MicroRNAs as Prognostic Biomarkers in Prostate Cancer

by

Aida Gordanpour

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Laboratory Medicine and Pathobiology
University of Toronto

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2012

ABSTRACT

Prostate cancer, one of the most common cancers among men, can be relatively harmless or extremely aggressive. The most widely used biomarker for the disease, the PSA test, is not independently diagnostic or prognostic of prostate cancer. One of the main challenges of prostate cancer research is to find reliable and effective prognostic biomarkers that will predict cancer recurrence following surgery, in order to identify clinically significant prostate cancer and improve management of the disease. In recent years, microRNAs (miRNAs) have been identified as master regulators of cellular processes, and dysregulated miRNAs have been associated with cancer development and progression. The intent of my PhD research program was to uncover novel miRNAs that contribute to prostate cancer pathogenesis in order to assess their potential as predictors of clinical progression. By analyzing a large cohort of primary prostate cancer samples, we have discovered that microRNA-221 (miR-221) is associated with metastasis and biochemical recurrence in prostate cancer, and is downregulated in TMPRSS2:ERG fusion gene-positive tumors. In addition, we have determined that microRNA-182 (miR-182) is overexpressed in prostate cancer and is associated with increased metastasis and clinical progression by targeting a tumors suppressor Forkhead box O1 (FOXO1). Overall, this work introduces novel candidate miRNA genes and downstream targets that are aberrantly expressed
in more aggressive prostate cancer, and presents a potentially significant role for miRNAs as prognostic biomarkers that are associated with clinical progression, and perhaps aids in defining how miRNAs might one day serve as anti-cancer therapeutic agents.
ACKNOWLEDGMENTS

To say I was fortunate to have pursued my PhD studies at Sunnybrook Hospital does not entirely capture the extent to which I recognize this to be true. The four years I spent there not only highlight my work as a cancer biologist, but they also represent a period of time that has profoundly influenced me personally. I am indebted to some of the finest people I have ever met for inspiring me and making Sunnybrook a positive environment in which I learned and grew. These individuals set an example of world-class scientists for their passion for cancer research, mentors who offered their time and expertise so generously, and best of friends that I was truly lucky to have met. The best way to honor their legacy would be to uphold their high calibre of professionalism and integrity in my future endeavours.

It is my fortune to gratefully express my appreciation to my supervisor Dr. Arun Seth for the continuous support of my PhD study and research, and for giving me the opportunities that opened so many doors for me. I also wish to extend my thanks to the members of my committee, lab members, and collaborators for their support.

I owe my most sincere gratitude to my closest friends; you know who you are, for bringing out the best in me, for being a source of friendship as well as inspiration, and mostly for the pure happiness that there was in times spent with you. The memories will last a lifetime…

Words fail to express my deep sense of appreciation to my family for all their unconditional love and support. To my parents who raised me with a love of science and education, and to my encouraging and caring sisters, brother-in-law, and little Audrina, Thank you!

Thank you God for giving me passion in my heart, inspiration in my life, showing me the way, giving me strength, and for teaching me to always trust in you!

I am now most eager to embark on the next chapter of my life as a medical student…
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>°C</td>
<td>Degree(s) Celsius</td>
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<tr>
<td>%</td>
<td>Percent</td>
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<tr>
<td>3’ untranslated region</td>
<td>3’ UTR</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
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<tr>
<td>AGO</td>
<td>Argonaute</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign prostatic hyperplasia</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>C. elegans</td>
<td>Caenorhabditis elegans</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DGCR8</td>
<td>DiGeorge critical region 8</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle's medium</td>
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<tr>
<td>ETS</td>
<td>E Twenty Six</td>
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<tr>
<td>ERG</td>
<td>ETS related gene</td>
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<td>Fig</td>
<td>Figure</td>
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<tr>
<td>FFPE</td>
<td>Formalin fixed paraffin embedded</td>
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<tr>
<td>FOXO1</td>
<td>Forkhead Box O1</td>
</tr>
<tr>
<td>FOXO3</td>
<td>Forkhead Box O3</td>
</tr>
<tr>
<td>Gb</td>
<td>Gigabases</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
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<tr>
<td>LnRNA</td>
<td>long non-coding RNA</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>μg</td>
<td>Microgram</td>
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<td>μl</td>
<td>Microliter</td>
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<td>mL</td>
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<tr>
<td>MiRNA</td>
<td>MicroRNA</td>
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<tr>
<td>MITF-M</td>
<td>Microphthalmia-associated Transcription Factor-M</td>
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<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>ncRNA</td>
<td>Non-coding RNA</td>
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<td>Ng</td>
<td>Nanogram</td>
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<td>NP-40</td>
<td>Nonidet P40</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PIN</td>
<td>Prostatic intraepithelial neoplasia</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate specific antigen</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>Pre-miRNA</td>
<td>Precursor miRNA</td>
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<tr>
<td>Pri-miRNA</td>
<td>Primary miRNA</td>
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<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
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<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
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<tr>
<td>RNase</td>
<td>Ribonuclease</td>
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<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
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<td>S</td>
<td>Second</td>
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"xi"
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecylsulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecylsulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SV</td>
<td>Seminal vesicle</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris buffered saline with tween 20</td>
</tr>
<tr>
<td>TCGA</td>
<td>The Cancer Genome Atlas</td>
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Chapter 1: GENERAL INTRODUCTION

A version of this chapter has been published in Prostate Cancer & Prostatic Diseases as

1.1 PROSTATE GLAND

Prostate is a sex accessory organ which plays a major role in the reproductive process by making part of the seminal fluid. The prostate is about the size of a large walnut, and is located close to the rectum, below the bladder at the base of the penis (Fig 1.1). The prostatic epithelium is composed of two major cell types, stromal cells and epithelial cells. The stromal compartment consists mainly of connective tissue, smooth muscle cells, and fibroblasts, and they mainly serve as structural support. Normal prostatic epithelium consists of five cell types: stem cells, basal epithelial cells, transit-amplifying cells, neuroendocrine cells, and secretory epithelial cells (1). Prostate specific antigen (PSA) is one of several serine proteases secreted by the prostate cells into the ejaculate, and although its main function is the regulation of semen coagulation, it has proved to be an invaluable, yet controversial marker of prostatic diseases.
Figure 1.1 - Male anatomy. Diagram adapted from medical-look.com
1.2 PROSTATE CANCER

Prostate cancer causes substantial clinical, social, and economical burden in the developed world. Identification of genes that are dysregulated in association with prostate cancer can provide molecular markers and clues relevant to disease etiology and progression. Prostate tumors display novel recurrent chromosomal translocations, and aberrant expression of specific miRNAs which can be used for elucidating prostate cancer biology, and better management of this disease.

1.2.1 Epidemiology and Etiology of Prostate Cancer

Prostate cancer is the most common cancer in men and the second leading cause of cancer death in North America, Europe and other developed regions of the world. According to Canadian Cancer Society, there were 25,500 cases of prostate cancer in Canadian men in 2011, with 4,100 deaths caused by this disease. On average, Canadian men have a 14% chance of being diagnosed with prostate cancer at some point in their lives, while their chance of dying from the disease is only 4%. The 5-year survival rate for prostate cancer is 96%. The prevalence of prostate cancer increases significantly with age (2), and studies suggest that by the age 80, more than 50% of men have some cancerous cells in their prostate (3). The introduction of PSA testing has substantially increased the rate of detection of local-regional disease, whereas the incidence of metastatic disease has decreased (4). Although the exact causes of prostate cancer development and progression are not yet known, substantial evidence suggests that both genetics and environment play a role in the origin and evolution of prostatic neoplasia, and several risk factors associated with this disease include: familial and genetic influences (5), chronic
inflammation and infection (6), sexual activity (7), diet (8), and various molecular biomarkers such as androgens (9), and vitamin D (10).

1.2.2 PSA Test

Since 1987, men have been undergoing a blood test called the PSA test to detect prostate cancer at its earliest stages (11). PSA is a protein produced by prostate epithelial cells and the majority of it is secreted in the semen and functions as a coagulase (12). Typically, a family doctor will refer a patient to a urologist if his PSA level is above 4 ng/mL, and the patient will then undergo a prostate biopsy for further evaluation. A small portion of PSA that is found in the circulation is immediately bound by alpha-1 antichymotrypsin, referred to as complexed-PSA (12). A small portion also exists as a free form. This unbound PSA may be caused by benign prostatic hyperplasia (BPH), a non-cancerous enlargement of the prostate, and not prostate cancer. Therefore the higher a man's percentage of free PSA, the less likely it is that prostate cancer is to blame. Catalona et al. was the first to show that patients with a low free: total PSA ratio were associated with a higher risk for prostate cancer (13). However, its ability to improve the positive predictive value is only when the PSA level is between 4 - 10 ng/mL (14), the grey zone in which BPH could be the culprit. Readings of greater than 25% free PSA suggest that much of the elevated PSA is caused by BPH, while a reading of less than 10% indicates that prostate cancer is most likely causing this elevation.
1.2.2.1 PSA Controversy

Despite routine application of PSA assays, PSA screening has been very controversial. As of October 2011, the United States Preventive Services Task Force (USPSTF) has recommended against the use of PSA as a screening tool for prostate cancer in asymptomatic men. Prostate Cancer Canada, however, continues to view PSA as a useful test when applied appropriately, and recommends PSA testing for the early detection of prostate cancer in Canada. The controversy around use of PSA as a screening tool stems from the fact that PSA is for the most part organ-specific and not disease-specific. This widely used test has been criticized for its high false-positive results, causing over-treatment of cancers that may have never caused harm.

There are several reasons, in addition to prostate cancer, for elevated levels of PSA found in a man's blood. These include: BPH, prostatitis (an inflammation of the prostate) (15), recent ejaculation, digital rectal exam, and prostate biopsy. Among men with PSA levels greater than 4 ng/mL, biopsies show that over two-thirds of patients do not have prostate cancer (16). Meanwhile, there are men with PSAs in the normal range (below 4ng/mL) who have prostate cancer (17).

Another important limitation of PSA as a biomarker is its inability to identify patients with aggressive and lethal forms of prostate cancer. Because many forms of prostate cancer are indolent and do not progress to metastasis and death, it would be important for new biomarkers to be able to distinguish those from aggressive prostate cancer. Over the last 25 years, no new blood test, genetic test or medical x-ray have been able to replace PSA. So we are in a midst of a biomarker crisis as lack of specificity of PSA requires supplementation in order to improve patient management, and to differentiate cancer from benign diseases of the prostate. Research has indicated that the therapeutic success rate for prostate cancer can be improved tremendously,
if this disease is diagnosed at an earlier stage. Currently, there remains a lack of reliable biomarkers that can specifically distinguish between patients who need to be treated adequately to halt the aggressive form of the disease and those who should avoid overtreatment of the indolent form. Thus, current biomarkers for prostate cancer are not ideal as they fail to replace PSA.

1.2.3 Pathology of Prostatic Neoplasia

Histologic grading is the clinically most useful prognostic factor for prostate cancer (18). The Gleason grading system is a method used to evaluate prostatic carcinoma based on the architectural pattern of the tumor as identified at relatively low magnification. This technique was developed in 1966 by Dr Donald F. Gleason, a pathologist, and members of the Veterans Administration Cooperative Urological Research Group (19). This grading system assesses the histologic pattern of cancer cells in Hematoxylin and Eosin (H&E)-stained sections (20). The primary (predominant) and the secondary (second most prevalent) patterns are identified and assigned a grade from 1 to 5, with 1 being the most differentiated and 5 being the least differentiated, and the Gleason score is calculated by the addition of the primary and secondary grades. The five different grade patterns used to generate a score are illustrated in a drawing by Dr Gleason (Fig 1.1). Due to different biopsy practices and also the widespread use of PSA test, diagnosis of prostate cancer today is significantly different from the time that Gleason grading was first used—when most men had advanced disease. Hence there have been modifications in how the Gleason grading is utilized, namely to restrict the definition of Gleason pattern 3 and expand the definition of pattern 4, therefore causing a general trend toward upgrading (18).
Figure 1.2- Gleason grades: original drawing. Adapted from Humphrey, *Modern Pathology*. (17) 292-306, 2004.
1.2.4 Treatment of Prostate Cancer

Because prostate cancer exhibits a wide spectrum of aggressiveness, different methods of treatment have been developed. Clinically localized prostate cancer is confined to the prostate, and the treatment options for it include surgery (radical prostatectomy), radiation therapy, and active surveillance (deferred treatment until necessary) (21). Locally advanced prostate cancer is defined as disease that shows evidence of spread outside of the prostate capsule, involvement of the seminal vesicles, or involvement of adjacent organs. Although the widespread use of PSA screening has caused a decline in proportion of men who are diagnosed with locally advanced prostate cancer, it remains a considerable fraction which contributes disproportionately to disease mortality. Hence better treatment options in these cohorts can have a major positive influence on overall morbidity and mortality of prostate cancer. It has been shown that only 25% of men with seminal vesicle invasion and none with lymph node metastases are biochemically progression-free at 10 years after radical prostatectomy (22).

Metastatic disease constitutes cancer that has spread beyond the prostate. The most frequent sites of metastatic prostate carcinoma are lymph node and bones, followed by lungs, bladder, liver and adrenal glands (23). Treatment options for metastatic prostate cancer include chemotherapy and hormonal therapy. In hormone refractory disease, prostate cancer continues to grow despite the fact that hormonal therapy suppresses male hormones androgen and testosterone. Patients with hormone refractory prostate cancer are given chemotherapy which may lengthen survival time and reduce the pain caused by cancer spreading to the bones.
1.3 RECURRENT CHROMOSOMAL ABERRATIONS IN PROSTATE CANCER

Chromosomal aberrations in haematological disorders such as leukemia and lymphoma were identified and characterized years ago, and are now routinely used for diagnosis, patient stratification, and drug development (24). Genomic aberrations were largely ignored in solid tumours, mostly due to technological limitations associated with cytogenetics, until recently recurrent gene fusions were found in epithelial tumours. Six years after the discovery of the first gene fusion in prostate cancer by Tomlins et al. (25), they are now considered to be an important and distinct class of mutations that occur in a high percentage of patients and can be powerful biomarkers of prostate cancer.

The most prevalent form of genetic aberration in prostate cancer is gene fusions. They can involve a variety of 5’ regulatory elements fused to ETS genes such as ERG, ETV1, ETV4, and ETV5, which are normally involved in self-renewal-associated proliferation (26). TMPRSS2:ERG, the most common gene fusion, is present in over 50% of prostate cancers (27), and is absent in normal and benign prostate tissue (28). It is characterized by fusion of the promoter region of an androgen-responsive, prostate-specific, trans-membrane serine protease (TMPRSS2), to v-ets erythroblastosis virus E26 oncogene homolog (ETS related gene; ERG), resulting in the over-expression of a transcript that encodes a truncated ERG protein product (Fig 1.3). ERG gene is a member of the ETS (E Twenty Six) family of transcription factors. In vitro studies have shown that overexpression of ERG stimulates cell migration and invasion, while its knockdown decreases the invasive properties of VCaP cells (29).

