothesis. Knudson hypothesis is a theory that a single cell must undergo a series of mutational events to achieve malignancy. The accumulation of genetic changes and insults will lead to DNA damage and ultimately genetic instability that will make normal cells more susceptible to malignant transformation. Thus, multiple genetic insults may increase RWPE1 cells susceptibility to malignant transformation via miR-186 overexpression.

Unexpectedly, miR-186-5p overexpression reduced the cellular proliferation of non-metastatic E006AA cells. We speculated that miR-186-5p overexpression would increase the aggressiveness of these non-metastatic cells. E006AA cells are androgen sensitive and possess a wild type p53 protein, but null for PTEN protein [134]. Although miR-186-5p was severely overexpressed in E006AA cells, it is possible that androgen receptor stimulation may be required to increase sensitivity or susceptibility to miR-186-5p overexpression to enhance proliferation. Furthermore, p53 may combat tumor aggressive effects exerted by miR-186-5p overexpression. Future studies should address whether miR-186-5p oncogenic function may be restricted to metastatic or advanced disease or require androgen receptor stimulation to exert its effects in PCa.

Interestingly, the suppression of three different cancer hallmarks (i.e., proliferation, invasion and anchorage independent growth) was primarily

observed in transiently miR-186-5p inhibited metastatic PC-3 cells. The PC-3 cells exhibited the highest expression of miR-186-5p compared to the other PCa cells. It is possible that this cell line was more sensitive to miR-186-5p inhibition due to a dependence on miR-186-5p expression to maintain its malignant transformation. Therefore, any reduction of miR-186-5p expression in PC-3 cells suppresses the tumor phenotype of these cells.

Anchorage independent growth and cell invasion are characteristics of advanced disease as well as assessments of the neoplastic nature of tumor cells. To our knowledge, there are no published reports on the influence of miR-186-5p on anchorage independent growth using cancer cell models. Transient inhibition of miR-186-5p alone was sufficient to decrease anchorage independent growth in androgen insensitive metastatic PC-3 cells, but not MDA PCa 2b cells. However, miR-186-5p inhibition of MDA PCa 2b cells after androgen receptor stimulation via 5-alpha DHT treatment (10 nM) lead to a reduction in anchorage independent growth. Normally androgen receptor stimulation induces cellular proliferation and/or growth. It may be possible that DHT treatment sensitizes MDA PCa 2b cells to the effects of miR-186-5p inhibition. This observation implicates an involvement of androgen receptor signaling in androgen sensitive cells for miR-186 to exert its effects on anchorage independent growth. Although, miR-186-5p expression does not appear to be dependent on androgen receptor sensitivity status based upon its expression in our PCa cell lines. But this observation implies miR-186-5p may be influenced by androgen sensitivity.

Moreover, overexpression of miR-186-5p in RWPE1 and non-metastatic E006AA cells did not promote anchorage independent growth in soft agar.

The current study revealed transient inhibition of miR-186-5p reduced cell invasion in androgen insensitive PC-3 cells. However, we did not observe a reduction in cell invasion of androgen sensitive MDA PCa 2b cells. However, we cannot rule out the possibility that androgen stimulation of miR-186 inhibited MDA PCa 2b cells will lead to a reduction in cellular invasion. Similar to the impact of DHT on anchorage independent growth of miR-186 inhibited MDA PCa 2b cells, it is plausible androgen receptor signaling is essential for miR-186-5p effects on tumor cellular behavior using androgen sensitive PCa cell models. MiR-186-5p expression has been previously shown to alter cell invasion in two studies. Contrary to our findings, these reports suggest a tumor suppressor role for miR-186-5p demonstrated by a decrease in cell invasion via miR-186 overexpression in bladder and NSCLC cancer. However, our study suggests an oncogenic role for miR-186-5p in PCa.

Collectively, *in vitro* studies in bladder and pancreatic cancer and our study findings support an oncogenic role for miR-186-5p. Overexpression of miR-186-5p in pancreatic and bladder cancer cells led to the enhancement of the tumor phenotype (i.e., increased proliferation, colony formation, invasion). Inhibition of miR-186-5p in pancreatic and PCa cells in the current study led to a reduction in the tumor phenotype. Although there is conflicting data on miR-186-5p expression in bladder cancer, more consideration was given to the report that suggested an up-regulation of miR-186 based their analysis of miR-186-5p in

tumor tissue carefully selected by NIH's The Cancer Genome Atlas and the procurement bladder cell lines from a reliable repository, ATCC [130, 131]. However, several studies propose a tumor suppressor role for miR-186-5p. Only two studies have evaluated miR-186-5p expression in NSCLC tissue and cell lines [106, 107]. Both reports failed to analyze miR-186-5p expression in laser captured micro-dissected tumor tissue. Moreover, one report does not explain the collection process for their matched tumor and normal tissue biospecimens as well. Additionally, both studies only evaluated miR-186-5p in 4 out of the 12 common NSCLC cell lines in the literature [106, 107, 135]. Additionally, each study evaluated miR-186-5p expression in different normal bronchial epithelial cell lines (16HBE, BEAS-2B) as controls to compare with miR-186-5p expression in NSCLC cell lines [106, 107, 135]. In another report, miR-186-5p was only evaluated in ovarian tumor tissue (stage IIIC, IV), but not normal adjacent tissue [112]. Furthermore, this study failed to analyze miR-186 in micro-dissected tumor tissue as well. Collectively based upon the aforementioned failures, these reports may have slightly skewed results in relation to miR-186.

We have considered the limitations, strengths and future directions of the current study. The characterization of miR-186-5p using PCa and normal epithelial cell models was restricted to the available cell lines in the current laboratory and laboratories of collaborators. In the current study, possible mechanisms responsible for miR-186-5p up-regulation in PCa were not evaluated. Therefore, future studies will elucidate potential biological mechanisms (e.g., dysregulation of miRNA machinery components,

hypomethylation) responsible for the up-regulation of mature miR-186-5p in serum and metastatic PCa cells. Previously, the up-regulation of miRNA machinery such as RNase enzymes, Dicer and/or Drosha, have been associated with miRNA dysregulation in cancer [136-139]. It is possible that the up-regulation of Dicer and Drosha may contribute to elevated levels of miR-186-5p observed in not only PCa, but several other malignancies (i.e., pancreatic, bladder, cervical, and endometrial) as well.

Epigenetic changes (i.e., global/regional hypomethylation and hyperacetylation) may also attribute to the up-regulation of oncogenic miRNAs.. In cancer, hypomethylation of promoter regions of oncogenic-related genes may allow high expression of oncomiRs such as miR-186-5p. Additionally, hyperacetylation may be another mechanism responsible for miRNA up-regulation. During hyperacetylation, the chromatin structure relaxes and exposes the DNA wrapped around histones. This may present an opportunity for transcription factors that positively regulate miR-186-5p to bind to DNA regulatory elements and induce miR-186-5p expression. Thus the investigation of miRNA biogenesis machinery and epigenetic changes may lead to additional biological explanations for miR-186-5p up-regulation in PCa.

In the current study, there was no change in anchorage independent growth of miR-186 inhibited MDA-PCA-2b cells; however, we observed a significant decrease in anchorage independent growth following stimulation of miR-185 inhibited MDA-PCA-2b cells with DHT (an androgen stimulator). Therefore, future studies will assess whether miR-186-5p inhibition suppresses

the cell growth, proliferation, invasion of androgen sensitive cells (e.g., LNCaP, MDA-PCa-2b) following DHT treatment. Moreover, additional *in vitro* and *in vivo* studies are needed to assess whether miR-186-5p overexpression and inhibition alters cellular proliferation, anchorage independent growth, cell invasion, tumor growth, and tumor metastasis using other androgen sensitive metastatic PCa cell lines (i.e., TSU-Pr1, MDA PCa-2a, ALVA-101) as well as xenograft cell lines (i.e., VCaP, DuCaP) Furthermore, our study suggests miR-186 may target genes involved in proliferation, anchorage independent growth and invasion in metastatic PCa cell models. Consequently, additional *in vitro* studies are needed, similar to those in Chapter 4, to identify and validate miR-186 targets responsible for attenuating aggressive tumor behavior in metastatic PCa cell models with and without androgen stimulation. Our laboratory also has interest in retrospective studies to assess whether miRNA-186 or other oncomiRs detected in serum or micro-dissected prostate tumor specimen may correspond with higher risk of biochemical or disease recurrence among PCa patients following a radical prostatectomy. Given the assumption that the aforementioned pre-clinical and serum/tissue based studies demonstrate miR-186 inhibition may reduce tumor burden and metastatic potential, future clinical trials can test the therapeutic benefit of miR-186 inhibitor alone or in combination with immuno- or chemotherapy. Although toxicity and efficacy of miRNA inhibitors have not been evaluated in clinical trial studies to treat metastatic PCa, miR mimics have transitioned from the bench to the bedside. Currently, there are two phase I clinical trials evaluating the toxicity and efficacy of tumor suppressor miRNA

mimics, miRs-16 and -34, as therapeutic agents to treat advance disease in liver and non-small lung cancer [NCT01829971, NCT02369198]. These clinical trials present promise for the utilization of miRNA inhibitors as potential therapeutic agents to treat cancer as well. Adverse side effects are expected with miRNA mimics or inhibitors; however, this concern is typical for any therapeutic agent. To potentially mitigate these adverse effects of miRNA mimics or inhibitors, these therapeutic agents should be directly administered to prostate tumor cells located in the prostate and/or distant organs using a biomarker targeting system. This system would consist of the miR-186-5p inhibitor encapsulated within a bio-degradable nanoparticle attached to a substrate and/or ligand that binds to a protein and/or receptor specifically and highly expressed in prostate tumor cells. Collectively, the aforementioned proposed studies may assist with the identification and validation of miRNAs as well their inhibitors/mimics as prognostic, therapeutic and clinical management tools necessary to reduce the burden of aggressive PCa.

CHAPTER IV

IDENTIFICATION AND VALIDATION OF MIR-186 GENE TARGETS

Introduction:

MicroRNAs regulate a vast number of mRNA targets to exert their effects on cellular processes in normal and tumor cells. Commonly, miRNAs bind to the 3'UTR of mRNA transcripts. However, some studies have revealed miRNAs also bind to the 5'UTR of as well and do not rely solely on complete complement binding to regulate target genes. Aberrant gene expression profiles in PCa are partially attributed to the dysregulated miRNAs that regulate them. MiR-186 has been implicated as a tumor suppressor in oral squamous, non-small cell lung, colorectal and ovarian cancer [106-112]. However, miR-186 has also been linked to an oncogenic role in several malignancies as well [63, 99-104, 106-108, 140, 141]. Although the aforementioned reports exhibit that miR-186-5p expression is up- and/or-down-regulated in some cancers, miR-186-5p characterization *in vitro* studies in our lab (Chapters 2 and 3) support an oncogenic role for miR-186 in PCa. Elevated levels of this miRNA are associated several cancers, which further support an oncogenic function for miR-186-5p. Moreover, some aforementioned reports have identified tissue specific targets of miR-186. However, targets for miR-186-5p in PCa cells have not been validated

[63, 113]. Thus, the identification of miR-186-5p targets in PCa would provide mechanistic evidence to elucidate the potential role of miR-186-5p in PCa.

miRNA-186 and its gene targets in cancer

Several miR-186 gene targets have been identified and validated in various solid tumors. Additionally, the biological outcomes via overexpression and/or inhibition of miR-186-5p were evaluated in different cancers as well. Forced expression of miR-186 down-regulates the gene and/or protein expression of pro-apoptotic gene, $P2X_7$, tumor suppressor-related genes (NR5A2, FOXO1, AKAP12, PPM1B), glucose transporter GLUT1, PTTG1, and oncogenic genes (ROCK1, NSBP1), which are direct miR-186 targets as shown in Table 4 [100, 101, 103, 105-108]. Among the cancers that under-express miR-186-5p, several targets have been validated in the literature [106-112, 131]. For instance, overexpression of miR-186 inhibits cellular migration, invasion and glucose uptake of cancer cells via down-regulation of pituitary tumor transforming 1 (PTTG1), Rho-associated protein kinase (ROCK1) and GLUT1 expression in NSCLC and cancer associated fibroblasts, respectively [106-108]. In one study, ectopic expression of miR-186 decreased ROCK1 and PTTG1 protein expression in HEK 293T and NSCLC cell line, A549, respectively. Sun and co-workers (2014) observed that miR-186 overexpression decreases GLUT1 gene and protein expression [108]. In another study, overexpression of miR-186 resulted in a down-regulation of oncogenic-related gene, NSBP1, in HT-1376 bladder cancer cells [131].