TMPRSS2 and ERG are both on the long arm of chromosome 21, and are separated by about 3 Mbp. In the case of this fusion gene, a deletion removes the intervening sequence.
Fusion genes with *ETV1*, *ETV4*, and *ETV5* are a lot less common, only present in about 10% of prostate tumours, but display great variability in fusion structure (Table 1.1). It is unclear what causes the formation of fusion genes, but there is emerging evidence that inherited polymorphisms can predispose individuals to develop *TMPRSS2:ERG* (30).
Figure 1.3- Schematic representation of TMPRSS2:ERG fusion gene.
<table>
<thead>
<tr>
<th>Fusion gene</th>
<th>Fusion type</th>
<th>Locus</th>
<th>Study</th>
</tr>
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<tr>
<td>TMPRSS2-ERG</td>
<td>Deletion/insertion</td>
<td>(21)(q22.2)</td>
<td>Tomlins et al. (25)</td>
</tr>
<tr>
<td>SLC45A3-ERG</td>
<td>translocation</td>
<td>(1;21)(q32.1;q22.2)</td>
<td>Esgueva et al. (31)</td>
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<tr>
<td>HERPUDI-ERG</td>
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<td>(16;21)(q13;q22.2)</td>
<td>Maher et al. (32)</td>
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<tr>
<td>NDRG1-ERG</td>
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<td>(8;21)(q24.22;q22.2)</td>
<td>Pflueger et al. (33)</td>
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<tr>
<td>TMPRSS2-ETV1</td>
<td>translocation</td>
<td>(7;21)(p21.2;q22.3)</td>
<td>Tomlins et al. (34)</td>
</tr>
<tr>
<td>HERVK-ETV1</td>
<td>translocation</td>
<td>(7;22)(p21.2;q11.23)</td>
<td>Hermans et al. (35)</td>
</tr>
<tr>
<td>C15orf21-ETV1</td>
<td>translocation</td>
<td>(7;15)(p21.2;q21)</td>
<td>Tomlins et al. (34)</td>
</tr>
<tr>
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<td>translocation</td>
<td>(7;7)(p21.2;p15)</td>
<td>Tomlins et al. (34)</td>
</tr>
<tr>
<td>SLC45A3-ETV1</td>
<td>translocation</td>
<td>(1;7)(q32;p21.2)</td>
<td>Han et al. (36)</td>
</tr>
<tr>
<td>ACSL3-ETV1</td>
<td>translocation</td>
<td>(2;7)(q36.1;p21.2)</td>
<td>Attard et al. (37)</td>
</tr>
<tr>
<td>FLJ35294-ETV1</td>
<td>translocation</td>
<td>(7;17)(p21.2;p13.1)</td>
<td>Han et al. (36)</td>
</tr>
<tr>
<td>EST14-ETV1</td>
<td>translocation</td>
<td>(7;14)(p21.2;q21)</td>
<td>Hermans et al. (35)</td>
</tr>
<tr>
<td>TMPRSS2-ETV4</td>
<td>translocation</td>
<td>(17;21)(q21;q22.3)</td>
<td>Tomlins et al. (38)</td>
</tr>
<tr>
<td>KLK2-ETV4</td>
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<td>(17;17)(q22;q25)</td>
<td>Hermans et al. (39)</td>
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<td>TMPRSS2-ETV5</td>
<td>translocation</td>
<td>(3;21)(q27;q22.3)</td>
<td>Helgeson et al. (40)</td>
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<tr>
<td>SLC45A3-ETV5</td>
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<td>(1;3)(q32;q27)</td>
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</tr>
<tr>
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<td>translocation</td>
<td>(1;7)(q32.1;q34)</td>
<td>Palanisamy et al. (41)</td>
</tr>
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<td>SLC45A3-ELK4</td>
<td>unknown</td>
<td>1(q32.1)</td>
<td>Rickman et al. (42)</td>
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<tr>
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<td>translocation</td>
<td>(3;8)(p25.1;q22.1)</td>
<td>Palanisamy et al. (41)</td>
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</table>
1.3.1 TMPRSS2:ERG as a Biomarker

PSA is the current standard of care in prostate cancer screening, but due to its lack of specificity and low sensitivity, it has become increasingly clear that other biomarkers are needed to complement PSA. Ever since the discovery of recurrent gene fusions in prostate cancer, TMPRSS2:ERG fusion gene has been considered a potentially suitable biomarker to supplement or even replace the PSA test, and many groups are working towards that goal. For example, a noninvasive, urine-based multiplex prostate cancer assay that uses TMPRSS2:ERG as one of the four genetic markers of cancer diagnosis was able to outperform the PSA test by achieving a specificity and positive predictive values of 76.0% and 79.8%, respectively (43). A similar study has also reported that a combined test for TMPRSS2:ERG and prostate cancer antigen 3 (PCA3) expression in urine can significantly improve the sensitivity of prostate cancer detection (44). For quite some time the ERG protein was not detectable due to lack of specific antibodies. But recently a rabbit anti-ERG monoclonal antibody (Epitomics, Burlingame, CA) has been shown reliable and sensitive, and can detect ERG-rearrangement positive prostate cancer (45). ERG protein expression, thus, can potentially be a valuable marker to identify patients’ TMPRSS2:ERG status with a prostate needle biopsy.

Another important aspect of TMPRSS2:ERG as a biomarker is whether it can act as a reliable prognosticator in prostate cancer. After years of investigation and numerous studies, there is still no clear consensus on gene fusion status and clinical outcome. While there are many studies that have linked fusion positive tumours to negative prognostic outcome (46-53), there are also studies that show no association (54-57), or even improved clinical outcome (58-60). The inconsistency between these studies highlights the likelihood that TMPRSS2:ERG interacts with additional factors to affect the progression of prostate cancer. There is much optimism that
*TMPRSS2:ERG* can effectively stratify patients for surveillance or treatment options, and potentially act as a direct and specific target for molecularly-based drugs.

### 1.3.2 Gene Fusions and Therapeutics

Gene fusions can be powerful and specific targets for anticancer drugs and some cancers are classified and treated based on a particular gene fusion status. Chronic myeloid leukemia patients with *BCR–ABL* fusion gene are treated with Imatinib mesylate (Gleevec®, STI-571) (61), and acute promyelocytic leukemia patients with *PML–RARα* fusion of the retinoic acid receptor-α are treated with all-trans retinoic acid in combination with chemotherapy (62). A recent study by our group, summarized in Chapter 2 of this thesis, showed *miR-221* is predictive of recurrence in prostate cancer, and most notably, we also found that *miR-221* is significantly down-regulated in patients with *TMPRSS2:ERG* fusion gene (63). That study was the first to show a clinical link between aberrant expression of a miRNA and TMPRSS2:ERG fusion gene status, and highlights the potential in identification of miRNAs regulating the oncogenic ERG. Fusion gene status can potentially pave the way for individualized therapy and better treatment options for men diagnosed with the disease.
1.4 NON-CODING RNAs

More than 98% of the human genomic size consists of non-coding sequences (64). Until recently, these sequences were called ‘junk DNA’ based on the fact that no specific function could be attributed to them. The discovery of non-coding RNAs (ncRNAs) as invaluable regulators of gene expression has fundamentally changed our understanding of the genome and gene expression. These RNA molecules play crucial roles in eukaryotic physiological and pathological processes. NcRNAs vary in size from miRNAs that are short in size, a mature miRNA is 18-25 nucleotides long, to long non-coding RNAs (lnRNAs) that are thousands of nucleotides long.

In 1965 the first ncRNA, alanine transfer RNA (tRNA), was discovered in baker’s yeast (65). tRNAs are adaptor molecules that acts as a bridge between the four-letter genetic code of mRNA and the twenty-letter code of amino acids in proteins (66). One end of the tRNA contains the three-nucleotide sequence called the anticodon which forms three base pairs with the matching codon in mRNA. On its other end, each tRNA is covalently attached to the amino acid that corresponds to the anticodon sequence. During protein synthesis, a tRNA is delivered to the ribosome where it attach the amino acid from its 3’ end to a growing polypeptide chain. Ribosomal RNA (rRNA) is the RNA component of the ribosome that forms 2 subunits that envelope mRNA during translation and catalyze the formation of a peptide bond between 2 amino acids (67). While tRNA and rRNA are two major house-keeping ncRNAs, there are several others that also play important roles in the cellular processes. For example, 7SL RNA is a component of signal recognition particle which binds to the signal peptide emerging from a ribosome and directs the traffic of the protein to the plasma membrane or the endoplasmic reticulum membrane for secretion or membrane insertion within the cell (68).
1.4.1 MicroRNAs

MiRNAs constitute a large family of naturally-occurring, endogenous, single stranded RNA molecules that regulate gene expression in metazoans and plants (69). MiRNAs are not translated into proteins. The canonical mechanism of miRNA-mediated gene regulation is by its binding to the 3’ UTR of an mRNA and inhibiting protein production (70). Nucleotides 2-7 from the 5’ end of the miRNA, called seed sequence, are essential to the binding of the miRNA to the target mRNA, and has to bind perfectly. All the other nucleotides of the miRNA can bind imperfectly. Initially, miRNAs were thought to regulate gene expression solely by inhibiting protein translation of a target mRNA (71). Conversely, recent studies have shown that miRNAs can also degrade their target mRNA by affecting its stability, thereby inhibiting protein synthesis indirectly (72;73) (Fig 1.4). Although the majority of published data has focused on miRNAs that act via the canonical pathway, there are no mechanistic requirements that restrict miRNA action to only the 3’ UTR. In fact, miRNAs have also been shown to use 5’ UTR and open reading frame (ORF) binding sites to regulate mRNA expression (74;75). In addition, there have been reports of miRNAs that influence gene expression by directly binding to DNA (76-78), and some miRNAs can even activate, rather than inhibit gene expression (79). Taken together, these findings highlight the complexity of gene regulation by the miRNAs.

The field of cancer genetics has rapidly expanded from identifying the first cancer-related miRNAs (80), to exploring their potential as therapeutic options in less than ten years. Based on the latest release 18 of miRBase database, the primary online repository for all miRNA sequences and associated annotations (81), there are 21643 mature miRNA products in 168 species, including 2,154 mature human miRNAs. MiRBase is available online at: http://www.mirbase.org/. It is estimated that 60% of mRNAs are regulated by miRNAs (82-85).
Figure 1.4- Overview of post-transcriptional gene regulation by miRNAs. MiRNAs regulate gene expression by binding to their target mRNAs, and thereby repress translation or promote degradation of the target mRNA, determined by the amount of complementarity that exists between the miRNA and its target mRNA.
MiRNAs have been associated with almost every cellular process, such as development (86), differentiation (87), apoptosis (88), and cell cycle regulation (89). Consequently, misregulation at any of these processes due to abnormal miRNA expression or mutations can potentially lead to cancer, as the aberrant expression of many miRNAs has been characterized in numerous cancer subtypes, including prostate cancer.
1.4.1.1 MiRNA History

The first miRNA was discovered in Caenorhabditis elegans (C. elegans) in 1993 by Lee et al., who observed that a non-coding 22-base RNA (lin-4) could bind to the 3’ untranslated region (3’UTR) of lin-14 messenger RNA (mRNA), and repress its translation (90). At that time, the scientific community did not know that a new class of RNAs had been discovered that would revolutionize our understanding of molecular biology. It was not until 7 years later, in 2000, that a second 21-base RNA let-7 was identified in C. elegans which could also bind to the 3’UTR of lin-41 mRNA, and repress its protein levels (91). Identification and functional studies of miRNAs then extended from C. elegans to other species, including humans. Since then, there has been an exponential growth of both miRNA discoveries as well as number of publication focusing on miRNAs.

1.4.1.2 The Canonical Pathway of MiRNA Processing

MiRNA biogenesis starts with transcription in the nucleus (Fig 1.5) (92). MiRNA genes are transcribed by RNA polymerase II or RNA polymerase III to form primary miRNA transcripts (pri-miRNA) (93;94). Many pri-miRNAs are polyadenylated and have a 5’ 7-methylguanylate cap (95). Usually, one miRNA gene codes for a single miRNA, but there are also miRNAs that are clustered together and are transcribed as one transcript (96). A typical human pri-miRNA consists of one or more hair pin structures, and each hairpin has a stem of 33 base-pairs, a terminal loop, and two single-stranded flanking segments upstream and downstream of the hairpin stem (97). The pri-miRNA is cleaved by the nuclear microprocessor complex formed by Drosha, a member of the RNase III family of enzymes, and the DGCR8 (DiGeorge
critical region 8) proteins (98). DGCR8 functions as an anchor which recognizes the junction between the single-stranded flanking regions and the double-stranded stem, and acts as a molecular ruler that guides Drosha to cleave the RNA molecule 11 base pairs away from the single-stranded/double-stranded RNA junction at the base of the hairpin stem (99). The product of this cleavage is the precursor miRNA (pre-miRNA) that has a 2 nucleotide 3’ overhang, and is exported from the nucleus to the cytoplasm by Exportin-5 (100). Exportin-5 recognizes the pre-miRNA regardless of sequence or structure of the pre-miRNA, but the 16 base pair minimal length of the double-stranded stem and single-stranded flanking regions are important for the proper binding pre-miRNA to Exportin-5 (101).

In the cytosol, RNase III Dicer cleaves off the pre-miRNA loop and generates an approximately 22-nucleotide miRNA duplex (102). This mature miRNA duplex is double stranded, and gets separated into two strands by helicases. A functional strand, the strand with the less stable base pair at its 5’ end, is incorporated into the RNA-induced silencing complex (RISC) and guides it to bind to the target mRNA, thereby inhibiting translation by degradation of the mRNA or by translational repression (103). The other strand, the passenger strand, will subsequently get degraded. Argonaute (AGO) proteins are important factors in the assembly and function of the RISC complex that mediate mRNA degradation or translational inhibition (104). The human genome encodes four AGO proteins (hAgo1-4) (105), of which only hAgo2 is endonucleolytically active (106). An Activated RISC complex consists of an AGO protein and a single-stranded miRNA, which acts as a guide to target complementary sequences within mRNAs, in a process which is thought to be driven by diffusion (107). When the seed sequence of miRNA and its target mRNA have high complementation, the RISC induces mRNA degradation via the RNase III catalytic domain of AGO proteins, followed by degradation by
enzyme XRN1, the exosome, and the SKI complex (108). If partial complementarity is formed between the two RNA molecules, mRNA degradation can occur by two pathways. Initially, the poly(A) tail of the mRNA is deadenylated which allows the cytoplasmic exonucleases to degrade the mRNA from the 3’ end (109). Another mRNA degradation mechanism is by removal of 5’ cap via decapping complex proteins (DCP1 and DCP2) and CAF1-CCR4-NOT complexes (110). The mRNA is then degraded from 5’ to 3’ end by XRN1 (111). Regardless of which of these mechanisms occur, there will be a decrease in the amount of protein synthesized by the mRNA.
**Figure 1.5** - The canonical pathway of miRNA processing. Adapted from Winter et al., Nature Cell Biology, (11) 3:228-234, 2009
1.4.1.3 MiRNAs in Diseases

Considering that miRNAs are vastly involved in various physiological and pathological processes in the cell, it is therefore not surprising that miRNA dysregulation is linked to numerous diseases including many different cancers. There are several different ways by which misregulation of miRNAs may lead to cancer. Misregulation by miRNA processing molecules such as Drosha and Dicer may lead to disruption of miRNAs and cancer (112;113). Changes in transcription of miRNAs by altered methylation, histone modifications, or transcription factors that regulates miRNA genes can lead to different regulation of miRNAs (114). Genomic instabilities such as translocation, deletion, amplification can also affect miRNA regulation. Approximately half of human miRNA genes are encoded in genomic regions prone to cancer-associated alterations (115). If a miRNA is located in a genomic region that is amplified, it can become oncogenic. In contrast, a miRNA that is located in a genomic region that is deleted in cancer can act as a tumor suppressor. Loss of miRNA binding site in the target mRNA, caused by a SNP, alternate splicing, or separation of 3’UTR due to a chromosomal translocation may also lead to misregulation of miRNAs (116). MiRNA expressions may also correlate with the clinical characteristics of cancer, such as the tumor aggressiveness and its response to therapy, which will be discussed more thoroughly in subsequent thesis chapters.