However, miR-186 also plays an oncogenic role in several cancers (i.e., pancreatic, cervical, bladder, and endometrial) via the down-regulation of anti-cancer-related genes [100, 101, 103, 105, 130]. For instance, overexpression of miR-186 was associated with a decrease in the mRNA expression of pro-apoptotic $P2X_7$ gene in HEK 293T cells. Furthermore in HEK 293T cells, miR-186 inhibition resulted in an increase of $P2X_7$ gene expression relative to controls. In another report, ectopic miR-186 expression suppressed the protein expression of tumor suppressor, FOXO1, in endometrial cancer HEC-1B cells [100]. Whereas, inhibition of miR-186 led to an increase in the protein expression of FOXO1 and p27, a mediator of cell cycle arrest and senescence, in endometrial cancer Ishikawa cells [100, 101]. Moreover, Goeppert and associates (2010) demonstrated that overexpression of miR-186 in HEK 293 cells reduced gene expression and luciferase activity of AKAP12, a tumor suppressor in liver cancer [105]. In two other reports, miR-186-5p overexpression in pancreatic (i.e., Panc-1, BxPC-3, and MiaPaca-2) and bladder (i.e., J82) cancer cells led to the down-regulation of tumor suppressor genes, NR5A2 and PPM1B, relative to controls, respectively [103, 130]. Unfortunately, no definitive miR-186-5p targets have been validated in PCa due to limited reports [99-112, 130, 131]. Therefore, we performed experiments to define targets of miR-186-5p in PCa cells.

Cancer Type	Expression	Specimen	Target	Reference
Pancreatic	↑	tissue, cell lines	n/a	2009 Zhang et al.
	↑	tissue, cell lines	NR5A2	2015 Zhang et al.
Endometrial	↑	tissue, Ishikawa cells	FOXO1	2010 Myatt et al.
Esophageal	↑	tissue	n/a	2013 Zhao et al.
Cervical	↑	cell lines	P2X7	2008 Zhou et al.
Bladder	↑	cell lines, tissue, in vivo	PPM1B	2015 Yang et al.
Prostate	↑	tissue		2008 Ambs et al.
Prostate	↓	tissue		2014 Erdmann et al.
Non-small cell lung	↓	tissue, cell lines, tumor xenografts	CDK2, CDK6, cyclin D1	2013 Cai et al.
	↓	tissue, cell lines	PTTG1	2013 Li et al.
	↓	tissue, cell lines	ROCK1	2014 Cui et al.
Oral squamous cell carcinoma	↓	whole blood	n/a	2014 Ries et al.
Ovarian	↓	tissue, cell lines	TWIST1	2015 Zhu et al.
Bladder	↓	tissue, cell lines	NSBP1	2015 Yao et al.

Table 4. Validated Gene Targets of miR-186 in Cancer. Aberrant expression of miR-186 detected via qRT-PCR in 14 different studies for several malignancies. Six studies show miR-186-5p up-regulated in pancreatic, endometrial, esophageal, and cervical cancer. In contrast, other studies detected miR-186-5p down-regulated in non-small cell lung, oral squamous, and ovarian cancer. However, conflicting miR-186-5p expression data exists in relation to bladder and PCa.

We hypothesized based our *in vitro* studies that overexpression of miR-186-5p would lead to the down-regulation of tumor suppressor genes in PCa. AKAP12 was validated as a direct target of miR-186-5p in a metastatic PCa cell line, PC-3. In previous reports, AKAP12 has been identified as a tumor suppressor in gastric and PCa [142, 143]. Transient ectopic expression of miR-186-5p down-regulated AKAP12 endogenous protein expression in HEK 293T cells. Additionally, transient miR-186-5p inhibition in PC-3 cells lead to a slight up-regulation of endogenous AKAP12 protein. Furthermore, this tumor suppressor gene may be considered as a potential target of future therapeutic agents against PCa.

Methods:

Cell Culture

Cell lines (PC-3, RWPE1) used to analyze gene expression profiles altered by miR-186-5p overexpression and/or inhibition were previously described in Chapter 3 methods.

HEK 293T is an immortalized and transformed human epithelial embryonic kidney cell line with a SV40 T-antigen derived from a human fetus. 293T cells were sub-cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium supplemented with 10% of FBS, 1% of 200nM L-glutamine [100x] (Catalog# 25030-081, Thermo Fisher Scientific, Waltham, MA) and 1% of antibiotic [10,000 I.U./ml of penicillin, 10,000ug/ml of Streptomycin, 25ug/ml Amphoterricin B] (Catalog # 30-004-Cl, Mediatech Inc., Manassas, VA).

microRNA Isolation from cell lines

miRNA isolation from cells protocol was previously described in Chapter 3 methods.

Transient and Stable Transfection

Transient and stable transfection protocols were previously described in Chapter 3 methods.

HEK-293T cells were at a cell density of $8x10^5$ into 60 mm dishes and incubated 37^oC overnight. Cells were washed twice in PBS before transfection.

Cells were transfected using mirVana miR-186 mimic (19 nM), mirVana mimic scramble control (19 nM) (Thermo Fisher Scientific, Waltham, MA, Carlsbad, CA) according to manufacturer's instructions. In brief, a 10 µM stock solution was made for the mirVana mimic scramble control (10 pmoles/µl) and mirVana miR-186 mimic (10pmoles/µl). Cells were transfected in 60mm dishes with 50pmoles of mirVana mimic scramble control and mirVana miR-186 mimic in 1ml of Opti-MEM Reduced Serum Media (Catalog# 11058921, Thermo Fisher Scientific, Waltham, MA), JetPrime reagent and buffer (Catalog# 114-01, Polyplus Transfection, New York, NY) according to manufacturer's instructions. After a 2hr incubation at 37^{o}C, 1 ml of growth medium was added each dish. After 24 hrs, cells were used western blot analyses.

miRNA Expression

miRNA expression procedure was p previously described in Chapter 3 methods.

Human Gene Expression Array

Total RNA was isolated from a total of 24 samples described as metastatic PC-3 cells with transient (n = 3) and stable inhibition of miR-186 (n = 3), normal epithelial RWPE1 cells with transient (n = 3) and stable overexpression of miR-186 (n = 3) and their respective controls, scramble control (transient) (PC-3 n = 3; RWPE1 n = 3) and empty vector control (stable) (PC-3 n = 3; RWPE1 n = 3). Ectopic and inhibition expression of miR-186 in the cell lines were performed independently in triplicate. RNA sample purity and integrity was assessed using

the Agilent 2100 Bioanalyzer instrument. RNA quality (A_{260}/A_{280}) was evaluated using a Nanodropper spectrophotometer.

According to the instructions of the 3'IVT Plus Reagent kit (Catalog# 902416, Affymetrix Inc., Santa Clara, CA), sample and stock preparations were performed in non-stick nuclease-free tubes. Serial dilutions for the poly-A control was prepared in the following order: 1st dilution was a 1:20 dilution of poly-A control stock (2µl) pipetted into poly-A control dilution buffer (38 µl), 2nd dilution was a 1:50 dilution of the 1st dilution (2 µl) pipetted into poly-A control dilution buffer (98 µl), 3rd dilution was 1:50 dilution of the 2nd dilution (2 µl) pipetted into poly-A control dilution buffer (98 µl), 4th dilution was a 1:4 dilution of the 3rd dilution (5 µl) pipetted into poly-A control dilution buffer (15 µl) and 2 µl of the 4th dilution poly-A RNA solution was added to each total RNA (250 ng) sample. The First-Strand Master mix [x26, 3' First-Strand buffer (4 µl), 3'First-Strand Enzyme (1 µl)] was made, mixed thoroughly by gently vortexing tubes, centrifuged briefly to collect mixture at the bottom of the tubes and placed on ice. First-Strand master mix (5 µl) was added to each sample, mixed thoroughly by gently vortexing tubes and centrifuged briefly to collect mixture at the bottom of the tubes. For the first-strand synthesis reaction, samples were incubated for 2 hrs at 42^{o}C and at least 2 mins at 4^{o}C. Immediately after incubation, samples were centrifuged briefly to collect cDNA at the bottom of tubes and placed on ice for 2 mins. The Second-Strand master mix [x26, 3' Second-Strand Buffer (5 µl), 3' Second-Strand Enzyme (2µl), Nuclease-free H_2O (13 µl)] was made, mixed thoroughly by gently vortexing tubes, centrifuged briefly to collect mixture at the bottom of the tubes

and placed on ice. Second-Strand master mix (20 µl) was added to each First-Strand cDNA sample (10 µl), gently vortexed and centrifuged briefly. Samples were incubated for 1hr at 16^{o}C, 10 mins at 65^{o}C and held at 4^{o}C for at least 2 mins in a thermocycler. After incubation, samples were immediately centrifuged and placed on ice. In Vitro Transcription (IVT) master mix [x26, 3' IVT Biotin Label (4 µl), 3' IVT Buffer (20 µl), 3' IVT Enzyme (6 µl)] was prepared at room temperature, gently vortexed and centrifuged briefly. IVT master mix (30 µl) was transferred to each Second-Stranded cDNA sample, gently vortexed and centrifuged briefly to collect reaction at the bottom of tube. Samples were incubated for 16hrs at 40^{o}C and held at 4^{o}C to synthesize complementary RNA (cRNA) using a thermocycler. After incubation, samples were centrifuged briefly and placed on ice. Purification beads and cRNA were equilibrated to room temperature. Purification beads (100 µl) were added to each cRNA sample (60 µl) in 96-well plate. Each mixture was pipetted up and down 10 times and incubated for 10 mins at room temperature to facilitate cRNA binding to beads. Plate was moved to a magnetic stand to capture beads. Supernatant was carefully aspirated off beads and discarded after 5 mins. Purification beads were washed with 80% EtOH (200µl) for an incubation period of 30 secs three times. After the final wash, 80% EtOH was slowly aspirated off and discarded without disturbing the purification beads. Beads were air dried on the magnetic stand for 5 mins until no liquid is visible. Plate was removed from magnetic stand and pre-heated (65^{o}C) nuclease-free H_2O (27 µl) was added to each sample. After a 1 minute incubation, samples were pipetted up and down 10 times or until beads

were fully resuspended into solution. Plate was moved to magnetic stand for 5 mins to capture purification beads. Supernatant (eluted cRNA) for each sample was transferred to nuclease-free tubes and placed on ice. The quality and yield of cRNA was measured using a nanodrop spectrophotometer. Purified cRNA was fragmented by divalent cations and elevated temperature. Labeled cRNA (12 µg) was transferred to nuclease-free tubes and brought up to required volume (25.6 µl) with nuclease free H_2O. 3' Fragmentation buffer (6.4 µl) was added to each sample for a total volume of 32 µl, gently vortexed and centrifuged briefly. Samples were incubated for 35 mins at 94^oC and held at 4^oC for at least 2 mins in a thermocycler. After incubation, samples were centrifuged briefly and placed on ice. Agilent Bioanalyzer was used to check fragmentation of cRNA. GeneChip PrimeView Human Gene Expression arrays were removed from 4^oC storage and allowed to equilibrate to room temperature. Hybridization oven was pre-heated at 45^oC, pre-hybridization buffer was allowed to equilibrate to room temperature and 20X hybridization controls (bioB, bioC, bioD, cre) were heated at 65^oC for 5 mins in a thermocycler. For the 100 format, a hybridization master mix [x25, control oligo B2 (3nM, 3.3 µl), 20X hybridization controls (10µl), 2X hybridization mix (100µl), DMSO (20 µl), nuclease-free H_2O (40 µl)] was made to add to fragmented and biotin-labeled cRNA (10 µg) to make hybridization cocktail for a total volume of 200ul. Hybridization cocktail was gently vortexed and centrifuged briefly. Each array was processed one at a time. Array was vented from the insertion of a pipette tip into the upper right septum and filled with pre-hybridization buffer (130 µl) from the bottom left septum and placed in pre-heated

oven (45^{o}C) for 10 mins. Pre-hybridization buffer was removed from array. Hybridization cocktail was incubated for 5 mins at 99^{o}C and 5 mins at 45^{o}C in a thermocycler. After incubation, samples were centrifuged briefly. Array was refilled from the lower bottom septum with hybridization cocktail (130 µl) avoiding insoluble matter. Both septa were covered with ½" tough spots to minimize evaporation and/or leaks. Array was loaded into the hybridization oven and incubated at a rotation of 60rpm for 16hrs at 45^{o}C. Array was removed from the oven and tough-spots were removed from the array. Hybridization cocktail was extracted from the array and refilled with wash buffer A. Array was allowed to equilibrate at room temperature before washing and staining protocol. In the fluidics station, stain cocktail 1 (600 µl) was placed in stain holder 1, statin cocktail 2 (600µl) was placed on stain holder 2 and array holding buffer (800 µl) was placed in buffer holder 3. Array was washed and stained according to array type, the type of hybridization components and fluidics protocol FS450_0002. Each array was analyzed by gene chip scanner (Affy.Command console Version 3.3).

Statistical Analysis of Human Gene Expression Array

Statistical analysis of gene expression data from arrays was performed using the Partek Genomics Suite 6.6 software (St. Louis, MO). Gene expression profiles differentially expressed by 1.2-fold change with their corresponding p-value were identified in metastatic PC-3 cells and normal epithelial RWPE1 cells. Genes overexpressed in PC-3 cells and under-expressed in RWPE1 cells were

identified as potential miR-186 targets. Next, gene expression profiles were adjusted by multiple hypothesis testing using a false discovery significance level of ≤ 0.05. Principal component analyses for gene expression in the PC-3 and RWPE1 cell lines and experimental groups were performed to determine the spread between biological replicates and each experimental group.

miR-186 Target Selection

MiR-186 potential targets were selected from differential expressed genes in stable anti-miR-186 PC-3 and stable ectopic miR-186 expressing RWPE1 cells. Genes down-regulated in stable RWPE1 cells and up-regulated in stable PC-3 cells by ± 1.2 fold change. Expression profiles were adjusted for multiple hypothesis testing using the FDR cut off p-value of 0.05. Genes that survived multiple hypothesis testing were further filtered using in silico tools (MetaCore, Ingenuity, microrna.org) and validated targets in the literature to determine direct and indirect targets of miR-186. After filtering, 21 genes were selected to be potential direct targets. Also, two additional targets were selected as potential indirect target of miR-186.

mRNA Expression

Total RNA (500ng or 1µg) was reverse transcribed using qScriptTM cDNA SuperMix (Catalog# 95048-025, Quanta Biosciences). Quantitative real-time PCR (qRT-PCR) of synthesized cDNA was performed using a 2hr cycling protocol on Applied Biosystems Step Up Real or 7900 PCR system and PerfeCTa SYBR Green FastMix ROX (Catalog# 95073-012, Quanta

Biosciences) according to the manufacturer's instructions. The relative expression of mRNA transcripts were normalized to GAPDH expression and calculated using $2^{-\Delta\Delta Ct}$ method. Primers used in gene expression analysis are the following: AKAP12 (Catalog# PPH06033A, Qiagen, Germantown, MD) and TRIB3 (Catalog# PPH05870C, Qiagen, Germantown, MD). Gene expression was measured using the following cycling conditions: 10 mins at 95^{o}C for 1 cycle, 15 secs at 95^{o}C and 1 minute at 60^{o}C for 40 cycles, then melting curve 15 secs at 95^{o}C, 1 minute at 60^{o}C, +0.3^{o}C per second and 15 secs at 95^{o}C.

Western Blot Analysis

HEK 293T and PC-3 cells were seeded in 60 mm dishes. HEK 293T cells were transiently transfected with negative scramble control (19 nM) and miR-186 mimic (19 nM). PC-3 cells were transiently transfected with negative scramble control (33 nM) and miR-186 inhibitor (33nM). Whole cell protein lysates were collected 24, 48, 72, 96 hrs post-transfection in Radio-Immunoprecipitation Assay (RIPA) buffer (Catalog #R0278, Sigma Aldrich, St. Louis, MO) supplemented with 1:100 dilution of 100mM sodium orthovanadate (Catalog# 567540-5GM, Sigma Aldrich, St. Louis, MO) and 1:100 dilution of protease inhibitor cocktail (Catalog# P8340, St. Louis, MO). Protein concentrations were determined by Bradford assay (Catalog# 5000001, Bio-Rad, Hercules, CA). Cell lysates were separated by MP TGX 4-20% gels (Catalog# 4561094, Bio-Rad, Hercules, CA) and transferred to PVDF membranes (Catalog# 1704272, Bio-Rad, Hercules, CA) using the Trans-Blot Turbo system (Bio-Rad, Hercules, CA). Protein expression

of AKAP12 and TRIB3 was measured using primary monoclonal mouse AKAP12 antibody (Catalog# WH0009590M1-100, Sigma Aldrich, St. Louis, MO), primary monoclonal mouse TRIB3 antibody (Catalog# sc-390242, Santa Cruz Biotechnology, Dallas, TX), and secondary anti-mouse antibody (Catalog# 7076, Cell Signaling, Danvers, MA). Anti-B-actin (Catalog# A5316, Sigma Aldrich, St. Louis, MO) at a 1:5,000 dilution was used as a loading control. Anti-AKAP12 (1:500) and Anti-TRIB3 (1:200) were detected in cell lysate (35ug) from HEK 293T and PC-3 cells. Densitometry analysis was performed using ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland).

Results:

Differential gene expression influenced by miR-186 inhibition and overexpression

To identify potential miR-186 targets in PCa, gene expression profiles were evaluated in total RNA extracted from transiently and stably miR-186 inhibited PC-3 cells and transiently and stably miR-186 overexpressing RWPE1 cells. The PC-3 cells were chosen as a cell model for miR-186-5p inhibition for the microarray analysis due to their pronounced expression of miR-186-5p relative to normal epithelial cells (Figure 9). Also, RWPE1 cells were used as a cell model for miR-186-5p overexpression in the analysis to provide information on the dysregulation of gene profiles impacted by miR-186 in the normal prostate cellular phenotype. Gene profiles were identified as potential miR-186 target based on the following selection criteria: 1.2 fold change, false discovery rate (FDR) p-value ≤ 0.05 and unique and/or common genes in PC-3 and RWPE1 cells (Figure 22). After filtering profiles according to the selection criteria, 222 genes were identified as up-regulated by ≥ 1.2-fold change in transient miR-186 inhibited PC-3 relative to scramble control (Data not shown). Before adjusting for multiple hypothesis testing, 344 targets were down-regulated (≥ 1.2-fold change) in transient miR-186-5p over-expressing RWPE1 cells relative to scramble control (Data not shown). However, gene profiles in the transient RWPE1 cells did not survived multiple hypothesis testing. In contrast, a total of 2,898 genes were down-regulated in stable RWPE1 cells and 4009 targets were up-regulated in stable PC-3 relative to the empty vector controls (Figure 23). After filtering according to the selection criteria for 2,343 genes were down-regulated in stable RWPE1 cells and 3,454 genes were up-regulated in stable PC-3 cells relative to

empty vector (Figure 23). Gene expression profiles altered in stable PC-3 and stable RWPE1 cells were further examined due to a larger selection of genes and being more physiological cell models compared to the transient cell models. Moreover, we identified potential miR-186 targets from microarray analyses using previously validated miR-186 targets and miR-186 targets predicted by *in silico* tools (e.g., miR Base, microRNA.org, Metacore and Ingenuity). A combination of these predicted targets were used to further filter differentially expressed genes from the human gene expression arrays. Twenty-three targets were selected for further validation via qRT-PCR, and western blot analysis (Figure 24).

miR Target Selection

The microarray analysis for the physiological cell models (i.e., stable anti-miR-186 PC-3 and stable miR-186 RWPE1 cells) revealed a wealth of informative gene expression profiles. Twenty-three genes (JUN, FN1, p53, WNT5A, CASP9, FOXO3, VEGFA, c-Myc, EGR1, AKAP12, FOXG1, STAT4, CCL20, PTEN, SMAD4, WASL, PAK1, YY1, TDG, IL1RL1, TRIB3) were selected as predicted targets of miR-186 from the gene expression profiles that survived multiple hypothesis testing and further filtering on gene targets predicted by *in silico* tools (11,000 genes), validated mRNA transcript targets and biological role in cancer. Previous reports demonstrated that miR-186 directly target genes involved in tumor suppression (i.e., FOXO1, NR5A2), oncogenesis (ROCK1), stimulation of cell migration and/or invasion, cell cycle regulation (i.e., CDK2, CDK6, cyclin D1), meschymal phenotype regulation (TWIST1) and apoptosis

(i.e., P2X7) [100, 101, 103, 105-108]. These twenty-three genes included two possible indirect targets of miR-186, IL1RL1 and TRIB3, due to their significant up-regulation (8 and 11-fold) in stable anti-miR-186 PC-3 cells. Furthermore, the microrna.org database was utilized to determine the binding sites of miR-186 for all 23 genes (Data not shown). This database uses mirSVR and PhastCons scoring methods to determine potential direct gene targets of miR-186. The PhastCons score is a probability that each nucleotide belongs to a conserved element. A negative value indicates faster-than expected evolution and a positive value imply conservation (threshold minimum = 0.566). mirSVR is a regression model that assesses a weighted sum of a number of sequence and context features of the predict miRNA-mRNA duplex to aid in the prediction of target sites of 6-mer or better seed site, or a mirSVR score ≤ -0.1. (www.microrna.org). The gene and protein expression of selected potential targets were further validated via qRT-PCR and western blot analyses.

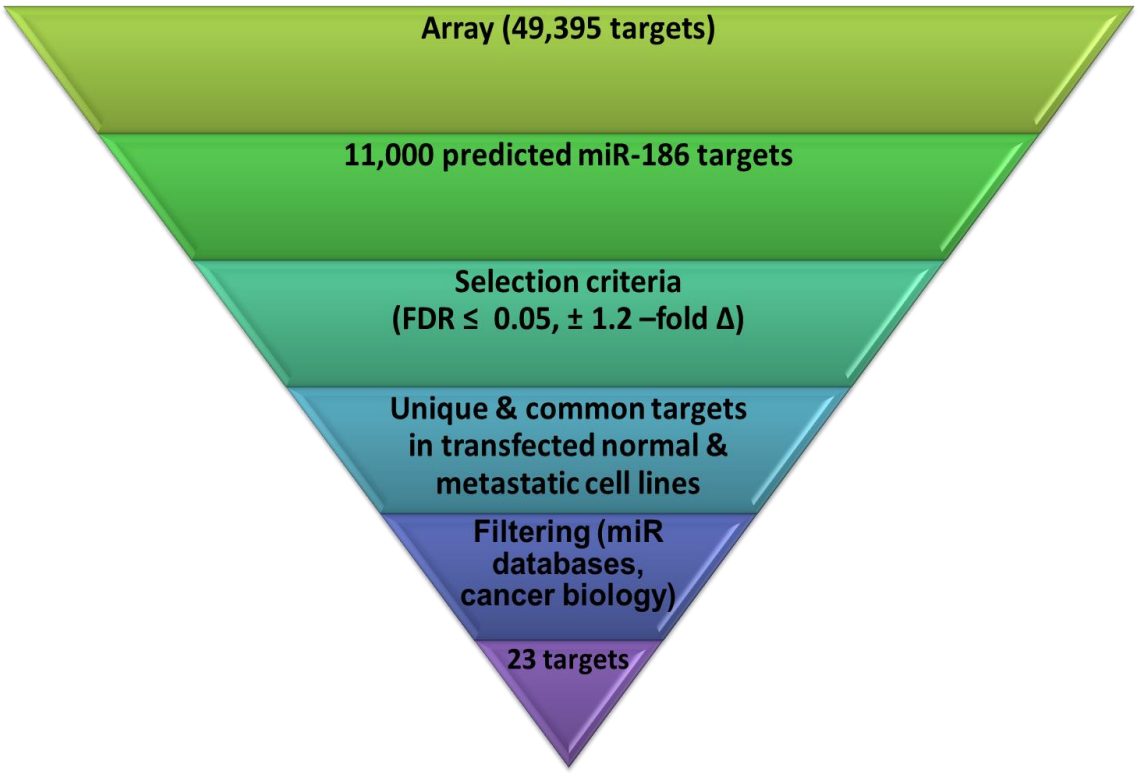

Figure 22. Gene selection criteria for miR-186 potential targets. Human GeneChip Primeview Human gene expression array contained 49,395 genes. Gene expression profiles were evaluated using the 3'UTR region of each gene via qRT-PCR. In silico tools (i.e., MetaCore, Mirbase.org, Ingenuity) were used to determine about 11,000 predicted targets for miR-186. Gene expression profiles of predicted targets that ± 1.2 fold change and false discovery rate (FDR) p-value ≤ 0.05 were identified as potential miR-186 targets in PC-3 and RWPE1 cells. Common and unique targets were identified in each cell line as well. Additional target selection was performed using identified and validated miR-186 targets in from miR databases and published reports to produce a list of 21 targets. Genes not identified as putative targets, but highly up-regulated in PC-3 cells were selected as potential indirect targets (≥ 5 fold change and FDR p-value ≤ 0.05).