1.4.1.4 Putative Targets of MiRNAs

The major functional role of miRNAs is marked by the protein-coding mRNAs that they target. Due to partial complementarity between a miRNA molecule and its binding sequence, every miRNA can potentially bind to numerous mRNA targets, and each mRNA could be
regulated by many different miRNAs. This makes elucidating the biological function of a miRNA challenging, as identification of a single target may not adequately reflect the entire function of a miRNA. Most studies thus far have used bioinformatic tools to predict targets of different miRNAs, and algorithms on the most frequently used bioinformatic programs often give hundreds of putative targets for each miRNA. Different computational target prediction software such as TargetScan, PicTar, miRanda, and miRBase can use varying methods combining thermodynamics-based models and sequence alignment of miRNA-mRNA molecules and their conservation in different species to name putative targets of miRNAs (117). While these algorithms are a good starting point for the prediction of miRNA targets, ultimately functional experiments must be used to validate a gene target.

Prediction of an miRNA-mRNA interaction is based on several factors (118). A conserved sequence known as the seed sequence, situated at nucleotides 2-7 from the miRNA 5´-end, is essential for the binding of the miRNA to the mRNA. Although base pairing between miRNA and its target mRNA is only partially complementary, the seed sequence has to be perfectly complementary. The complementarity between the 6 base pairs in the seed sequence is the minimum for efficient binding, and the probability that a mRNA sequence is a target of a miRNA is higher if there are more than 6 paired bases. Their specificity is also increased by cross species binding site conservation, binding site structural accessibility (119), and proximity of one binding site to another within the same 3' UTR (120).
1.4.1.5 MiRNAs as Biomarkers in Prostate Cancer Pathogenesis

The initial studies of miRNA deregulation in prostate cancer were performed by miRNA microarray profilings, and since then several studies have analyzed prostate cancer-specific miRNA profiles by using genome-wide screenings and validation by quantitative PCR technology (121-126). Examination of prostate tumor miRNA expression profiles has revealed widespread dysregulation of miRNAs in primary prostate cancer compared to normal prostate tissue. In addition there are miRNAs that are abnormally expressed in different stages of disease progression and metastasis, and are hence implicated as prognostic markers of clinical aggressiveness and recurrence. There are some miRNAs that have been found to influence tumor microenvironment (127). This extensive body of knowledge points to miRNAs as potentially being novel cancer biomarkers, and as such they have several advantageous features. The small size and distinctive biochemical structure of miRNAs makes them resistant to endogenous RNase activity (128). MiRNAs are easily detected in tissue, blood, urine, saliva, and oral mucosa by the sensitive and easy detection methods such as quantitative Real-Time PCR (qPCR). Small RNAs such as miRNAs are extremely stable in formalin-fixed tissues, and that is a critical factor in the detection of a biomarker (129). Although there is a decrease in the level of miRNA molecules after the Formalin fixed paraffin embedded (FFPE) process, this diminishment is proportional to, and correlates with the corresponding miRNA levels in cryopreserved specimens (130;131). miRNAs are also detectable in plasma and serum fluids, as Mitchell et al. were able to distinguish patients with prostate cancer from healthy controls by measuring miR-141 levels in serum (128). This remarkable stability allows miRNAs to be readily detectable and makes them potentially invaluable biomarkers.
1.4.1.5.1 MiRNAs as Oncogenes or Tumor Suppressors

MiRNA expression is deregulated in cancer cells compared to the corresponding normal tissue, and depending on the specific cell type and stage of development, miRNAs can function as either tumor-suppressors or oncogenes. Together, they have been termed as oncomirs. Table 1.2 shows numerous miRNAs that have been reported to be abnormally expressed in primary prostate cancer in comparison to normal tissue. Some miRNAs, specifically marked in Table 1, have shown conflicting results—having been reported to be up-regulated in some studies and down-regulated in others. Different methods of sample collection, varied study designs, and diverse specificity of the platforms used, could explain some of these inconsistencies. Aberrant expressions of some miRNAs such as miR-25, miR-34a, miR-145 and miR-205 have been reported in several miRNA studies. Oncogenic miRNAs that are up-regulated in cancer may promote tumorigenesis by negatively regulating tumor suppressor genes inhibiting proliferation, or by repressing genes associated with apoptosis and differentiation. In this case, inhibition of miRNA activity can be achieved by antisense synthetic oligonucleotides (antagomirs) or by drugs that can inhibit the oncogenic activity of the miRNA. Conversely, down-regulation of a miRNA that functions as a tumor-suppressor can promote neoplastic development by enhancing proliferation. Restoration of these miRNAs with synthetic mimics or by using viral vectors encoding the miRNAs could have a therapeutic benefit by halting or even reversing tumor growth. It is important to note that there is a vast number of miRNAs that have been linked to prostate cancer in genome-wide screening studies, but there is still no consensus on exactly which miRNAs are involved in prostate cancer formation and progression. Hence further experimental validation is critical in better elucidating miRNAs’ biological function and role in the pathogenesis of prostate cancer.
Table 1.2 - MiRNAs associated with primary prostate cancer

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<th>Down-regulated in prostate tumors</th>
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<td>let-7a*</td>
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<td>miR-181a-1(122)</td>
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<td>miR-182(122;138)</td>
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<td>miR-182*(124)</td>
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*Opposite expression of miRNAs in different studies
1.4.1.5.2 Metastasis-regulatory MiRNAs

Cancer metastasis, rather than the primary tumor itself, is the cause of death in prostate cancer patients. In addition to their role as potential oncogenes and tumour-suppressors, miRNAs can also be involved in the regulation of metastasis (142). These “metastamirs” could be regulating various steps of the metastatic cascade such as epithelial to mesenchymal transition (EMT), migration, invasion, angiogenesis, adhesion, and colonization of distant organs. So far, only a few studies have investigated miRNAs associated with metastatic prostate cancer. One recent study by Watahiki et al. has used next generation sequencing to compare the miRNA profiles of a transplantable metastatic versus a non-metastatic prostate cancer xenograft, and has identified many miRNAs that are differentially expressed in prostate cancer metastasis (143). Table 1.3 lists miRNAs that have been linked to metastasis in prostate cancer as either pro-metastatic or anti-metastatic. Some miRNAs such as miR-21, miR-331-3p, miR-205, and miR-203 have been associated with prostate cancer metastasis. For instance, miR-21 has been shown to be up-regulated in the majority of human cancers, including prostate, and has been associated with increased invasiveness of prostate LNCaP cells (144). MiR-205 has been reported by Gandellini et al. to inhibit EMT and reduce cell migration and invasion through down-regulation of protein kinase Cepsilon (140).
### Table 1.3- MiRNAs associated with metastatic prostate cancer

<table>
<thead>
<tr>
<th>Pro-metastatic miRNAs</th>
<th>Anti-metastatic miRNAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>let-7d(143)</td>
<td>let-7c(145)</td>
</tr>
<tr>
<td>let-7g(143)</td>
<td>miR-1-1(148)</td>
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<td>let-7g*(143)</td>
<td>miR-7-1*(143)</td>
</tr>
<tr>
<td>let-7l(143)</td>
<td>miR-15a(137)</td>
</tr>
<tr>
<td>miR-7(143)</td>
<td>miR-15b(143)</td>
</tr>
<tr>
<td>miR-9(143)</td>
<td>miR-16(143;152)</td>
</tr>
<tr>
<td>miR-9*(143)</td>
<td>miR-16-1(137)</td>
</tr>
<tr>
<td>miR-17(143)</td>
<td>miR-17-3p(153)</td>
</tr>
<tr>
<td>miR-18a(143)</td>
<td>miR-24(143)</td>
</tr>
<tr>
<td>miR-18b(143)</td>
<td>miR-24-2*(143)</td>
</tr>
<tr>
<td>miR-20b*(143)</td>
<td>miR-26b(143)</td>
</tr>
<tr>
<td>miR-21(144;150)</td>
<td>miR-28-5p(143)</td>
</tr>
<tr>
<td>miR-27a(143)</td>
<td>miR-29a(143)</td>
</tr>
<tr>
<td>miR-27b(143)</td>
<td>miR-29b(155)</td>
</tr>
<tr>
<td>miR-30a(143)</td>
<td>miR-29c(143)</td>
</tr>
<tr>
<td>miR-30a*(143)</td>
<td>miR-203(139;143;156)</td>
</tr>
<tr>
<td>miR-31(143)</td>
<td>miR-333b(143)</td>
</tr>
<tr>
<td>miR-34c-5p(143)</td>
<td>miR-34a(143;158)</td>
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<tr>
<td>miR-99a(143)</td>
<td>miR-95(143)</td>
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<tr>
<td>miR-106a(143)</td>
<td>miR-100(145)</td>
</tr>
<tr>
<td>miR-125b(143)</td>
<td>miR-101*(143)</td>
</tr>
<tr>
<td>miR-125b-2*(143)</td>
<td>miR-106b(143)</td>
</tr>
<tr>
<td>miR-126(143)</td>
<td>miR-126*(143;161)</td>
</tr>
<tr>
<td></td>
<td>miR-133a(148)</td>
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<tr>
<td></td>
<td>miR-339-5p(143)</td>
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<tr>
<td></td>
<td>miR-339-5p(143)</td>
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<td>miR-342-3p(143)</td>
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<td>miR-361-5p(143)</td>
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<td>miR-324-5p(143)</td>
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<td>miR-324-5p(143)</td>
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<td>miR-335(143)</td>
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<td></td>
<td>miR-3065-5p(143)</td>
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</table>
1.4.1.5.3 MiRNAs in Preclinical Studies

There is a small but increasing number of preclinical studies focusing on the role of miRNAs in prostate cancer, and have been reported in Table 1.4. Bonci et al. have shown that \textit{miR-15a} and \textit{mir-16-1} are down-regulated in cancer cells of advanced prostate cancer, resulting in BCL2, CCND1, and WNT3A up-regulation, which leads to increased cell survival and invasion (137). They showed that the loss of function of \textit{miR-15a} and \textit{miR-16} causes the nontumorigenic prostate cell line RWPE-1 to form tumours in NOD-SCID mice. The authors propose that \textit{miR-15a} and \textit{miR-16} may have significant therapeutic potential, as single agents or in combination with chemotherapy, since delivery of \textit{miR-15a} and \textit{miR-16} to prostate cancer xenografts caused tumour regression. Interestingly, Takeshita et al. reported that systemic delivery of \textit{miR-16} by atelocollagen inhibits the growth of metastatic prostate cancer in bone of nude mice, and may potentially be used for the treatment of metastatic prostate cancer (152). Zhang et al. observed that \textit{miR-17-3p} regulates the expression of vimentin and altered expression of \textit{miR-17-3p} decreases the tumourigenic behaviour and reduces tumour size in nude mice (153). Saini et al. also noted that relative \textit{miR-203} expression is lower in prostate cancer cell lines derived from bone metastasis, and over-expression of \textit{miR-203} has a negative effect on the development of metastases in nude mice (156). Significantly, \textit{miR-203} has been shown to repress bone-specific transcriptional regulators such as Runx2 and Dlx5, in addition to regulating pro-metastatic genes ZEB2, Bmi, and Survivin. A recent preclinical study by Liu et al. showed convincing preclinical therapeutic evidence, implicating \textit{miR-34a} in prostate cancer metastasis (162). Intratumoral injection of \textit{miR-34a} into subcutaneous tumours inhibited tumour growth, and systemic delivery of \textit{miR-34a} into the tail vein could reduce tumour burden by half. In orthotopic LAPC9 tumors, they showed that \textit{miR-34a} can reduce lung metastasis without
changing tumour growth, and can increase survival of mice. These results make a strong case for the effectiveness of miR-34a as a therapeutic agent.
<table>
<thead>
<tr>
<th>miRNA</th>
<th>Target</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-15a, miR16-1</td>
<td>BCL2, CCND1, WNT3A</td>
<td>Bonci et al. (137)</td>
</tr>
<tr>
<td>miR16</td>
<td>CDK1, CDK2</td>
<td>Takeshita et al. (152)</td>
</tr>
<tr>
<td>miR-17-3p</td>
<td>Vimentin</td>
<td>Zhang et al. (153)</td>
</tr>
<tr>
<td>miR-203</td>
<td>Runx2, Dlx5, ZEB2, Bmi, survivin</td>
<td>Saini et al. (156)</td>
</tr>
<tr>
<td>miR-34a</td>
<td>CD44</td>
<td>Liu et al. (162)</td>
</tr>
<tr>
<td>miR-221, miR-222</td>
<td>P27</td>
<td>Mercatelli et al. (136)</td>
</tr>
</tbody>
</table>
1.4.1.6 MiRNAs as Therapeutic Agents—Breakthroughs and Challenges

The association between the differential expression of miRNAs and cancer is now so convincing that there has been considerable attention focused on applying these gene regulators as therapeutic agents. Although the utilization of miRNA-targeted therapy has not yet been implemented in clinical practice, miRNA-based therapeutics can potentially be applied as effective therapeutics by utilizing them as either drugs or drug targets: miRNA molecules that act as tumor suppressors or antagonirs of oncogenic miRNAs may serve as cancer treatments. This approach offers a new paradigm for treating human diseases, one that can utilize the entire human genome for therapeutic purposes. After establishment of a miRNA’s role in cancer pathogenesis and identification of its targets, expression of the specific transcripts bearing the complementary sequence of the miRNA can be manipulated by small nucleic acids that mimic or antagonize miRNAs, using endogenous miRNA machinery.

MiRNAs are naturally occurring molecules, and so they benefit from million years of evolutionary “fine tuning” of their function, and there are likely distinct advantages in applying miRNAs as therapeutic agents over the current conventional drugs. Unlike other nucleic acid therapeutics such as siRNAs, specificity for a single target is not the purpose for miRNA drugs, since one miRNA can target multiple downstream effectors (163). Consequently miRNA-based drugs have the advantage that they can target multiple genes in a pathway concurrently by either inhibiting or mimicking a single miRNA. Also, multiple tumor-suppressive miRNAs can be used simultaneously on one or a group of target genes to augment the effect of the therapy. For example, if there is a mutation in the binding sequence of an oncogenic target, one miRNA may not be able to bind to it, but combination of several miRNAs that target the same gene would
reduce the probability of mutation-induced resistance (164). Another advantage of using miRNAs as drugs is that they are small in size, and are therefore less antigenic than protein-coding gene replacement therapies (164).

Despite the great potential of miRNAs as anticancer drugs, there are some challenges that need to be addressed in order to bring miRNAs into the clinic. The development of miRNA-based therapies requires that miRNAs pertinent to cancer formation and progression be first identified and validated. As previously mentioned, oligonucleotides or virus-based constructs can be used to directly introduce miRNAs to the system, or drugs can be used to regulate miRNA expression and processing (165). Perhaps the main hurdle in using miRNA-based therapy is delivering the drug to cancer cells, whether locally in the prostate or to metastatic cells disseminated throughout the body. Several delivering strategies are considered plausible, such as incorporating the miRNAs within liposomes, or conjugating them to peptides that can penetrate the plasma membrane (166). For example, a lipid-based system was used to deliver chemically synthesized miR-34a, resulting in inhibition of tumor growth in mouse models of non-small-cell lung cancer (167). Also, tandem arrays of miRNA mimics delivered by lentiviral vectors proved effective against Bcr-Abl lymphoid leukemia (168). Although the multispecific nature of miRNAs makes them very effective in regulating cellular processes associated with normal cell function and neoplastic development, this property can also be a weakness of miRNA-based therapy, as perturbing miRNA levels may affect the expression of unintended mRNA target. Another major concern would be that the introduction of exogenous miRNAs may overwhelm the RNA-induced silencing complex and inhibit the processing of other miRNAs that are involved in normal cellular function (169). An additional challenge for developing miRNA-based therapies is the issue of toxicity and safety. There is concern that miRNAs would affect
off-target genes, delivery with liposomes might be toxic (170), and high concentration of small RNAs may cause liver damage (171). Ongoing and future clinical trials will be crucial in assessing the safety of miRNAs as therapeutics.