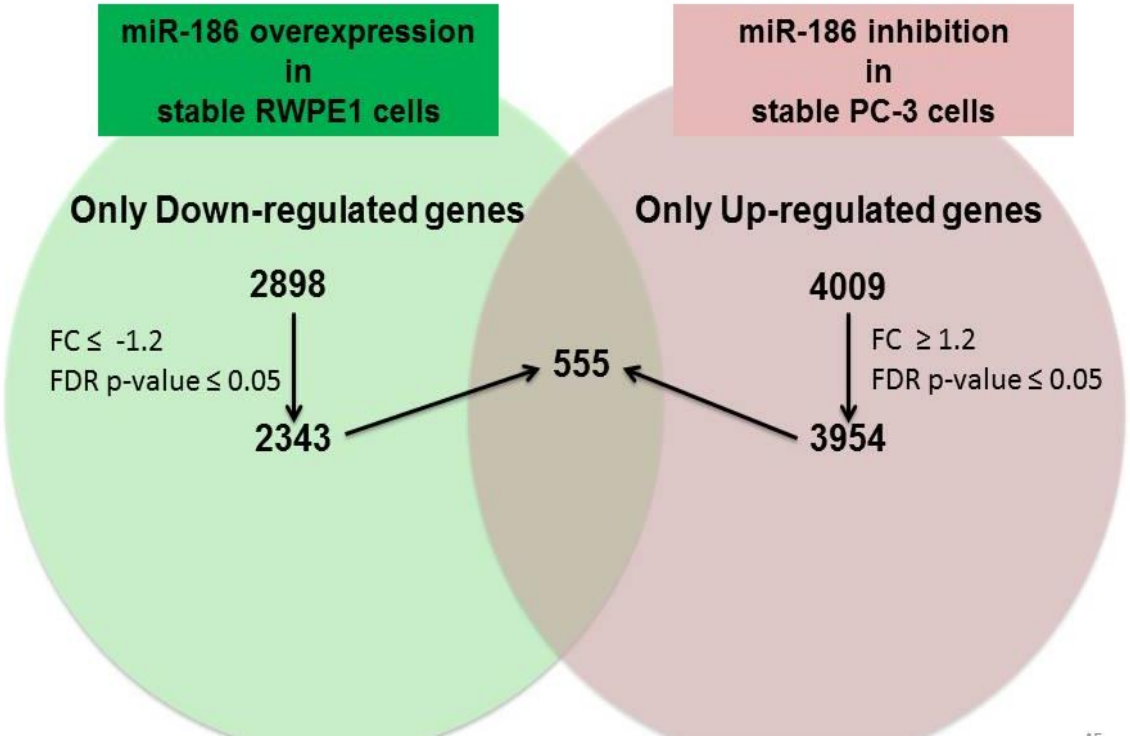

Figure 23. Identification of potential miR-186 targets in PCa. Gene expression profiles of 50,000 targets in stable miR-186 overexpression in RWPE1 cells and stable miR-186 inhibition in PC-3 cells were evaluated by human gene expression Affymetrix microarrays. The down-regulation of 2343 differentially expressed gene targets were identified in RWPE1 cells relative empty vector. In stable PC-3 cells, 3454 gene targets were up-regulated relative to empty vector. There were 555 common genes identified as potential targets of miR-186 between PC-3 and RWPE1 cells.

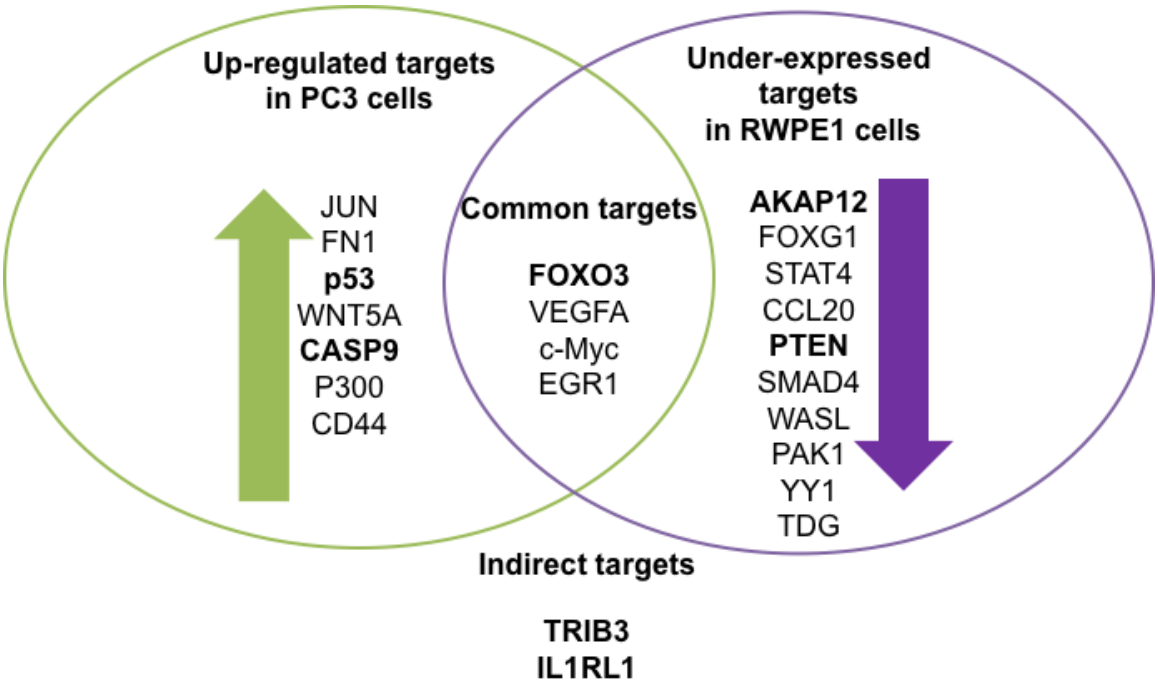

Figure 24. Gene list of predicted direct targets of miR-186. In the microarray analysis, a total of 21 targets (JUN, FN1, p53, WNT5A, CASP9, FOXO3, VEGFA, c-Myc, EGR1, AKAP12, FOXG1, STAT4, CCL20, PTEN, SMAD4, WASL, PAK1, YY1, TDG) were identified as differentially expressed genes due to alternate miR-186-5p expression based on the gene selection criteria (± 1.2 fold change and false discovery rate (FDR) p-value ≤ 0.05). The above common and unique gene targets in PC-3 and RWPE1 cells were identified as direct miR-186 targets via in silico tools (Ingenuity, MetaCore, www.microrna.org, MirBase). In addition, we identified as a potential indirect miR-186-5p target, TRIB3, due to its significant up-regulation (8-fold) in stable PC-3 cells.

AKAP12 is a direct target of miR-186 in metastatic PCa

Only a select number of miR-186 targets (p53, PTEN, CASP9, FOXO3, AKAP12, PTEN, TRIB3, IL1RL1) were validated from the microarray analysis based on time and budget constraints. Gene expression of potential miR-186 targets were analyzed in miR-186-5p inhibited PC-3 cells and miR-186-5p overexpressing RWPE1 cells. PC-3 cells were transiently transfected with miR-186-5p inhibitor (33 nM) and scramble control (33 nM). RWPE1 cells were transiently transfected with miR-186-5p mimic (19 nM) and scramble control (19 nM). Cell lysates were collected 24, 48, 72, and 96 hrs post transfection. Total RNA was isolated from cells at the indicated time points using the mirVana microRNA isolation kit. Total RNA was reverse transcribed into cDNA and expression of miR-186-5p and selected genes were validated by qRT-PCR. Next, whole cell protein lysates from transient miR-186-5p inhibited PC-3 cells and miR-186-5p overexpressing HEK 293T cells were collected 24, 48, 72, 96hrs post transfection to measure the endogenous protein expression of gene targets. No significant difference was detected in the transcript expression of p53, CASP9, FOXO3, PTEN, and IL1RL1. The gene expression of pro-apoptotic gene, TRIB3, was up-regulated after 48 hrs post-transfection in metastatic PC-3 cells relative to scramble control (Figure 25A). However, this up-regulation was not significant. Tumor suppressor, AKAP12, was up-regulated by 1.78-fold 72 hrs post-transfection in PC-3 cells relative to scramble controls (Figure 25B). Additionally, miR-186 has three different binding sites on the AKAP12 transcript (Figure 26). In stable anti-miR-186 PC-3 cells, AKAP12 gene expression was up-regulated by 1.6-fold relative to empty vector (Figure 27A). Furthermore, AKAP12

transcript expression was extremely under-expressed in metastatic MDA PCa 2b and LNCaP cells relative to normal epithelial RWPE1 cells (Figure 27B). However, AKAP12 was up-regulated by 2-fold in PC-3 cells relative to RWPE1 cells (Figure 28B). Although AKAP12 was up-regulated in PC-3 cells, miR-186-5p expression in PC-3 cells (16-fold) is 8 times more than the expression of AKAP12 expression.

To determine the effect of miR-186-5p expression on the endogenous protein of AKAP12 and TRIB3, immunoblotting analyses were performed in HEK 293T and PC-3 cells. HEK-293T cells were transiently transfected with miR-186 mimic (19 nM) and scramble control (19 nM) for 24-72 hrs. After 72hrs post transfection, AKAP12 protein expression was decreased in HEK 293T cells (Figure 28A). Transient miR-186-5p inhibition in PC-3 cells with miR-186 inhibitor (33nM) and scramble control (33 nM) resulted in the slight up-regulation of AKAP12 protein expression in PC-3 cells 48hrs post-transfection relative to scramble control. Unfortunately, TRIB3 exhibited no protein expression changes in HEK293T and PC-3 cells (Figure 28B).

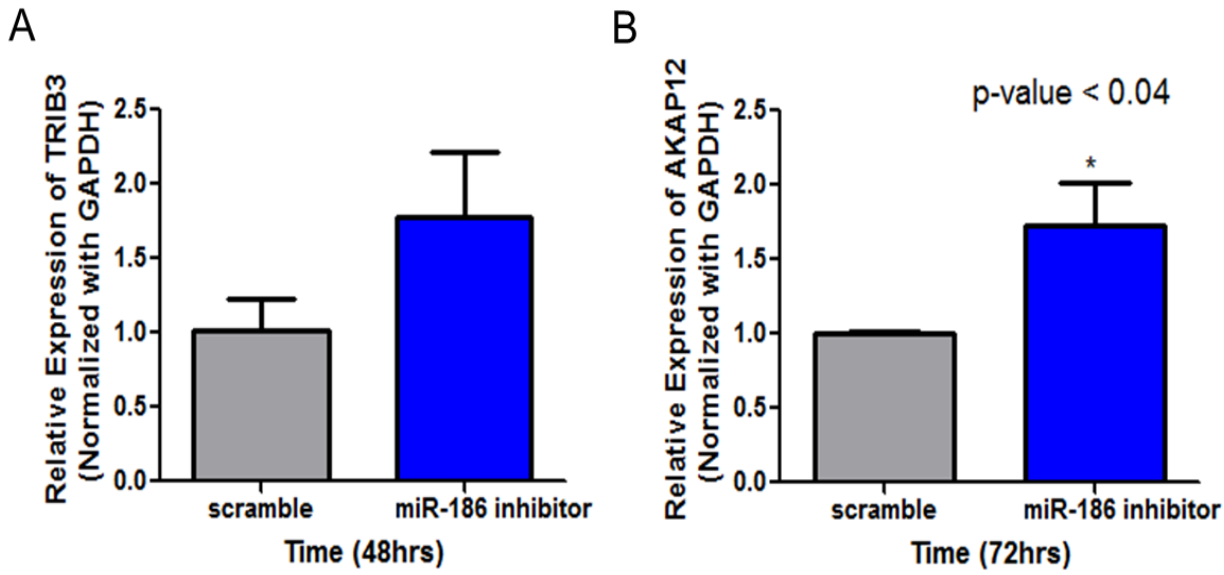

Figure 25. Expression of potential miR-186 targets, TRIB3 and AKAP12, in metastatic PC-3 cells. Transiently transfected PC-3 cells with miR-186 inhibitor and scramble negative control. Cell lysates were collected 24, 48, 72, 96 hrs post-transfection and total RNA was isolated from lysates using mirVana microRNA isolation kit. TRIB3 and AKAP12 gene expression in PC-3 cells was examined by qRT-PCR. A) Pro-apoptotic gene, TRIB3, expression was up-regulated by 1.72-fold increase after 48 hrs relative to negative control (p-value = 0.0769). B) Tumor suppressor AKAP12 gene expression was up-regulated 1.78-fold increase after 72 hrs relative to negative control (p-value = 0.0346). Data was quantitated from at least three independent experiments. Statistical analysis was performed using an Unpaired Student's T test and significance cut-off p-value ≤ 0.05.

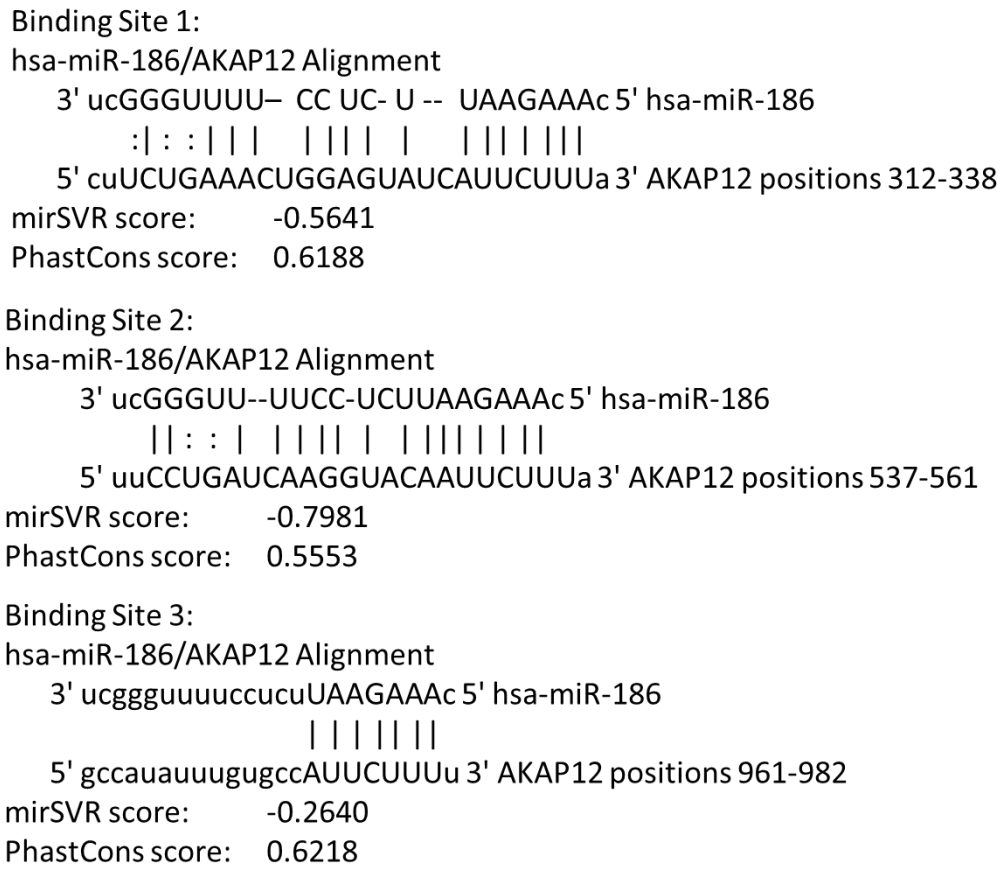

Figure 26. Predicted binding sites for hsa-miR-186 on the AKAP12 transcript. Mir-186 has three potential binding sites for the AKAP12 gene as predicted by *in silico* tool www.microrna.org. mirSVR score is the result of a regression model that computes a weighted sum of a number of sequence and context features of the predicted miRNA::mRNA duplex [144]. Context features are divided into duplex, sequence and global. The duplex features include base pairing at the seed region, and 3'end of the miRNA. The sequence features include A/U composition near the target sites and secondary structure accessibility. Global features are the length of the UTR, relative position of the target site in the UTR and conservation score. PhastCons score is a probability that each nucleotide belongs to a conserved element. A negative value indicates faster-than expected evolution a positive value imply conservation (Threshold minimum = 0.566) (www.microrna.org).

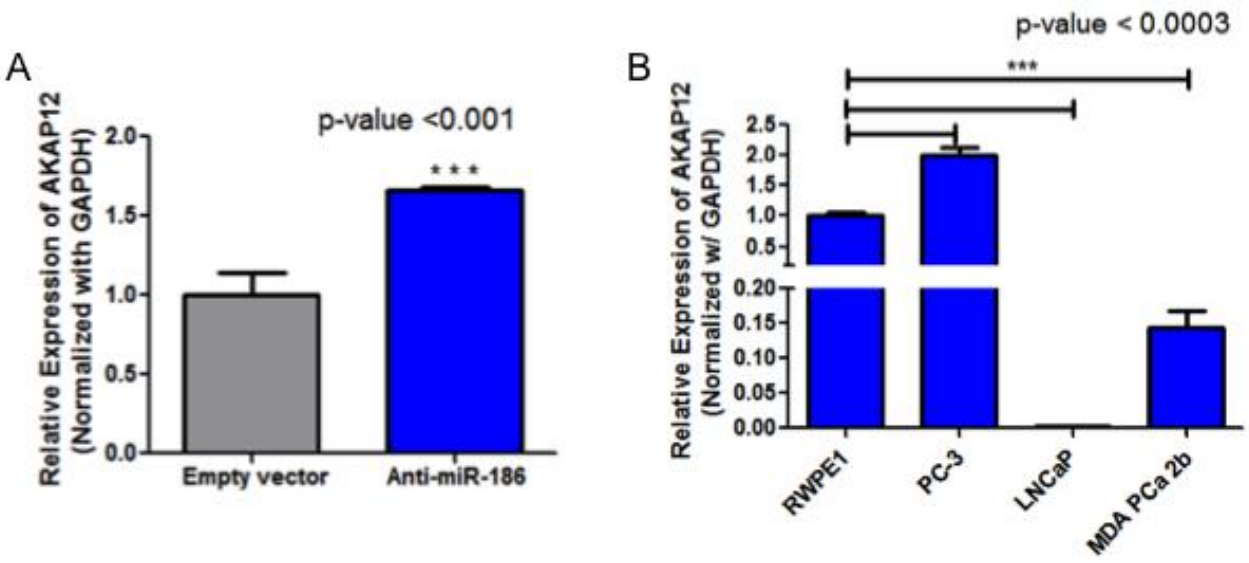

Figure 27. Endogenous AKAP12 expression in stable anti-miR-186 PC-3 cells and metastatic PCa. Total RNA was isolated from cell lysates collected from stable anti-miR-186 PC-3 cells and normal epithelial and PCa cells using mirVana microRNA isolation kit. A) AKAP12 expression was up-regulated by 1.66-fold change in stable PC-3 cells relative to empty vector (p-value= 0.0006). B) Relative to RWPE1 cells, AKAP12 was under-expressed in androgen sensitive metastatic PCas cells (i.e., LNCaP, MDA PCa 2b). However, AKAP12 was up-regulated by 2.0 in PC-3 cells. Data was quantitated from at least three independent experiments. Statistical analysis was performed using an Unpaired Student's T test and significance cut-off p-value ≤ 0.05.

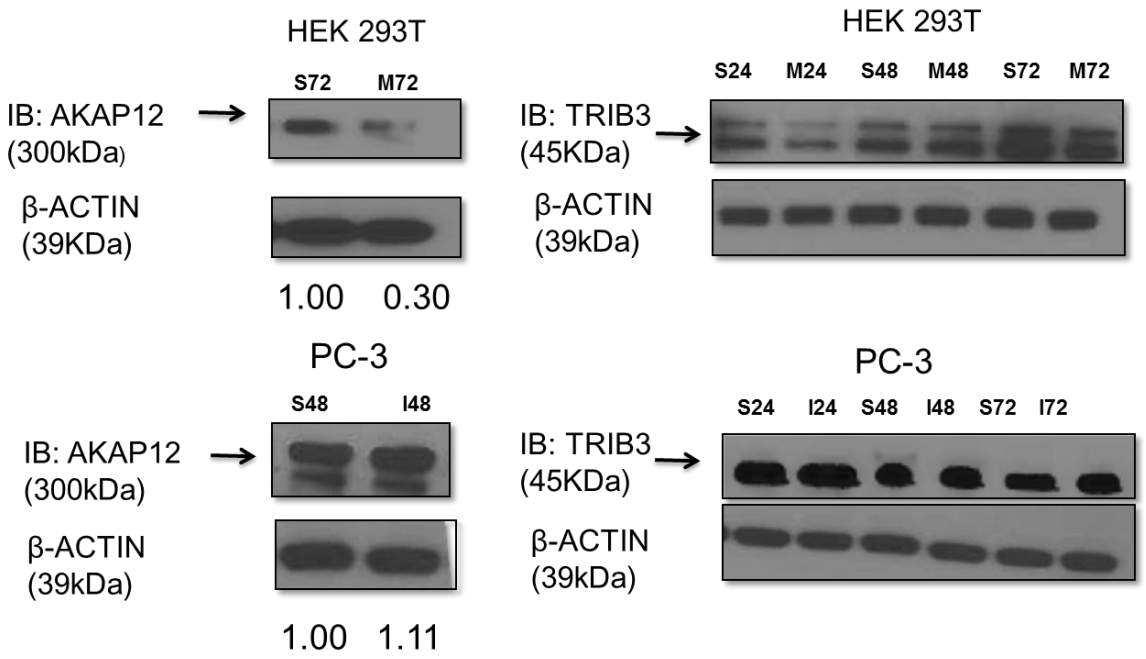

Figure 28. Protein expression of AKAP12 and TRIB3 in 293T and PC-3 cell lysates. A) Hek-293T cells were transiently transfected with miR-186 mimic (19 nM) and scramble control (19 nM). Protein expression of AKAP12 was decreased (70%) to scramble 72 hrs post-transfection. Next, TRIB3 protein expression was examined in HEK 293T cells, however no significant difference was detected between scramble and miR-186 mimic. B) PC-3 cells were transiently transfected with miR-186 inhibitor (33nM) and scramble control (33 nM). AKAP12 protein expression was slightly up-regulated by 11% in PC-3 cells 24 hrs post-transfection relative to scramble. However, no difference was detected for TRIB3 protein expression. Data was quantitated from at least two independent experiments. Densitometry analysis was performed using ImageJ software.