1.4.1.6.1 MiRNAs in Clinical Trials

There are currently a number of major pharmaceutical companies that have miRNA therapeutics programs. The most advanced miRNA therapy program is being done in liver, benefiting from the fact that oligonucleotides administered systematically will largely localize to the liver. In 2010, Santaris Pharma A/S, a biopharmaceutical company that focuses on the development of RNA-targeted therapies announced that their drug miravisen (SPC3649), a \(\text{miR}-122\) inhibitor, has been advanced into Phase II studies. This drug is being used to treat Hepatitis C virus (HCV) infections by sequestering \(\text{miR}-122\), and thereby inhibiting the replication of Hepatitis C virus. Miravirsen was the first miRNA-targeted drug to enter clinical trials. This drug was developed using Santaris Pharma A/S Locked Nucleic Acid (LNA) Drug Platform. Data from the drug’s phase I trial on healthy volunteers showed that this miRNA-targeted therapy is well tolerated. Recently, the FDA gave the miRagen Therapeutics’ compound MGN—4893 that targets miR-451 orphan drug status, a designation intended for drugs used for treatment of rare conditions, and clinical trials for this compound are scheduled to begin in 2012. This drug is being used to treat polycythemia vera, a myeloproliferative disease which is characterized by the abundance of blood cells and platelets in the body.
1.4.1.6.2 Prostate Cancer-associated MiRNAs Currently in Clinical Trials

Recently, Mirna Therapeutics and researchers at the University of Texas MD Anderson Cancer Center have had success in inhibiting prostate cancer tumour growth, decreasing lung metastasis, and extending survival in mice by using miRNAs. This represents a very exciting step towards the clinical use of miRNA-based drugs. As previously mentioned, Liu et al. showed that miR-34a is under-expressed in prostate cancer stem cells, and systemic delivery of miR-34a using a liposome-based delivery agent inhibited prostate cancer stem cells from replicating by suppressing the adhesion molecule CD44 (162). This group hopes to advance miR-34a as a treatment option for prostate cancer patients. Although impeding cancer in mice might be a lot easier than in human patients, these results are indeed promising as miRNA-targeted therapeutic tools that have clinical value.

Currently there are several observational clinical trials that aim to study miRNAs in prostate cancer (Table 1.5) (clinical.trials.gov). ‘Micro-RNA expression profiles in high risk prostate cancer’, NCT01220427, is being conducted at Wurzburg University hospital. Its main goal is to examine whether specific miRNA expression profiles are correlated with prostate cancer outcome. Another trial ‘Molecular correlates of sensitivity and resistance to therapy in prostate cancer’, NCT01050504, is based at the University of Washington. It aims to study differences in gene expression patterns of miRNAs, in addition to other genes, in order to discover new biomarkers and drug targets. Both of these trials are currently recruiting participants. There are also two clinical trials that briefly study miRNA expression as a secondary objective of their study. While there are no clinical trials that use miRNAs as a treatment option for prostate cancer, the emerging field of miRNA-targeted therapeutics is
undoubtedly progressing and the vast body of knowledge from basic science and pre-clinical work is reassuring.
<table>
<thead>
<tr>
<th>Trial title</th>
<th>Study type</th>
<th>Institution</th>
<th>Trial Identifier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micro-RNA expression profiles in high risk prostate cancer</td>
<td>Observational</td>
<td>Wuerzburg University Hospital, Germany</td>
<td>NCT01220427</td>
</tr>
<tr>
<td>Molecular correlates of sensitivity and resistance to therapy in prostate cancer</td>
<td>Observational</td>
<td>University of Washington</td>
<td>NCT01050504</td>
</tr>
<tr>
<td>Trial of vaccine therapy in curative resected prostate cancer patients using autologous dendritic cells loaded with mRNA from primary prostate cancer tissue, hTERT and Survivin</td>
<td>Treatment</td>
<td>Rikshospitalet University Hospital, Norway</td>
<td>NCT01197625</td>
</tr>
<tr>
<td>Phase II randomized study of combined androgen deprivation comprising Bicalutamide and Goserelin or Leuprolide Acetate with versus without Cixutumumab in patients with newly diagnosed hormone-sensitive metastatic prostate cancer</td>
<td>Biomarker/Laboratory analysis, Treatment</td>
<td>Saint Anthony's Hospital at Saint Anthony's Health Center, Illinois</td>
<td>NCT01120236</td>
</tr>
</tbody>
</table>
Characteristics of Malignant Transformation

Tumorigenesis is a multi-step process caused by genetic changes that result in the progressive conversion of normal human cells into extremely malignant derivatives (172). This cellular transformation is depended on an inherent or acquired genetic instability that leads to numerous mutations, thus allowing the cell to acquire multiple malignant characteristics which confer uncontrollable proliferative advantage to the malignant cell. These characteristics or ‘acquired capabilities’ that govern the transformation of normal human cells into cancer cells were outlined by Hanahan and Weinberg in “The Hallmarks of Cancer” (173). There are over 100 different types of cancer, and these capabilities are shared by the majority of these cancers. These features are: self-sufficiency in proliferative signals, evading anti-growth signals, evading programmed cell death (apoptosis), increased replicative potential, sustained angiogenesis, and invasion and metastasis (173) (Figure 1.2). Recently the authors have added four additional capabilities as hallmarks of cancer. They are: genomic instability, inflammation, deregulation of energy metabolism, and evading immune destruction (174).

Normal cells require stimulatory signals in order to transition from a resting state into a proliferative state (173). These signals that are induced by growth factors, extracellular matrix components and cell-to-cell adhesion molecules are transmitted into the cell by trans-membrane receptors. Although neoplastic cells divide at a fast rate, they however have significantly less dependence on external growth signals since they have achieved autonomy from their normal microenvironment. They are multiple ways in which increased proliferation is sustained. The growth factor cell-surface receptors with intracellular tyrosine kinase domains that stimulate cell cycle progression are over-expressed in many cancers (175;176). Also, mutations can cause structural changes in the receptors that causes sustained signalling independent of ligand
Figure 1.5- The Hallmarks of Cancer. The features involved in enabling the conversion of normal human cells into malignant cancers. These traits are: self-sufficiency in growth signals, insensitivity to anti-growth signals, evading programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Adapted from Hanahan and Weinberg, 2000(177))
stimulation (178). Additionally, changes in the different downstream components of complex signalling pathways may lead to growth signal autonomy (173). For example, in 25% of human tumours, Ras proteins are structurally altered so that they can release mitogenic signals into cells without stimulation from their upstream regulators (179).

Within a normal tissue, antigrowth signals bind to transmembrane cell surface receptors that are coupled to intracellular signaling circuits to maintain cellular quiescence (173). Neoplastic cells are insensitive to anti-proliferative signals, mostly through pathways involving the retinoblastoma protein (pRb). When pRb is hypophosphorylated, it blocks proliferation by sequestering E2F transcription factors that control the expression of genes associated with progression from G1 into S phase (180).

The ability of cancer cells to resist programmed cell death—apoptosis—is also a hallmark of cancer. Evading apoptosis can be acquired by cancer cells by a number of strategies. One of the most common ways involves mutation of the p53 tumor suppressor gene, resulting in the inactivation of the p53 protein in more than 50% of human cancers (173). The BCL-2 family of proteins are also important apoptotic inhibitors that are over-expressed in cancers (181).

In order for a clone of cells to form a macroscopic tumor, cancerous cells need to have unlimited replicative potential, contrary to normal cells that have a finite cell division cycles before cell death (173). Neoplastic cells achieve unlimited multiplication by maintaining their telomeres at a length above a critical threshold (182). Telomeres are region of repetitive nucleotide sequences that protect the ends of eukaryotic chromosomes and are progressively shortened during each round of DNA replication. Malignant cells maintain their telomere length which permits the accumulation of mutant oncogenes and tumour suppressors and this genomic instability can lead to immortalized cells.
Metastasis, the spread of cancer cells from the primary tumor mass to new parts in the body, is the cause of 90% of human cancer deaths (183). The metastatic process occurs in successive and multiple steps, starting with local invasion, followed by intravasation of cancer cells into blood and lymphatic vessels, transportation of cancer cells through such vessels, breaking out of cancer cells from the vessels into the parenchyma of distant tissues, formation of micrometastases, and ultimately development of macroscopic tumors (184). Numerous classes of transcription factors and proteins have been associated with increased invasiveness and migratory capabilities of cancer cells, such as cell-cell adhesion molecule, cadherin (173).

The cells that make up the body’s tissues require the oxygen and nutrients supplied by the vasculature in order to proliferate; consequently the processes that control new blood vessel formation are critical for normal physiological functions, as well as some pathological conditions including cancer (173). The development of new blood vessels during embryonic development requires the assembly of angioblasts, immature endothelial cell precursors, into a de novo capillary network. This process is called vasculogenesis and is distinguished from angiogenesis, a process by which new blood capillaries are formed from pre-existing vasculature. It was first reported by Judah Folkman in 1971 that tumors require vascular networks to grow beyond 1-2 mm in diameter (185). The ability of a malignant cell to influence its microenvironment to favor the formation of new blood vessels, and the potential of angiogenic processes as an effective anti-cancer therapy have been an area of intense interest. The most well studied of the positive regulators of angiogenesis is vascular endothelial growth factor (VEGF), which can directly activate mitogenic, survival, and anti-apoptotic properties in vascular endothelial cells (186), mobilize hematopoietic cells including monocytes and granulocyte-macrophage progenitor cells (187), which can promote angiogenesis (188). Also,
VEGF can cause vascular permeability, potentially resulting the formation of edema and thereby facilitating the distant spread of metastases (189).

The emerging hallmarks of cancer whose validity needs to be further established include: the ability to evade destruction by the immune system, and reprogramming of energy metabolism in cancer cells in order to maintain the uncontrollable cell proliferation and division (184). In addition, genomic instability and inflammation are considered enabling characteristics of neoplasia (184). The body’s genomic surveillance system has the ability to identify and repair mutations in the DNA, therefore spontaneous mutations are typically rare. However, some mutant genotypes can give selective advantage to the cells harboring them, and cancer cells often increase the rate of mutations (190). They can do so by increasing the sensitivity of cells to mutagenic agents, and/or by disrupting genes that identify DNA damage and enable DNA repair (184). Tumors are heavily infiltrated by the innate and adaptive cell types of the immune system and thus mimic non-neoplastic inflammatory conditions (184). Inflammation can enhance hallmark capabilities of tumorigenesis and progression by providing molecules to the tumor microenvironment that promote proliferation, survival factors that limit cell death, pro-angiogenic factors, and metastasis signals that lead to activation of EMT (191).
1.6 PROJECT RATIONAL

Given the importance of miRNAs in regulating gene expression in cells and their involvement in tumor development and progression as demonstrated by the aberrant miRNA expressions in various cancer subtypes, the hypothesis I studied at the onset of this thesis project was that some miRNAs might play an integral role in the biology of prostate cancer. If such a miRNA existed, and analysis of the miRNA and its downstream targets could establish this, it would allow for the further studies into the possible role of the miRNA as a prognostic biomarker of prostate cancer. While the importance of such a miRNA would be studied to investigate its role during tumor progression, it would also be important to assess its potential as a therapeutic agent — either as a drug or drug target. These considerations have significant implications for current testing of miRNAs as the new class of anti-cancer agents in the clinical setting.
1.7 THESIS OBJECTIVES AND ORGANIZATION

The objectives of this thesis were as follows: a) to establish whether a relationship could be found between miR-221, prostate cancer prognosis, and TMPRSS2:ERG fusion gene; b) and to investigate whether miR-182 is associated with enhanced metastasis and clinical progression in prostate cancer, and to identify its target that could result in phenotypic changes.

Chapter 2 details studies demonstrating for the first time that miR-221 is down-regulated in TMPRSS2:ERG fusion-positive prostate cancer. This study, published in 2011 (63), was the first of its kind by providing a link between a miRNA and gene fusion expression, and it also demonstrated a link between miR-221 and clinical progression in prostate cancer.

Chapter 3 demonstrates studies that validate miR-182 overexpression in prostate cancer, and analyze its merit as a prognostic biomarker. These results illustrate the effects of miR-182 overexpression on metastatic potential in prostate cancer cells, and identify FOXO1 as a target of miR-182. This study may be pertinent to further clinical exploitation of miR-182 as a biomarker or perhaps therapeutic tool for prostate cancer.

Finally, appendix 1 which was recently published in the Journal of visualized experiments(192), presents quantitative Real-Time PCR methodology as an assay for accurate quantification of miRNA levels in prostate tumor tissues.
CHAPTER 2: MIR-221 IS DOWNREGULATED IN TMPRSS2:ERG FUSION-POSITIVE PROSTATE CANCER

A version of this chapter has been published in *Anticancer Research* as Gordanpour A, Stanimirovic A, Nam RK, Moreno CS, Sherman C, Sugar L, Seth A. (2011) 31(2):403-10.
2.1 ABSTRACT

Expression profiling studies using microarrays and other methods have shown that miRNAs are dysregulated in a wide variety of human cancers. The up-regulation of miR-221 has been reported in carcinomas of the pancreas, breast, and papillary thyroid, as well as in glioblastoma and chronic lymphocytic leukaemia. In prostate cancer, however, down-regulation of miR-221 has been repeatedly confirmed in miRNA expression studies. Also unique to prostate cancer, and found in more than 50% of patients, is the aberrant expression of a known oncogene, the TMPRSS2:ERG fusion. To date, there has been no published study describing miRNA associations in prostate tumours that over-express the ERG oncogene from the TMPRSS2:ERG fusion transcript. Herein we report that in a large and diverse cohort of prostate carcinoma samples, miR-221 is down-regulated in patients with tumours bearing TMPRSS2:ERG fusion transcripts, thus providing a link between miRNA and gene fusion expression.
2.2 INTRODUCTION

Prostate cancer exists along a biological continuum that ranges from clinically insignificant to extremely aggressive disease. Although clinically localized prostate cancer is largely manageable by surgery, with patients rarely developing clinical recurrence, recurrent disease remains essentially incurable. Because of this difference in treatment options, it is critical to establish specific biomarkers to differentiate between different stages of disease not only to determine presence of disease, but also to differentiate between indolent and aggressive cancer.

The most common class of prostate cancer biomarker thus far is gene fusions resulting from chromosomal rearrangements. Numerous recurrent chromosomal rearrangements have been identified that are generally characterized by the fusion of various 5’ regulatory elements to E twenty-six (ETS) transcription factors, leading to high expression of these oncogenic transcription factors. Transmembrane protease serine 2: ETS-related gene (TMPRSS2:ERG), present in over 50% of all prostate cancer, is the most commonly identified fusion gene (193).

TMPRSS2 is an androgen-responsive, prostate-specific serine protease of unknown function, and ERG is a member of the ETS transcription factor family and is rarely detected in normal prostate tissue (194). The consequences of ERG over-expression, and its correlation to the progression of prostate cancer remains unclear. What is known is that androgen stimulation induces the over-expression of an mRNA containing the ERG ORF and 3’ UTR when ERG is fused to the TMPRSS2 5’ UTR. In vitro studies have shown that over-expression of ERG stimulates cell migration and invasion, while its knockdown decreases the invasive properties of VCaP cells (195). In addition, it has been shown by our laboratory and others that TMPRSS2:ERG fusion mRNAs are present in prostate tumours but seldom in normal prostate cells. Thus, detection of abnormally high ERG expression could, at least theoretically, be a
potential diagnostic and/or prognostic marker for prostate cancer. Although the chromosomal alterations seem to be important in the development of prostate neoplastic development, they alone may not be sufficient to induce cancer formation (194).

An active area of prostate cancer research is to find biomarkers that are predictive of recurrence in patients in order to aid oncologists in treatment or non-treatment decisions. TMPRSS2:ERG has been repeatedly, but not unanimously, associated with a poorer prognosis in prostate cancer patients and our laboratory, in collaboration with other groups, has previously demonstrated that prostate cancer patients with the TMPRSS2:ERG gene fusion have a higher risk of recurrence (196), while others have reported no association between this chromosomal rearrangement and clinical outcome (197). The reasons for this lack of congruity between findings are unclear and the value of TMPRSS2:ERG as an independent prognostic biomarker of prostate cancer remains contentious; however, it is possible technical discrepancies may be an important factor, such as institutional inconsistencies in disease staging, or statistical variations, such as disparate cohort sizes (193). In fact, studies that show an absence of clinical correlation between fusion and prognosis highlight the importance of finding out how the fusion may be epigenetically regulated.

MiRNAs have received considerable attention in recent years as possible biomarkers not only in prostate cancer, but also in various cancer subtypes. They are small, single-stranded, noncoding RNA molecules that regulate mRNA function by binding to the 3’ UTR of mRNAs to which they are partially complementary, thereby repressing translation (198). MiRNAs are known to be involved in almost every cellular function, including early development (86), differentiation (199), apoptosis (200), and cell cycle regulation (201); as such, it is not surprising that miRNAs have also been linked to cancer, since misregulation of any of these important
cellular functions can lead to cancer (202). Aberrant expression of miRNAs has been found in prostate cell lines, xenografts, and clinical tissues. Since the majority of cancer deaths are caused by complications from metastasis, miRNAs that specifically regulate cancer metastasis (metastamirs) are of particular interest. The study of miRNAs as biomarkers and their exact involvement in the formation and/or progression of prostate cancer is still at its early stages, and more research is needed to evaluate the potential use of miRNAs as diagnostic and prognostic markers of prostate cancer.

Given that miRNAs and fusion genes have been independently linked to prostate disease and progression, we set out to study the possible connection between miRNA regulation, prostate cancer recurrence, and TMPRSS2:ERG gene fusion status. We used a large cohort of men with clinically localized prostate cancer who were treated with radical prostatectomy and had long-term follow-ups. We assessed whether miR-221, a metastasis-promoting miRNA (203) located on the X chromosome that is differentially expressed in recurrent prostate cancers, is also associated with TMPRSS2:ERG fusion gene. Thereby, our study may prove applicable to future use of these regulators as surrogate biomarkers of prostate cancer.
2.3 MATERIALS AND METHODS

2.3.1 Study Participants and Prostate Sample Collection

Tumour samples were obtained after radical prostatectomy from prostate cancer patients who had surgery at Sunnybrook Health Sciences Centre (Toronto, Canada) between 1998 and 2006. As described by Nam et al., following radical prostatectomy, a midsection of the specimen was snap-frozen in liquid nitrogen, and stored at −80°C until extraction of RNA (196). Most tumours were not visible within the prostatectomy specimen, and thus, the samples obtained from the prostate were considered to be random. The banked slices of specimens were photocopied, oriented (anterior, posterior, right and left), quadrisected and cut into 5 mm sections on a cryostat. The sections were stained with hematoxylin and eosin (H&E) and then reviewed by the pathologist. The areas of tumour were marked on the stained slides and on the photocopied diagram. The marked areas were used to extract the tissue for total RNA extraction. All research was conducted with the approval of Sunnybrook Health Sciences Centre Research Ethics Board.

2.3.2 Patient Follow-up

In 1998, a prostate tumour tissue bank was established at Sunnybrook hospital. Clinical data and follow-up information were collected prospectively. The medical records were thoroughly reviewed using standardised data entry forms by trained data abstractors and stored within a prostate cancer-specific database. Clinical follow up consisted of four assessments in the year following surgery, two assessments in the second year and one assessment every year thereafter. At each follow-up, patients had a prostate-specific antigen (PSA) test, and clinical evaluation. Biochemical recurrence was defined as a rise in blood levels of PSA in prostate
cancer patients on two consecutive measurements after radical prostatectomy. Data on the following characteristics were available for each patient: Age, family history of prostate cancer, PSA score, Gleason grade, tumour stage, seminal vesicle invasion, surgical margins (categorized as positive or negative), metastasis (categorized as absence or presence), and *TMPRSS2:ERG* translocation (categorized as presence or absence). The clinical demographics of the patients used in the study are summarized in Table 2.1.

2.3.3 **Quantitative Real-time Polymerase Chain Reaction (PCR)**

Total RNA from prostate tumours was extracted with TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturers’ instructions. One microgram of total RNA was reverse-transcribed using the QuantiTect Rev. Transcription Kit (Qiagen GmbH, Hilden, Germany). Quantitative real-time PCR was performed in triplicate by using QuantiTect SYBR Green PCR Kit (Qiagen GmbH, Hilden, Germany) on LightCycler Real-time PCR system (Roche Applied Science, Mannheim, Germany). The miRNA level was normalized by housekeeping gene *RNU6B*. Optimized miRNA-specific primers for *miR-221* as well as for the endogenous control *RNU6B* are also commercially available (miScript Primer Assays; Qiagen). The relative amount of *miR-221* in each sample was calculated based on the crossing-point analysis (Relquant, version 1.01).

2.3.4 **RT–PCR**

Total RNA was extracted from the frozen prostate cancer tissue by homogenization in Trizol (Invitrogen Corporation) followed by ethanol precipitation. RNA pellets were dissolved in RNase-free H2O and quality determined using 2100 Bioanalyzer (Agilent Technologies, Inc.,
Santa Clara, CA, USA). The presence of *TMPRSS2:ERG* was assayed using RT-PCR as previously described (196). All reactions were performed with two primer sets that yield a 125-bp (F-`TAGGCGCGAGCTAAGCAGGAG`, R-`GTAGGCACACTCAAAACAACGACTGG`) and 595-bp (F-`CAGGAGGCAGGAGCAGG`, R-`GGCGGTGCTGGGCTGGAG`) product.

2.3.5 Statistical Analysis

Results were statistically analyzed using Prism v4.0 software (GraphPad Software Inc., La Jolla, CA, USA). Scatter plots were analyzed using Student’s *t*-tests which were two-tailed and unpaired. *P*-values less than 0.01 were considered statistically significant.
2.4 RESULTS

2.4.1 Patient Demographics

RNA was extracted from and TMPRSS2:ERG status analyzed in 170 radical prostatectomy samples. The distribution of clinical characteristics such as PSA, Gleason grade, pathological stage, and surgical margin status are described in Table 2.1. Of these 170 patients, we opted to eliminate 17 from our study because certain clinical information was missing. Table 2.2 summarizes the fusion gene status and complete clinical information of the 153 remaining patients. The age range at diagnosis of the 153 patients was 31–75 years. The average follow-up lasted 5.4 years in the fusion-negative group, and 4.69 years in the fusion-positive group. Among the cohort, 54.2% of the patients had tumours confined to the prostate gland and 69.3% of tumours were of Gleason score 7.

2.4.2 miR-221 is Downregulated in TMPRSS2:ERG Fusion Gene-positive Prostate Tumours

Due to the high prevalence of TMPRSS2:ERG in prostate cancer and its association with higher chances of recurrence and poorer prognosis, we hypothesized that miR-221, an miRNA previously linked to metastasis and recurrence in prostate cancer, may be associated with the fusion gene. To test our hypothesis, TMPRSS2:ERG status was determined for 153 radical prostatectomy samples by using RT–PCR and sequencing using random and oligo-dT primers. Prostate samples from 83 out of 153 (54.2%) patients were found to be positive for transcripts of TMPRSS2:ERG, while 70 (45.8%) lacked the fusion gene (Table 2.2). TMPRSS2:ERG fusion-positive samples produced the expected 125 or 595 bp bands depending upon which primer set was used, as previously described (196). To analyse whether miR-221 expression was associated with the presence of the fusion gene, quantitative RT-PCR was performed, which showed that
the mean expression level of miR-221 was significantly lower (p<0.01) in fusion-positive (4.52±0.34) compared with fusion-negative (7.70±1.09) tumours (Figure 2.1). Gleason grade and surgical margin status were not different between fusion-positive and fusion-negative populations (Table 2.2).
Figure 2.1 - MiR-221 is suppressed in prostate tumours with TMPRSS2:ERG
2.4.3 Low MiR-221 Expression is Associated with Metastasis and Biochemical Recurrence of Prostate Tumour

To further analyse the association of miR-221 levels with the aggressiveness of prostate cancer, we categorized the tumour samples into two subgroups: those from patients who had metastasis at the time of radical prostatectomy and/or had biochemical recurrence in follow-up years and those from patients with non-metastatic and non-recurrent disease. Long-term follow-up information on all 153 patients was not available, as some had moved to other hospitals or did not follow-up with their appointments. Of the 153 tumours, 99 were from patients with sufficient follow-up, more than 5 years on average (Table 2.3). These 99 patients were divided into two groups: those with recurrent and/or metastatic tumours, and those with non-recurrent and non-metastatic tumours.

Quantitative RT-PCR was carried out in order to analyse the expression levels of miR-221 in prostate tumours. The qRT-PCR analysis using the 55 recurrent and/or metastatic samples and 44 non-recurrent and non-metastatic samples confirmed that the mean expression level of miR-221 was down-regulated ($p<0.01$) in the tumours with metastasis and/or recurrence (3.89±0.39) compared to tumours with no metastasis or recurrence (6.32±0.73) (Figure 2.2).
Figure 2.2- MiR-221 is suppressed in prostate tumours with metastasis and/or biochemical recurrence.
2.4.4 MiR-221 Levels are Lower in Recurrent/Metastatic TMPRSS2:ERG Fusion-negative Tumours

We wanted to further analyse the differential expression of *miR-221* in relation to both genetic aberrations and clinical parameters: specifically, *TMPRSS2:ERG* fusion status, metastasis and recurrence. All 99 tumour samples from patients with long-term follow-ups were studied. We found that tumours positive for the oncogenic *TMPRSS2:ERG* had down-regulated *miR-221* levels with or without metastasis and biochemical recurrence (4.20 and 4.22, respectively) (Figure 2.3). In *TMPRSS2:ERG* fusion-negative tumours, on the other hand, *miR-221* expression differs with clinical status: *miR-221* levels are significantly lower in patients with recurrence and/or metastasis (3.52) than in those with no recurrence or metastasis (8.62) (Figure 2.3).
Figure 2.3- MiR-221 levels are lower in recurrent/metastatic TMPRSS2:ERG fusion-negative tumours
2.5 DISCUSSION

The potential utility of the *TMPRSS2:ERG* fusion product as an independent prognostic marker for patients with clinically localized prostate cancer is becoming clearer every year. Initial studies comparing clinico-pathological parameters (196;204-207) and prognostic significance (196;204;205;208-210) of this fusion gene showed conflicting results. Some studies showed no correlation between histological grade (Gleason score) (204), while others found positive associations (207;208), and yet others demonstrated correlations between fusion status and tumour stage (205;206). Wang *et al.* examined 119 patients for fusion status from a case control approach and found significant correlations with tumour stage, but no associations were found with early recurrence (48). Furthermore, Lapointe *et al.* in another case control study found no correlations with any clinico-pathological parameter and recurrence-free survival (204). However, two cohort studies of men with clinically localized prostate cancer who did not undergo treatment (*i.e.* watchful waiting) showed that men who had *TMPRSS2:ERG* fusion had lower prostate cancer-specific survival compared to men without fusion expression (208;209). Patients managed and selected for watchful waiting from these cohorts have different baseline distributions in grade, stage and PSA level to patients treated with surgery and may not be comparable. Initially, it was unclear whether the *TMPRSS2:ERG* gene fusion is only a surrogate marker for established prognostic factors of grade and stage, or whether it is an independent molecular-based marker for disease recurrence with no association with grade or stage, particularly for patients who are candidates for surgery for clinically localized prostate cancer.

Since that time, other groups, including our own, have found robust associations. More than two dozen high-impact reports have found clinically significant links to fusions in greater
than 50% of over 1500 samples of clinically localized prostate cancer (193). Furthermore, a recent publication by Carver et al. showed that prostatic intraepithelial neoplasia (PIN) is induced in host prostates by transgenic over-expression of ERG (211), suggesting that high frequency of ETS genetic rearrangements and subsequent overexpression of ETS factors may represent a crucial event in prostate tumourigenesis (212).

In addition to TMPRSS2:ERG, there are increasing reports of miRNAs or specific miRNA signatures that correlate with a wide range of clinico-pathological features in prostate cancer. Many miRNAs are found to be predictive of patient clinical outcome and/or response to treatment suggesting that miRNAs can be used as diagnostic or prognostic/predictive biomarkers (213). Investigations of miRNA dysregulation in prostate cancer typically compare miRNA expression profiles in prostate cancer versus normal/benign tissues. Two studies have included prostate cancer among other human cancer types, finding overall down-regulation of miRNAs in solid tumours compared with normal tissues (214;215). A study comparing prostate tumours with benign prostate tissue found 37 down- and 14 up-regulated miRNAs (216). Others have found either overall down-regulation of miRNAs (217) or general up-regulation of miRNAs (218). Most significantly for prostate cancer, Spahn et al. assessed miRNA expression profiles in lymph node metastasis of prostate carcinoma and found that miR-221 down-regulation was a hallmark of metastasis (219). In a larger patient group, they found that the expression of miR-221 was associated with prostate cancer progression and clinical recurrence.

miR-221 is known to be over-expressed in tumours of the breast (220), pancreas (221), glioblastoma (222), papillary thyroid carcinoma (223), and chronic lymphocytic leukaemia (224). Up-regulation of miR-221 in chronic lymphocytic leukaemia was found to be associated with a poor prognosis. Functional studies have found that miR-221 induces down-regulation of
p27 and that inhibition of miR-221 impairs tumour formation in xenografted mice (225). Indeed, high levels of miR-221 are required in many different cancer types to inhibit the expression of p27, and stimulate proliferation.

However, none of these studies have looked for miRNA associations in prostate tumours that overexpress the ERG oncogene from the TMPRSS2:ERG fusion transcript. To that end, we investigated miR-221 expression in tumours bearing the TMPRSS2:ERG fusion transcript. Using a large and diverse cohort of prostate carcinoma samples, including patients with or without the fusion gene, as well as patients with different clinical progressions, we found that miR-221 is down-regulated in both patients with TMPRSS2:ERG fusion gene, and in patients with more aggressive tumours.

As a major centre for prostate cancer, we assembled a large database and tissue bank from patients undergoing a prostate biopsy to determine the presence of prostate cancer and surgery for clinically local prostate cancer. This resource has been used for study of multiple genetic and serological markers for prostate cancer diagnosis and prognosis (196;205;226-230). The resource includes men who underwent a prostate biopsy because of an abnormal PSA or digital rectal examination and preserves outcome data, along with DNA, plasma, and paraffin-embedded tumour samples. TMPRSS2:ERG status was determined as presence or absence by RT-PCR of RNA for samples used in this study and Table I shows the range of clinical and molecular characteristics.