Discussion:

Nearly 2,000 human miRNAs promiscuously regulate 30-60% of gene expression, which proposes a great dilemma for miRNA target selection and validation. Although multiple miRNAs may regulate the same mRNA transcript, the utilization of *in silico* tools (i.e., MetaCore, Ingenuity MirBase, TargetScan and MicroRNA.org), deep sequencing technologies and transient/stably transfected cell models aid in the identification of direct miRNA targets. In the current study, microarray analyses, *in silico* tools, specific statistical parameters, published reports and PCa *in vitro* studies in Chapter 3 were utilized to identify 23 putative miR-186 targets using physiological stable miR-186 overexpressing RWPE1 and miR-186 inhibited PC-3 cell models. Although over 2,000 targets were either up-regulated or down-regulated in the transfected RWPE1 and PC-3 cell lines, respectively, we only focused on 23 targets due to their biological function in cancer as well as time and budget constraints. To our knowledge, our lab is the first to demonstrate AKAP12 as a direct target of miR-186-5p in a metastatic PCa cell line (PC-3). Following miR-186 inhibition, we demonstrated AKAP12 protein was up-regulated by 11% in the PC-3 cells. Although this up-regulation of AKAP12 was modest, AKAP12 is known to be hypermethylated in a number of cancers including prostate [145-151]. It is possible this may partially attribute to the modest up-regulation of AKAP12 in PC-3 cells.

AKAP12 (A-kinase anchor protein 12) also known as the mouse homolog SSecKS functions as a scaffold protein in signal transduction that

associates with protein kinases A (PKA) and C and phosphatase [152]. Specifically, it binds to the regulatory subunit of protein kinase A and presumably alters various cancer hallmarks [143, 153-155]. Previously, several investigators demonstrate the down-regulation of AKAP12, a tumor suppressor, in several cancers, including prostate [145-151]. Overexpression of AKAP12 *in vitro* corresponds with a decrease in cell invasion, and anchorage independent growth of mouse PCa cell models, MLL [143, 153]. Furthermore, AKAP12 knockout *in vivo* resulted in prostatic hyperplasia [156]. However, additional studies are warranted to elucidate the impact of loss of AKAP12 on tumor growth and cellular behavior using PCa cell models. Also, AKAP12 was identified as a direct target of miR-186 in only one other report other than the current study. A study by Goppert et al reported a down-regulation of the AKAP12 transcript due to miR-186 overexpression in HEK 293T cells [105].

In the current study, we demonstrate miR-186-5p overexpression down-regulates endogenous AKAP12 protein for the first time to our knowledge. Additionally, miR-186-5p inhibition in metastatic PC-3 cells resulted in an up-regulation of the AKAP12 transcript and endogenous protein as well. We speculate AKAP12 may partially, but not exclusively mediate the biological effects of miR-186 on tumor behavior. Therefore, additional studies are needed to identify other miR-186-5p targets that may contribute to the effect of miR-186 inhibition on cell proliferation, anchorage independent growth and invasion. Collectively, the findings of our study and other studies suggest miR-186-5p expression impacts the tumor aggressive phenotype.

Fourteen studies have identified and validated miR-186s as either an oncomiR or tumor suppressor in several different cancers (Table 3). It's specific role in cancer appears to be tumor specific. Seven out of 14 reports suggest miR-186-5p plays a tumor suppressor role and targets oncogenic-related genes for which cancers [106-112, 130, 131]. For instance, two independent studies revealed ectopic expression of miR-186-5p in NSCLC cell models has led to the down-regulation of oncogenic ROCK1 and motility promoting PTTG1 protein expression [106, 107]. However in both studies, the β-actin loading controls (i.e., b-actin, GAPDH) were not evenly distributed comparing the negative control and miR-186 over-expressing cell lines. In contrast, six studies support an oncogenic role for miR-186-5p and have validated tumor-suppressor-related genes as miR-186-5p targets as well. Overexpression of miR-186-5p resulted in the repression of tumor suppressors, FOXO1, N5RA2, and PPM1B in endometrial, pancreatic and bladder cancer cell models [100, 105, 130]. Additionally, these targets have been shown as down-regulated at same time miR-186-5p was up-regulated in tumor tissue relative to normal tissue.

We have considered the limitations, strengths and future directions of this study. Although we hypothesized that primarily tumor suppressor genes would be altered by oncogenic miR-186, a mixture tumor suppressor and oncogenic-associated genes were affected by miR-186 expression. Microarray analyses revealed ectopic expression of RWPE1 cells led to the down-regulation of tumor suppressor genes, namely (i.e., PTEN, FOXO3, AKAP12, FOXG1, SMAD4),

transcription factor (YY1), pro-angiogenic (VEGFA), oncogenic (c-Myc) and chemokine (i.e., CCL20) genes. In contrast, miR-186 inhibition led to the up-regulation of tumor suppressor (i.e., FOXO3, p53), oncogenic (i.e., JUN, P300, c-Myc), cell adhesion (i.e., WNT5A), cytokine (i.e., IL1R1), pro-apoptotic (i.e., TRIB3) and cellular death-related (CASP9) genes. We speculate that the up-regulation and down-regulation of oncogenic genes in both PC-3 and RWPE1 cells may represent a residual effect of normal epithelial and tumor cells attempting to retain their original phenotype. For instance, well known oncogenes, c-Myc and JUN, were slightly up-regulated in stably miR-186 inhibited PC-3 cells. We speculate up-regulation of these oncogenes may trigger a survival mechanism to help this metastatic PC3 cell line retain its tumor phenotype. Unfortunately, in our study the up-regulation tumor suppressor genes involved in cell proliferation, anchorage independent growth and cellular invasion apparently outweighed the tumor promoting properties of up-regulated oncogenes. In the case of the over-expressing miR-186 normal epithelial cell lines, we evaluated whether miR-186 over-expression or inhibition would modulate a limited number of mRNA transcripts (n = 11,000). However, future analyses of the entire human transcriptome may elucidate novel miR-185-5p targets using RNA-seq or next generation sequencing of transfected PCa cell models and micro-dissected tissue-based studies.

Based upon the aforementioned reports and our study findings, AKAP12 may be a potential therapeutic target against PCa using pre-clinical studies. Based on the direct impact of AKAP12 on cell proliferation, invasion and

anchorage independent growth [143, 153-155], future studies may assess whether ectopic or knock-in AKAP12 expression alone and/or in combination with chemopreventive and/or chemotherapeutic agents may inhibit prostate tumor growth and metastasis. Ultimately, we speculate that ectopic expression of AKAP12 will reduce tumor growth and the metastatic potential of prostate tumor cells as well as demonstrate a synergistic interaction with PCa therapeutic agents. In closing, miRNAs may serve as valuable tools to find potential therapeutic targets for the effective monitoring and treatment of aggressive PCa.

CHAPTER V

DISCUSSION, STRENGTHS AND LIMITATIONS AND CLINICAL RELEVANCE

Discussion

Aberrant expression of miRNAs is commonly associated with various malignancies, including PCa. MiRNAs regulate a vast number of cancer-related genes that regulate cell survival, proliferation, differentiation, migration, epithelial mesenchymal transition, immune surveillance, angiogenesis, invasion and metastasis [53, 54]. Their expression is not only detectable in mammalian cells and tumor tissue, but also biological fluids (e.g., serum, plasma, urine) [52-54, 117, 121]. Consequently, miR expression in biological specimens offers an opportunity to find and validate new clinical biomarkers as ideal cancer diagnostic, prognostic and ultimately clinical management tools.

The primary objectives of the current study were to identify, validate and characterize an oncogenic miRNA, miR-186-5p, using serum and cancer cell models collected from PCa patients and appropriate controls. We assessed whether 377 miRNAs were differentially expressed comparing non-cancerous controls to PCa patients with non-metastatic and bone-specific metastatic disease using Taqman array and qRT-PCR data. Out of the

377 miRNAs, miRNA-106b-5p and -186-5p were up-regulated in the serum of European American men diagnosed with PCa (tumor stage I, III, IV) relative to disease free individuals. Since the literature on the role of miR-186 and PCa was understudied relative to miR-106b, we focused on the quantitation and characterization of miR-186 in immortalized PCa cell models and normal epithelial cells. Relative to normal epithelial cells, miR-186 was significantly up-regulated in a few metastatic PCa cell lines (i.e., PC-3, MDA PCa-2b and LNCaP), especially the PC-3 cells. In addition, inhibition of miR-186-5p in PC-3 and MDA-PCA-2b cells resulted in the reduction of cell proliferation, colony formation and cell invasion by 27-66% relative to appropriate controls for transient transfections. In terms of a putative miR-186 target, we demonstrate inhibition of miR-186 in PC-3 cells corresponds with an upregulation of a tumor suppressor gene AKAP12 based on micro-array data, quantitative real time PCR and western blot analysis. These findings were further confirmed upon ectopic expression of miR-186, which led to down-regulation of AKAP12 in HEK 293T cells. Lastly, previous empirical, text mining or *in silico* studies suggest AKAP12 is a likely miR-186 target based on bioinformatics analysis, immunohistochemistry staining, micro-array, qRT-PCR western blot and a luciferase reporter assay [105] (mirtarbase.mbc.nctu.edu; microrna.org).

There is a lot of controversy surrounding the role of miR-186 in relation to tumorigenesis. Six published studies suggest miR-186 has tumor suppressing potential; where as other studies indicate an oncogenic role [106-112]. Six other

studies propose miR-186 has oncogenic potential. MiR-186 expression is up-regulated in endometrial, pancreatic, esophageal, cervical, head/neck and non-melanoma skin cancer [99-104]. Controversy for miR-186 also exists for PCa [63, 113]. In 2008, Ambs et al. observed miR-186 expression was up-regulated in PCa relative to controls; whereas, Erdmann and co-workers (2014) indicate miR-186 was down-regulated in PCa patients with non-organ confined PCa and metastases. Neither of these two studies characterized the role of miR-186 in PCa using pre-clinical models. We addressed this gap in the literature by inhibiting the expression of miR-186 in metastatic PCa cell lines and monitoring the effects on several aggressive cancer phenotypes. Commensurate with the Ambs et al. report, our results are consistent with miR-186's role as an oncomiR. In short, inhibition of miR-186 leads to a reduction of cell proliferation, colony formation and invasion as well as re-expression of a tumor suppressor gene, (i.e., AKAP12), which is intimately involved in the aforementioned transformative tumor processes.

AKAP12 as a miR-186 gene target based on empirical and *in silico* evidence from published reports and our own studies

AKAP12 has been identified as a putative target of miR-186 in two studies including this study. Additionally, we observed an increase in the transcript and protein expression levels of AKAP12 after miR-186-5p inhibition in metastatic PC-3 cells. Our findings and published reports on AKAP12 taken together

suggest AKAP12 may be partially responsible for the suppression of proliferation, invasion and anchorage independent growth in PC-3 cells [143, 153-155].

The Role of AKAP12 in Tumorigenesis

Based on our data, several published reports and *in silico* evidence, we speculate AKAP12, a miR-186 gene target, may contribute to the observance of significant reductions in cell proliferation, anchorage independent growth and cell invasion in MiR-186 inhibited metastatic PCa cell models. AKAP12 (a.k.a., SSeCKs), a major protein kinase C substrate, is a tumor suppressor gene that is down-regulated in many solid tumors, including PCa [145-151]. Several independent studies demonstrate AKAP12's ability to suppress cancer phenotypes such as cell proliferation, anchorage independent growth, and cell invasion [143, 153-155]. Transient co-expression of AKAP12 significantly reduced colony formation and focus formation in v-Src NIH3T3 cell lines (genetically modified to have enhanced anchorage independent growth) [154, 155].

Six out of seven published reports indicate AKAP12 plays a role in cell proliferation [145, 152, 156-160]. Early passaged AKAP12 -/- or knock out MEFs cells exhibited a higher cellular proliferation than wild type MEFs [158]. However in later passages, AKAP12 KO MEFs lost capacity to proliferate and underwent pre-mature senescence due to the hyperactivity of PKC α isoform, which induces $p16^{Ink4a}$/Rb via the mitogen-activated protein kinase kinase 1 (MEK) signaling pathway. In another study, overexpression of AKAP12 significantly reduced cell proliferation in S2-6 cells (i.e., equine fibroblasts transformed with the BPV-1

genome), after 6 -10 days relative to Tet-regulated S2-6 clone [157]. This overexpression of AKAP12 also leads to G1 cell cycle arrest via down-regulation of the cyclin D transcript. Moreover, AKAP12 null mice had higher levels of cellular proliferation within the anterior and ventral prostatic lobes relative wild type mice [156]. Although higher levels of cell death within the anterior prostatic lobe were detected in knock out mice, the number of newly proliferated cells far exceeded the apoptotic cells.