Almost half of the samples (54.2%) bear TMPRSS2:ERG fusion transcripts (Table 2). We found that miR-221 expression in fusion-positive tumours is 1.7-fold lower than in fusion-negative samples, with a tighter distribution (Figure 2.1, Table 2.2). Over-expression of the ERG transcription factor is a powerful inducer of prostate tumourigenesis, as we and others have
reported. Similarly, one would also expect to see down-regulation of tumour suppressors, such as miR-221 in at least some prostate tumours. It is therefore possible that the ERG transcription factor or one of its target genes could directly down-regulate miR-221.

A subset of 99 cases from the full cohort had enough clinical follow-up with which to examine the long-term consequences of miR-221 dysregulation in prostate cancer patients (Table 2.3). Average miR-221 expression was 1.6-fold lower in samples from tumours that recurred or metastasized after surgery (Figure 2). Previously we found that the TMPRSS2:ERG fusion is also strongly associated with recurrence. That report and our finding here that miR-221 is reduced in fusion-positive tumours suggests that they have a cumulative effect on tumour aggressiveness. It may be that TMPRSS2:ERG fusion has the stronger effect as we found that tumours positive for the oncogenic TMPRSS2:ERG have lower levels of miR-221 regardless of recurrence status (Figure 2.3).

In numerous other cancer types, miR-221 is up-regulated and acts to induce proliferation and tumour formation by down-regulating the p27 tumour suppressor. The role of miR-221 in prostate cancer appears to be different than in other cancer types, as reported here and by others (219). Although the Spahn group did not examine the mechanisms, they found that miR-221 is down-regulated in metastatic tumours (219). We also found down-regulation of miR-221 in prostate tumours and that such down-regulation can occur in the absence of TMPRSS2:ERG. Although results shown in Figure 2.1 suggested a link between fusion status and miR-221 status, direct dependence of miR-221 expression on TMPRSS2:ERG in every tumour seems unlikely given the independence of miR-221 down-regulation in fusion-negative tumours.

This is the first study to reveal that miR-221 down-regulation in prostate cancer is associated with the presence of the oncogenic TMPRSS2:ERG fusion transcript. Understanding
how this association leads to greater metastasis and biochemical recurrence will facilitate understanding of prostate cancer biology. More practically, a deeper molecular understanding of the mechanisms of prostate cancer genesis and maintenance will eventually provide therapeutic interventions designed to affect the unique characteristics of individual prostate tumours.
Table 2.1 - Clinical demographics of prostate cancer cases used in this study

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cohort size (n)</td>
<td>170</td>
</tr>
<tr>
<td>Biochemical recurrence</td>
<td>58</td>
</tr>
<tr>
<td>Range of Follow-up (years)</td>
<td>1-11</td>
</tr>
<tr>
<td>Range of Age (years)</td>
<td>38-83</td>
</tr>
<tr>
<td>Preoperative PSA (ng/ml)</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>9.2</td>
</tr>
<tr>
<td>Range</td>
<td>0.8-43.0</td>
</tr>
<tr>
<td>Gleason score</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>31 (18.2%)</td>
</tr>
<tr>
<td>7</td>
<td>111 (56.3%)</td>
</tr>
<tr>
<td>8-9</td>
<td>14 (8.2%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>14 (56.3%)</td>
</tr>
<tr>
<td>Pathologic stage</td>
<td></td>
</tr>
<tr>
<td>Seminal vesicle invasion</td>
<td>17 (10%)</td>
</tr>
<tr>
<td>Positive margins</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>86 (50.6%)</td>
</tr>
<tr>
<td>Yes</td>
<td>84 (49.4%)</td>
</tr>
</tbody>
</table>

Table 2.1 - Clinical demographics of prostate cancer cases used in this study. Cohort clinical characteristics for 170 prostate cancer patients initially used in this study are summarized. Data on the following characteristics were available for each patient: Age, PSA score, Gleason grade, seminal vesicle invasion, surgical margins (categorized as positive or negative), and biochemical recurrence.
### Table 2.2- MiR-221 and TMPRSS2:ERG fusion gene status

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cohort size (n)</strong></td>
<td>153</td>
<td>83</td>
<td>70</td>
</tr>
<tr>
<td><strong>Age at diagnosis, Range (years)</strong></td>
<td>31-75</td>
<td>44-75</td>
<td>31-73</td>
</tr>
<tr>
<td><strong>Average follow-up (years)</strong></td>
<td></td>
<td>4.69</td>
<td>5.4</td>
</tr>
<tr>
<td><strong>Gleason score</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>33 (21.6%)</td>
<td>19 (22.9%)</td>
<td>14 (20%)</td>
</tr>
<tr>
<td>7</td>
<td>106 (69.3%)</td>
<td>58 (69.9%)</td>
<td>48 (68.6%)</td>
</tr>
<tr>
<td>8-9</td>
<td>14 (9.1%)</td>
<td>6 (7.2%)</td>
<td>8 (11.4%)</td>
</tr>
<tr>
<td><strong>Positive margin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>83 (54.2%)</td>
<td>45 (54.2%)</td>
<td>38 (54.3%)</td>
</tr>
<tr>
<td>Yes</td>
<td>70 (45.8%)</td>
<td>38 (45.8%)</td>
<td>32 (45.7%)</td>
</tr>
<tr>
<td><strong>Initial treatment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surgery only</td>
<td>111 (72.5%)</td>
<td>60 (72.3%)</td>
<td>51 (72.9%)</td>
</tr>
<tr>
<td>Surgery + adjuvant treatment</td>
<td>42 (27.5%)</td>
<td>23 (27.7%)</td>
<td>19 (27.1%)</td>
</tr>
<tr>
<td><strong>Metastasis</strong></td>
<td></td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td><strong>Average miR-221 level</strong></td>
<td>4.52</td>
<td>7.70</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.2- miR-221 is associated with TMPRSS2:ERG fusion gene status.** Cohort clinical characteristics for 153 prostate cancer patients are listed for TMPRSS2:ERG fusion positive and negative patients. Age, years of follow-up, Gleason score, surgical margins, treatments, and number of patients with metastasis in both fusion-positive and -negative groups, along with miR-221 expression levels are listed.
Table 2.3- miR-221 as a prognostic factor of prostate cancer recurrence

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Recurrent and/or metastatic</th>
<th>Non recurrent and Non-metastatic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cohort size (n)</td>
<td>99</td>
<td>54</td>
<td>45</td>
</tr>
<tr>
<td>Age at diagnosis, range (years)</td>
<td>44-75</td>
<td>50-75</td>
<td>44-70</td>
</tr>
<tr>
<td>Average follow-up (years)</td>
<td>5.33</td>
<td>5.23</td>
<td>5.45</td>
</tr>
<tr>
<td>Preoperative PSA (ng/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>9.24</td>
<td>10.36</td>
<td>7.73</td>
</tr>
<tr>
<td>Range</td>
<td>0.8-42.99</td>
<td>3-42.99</td>
<td>0.8-27</td>
</tr>
<tr>
<td>Gleason score</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>17</td>
<td>2</td>
<td>15</td>
</tr>
<tr>
<td>7</td>
<td>73</td>
<td>43</td>
<td>30</td>
</tr>
<tr>
<td>8-9</td>
<td>9</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Positive margins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>61</td>
<td>47</td>
<td>14</td>
</tr>
<tr>
<td>No</td>
<td>38</td>
<td>7</td>
<td>31</td>
</tr>
<tr>
<td>Seminal vesicle invasion T3b</td>
<td>16</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Average miR-221 level</td>
<td>3.89</td>
<td>6.32</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.3- miR-221 is predictive of prostate cancer recurrence. Cohort clinical characteristics for 99 prostate cancer patients are listed for patients with recurrent and/or metastatic disease and those with non-recurrent and non-metastatic disease. Age, years of follow-up, PSA, Gleason score, surgical margins, seminal vesicle invasion, and miR-221 expression levels are summarized.
Chapter 3: ABERRANT MIR-182 EXPRESSIONEnhances the metastatic potential ofprostate cancer and predicts clinicalrecurrence
3.1 ABSTRACT

Despite great advancements in prostate cancer management, finding new biomarkers to accurately evaluate the aggressiveness of the disease and to determine which prostate cancers are likely to recur following radical prostatectomy remains a major challenge. Using a large cohort of primary prostate carcinomas, we determined here that miR-182, which has been previously linked to increased metastatic potential in other cancer subtypes, is highly expressed in prostate tumor samples compared to normal prostate tissue, and is its elevation is associated with higher Gleason grade, seminal vesicle invasion, and biochemical recurrence. Ectopic expression of miR-182 in prostate cell line LNCaP enhanced their motility and invasiveness, demonstrating its direct involvement in metastatic progression. The effects of miR-182 are likely mediated, at least in part, through downregulation of a tumor suppressor Forkhead box O1 (FOXO1), which has been previously reported to be a direct target of miR-182. Herein, we show that miR-182 negatively regulates FOXO1 in LNCaP cells, and FOXO1 is inversely correlated with miR-182 expression in primary prostate cancer specimens. Our findings suggest a mechanism for increased aggressiveness and recurrence in prostate cancer by miR-182 which may be valuable in future therapeutic interventions.
3.2 INTRODUCTION

Cancer metastasis is the major cause of morbidity and death in patients with prostate cancer. The current prognostic indicators for prostate cancer survival, blood PSA level and Gleason grade are not precise in predicting clinical progression following surgical radical prostatectomy. It is thus imperative to identify genes or gene products that may be used as prognostic biomarkers. MicroRNAs (miRNAs) are involved in critical steps of metastatic progression, such as migration, invasion, and angiogenesis, and their aberrant expressions have been reported in numerous cancer subtypes—including prostate cancer (231). MiRNAs are endogenously expressed, ~18-25 nucleotide, single-stranded RNA molecules. They regulate gene expression at the post-transcriptional level by mainly binding to the 3’-untranslated region (UTR) of a target messenger RNA (mRNA). MiRNAs can regulate gene expression by inhibiting protein translation of a target mRNA (71), and also by degrading their target mRNA by affecting its stability, thereby inhibiting protein synthesis indirectly (72;73). MiRNAs that are overexpressed in tumors often target tumor suppressors associated with various steps of the tumor progression, and identifying these miRNAs may be vital in the management of disease.

MiR-182, a member of the miR-96/-182/-183 cluster (232), has been associated with metastatic progression in various tumor types. In particular, miR-182 has been shown to promote invasion and migration of melanoma cells, and to enhance their metastatic ability by directly repressing Forkhead Box O3 (FOXO3) and Microphthalmia-associated Transcription Factor-M (MITF-M) (233). In breast cancer cells, miR-182 appears to regulate cell proliferation and survival by directly targeting Forkhead box O1 (FOXO1) (234). Although some genome-wide profiling studies of miRNA expressions have revealed that miR-182 may be aberrantly expressed
in prostate tumors (235;236), these findings are yet to be validated. Moreover, there is yet no evidence that miR-182 regulates metastasis and affects clinical progression in prostate cancer.

For these reasons, we investigated the possible role of miR-182 in prostate cancer progression, with the hope of gaining more insights into the mechanisms of prostate cancer pathogenesis. We analyzed the expression levels of miR-182 in a large cohort of primary prostate tumors in various clinical states, normal prostate tissues, as well as several prostate cell lines. We found that miR-182 is overexpressed in human prostate tumors compared to normal prostate tissue, and miR-182 levels rise with increasing Gleason grades in tumor samples. Further analysis of human tumor specimens revealed that miR-182 levels are significantly higher in patients who have seminal vesicle (SV) invasion and biochemical recurrence after prostatectomy. Furthermore, we demonstrate that ectopic expression of miR-182 enhances the tumorigenic, migratory, and invasive properties of LNCaP cells. We identified a tumor suppressor FOXO1, which has been linked to regulation of migration and invasion in prostate cancer (237), as a miR-182 target in prostate cancer. Indeed, in LNCaP cells miR-182 overexpression reduced FOXO1 protein levels similar to FOXO1-specific siRNA. Furthermore, in primary prostate carcinoma specimens miR-182 expression has an inverse correlation with FOXO1 expression. We also show that FOXO1 mRNA levels are lower in recurrent prostate cancer, and higher FOXO1 levels correlate with increased time of recurrence free survival. These findings may be applicable to future exploitation of miR-182 as a prognostic biomarker of prostate cancer, as well as a potential therapeutic target.
3.3 MATERIALS AND METHODS

3.3.1 Cell Lines

The nontumorigenic human prostatic epithelial cell line, RWPE-1, and human prostate cancer PC-3, Du145, LNCaP, and VCaP cell lines were obtained from the American Type Culture Collection. LNCaP cells were stably transduced with the pLemiR lentiviral vector (Open Biosystems) using standard techniques. The base medium for RWPE-1 cells is provided by Invitrogen as Keratinocyte Serum Free Medium (K-SFM; Gibco). This kit is supplied with the two additives required to grow this cell line: bovine pituitary extract and human recombinant EGF. PC-3 cells are maintained in F-12K Medium, DU145 in Eagle's Minimum Essential Medium, VCaP in Dulbecco's Modified Eagle's Medium, and LNCaP and its transduced derivative cells in RPMI-1640 Medium, all supplemented with 10% fetal bovine serum (FBS) (Life Technologies-Invitrogen Corp.). Cells were incubated at 37°C and 5% CO₂ in a humidified incubator.

3.3.2 Quantitative Real-Time PCR

Total RNA was extracted from prostate tissues using TRIzol Reagent (Invitrogen) according to the manufacturers’ instructions. 1 µg of total RNA was reversed transcribed using QuantiTect Reverse Transcription Kit (Qiagen). Quantitative Real-Time PCR analysis of miR-182, miR-93, and miR-98 was performed in triplicate using miScript SYBR Green PCR Kit (Qiagen) on LightCycler Real-time PCR system (Roche applied science). The miRNA level was normalized by a reference gene, RNU6B. Primers for each miRNA as well as for RNU6B were commercially purchased (miScript Primer Assays, Qiagen). For genes other than miRNAs, two primer sets were used. The Primers used are as follows: FOXO1 (Forward: AAGAGCGTGCCCTACTTCAA, Reverse: CTGTTGTGTCCATGGATGC), Beta-actin
(Forward: GGAGAATGGCCACGTCTC, Reverse: GGGCACAAGGCTCATCAT). The relative amount of genes in each sample was calculated based on the crossing-point analysis (RelQuant, version 1.01). For semiquantitative end point PCR analysis, cDNA was subjected to 40 rounds of amplification, and PCR products were electrophoresed and visualized in ethedium bomide-stained 1.5% agarose gels.

3.3.3 Soft Agar Assay

The bottom layer was obtained by covering 6-well dishes with 1 ml of 0.8% agarose in Dulbecco’s Modified Eagle Medium 2X (DMEM 2X) containing 10% Fetal bovine serum (FBS). After an hour, 1.2x10^4 stably transduced LNCaP cells with either empty vector or with miR-182-expressing vector were seeded in triplicate in 1 ml DMEM 2X containing 0.5% agarose and 10% FBS. After two weeks, colonies were photographed and scored.

3.3.4 Wound Healing Assay

LNCaP Cells were seeded in six-well culture plates and grown to confluence forming a monolayer covering the surface of the entire well. After cells were serum-starved in serum-free RPMI for 18 h, the wound was created in the center of the cell monolayer by the gentle removal of the attached cells with a 10 µl pipette tip. Debris was removed by PBS wash, and the cells received fresh RPMI with 10% FBS and 10 mmol/L of hydroxyurea. Cells were photographed with a Zeiss Axiovert 200M inverted microscope at the intervals of 0, 24, and 72 hours.