Two studies suggest ectopic expression of SSeCKS (AKAP12 in humans) reduces tumor invasion *in vitro* [143, 153]. In one study, ectopic expression of SSeCKS in v-Src NIH3T3 cells (genetically modified to have enhanced aggressive cancer behavior) significantly reduced the cellular invasion of NIH3T3 cells relative to v-Src NIH3T3 cells with basal levels of AKAP12 via RhoA- and Cdc42-Dependent Pathways [153]. Notably, activated RhoA and Cdc42 could reverse SSeCKS suppression of cytoskeletal architecture. In another study, re-expression of SSeCKS in MAT-LyLu (MLL) PCa cell line derived from rodents decreased the chemotaxis and cell invasion through a matrigel by 4-6-fold relative to MLL cells with endogenous levels of SSeCKS [143]. This increased invasive potential mediated through overexpression of AKAP12 in MLL cells is primarily dependent on MMP-2 expression and to a lesser extent MMP-9. Treatment of MML cells with MMP inhibitor, GM6001, reduces cellular invasion and the activity of MMP-2/9 [143]. Moreover, SSeCKS re-expression reduces chemotaxis, cell invasion and MMP-2 transcript levels in MML cells. Matrix

metalloproteinases (MMPs), including MMP2, play a role in the degradation of the extracellular matrix proteins, necessary for the initiation of aggressive cellular behaviors, including cell proliferation, migration, invasion, differentiation, angiogenesis and apoptosis. The SSeCKS mediated reductions in chemotaxis and cell invasion appear to be influenced by SSeCKS's inhibitory effect on MEK, which in turn blocks MEK/ERK signaling activation and subsequently MMP2 transcription. SSeCKS appears to regulate MEK/ERK signaling via inhibition of PKC-induced activation of Raf, presumably through direct scaffolding of PKC by SSeCKS [143]. The influence of SSeCKS (AKAP12) on aggressive cancer behavior can be reversed by co-expressing CA-MEK1, MEK2 or ERK2 in constitutively SSeCKS expressing MLL cells, which increases chemotactic motility. Re-expression of SSeCKS in MLL cells does not appear to alter RNA levels of MMP-14 and TIMP-2, which are both required for MMP-2 activation [143]. Collectively, these findings suggest SSeCKS potentially inhibit motility parameters related to metastasis, presumably by deactivating Raf/MEK/ERK pathways, which result in the down-regulation of MMP-2.

Commensurate with the aforementioned *in vitro* studies, our results demonstrate that transient inhibition of miR-186 leads to an increase in AKAP12 transcript expression as well as a decrease in cell proliferation (PC-3, MDA PCa 2b), anchorage dependent growth (PC-3, MDA PCa 2b) and cell invasion (PC-3, MDA PCa 2b) in metastatic PCa cells. The influence of miR-186 inhibition on cellular behavior was more pronounced for the PC-3 cells and MDA PCa 2b cells

derived from European-American and African-American men diagnosed with bone-specific metastatic PCa, respectively. Interestingly, miR-186 inhibition did not significantly influence anchorage independent growth of the MDA PCa 2b cells unless cells were treated with DHT. This suggests androgen sensitivity may increase susceptibility of cells to the tumor suppressing effects of miR-186-5p inhibition. This androgen receptor-mediated change in anchorage independent growth for MDA PCa 2b cells did not reach the level of significance observed for miR-186-5p inhibited PC-3 cells. Although androgen-insensitivity may contribute toward the cancer cell lines responsivity to miR-186 inhibition, we cannot rule out the possibility of other genomic differences between these two cell lines. We speculate the presence of a mutant P53 and null PTEN tumor suppressor genes within PC-3 may make this cell line more dependent on miR-186's oncogenic potential to maintain a metastatic phenotype. Future studies will test this hypothesis by inhibiting or re-expressing P53 and/or PTEN within PC-3 cells, respectively.

Limitations and Strengths

We have considered the strengths, limitations and future directions of the current study. The current study demonstrates that over expression of miR-186-5p in serum collected from PCa patients relative to controls, even in the presence of a small sample size. Our limited sample size may have compromised our capacity to detect other oncomiRs that were differentially expressed in serum from PCa patients. Future studies are needed to confirm up-regulation of miR-

186 in serum and matched micro-dissected PCa tissue specimens collected from ethnically/racially diverse populations. Such studies require appropriate sample sizes to have adequate statistical power to detect significant differences in miR-186 expression comparing lethal and non-lethal PCa.

Moreover, we may have limited our capacity to identify additional oncomiRs, since we did not include an acceptable internal control (i.e., *C. elegans* miR-39) within the Taqman array cards designed to analyze 377 miRs. We attempted to address this issue using a global normalization technique. Global normalization is a technique that takes the median Ct value of all miRNA profiles and screens for differentially expressed miRNAs that are down- or up-regulated relative to the median Ct values. Although global normalization led us to two important oncomiRs detected in our cell models transiently transfected with a miR-186 inhibitor, global normalization can lead to false positives and false negatives. For instance, miR-186 upregulated in serum collected from PCa patients relative to disease-free individuals following normal globalization of the array data; however, this same miR was down-regulated during validation using qRT-PCR and normalization with an external control (i.e., *C. Elegans* miR-39). To minimize discordant findings attributed to varied normalization methods, we will add miR-39 as an external control prior to serum processing and analysis using taqman array and qRT-PCR. However, given what we learned about serum-based miRNAs, miR-39 is an appropriate control. Since there is no appropriate internal control for serum-based miRNAs, we used a non-human derived miRNA (miR-39) for our qRT-PCR validation of miR-186, prior to the

isolation of total RNA and conversion of RNA to cDNA [52]. *C. Elegans* miRNAs (e.g., miR-39) may serve as ideal external controls for the analysis of serum-based human miRNAs because they help to account for any miRNA losses during biospecimen processing [52, 122]. The use of miR-39 was ideal for our serum based analysis, since C. elegans miR-39 is not detected in humans and is unaffected by disease states.

Despite the aforementioned limitations, we demonstrated miR-186-5p was significantly up-regulated in three metastatic PCa cell lines (i.e., PC-3, MDA PCa 2b and LNCaP cells) relative to normal epithelial cells. More importantly, we established a link between miR-186 and several hallmarks of cancer (i.e., proliferation, anchorage independent growth and invasion). Inhibition of mIR-186-5p reduced cellular proliferation, anchorage independent growth and cell invasion in metastatic PCa cells. This decrease in aggressive cancer behavior may be partially attributed to up-regulation of AKAP12 via miR-186 inhibition. However, the up-regulation of other miR-186-5p gene targets may also contribute this decrease in aggressive behavior as well. Our data demonstrates an over-expression of AKAP12 following inhibition of miR-186 *in vitro*. Since AKAP12 appears to regulate cellular proliferation, anchorage independent growth, and cell invasion in other cancers, we speculate its loss in PCa may contribute toward enhanced cellular proliferation, hyperplasia and ultimately prostate tumorigenesis. However, additional studies are needed to assess whether ectopic expression of AKAP12 leads to a decrease in cell proliferation, anchorage independent growth, and cell invasion using various PCa pre-clinical

studies. In addition, future pre-clinical or human tissue-based will assess whether AKAP12 expression levels are inversely related to markers involved in cellular proliferation (e.g., e-Cadherin, PTK2), anchorage independent growth (e.g., PTK2), and cell invasion (e.g., MMP-2, MMP-9) in cell models with ectopic expression of AKAP12 or immunohistochemistry staining of cancerous tissue derived from animal models or humans. In addition, we will assess whether over expression of AKAP12 will inhibit tumor growth and metastasis using a PCa animal model.

In closing, we added new insights toward a definitive role of miR-186 in PCa. Commensurate with previously mentioned studies [143, 145, 152, 153, 156-160], we provide convincing data that reveals miR-186 inhibition leads to up-regulation of AKAP12 as well as a 27-46% reduction of cell proliferation, anchorage independent growth and invasion in metastatic PC-3 and/or MDA PCa-2b cells. Ultimately, our study findings serve as a foundation for future studies focused on the identification and characterization of a new miRNA biomarkers needed to distinguish between lethal and non-lethal PCa. In addition, we offer guidance toward potential targets (e.g., AKAP12) that can be tested as a therapeutic target for the effective treatment of aggressive PCa. These efforts, if successful, may help to reduce the burden of PCa among all men.

Clinical Relevance

MiRNAs differentially expressed in cancer are steadily beginning to be accepted as potential prognostic and diagnostic tools in PCa. Due to their regulation of a vast number of gene targets and stable expression in biological

fluids and tissue, miRNAs show great promise as potential biomarkers to aid current screening tools. Clinical trial studies have yet to identify a procedure that can completely replace the PSA test and digital rectal exam.

PCa detection has been solely dependent on procedures such as the PSA test, digital rectal exam (DRE) and tumor biopsies. Unfortunately, alone the PSA test is not a reliable tool in disease due to various biological stimulants [39, 40, 44, 46, 161]. The DRE is an invasive technique that also is insufficient alone to detect PCa [40, 44, 46]. The combinatorial effort of both tools do not drastically improve detection and can lead to false positive test results. Consequently, there is a great need for biomarkers to aid in PCa detection and improve the overall accuracy of these tools. To definitively diagnose PCa, physicians must collect a biopsy from the prostate for histological evaluation. Tumor biopsies are very invasive for patients and associated with urological problems. The measurement of miRNA expression in routinely collected biological fluids may serve as an alternative to these extremely invasive procedures.

The research presented in this dissertation is clinically relevant due to the number of phase I and II clinical trials evaluating the miRNA expression in various malignancies including PCa [NCT01220427, NCT02366494, NCT01503229, NCT02471469, NCT01444820, and NCT02391051]. Some trials are even investigating the use of tumor suppressor miRNA mimics as therapeutic agents in cancer [NCT01829971, NCT02369198]. In the current study, miR-186 has been identified as a potential oncogenic miRNA in PCa. Furthermore, inhibition of this miRNA has shown to repress the cellular behavior associated

with the tumor phenotype. Ultimately, therapeutic agents that inhibit miR-186 expression may impede disease progression in PCa.

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CURRICULUM VITA

Dominique Z. Jones-Reed, M.S.

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PROFESSIONAL SUMMARY

- Research scientist with experience in developing, leading, and conducting multi-disciplinary scientific studies in the areas of cancer and genetic epidemiology.
- Proficient communicator with the ability to provide results by preparing comprehensive written and oral summaries of research development and research findings.
- Adept at evaluating research studies through collaborations with basic scientists and community partners.
- Skilled at training and providing guidance to colleagues and students in research-related tasks.