3.3.5 Transwell Migration Assay

A suspension of 1x10^5 stably transduced LNCaP cells with either empty vector or with miR-182-expressing vector in 0.5% FBS medium was added to BD cell culture inserts with 8-µm porous membrane coated with 20% FBS (BD Biosciences, Bedford, MA). Cells were incubated
for 2-5 days at 37°C in a 5% CO₂ incubator. To quantify migrating cells, cells remaining on the top-side of the membrane were removed using a cotton-tipped swab, and cells that had migrated to the underside were visualized with a Zeiss Axiovert 200M inverted microscope and photographed.

3.3.6 Invasion Assay

A suspension of 1x10^5 cells in RPMI media (600 µL) containing 1% serum was added to transwell Boyden chamber containing 8.0 µm pore size filters coated with matrigel (BD Biosciences, Bedford, MA). The lower chamber was filled with 1.5 mL of media with 10% FBS as a chemo-attractant. Cells were incubated for 5 days under standard culture conditions. Tumor cells remaining on the top-side of the membrane were removed and cells that had migrated to the underside were visualized under a Zeiss phase contrast microscope, and photographed.

3.3.7 Western Blotting

Total cell extracts were prepared from subconfluent cells using Nonidet P-40 lysis buffer (20 mM HEPES-KOH [pH 7.6], 100 mM KCl, 5 mM MgCl₂, 2 mM dithiothreitol, 0.25% Nonidet P-40, 2 µg/ml leupeptin, 2 µg/ml pepstatin, and 102 µg/ml cycloheximide). Lysates were mixed with 2X SDS-loading buffer containing 10% β-mercaptoethanol, boiled for 5 min, and loaded onto 12% SDS-polyacrylamide gel for electrophoretic separation. The proteins were transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, CA) at 300 miliamps for 40 minutes at room temperature (RT). Membranes were then blocked for nonspecific binding with 5% non-fat dry milk in TBST (TBS with 0.1% Tween 20; pH 7.4) for 1 hour, and incubated with the primary antibodies overnight at 4°C. The following monoclonal and polyclonal antibodies were used: anti-FOXO1 and anti-FOXO3a (Cell Signaling, USA); anti-MiTF (abcam, USA);
anti-β-Tubulin (Sigma). After washing with TBST, membranes were incubated with species-specific secondary antibody conjugated to horseradish peroxidase enzyme for 1 hour at RT, and washed three times with TBST. Proteins were detected using an enhanced chemiluminescence detection reagent kit (Amersham Biosciences Inc.) and Kodak X-OMAT-AR film for autoradiography.

3.3.8 Transfection of SiRNA and MiRNA Inhibitors

1.5 x 10^5 cells were seeded per well in a 24-well plate. 5 nM of siRNA (control and FOXO1 siRNA) and 50 nM of miRNA inhibitor (control and anti-miR-182) from Qiagen were diluted into 100 μl of serum free medium. Subsequently, 3 μl of HiPerFect Transfection Reagent (Qiagen) was added to the mixture and incubated at room temperature for 10 min. The transfection complexes formed were then added drop-wise onto cells and incubated under normal growth conditions. Cells were harvested 48 hours after the transfections.

3.3.9 Prostate Sample Collection

Tumor samples were obtained, with the approval of the research ethics board, from prostate cancer patients who had surgery at Sunnybrook Health Sciences Centre (Toronto, Canada) between 1998 and 2006. As described by Gordanpour et al. (238), following radical prostatectomy, the specimen was oriented using anatomic landmarks and the prostate and seminal vesicles were painted right side green and left side blue. A random transverse midsection of the prostate was taken perpendicular to the rectal surface, frozen in liquid nitrogen, and stored at -80°C. Banked slices of specimens were photocopied, oriented (anterior, posterior, right and left), quadrisectioned, and cut on the cryostat. Sections were stained with H&E and reviewed by a pathologist to delineate tumor versus normal areas on the stained slides and a corresponding
image. The marked areas were used as a guide to indicate areas from which to extract the tumor tissue from which RNA was later extracted. All research was conducted with the approval of Sunnybrook Health Sciences Centre research ethics board.

3.3.10 Patient Follow-up

The medical records and follow-up information of patients were thoroughly reviewed using standardised data entry forms by trained data abstractors and stored within a prostate cancer-specific database. Biochemical recurrence was defined as a rise in blood levels of PSA in prostate cancer patients on three consecutive measurements after radical prostatectomy. Data on the following characteristics were available for each patient: age, months of follow-up, PSA level, Gleason grade, biochemical recurrence status after 60 months of follow-up, and presence of seminal vesicle invasion. The clinical demographics of the patients used in the study are summarized in Table 1.

3.3.11 Recurrence Analysis

Patient data and mRNA data from DASL assays were published previously (239). Cox survival regression analysis was performed using data from 68 patient specimens with recurrence at <48 months or without recurrence for >48 months.

3.3.12 Statistical Analysis

Results were statistically analyzed using GraphPad Prism v4.0 software. Error bars represent the standard error of the mean (SEM). Scatter plots were analyzed using student’s t tests which were two-tailed and unpaired. Nonlinear regression (curve fit) and Pearson product-moment correlation coefficient were used for correlation analysis of miR-182 and FOXO1.
3.4 RESULTS

3.4.1 MiR-182 Expression is Increased in Human Prostate Tumors, and Its Upregulation is Associated with Clinical Progression in Prostate Cancer.

MiR-182 expression was analyzed in 164 primary prostate cancer specimens that were obtained after radical prostatectomy, and in 5 normal prostate tissue samples by qPCR. As shown in Fig. 3.1A, miR-182 expression was progressively upregulated with increasing Gleason grades, and was significantly higher in tumors with Gleason 8 or 9 as compared with normal prostate tissue.

Based on the aforementioned results, we postulated that dysregulation of miR-182 is associated with clinicopathological features and clinical progression of prostate cancer. The clinical demographics of the patients in this study cohort are summarized in Table 3.1. We found that the mean expression level of miR-182 was significantly higher (p = 0.03) in patients who developed biochemical recurrence (0.92 ± 0.10) compared to those who remained recurrence-free five years following prostatectomy (0.63 ± 0.07) (Fig. 3.1B). Moreover, we established that miR-182 expression was higher in patients with seminal vesicle invasion at the time of radical prostatectomy (Fig. 3.1C). We also analyzed the expression levels of miR-98 and miR-93 which were thought to possibly be associated with prostate cancer. Levels of miR-98 and miR-93, however, did not differ significantly between different Gleason grades (Fig. 3.1A), and in relation to biochemical recurrence (data not shown).
Figure 3.1- MiR-182 is overexpressed in human prostate tumors, and its upregulation is associated with clinical progression. (A), miR-182 expression was measured using qPCR in various prostate tumor samples representing different Gleason grades compared with normal prostate tissue. Sample categories were distributed as follows: Normal (n=5), Gleason 5 or 6 (n=35), Gleason 7 (n=111), Gleason 8 or 9 (n=14). All samples were normalized to RNU6B expression. Expression levels of miR-93 and miR-98 were also assessed, but did not show a significant difference. *, p < 0.05.

[Continued on next page]
Figure 3.1- miR-182 is overexpressed in human prostate tumors, and its upregulation is associated with clinical progression. [Continued from previous page] B) expression levels of miR-182 were assessed using qPCR in groups of recurrent (n=64) and non-recurrent (n=48) prostate tumors. All samples were normalized to RNU6B expression. *, significant difference in the median expression levels (black lines) between subgroups (p < 0.05). p-Values were calculated with two tailed t-test.

[Continued on next page]
Figure 3.1- miR-182 is overexpressed in human prostate tumors, and its upregulation is associated with clinical progression. [Continued from previous page] C) expression levels of miR-182 were assessed using qPCR in groups patients with seminal vesicle invasion (n=19) and no seminal vesicle invasion (n=145) at the time of prostatectomy. All samples were normalized to RNU6B expression. *, significant difference in the median expression levels (black lines) between subgroups (p < 0.05). p-Values were calculated with two tailed t-test.
3.4.2 MiR-182 Overexpression Enhances the Tumorigenic Properties of LNCaP Cells.

We next assessed the expression levels of miR-182 by semi-quantitative end point PCR analysis in a series of prostate cell lines (Fig. 3.2A). The expression of miR-182 appears to be highest in highly aggressive PC3 prostate carcinoma cells, compared to less invasive cell lines, such as DU-145 and LNCaP. To assess the biological effects of miR-182, we used lentiviral pLemiR vectors (Figure 3.2B) to increase miR-182 expression in prostate cancer cell line, LNCaP. LNCaP cells stably transduced with miR-182 were found to robustly upregulate miR-182 levels to express more than 4-fold higher levels of miR-182 than the empty vector control (Fig. 3.2C). To analyze tumorigenic potential of these cells, we tested their anchorage-independent growth in soft agar as a marker of tumorigenicity. As shown in figure 3.3A, miR-182 cells developed more colonies in soft agar compared to vector control ($p = 0.0007$), indicating that miR-182 supports anchorage-independent growth of LNCaP cells. Importantly, miR-182 cells exhibited sharp morphological differences in culture (Fig. 3.3B). They appear to lose contact inhibition and form aggregates. The appearance of these irregular spherical structures is dense and individual cells are difficult to visualize. In contrast, vector control LNCaP cells exhibit a typical LNCaP morphology in bright field and fluorescent microscopy: growing as loosely adherent cells that do not form a uniform monolayer. Therefore, miR-182 overexpression enhances the tumorigenic properties of LNCaP cells.
Table 3.1- Clinical demographics of prostate cancer cases used in this study

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cohort Size (n)</strong></td>
<td>164</td>
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<tr>
<td><strong>Age at Diagnosis, Range</strong></td>
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</tr>
<tr>
<td><strong>Average Follow-up (years)</strong></td>
<td>5.86</td>
</tr>
<tr>
<td><strong>Preoperative PSA (ng/mL)</strong></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>9.36</td>
</tr>
<tr>
<td>Range</td>
<td>0.8-42.99</td>
</tr>
<tr>
<td><strong>Gleason Score</strong></td>
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</tr>
<tr>
<td>5-6</td>
<td>35</td>
</tr>
<tr>
<td>7</td>
<td>111</td>
</tr>
<tr>
<td>8-9</td>
<td>14</td>
</tr>
<tr>
<td><strong>Biochemical Recurrence</strong></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>64</td>
</tr>
<tr>
<td>No</td>
<td>48</td>
</tr>
<tr>
<td>Unknown</td>
<td>52</td>
</tr>
<tr>
<td><strong>Seminal Vesicle Invasion T3b</strong></td>
<td>19</td>
</tr>
</tbody>
</table>

Cohort clinical characteristics for 164 prostate cancer patients are listed. Data includes: age, years of follow-up, PSA, Gleason score, biochemical recurrence, and seminal vesicle invasion. PSA: Prostate Specific Antigen.
Figure 3.2- MiR-182 expression in LNCaP cells. (A), Expression levels of miR-182 was analyzed by semi-quantitative end point PCR in a series of Human Prostate Cell Lines. [Continued on next page]
Figure 3.2- MiR-182 expression in LNCaP cells. [Continued from previous page]

(B), Lentiviral pLemiR construct used to deliver miR-182 expression in LNCaP cell line. (C) Relative expression of miR-182 in stably transduced LNCaP cells with empty vector control (Vector) or with miR-182-expressing vector (miR-182). The level of the mature miR-182 was detected using qPCR, and the expression level was normalized to the RNU6B control.
Figure 3.3- MiR-182 overexpression enhances the tumourigenic properties of LNCaP cells. (A), Growth in soft agar of vector and miR-182 LNCaP cells. Graph is representative of 3 independent experiments (n=3). (*p<0.05; **p<0.01, ***p<0.001).

[Continued on next page]
Figure 3.3- MiR-182 overexpression enhances the tumourigenic properties of LNCaP cells. [Continued from previous page]

(B) Morphological transformation in culture of LNCaP cells with empty or miR-182-expressing vector. RFP: Red Fluorescent Protein.
3.4.3 miR-182 Enhances the Migratory and Invasive Abilities of LNCaP Cells.

Since aggressive and recurrent prostate tumors exhibit higher expression levels of miR-182, we questioned whether miR-182 expression levels might be involved in increased metastasis. We therefore investigated the role of miR-182 in cell migration of stably transduced LNCaP cells. When these cells were analyzed in a trans-well migration assay, the results shown in figure 3.4A indicate that miR-182 increased the migration of LNCaP cells by more than 10-fold ($p = 0.0012$). MiR-182 cells were also more proficient than equivalent vector-transduced cells at closing an artificial wound created over a confluent monolayer (Fig. 3.4B), further reaffirming the conclusion that miR-182 enhances the migratory of LNCaP cells.

Invasive potential of miR-182 cells was analyzed using Matrigel-coated cell culture inserts. As seen in figure 3.4C, miR-182 upregulation dramatically enhanced the invasive capacity of normally poorly invasive LNCaP cells ($p = 0.0006$). Therefore, miR-182 upregulation enhances migratory and invasive properties of LNCaP prostate cells. Together with the observed higher miR-182 expression levels in aggressive and recurrent prostate tumors, these findings implicate miR-182 in metastatic progression.
Figure 3.4- MiR-182 enhances the migratory and invasive abilities of LNCaP cells. (A), Trans-well migration assay on LNCaP cells stably transduced with either empty vector (Vector) or miR-182 (miR-182) (n=3). (*p<0.05; **p<0.01, ***p<0.001).

[Continued on next page]
Figure 3.4- MiR-182 enhances the migratory and invasive abilities of LNCaP cells. [Continued from previous page]

(B) Wound-healing assay on LNCaP cells transduced with empty or miR-182 vectors. Pictures were taken upon making the wound and at day 1, day 3, and day 4 (n=2). [Continued on next page]
Figure 3.4- MiR-182 enhances the migratory and invasive abilities of LNCaP cells. [Continued from previous page]

(C), Trans-well invasion assay of LNCaP transduced with empty or miR-182 vectors (n=2). Cells were added to cell culture inserts containing a filter with pores coated with matrigel. (*p<0.05; ** p<0.01, ***p<0.001).
3.4.4 MiR-182 Negatively Regulates FOXO1 in LNCaP Cells and Primary Prostate Tumors.

To identify targets that when inhibited by miR-182, could result in enhanced metastatic properties that we saw in LNCaP cells, we searched the literature and prediction algorithms such as miRBase and TargetScan, and found FOXO1, FOXO3, and MITF among the putative gene targets of miR-182. FOXO1 protein is dramatically reduced in LNCaP-miR-182 cells as compared to parental and vector alone cells, as shown by Western blot analysis (Fig. 3.5A). We also found a slight diminishment in MITF protein levels in response to miR-182 overexpression (Fig. 3.5B). We did not, however, detect any changes in FOXO3 protein levels.

To further confirm that miR-182 blocks FOXO1 expression, LNCaP-miR-182 cells were transfected with antisense miR-182 or negative control inhibitors. These are single-stranded RNA molecules which, after transfection, specifically inhibit miRNA function. A non-targeting inhibitor was used as a negative control. As seen in Fig. 3.5A, inhibition of miR-182 resulted in elevation of FOXO1 protein levels, while negative control miRNA inhibitors had no effect (compare lanes 3, 4, and 5). Increased expression of FOXO1 after transfection of miR-182 inhibitor proves that miR-182 is regulating FOXO1 expression. LNCaP-miR-182 cells were also transfected with FOXO1 siRNA, and miR-182-mediated downregulation of FOXO1 was comparable to that achieved by FOXO1-specific siRNA (Fig 3.5C, compare lanes 4 and 5). While over-expression of miR-182 resulted in 76% knockdown of FOXO1, concomitant over-expression of miR-182 and FOXO1 siRNA increased the FOXO1 knockdown efficiency to 94%.