EDUCATION

University of Louisville, Louisville, KY

Ph.D., Pharmacology and Toxicology May 2016

M.S., Pharmacology and Toxicology December 2014

Smith College, Northampton, MA May 2010

B.A., Biochemistry

RESEARCH AND PROFESSIONAL EXPERIENCE

Doctoral Candidate, Pharmacology and Toxicology Department

08/2011-05/2016

University of Louisville, Louisville, KY

P.I.: Dr. LaCreis Kidd

- Received training in statistical analysis software (SAS), Linux operating system programming, Collaborative Institutional Training Initiative (CITI) in Human Subject Research, Health Insurance Portability and Accountability Act (HIPPA) Privacy, Bloodborne Pathogens, and Laboratory Safety and Hazardous Waste
- Developed written evaluations and peer-reviewed manuscripts to disseminate research findings and study progress
- Developed laboratory protocols and facilitated the training of undergraduates and medical students
- Mentored and aided in the development of research projects for summer research students
- Investigating inflammatory and immune response related genetic variants linked to PCa risk through data mining techniques (e.g., logistic regression and multi-factor dimensionality reduction (MDR) modeling)

- Evaluating miRNA expression profiles in PCa serum and cell lines as potential prognostic tools for PCa through the utilization of molecular and cellular biology techniques to examine the impact of microRNA expression on cellular behavior in normal prostate and PCa cells
- Presenting research findings at local/national scientific meetings
- Managing and maintaining human cell culture lines for experiments, laboratory inventory, and administrative documents

Summer Research Intern, James Graham Brown Cancer Center

06/2011—08/2011

University of Louisville, Louisville, KY

P.I.: Dr. LaCreis Kidd

- Evaluated chemokine-associated genetic variants as predictors of PCa risk among men of African Descent though SAS programming and MDR modeling
- Routinely met with research team members and principal investigator to discuss study progress and propose solutions to methodological problems in order to achieve research goals in a timely manner
- Presented research findings at the regional Research Louisville symposium

Research Technician, Biomedical and Biotechnology Research Institute (BBRI)

11/2010-05/2011

North Carolina Central University, Durham, NC

PIs: Dr. Maxwell Gyamfi and Dr. Sean Kimbro

- Received American Association for Laboratory Animal Science (AALAS) certification training in animal care for research and education through the AALAS Learning Library
- Evaluated the role of mouse and human xenobiotic nuclear receptor pregnane X receptor (PXR) in obesity in vivo through protein analysis of targets involved in S-Adenosyl methionine (SAM) synthesis and metabolism, measurement of triglyceride, cholesterol and non-esterified free fatty acid in hepatic tissue as well as glucose tolerance, lipid profiling (triglycerides, HDL, LDL, and VDL) and liver toxicity (ALT/AST levels) in the serum

AWARDS AND HONORS

- Dean's Citation, University of Louisville 2016
- AACR MICR Scholar in Cancer Research Travel Award 2016
- ASPET Dolores C. Shockley Best Abstract Award 2nd Place 2016
- ASPET Underrepresented Graduate Student Travel Award 2016
- ASPET Dolores C. Shockley Best Abstract Award 1st Place 2015
- ASPET Underrepresented Graduate Student Travel Award 2015
- Alice Eaves Barns Award, University of Louisville 2014
- Dean's Citation, University of Louisville 2014
- Fall Graduate Research Symposium Poster Winner, University of Louisville 2014

- Southern Regional Education Board Doctoral Scholar Fellowship, University of Louisville 2012-2015
- Integrated Programs in Biomedical Sciences Fellowship (IPIBS), University of Louisville 2011-2012
- Dean's List, Smith College 2009-2010
- Jackie Robinson Foundation Scholar, Smith College 2006-2010
- American Chemical Society Scholar, Smith College 2006-2010

PROFESSIONAL MEMBERSHIP

- Sigma Xi, The Scientific Research Society, Member, 2010
- American Association of Cancer Research (AACR), Member, 2012-2016
- Black Biomedical Graduate Student Organization (BBGSO), President, 2012-2016
- Golden Key International Honour Society, Member, 2012-2016
- Minority Association of Graduate Students (MAGS), Vice President, 2015-2016
- Society of Toxicology, Member, 2014-2016
- American Society for Pharmacology and Experimental Therapeutics (ASPET), Member, 2014-2016

INVITED ORAL PRESENTATIONS

- American Society of Investigative Pathology (ASIP)/ American Association of Anatomists (AAA) Career Development & Mentoring Program and Lunch:

Fundamentals of Success: How to Give an Award Winning Presentation, March 29, 2015.

- American Society of Investigative Pathology (ASIP), Breast and PCa Mini-Symposium, March 30, 2015.
- Translating MicroRNA Cancer Biology to Therapy Symposium, April 6, 2016.

PEER REVIEWED MANUSCRIPTS

- Kidd L.R., Jones D.Z., Beache S., Rogers E.N., Ragin C., Jackson M., McFarlane-Anderson N., Tulloch-Reid M, Flores-Obando R., Barve S.S., Rudd J.E., and Kimbro K.S.. Chemokine Ligand 5 (CCL5) and Chemokine Receptor (CCR5) Genetic Variants and PCa Risk among men of African Descent. Hereditary Cancer in Clinical Practice, 10(1):16, 2012.
- Rogers E.N., Jones D.Z., Yeyeodu, S.T, Ragin C., Jackson M., McFarlane-Anderson N., Tulloch-Reid M, Kimbro K.S., and Kidd L.R. Toll-like Receptor (TLR)-Associated Sequence Variants as Predictors of PCa Risk Among Men of African Descent. Genes and Immunity, 10.1038/gene.2013.22, 2013.
- Kidd, L.R., Rogers, E.N., Yeyeodu, S.T., Jones, D.Z., Kimbro, K.S. Contribution of Toll-like Receptor Signaling Pathways to Breast Tumorigenesis and Treatment. Breast Cancer: Targets and Therapy, 10.2147/BCTT.S29172, 2013.
- Jones D.Z., Ragin C., Jackson M.,Kidd N.C., Flores-Obando R.E.McFarlane-Anderson N., Tulloch-Reid M, Kimbro K.S., and Kidd L.R. The

Impact of Genetic Variants in Inflammatory-related Genes on PCa Risk among men of African Descent. Hereditary Cancer in Clinical Practice,11(1):19, 2013.

- Spruiell K., Jones D.Z., Cullen J.M., Awumey E.M., Gonzalez F. J., and Gyamfi, M.A. Role of human pregnane X receptor in high fat diet-induced obesity in pre-menopausal female mice. Biochemical Pharmcology, 2014 Apr 7. pii: S0006-2952(14)00207-X. doi: 10.1016/j.bcp.2014.03.019.

ABSTRACTS AND PRESENTATIONS: NATIONAL/LOCAL MEETINGS

- Jones D.Z., Ragin C., Jackson M., McFarlane-Anderson N., Morrison S., Flores-Obando F., Kimbro K.S., and Kidd, L.R. Chemokine-associated genetic variants as predictors of PCa risk among men of African Descent. Research Louisville!, Louisville, Kentucky, September 28, 2011.
- Kidd L.R., Jones D.Z., Ragin C., Jackson M., McFarlane-Anderson N., Morrison S., Flores-Obando F., Kimbro K.S. Chemokine-associated genetic variants as predictors of PCa risk among men of African Descent. American Association for Cancer Research (AACR) Cancer Health Disparities, Washington, D.C. July 19, 2011.
- Ragin C., Jones D.Z., Ragin C., Jackson M., McFarlane-Anderson N., Morrison S., Flores-Obando F., Kimbro K.S., Kidd L.R. Inflammatory Cytokine SNPs and PCa in Black men. Caribbean Exploratory Research Center (CERC) Health Disparities Institute, St. Thomas Virgin Islands, USA, October 19-21, 2011.

- Kidd L.R., Jones D.Z., Ragin C., Jackson M., McFarlane-Anderson N., Morrison S., Flores-Obando F., Kimbro K.S. Chemokine-associated Loci and PCa Among Men of African Descent. American Association for Cancer Research (AACR), Chicago, IL, November 15, 2011.
- Jones D.Z., Anene D., Aloway A., Anene P., Avila D.V., Gobejishvili L., Barve S.S., McNally L.R., and Kidd L.R. Potential micro-RNA Biomarkers Associated with Cell Migration and Metastasis. Research Louisville!, Louisville, Kentucky, August 24, 2012.
- Anene D., Jones D.Z., Aloway A., Anene P., Avila D.V., Gobejishvili L., Barve S.S., McNally L.R., and Kidd L.R. Are Cell Adhesion Associated Micro-RNAs Linked With Metastatic PCa? Research Louisville!, Louisville, Kentucky, August 24, 2012.
- Aloway A., Jones D.Z., Anene D., Anene P., Avila D.V., Gobejishvili L., Barve S.S., McNally L.R., and Kidd L.R. Cell Survival miRNAs (29a, 29c, and 221) and Pre-metastatic PCa? Research Louisville!, Louisville, Kentucky, August 24, 2012.
- Jones D.Z., Anene D., Aloway A., Anene P., Avila D.V., Gobejishvili L., Barve S.S., McNally L.R., and Kidd L.R. Potential micro-RNA Biomarkers Associated with Cell Migration and Metastasis. James Graham Brown Cancer Center Retreat, Louisville, Kentucky, October 3, 2012.
- Jones D.Z., Anene D., Aloway A., Anene P., Avila D.V., Gobejishvili L., Barve S.S., McNally L.R., and Kidd L.R. Reduced Expression of miR-342-3p in

stage I, III, and IV PCa. American Association for Cancer Research (AACR), Washington, D.C., November 15, 2012.

- Jones D.Z., Linder J., Avila D.V., Gobejishvili L., Barker D., Schmidt M.L., Hobbing K., Clark G., and Kidd L.R. TGF-beta Signaling in PCa cell lines derived from European- and African-American men. Research Louisville!, Louisville, Kentucky, September 24, 2013.
- Linder J., Jones D.Z., Avila D.V., Gobejishvili L., Barker D., Schmidt M.L., Hobbing K., Clark G., and Kidd L.R. microRNA-885-5p and its role in the TGF-beta pathway using PCa cell lines derived from European- and African-American men. Research Louisville!, Louisville, Kentucky, September 24, 2013.
- Jones D.Z., Linder J., Avila D.V., Gobejishvili L., Barker D., Schmidt M.L., Hobbing K., Clark G., and Kidd L.R. TGF-beta Signaling in PCa cell lines derived from European- and African-American men. James Graham Brown Cancer Center Retreat, Louisville, Kentucky, October 25, 2013.
- Jones D.Z., Hobbing K., Schmidt M.L., Clark G. and Kidd L.R. The Effect of miR-186 Inhibition on Cell Viability and Colony Formation in PCa. Research Louisville!, Louisville, Kentucky, September 16, 2014.
- Jones D.Z., Hobbing K., Schmidt M.L., Clark G. and Kidd L.R. The Effect of miR-186 Inhibition on Cell Viability and Colony Formation in PCa. James Graham Brown Cancer Center Retreat, Louisville, Kentucky, October 17, 2014.
- Jones D.Z., Hobbing K., Schmidt M.L., Clark G. and Kidd L.R. The Effect of miR-186 Inhibition on Cell Viability and Colony Formation in PCa. Fall 2014 Graduate Research Symposium, Louisville, Kentucky, November 14, 2014.

- Jones D.Z., Hobbing K., Schmidt M.L., Clark G. and Kidd L.R. MicroRNA-186 inhibition alters cell proliferation and colony formation in PCa. Experimental Biology Meeting, Boston, MA, March 29, 2015.
- Jones D.Z., Hobbing K., Schmidt M.L., Clark G. and Kidd L.R. MicroRNA-186 inhibition alters cell proliferation and colony formation in PCa. American Association for Cancer Research (AACR), Philadelphia, PA, April 21, 2015.
- Jones D.Z., Hobbing K., Schmidt M.L., Clark G. and Kidd L.R. MicroRNA-186 inhibition alters cell proliferation and colony formation in PCa. Ohio Valley Society of Toxicology (OVSOT), Cincinnati, OH, June 29, 2015.
- Packer T.A., Jones D.Z., and Kidd, L.R. Impact of Quercetin on miR-21, Cell Proliferation and Migration of Metastatic and Non-Metastatic PCa Cell lines. Undergraduate R25 Poster Session, Louisville, Kentucky, July 30, 2015.
- Jones, D.Z., Schmidt M. Lee, Hobbing, K.R., Clark G. and Kidd L.R. miR-186 suppresses cell proliferation and anchorage-independence in a metastatic PCa cell line. Research Louisville!, Louisville, Kentucky, October 27, 2015.
- Jones, D.Z., Schmidt M. Lee, Hobbing, K.R., Clark G. and Kidd L.R. miR-186 inhibition suppresses cell proliferation and anchorage-independence in a metastatic prostate cancer cell line. Experimental Biology Meeting, San Diego, CA, April 4, 2016.
- Jones, D.Z., Schmidt M. Lee, Hobbing, K.R., Clark G. and Kidd L.R. Inhibition of miR-186 and repression of aggressive prostate cancer phenotype using a metastatic cell model. American Association for Cancer Research (AACR) Meeting, New Orleans, LA, April 18, 2016.

SERVICE TO UNIVERSITY

- University of Louisville School of Medicine Diversity Committee, Graduate Student Representative 2014-2016

SERVICE TO PROFESSION

- American Society for Pharmacology and Experimental Therapeutics (ASPET) Mentoring and Career Development Committee, Graduate Student Representative 2015-2016