To compare paired expression levels of FOXO1 and miR-182, we analyzed the expression of miR-182 and FOXO1 by qPCR in groups of prostate cancer samples, characterized by recurrent or non-recurrent prostate cancer. As shown in figures 3.6, we find that there is a significant
inverse correlation between FOXO1 and miR-182 expression levels (Pearson’s Test; \( p=0.0054 \)). Together, these results identify FOXO-1 as a target of miR-182.
Figure 3.5- MiR-182 negatively regulates FOXO1 in LNCaP cells. (A), Western blot analysis of FOXO1 protein expression in LNCaP cells, stably transduced LNCaP cells with empty vector (Vector), and with miR-182-expressing vector (miR-182). FOXO1 levels inversely correlate with miR-182 expression. LNCaP-miR-182 cells were also transfected with antisense miR-182 or control inhibitors. Inhibition of miR-182 increases FOXO1 levels. Densitometric quantification of FOXO1 relative to β-Tubulin from three independent experiments is graphically depicted. [Continued on next page]
Figure 3.5- MiR-182 negatively regulates FOXO1 in LNCaP cells. [Continued from previous page] (B), Western blot analysis of putative targets of miR-182, FOXO3 and MITF, in LNCaP, Vector, and miR-182 cells. Representative immunoblotting from two independent experiments are shown. [Continued on next page]
Figure 3.5- MiR-182 negatively regulates FOXO1 in LNCaP cells. [Continued from previous page] (C), LNCaP-miR-182 cells transfected with FOXO1 siRNA shows that miR-182-mediated downregulation of FOXO1 was comparable to that achieved by FOXO1-specific siRNA. Densitometric quantification of FOXO1 relative to β-Tubulin from three independent experiments is graphically depicted.
Figure 3.6- MiR-182 negatively regulates FOXO1 in primary prostate tumors. (A), FOXO1 levels inversely correlate with miR-182 expression in primary prostate tumor tissues, organized in aggressive and non-aggressive subgroups. Relative expression levels of miR-182 and FOXO1 were measured by qPCR. [Continued on next page]
Figure 3.6- MiR-182 negatively regulates FOXO1 in primary prostate tumors. [Continued from previous page] (B) Nonlinear regression (curve fit) and Pearson product-moment correlation coefficient were used for correlation analysis.
3.4.5 FOXO1 Downregulation is Associated With Biochemical Recurrence in Prostate Cancer.

Considering that FOXO1 is a tumor suppressor that has been linked to increased metastasis in prostate cancer cells, we sought to ascertain whether FOXO1 is associated with biochemical recurrence and clinical progression in prostate cancer. FOXO1 genomic loss has been detected in prostate cancer by The Cancer Genome Atlas (TCGA) consortium (p = 7.128E-8) (Fig. 3.7A). Furthermore, analysis of the Oncomine database (240) determined that FOXO1 mRNA levels were significantly decreased in cancerous versus normal prostate tissue, as reported by Lapointe (p = 3.34E-10, Fig. 3.7B) (241), LaTulippe (p = 9.22E-6, Fig. 3.7C) (242), and Tomlins (p = 1.98E-4, Fig. 3.7D) (243), among others.

We subsequently used qPCR analysis on primary prostate cancer specimens to assess whether FOXO1 expression levels are associated with clinicopathological features. We found that the mean expression level of FOXO1 mRNA was significantly lower (p=0.0014) in tumors from patients who developed biochemical recurrence (1.95 ± 0.13) compared to tumors from patients who were recurrence-free five years after their radical prostatectomy (3.00 ± 0.34) (Fig. 3.7E). Furthermore, higher FOXO1 levels were associated with increased time of recurrence-free survival using Cox Survival Analysis (Fig. 3.7F). These results confirm that FOXO1 acts as a tumor suppressor in prostate cancer.
**Figure 3.7-** FOXO1 downregulation is associated with prostate cancer progression. [Refer to next page] (A), box plot of FOXO1 DNA profile analysis in normal prostate tissue and prostate adenocarcinoma from The Cancer Genome Atlas (TCGA) consortium. Oncomine (Compendia Bioscience) was used for analysis and visualization. Analysis of FOXO1 mRNA expressions in normal versus prostate cancer tissue as reported by Lapointe (B), LaTulippe (C), and Tomlins (D), using the Oncomine database.
Figure 3.7. FOXO1 down-regulation is associated with prostate cancer progression. [Continued from previous page] (E), mRNA expression levels of FOXO1 were assessed using qPCR in groups of patients with biochemical recurrence or patients who were recurrence-free five years after surgery. All samples were normalized to β-actin expression. *, significant difference in the median expression levels (black lines) between subgroups (*p<0.05; **p<0.01, ***p<0.001). P-values were calculated with two tailed t-test. [Continued on next page]
Figure 3.7 - FOXO1 down-regulation is associated with prostate cancer progression. [Continued from previous page] (F), Cox survival regression analysis of FOXO1.
3.5 DISCUSSION

miRNAs have been implicated in the post-transcriptional regulation of gene expression in diverse cellular processes. Recent studies have evaluated miRNAs as potential biomarkers of disease formation and progression in numerous cancer subtypes, in part based on the recognition that some miRNAs are abnormally expressed in tumor versus normal tissue (244), and in many cases, in metastatic and aggressive disease (245). One of the miRNAs that shows great potential as a regulator of cancer metastasis is miR-182. Although several studies, including in melanoma and breast cancer, suggest that miR-182 is an important oncogenic miRNA which is closely linked to tumor progression and metastasis, however, so far there had been no studies examining the role of miR-182 in prostate pathogenesis. Herein we show, using a large cohort of primary prostate carcinoma, that higher expression of miR-182 promotes metastasis and clinical recurrence in Prostate cancer.

In these studies, we have demonstrated several novel findings. First, miR-182 was shown to be significantly up-regulated in primary tumors from patients who develop biochemical recurrence post-surgery. This observation can be utilized in potential future use of miR-182 as a prognostic biomarker of Prostate cancer. Next, we show that up-regulation of miR-182 causes prostate cancer cells to be more proficient at anchorage-independent growth and losing contact inhibition, thus displaying dramatic morphologic changes. These cells also show increased migratory and invasive abilities. Finally, our studies demonstrate that miR-182 targets FOXO1 in prostate cells, and down-regulates its mRNA and protein levels. This inverse correlation was shown in the expression levels of mir-182 and FOXO1 in LNCaP prostate cells, as well as over a hundred primary prostate tumors.
Malignant tumors are associated with altered tumour-host interactions leading to the ability to invade and metastasize. To identify specific gene targets of miR-182 through which it can promote invasive behaviour, we searched public target prediction algorithms such as TargetScan and miRANDA for putative target genes whose down-regulation could mediate the observed effects of miR-182 over-expression. These computational target prediction programs point to 3’ UTR of FOXO1 mRNA as a direct target of miR-182, and this has already been confirmed by several groups using luciferase assay (246-248).

The Forkhead Box O family of transcription factors with three main members (FOXO1, FOXO3, and FOXO4) are involved in many important cellular processes such as cell cycle regulation, cell differentiation, glucose metabolism, and other cellular functions (249). FOXO proteins are mainly located in the nucleus and regulate expression of an extensive array of tumor suppression genes. FOXO1 is itself a tumor suppressor that negatively regulates the highly oncogenic phosphatidylinositol 3-kinase (P13K)/AKT signaling pathway (250). It is located on the q14 band of chromosome 13 (13q14), where chromosomal deletions in cell lines, xenografts, and clinical specimens of prostate cancer are common (251). Here, we report that FOXO1 is also post-transcriptionally down-regulated via miRNA regulation. Our qPCR analysis of miR-182 and FOXO1 mRNA levels in primary samples of prostate carcinoma show a direct correlation between overexpression of miR-182 and reduction of FOXO1 mRNA. This inverse correlation is especially intriguing when considering the established role of FOXO1 as a tumor suppressor in prostate cancer. Recent data suggests that FOXO1 binds to and suppresses another transcription factor, Runx2, which plays a critical role in osteoblast maturation, bone formation, and prostate cancer cell metastasis (237). Thus overexpression of miR-182, causing degradation of FOXO1 mRNA, may lead to enhanced Runx2 and increased migration and invasion in prostate cancer.
cells. While our studies validate FOXO1 as a target of miR-182, future studies are needed to identify and functionally evaluate downstream targets of FOXO1 that are related to prostate cancer progression and metastasis, and are affected by miR-182 expression levels.

There are indeed promising breakthroughs in the field of miRNA research, such that we can now envision miRNA-based therapeutics in the near future. In prostate cancer, for example, systemic delivery of a synthetic mimic of miR-34a has been used to inhibit prostate cancer metastasis and extend survival of tumor-bearing mice (252). However, more research is needed in order to elucidate the interplay of various miRNAs involved in prostate cancer development and progression, and the exact mechanism of their action through their target genes. Our study presents a model of prostate cancer progression, in which increased miR-182 expression enhances cancer aggressiveness by promoting oncogenic and invasive characteristics. According to this model, upregulation of miR-182 promotes metastasis by affecting tumor suppressor FOXO1. These findings demonstrate that miR-182 and its downstream effectors could prove to be valuable in future exploitation of miR-182 as a prostate cancer biomarker and therapeutic agent.
CHAPTER 4: GENERAL DISCUSSION AND FUTURE DIRECTIONS
4.1 SUMMARY AND IMPACT OF MAJOR FINDINGS

The preceding chapters in this thesis introduce miRNAs previously undescribed in prostate cancer, confirm their aberrant expression in human prostate tumors, and outline possible modes of action for these miRNAs. In Chapter 2 of this thesis, we described the association of miR-221 downregulation and recurrent prostate fusion gene, TMPRSS2-ERG, and found miR-221 to be downregulated in recurrent prostate cancer. In Chapter 3, we implicated miR-182 as a novel prostate cancer oncogene – we showed that miR-182 is overexpressed in the more aggressive prostate tumors. Evidence is presented demonstrating that the ectopic expression of miR-182 corresponds to increased invasive and metastatic potential in prostate cells. Despite these important findings, several questions pertaining to the role of miR-221 and miR-182 in the biology of prostate cancer remain to be elucidated. These questions are discussed in more detail in the Future Directions section.

One of the major themes of this thesis is the examination of miRNAs as potential biomarkers for prostate cancer. Biomarkers can be used for purpose of screening, diagnosis, prognosis, as an imaging marker, in predicting drug efficacy, as well as an indicator of cancer recurrence. The current biomarker for prostate cancer, the PSA test, while limited in diagnosis and screening, its rise post-surgery is a very effective indicator that cancer has returned. But we are still in need of a biomarker that would predict which cancers are likely to recur in order to improve management of the disease. Aberrant expression of miRNAs in cancer may potentially be a useful predictor of clinical progression. We have analyzed miR-221 and miR-182 as biomarkers capable of predictive prognosis and cancer recurrence. Still, many more questions remain about the potential functional role of miR-221 and miR-182 as biomarkers of prostate
cancer progression, and also their potential utility as anticancer therapeutics. Although it remains unclear exactly how ectopic expression of one miRNA may influence the expression of many potential targets of that miRNA, and this certainly presents a challenge, it does not diminish the potential of miRNAs as biomarkers.

One important feature of the studies presented in this thesis deals with use of a large cohort of primary human prostate tumors to conduct translational research. For investigations into the mechanisms of gene regulation by miRNAs, it is imperative that a sufficient sample size is studied. In addition, in our identification and analysis of miRNA targets, we have utilized large multi-institutional cancer genomics databases such as The Cancer Genome Atlas (TCGA) and Oncomine database. These sources are particularly valuable for profiling large numbers of tumours in order to analyze abnormalities in DNA sequence, genomic copy numbers, chromosomal rearrangements, as well as gene expressions. Results from large-scale collaborative efforts such as TCGA can offer a more comprehensive characterization of cancer genome than ever before, and new candidate biomarkers can be discovered. These collaborative approaches should continue to advance our ability to diagnose, stratify, and treat prostate cancer, ultimately leading to decreased morbidity and mortality and enhanced quality of life for patients.
4.2 FUTURE DIRECTIONS

Many questions remain about the specific nature and potential functional role of miRNAs in cancer. This includes identification of all the miRNAs that are involved in regulation of cancer progression, the exact molecular mechanisms involved in various pathways, and feasibility of employing miRNAs that are specific and sensitive enough to be used as biomarkers and/or therapeutics. While this lack of information does present a challenge for more detailed understanding of miRNAs, it does not detract from their potential importance in tumour biology.

In a direct sense miRNAs function as post-transcriptional regulators of the cell’s gene expression. However, our work and others show that such regulation of transcription factors will cause altered regulation of transcription as well. It is, therefore, of critical importance to identify the magnitude of mRNAs targeted by a given miRNA in order to fully understand the influence of the miRNA on various cellular processes. By validating the mRNA targets of a given miRNA, the effects of deregulated miRNA expression can be put into context. Given the breadth and diversity of targets of miRNAs, there are numerous significant consequences that aberrant miRNA expression could have. Presently there are no confirmed targets of miR-221 and miR-182 in the context of prostate cancer. MiR-221 has been implicated by Galardi et al to increase proliferation in prostate cell lines by targeting a tumor suppressor p27kip1 (253). However, Spahn et al has suggested that miR-221 is a tumor suppressor that negatively regulates the proto-oncogene c-kit, though they did not find any correlation with p27Kip1 expression levels (159). As for miR-182, FOXO3 and MITF have been validated as direct targets of miR-182 in other cancer subtypes (247). In order to better understand the oncogenic effect(s) of aberrant miR-182 expression in prostate cancer, approaches aimed at identifying its mRNA targets in this tumour
are warranted. Although it has not yet been fully elucidated how many and which mRNAs are regulated by miR-182, it is conceivable that miR-182 could influence several targets. Using overexpression and/or knockdown strategies combined with array-based expression profiling, *bona fide* targets of miR-182 in prostate cancer may be discovered, bringing a more comprehensive insight into miR-182 function in prostate pathogenesis. We have shown that FOXO1 is a target of miR-182 in prostate cancer. Nonetheless, as promising as FOXO1 appears as a target of miR-182, it is likely that additional genes and pathways affected by miR-182 are involved in prostate cancer progression. In this regard, future experiments will require a more exhaustive look at other genes that may act as potential targets of miR-182.

In our characterization of the miRNAs that are aberrantly expressed in prostate cancer using array-based profiling of miRNAs as described in Chapter 3, miR-182 and miR-221 were among the six candidates that emerged. MiR-221 exhibited widespread downregulation and miR-182 showed upregulation in recurrent prostate cancer compared to non-recurrent cancer – thus making these two miRNAs a logical choice for follow-up studies. Other than miR-221 and miR-182, our miRNA microarray identified four additional candidate miRNAs that were aberrantly expressed in recurrent prostate cancer. MiR-103, miR-339, and miR-183 were also found to be upregulated and miR-136 was downregulated when comparing recurrent versus non-recurrent prostate cancer. To best of our knowledge, there are yet no studies that have analyzed the expression of miR-103, miR-339, and miR-136 in prostate cancer. As previously mentioned, miR-183, miR-182, and miR-96 are expressed as a cluster. Recently, Mihelich et al described overexpression of the entire miR-183-96-182 cluster in prostate cancer and reported that their upregulation suppressed five zinc transporters including hZIP1 (254). Prostate tumors are known to have lower zinc levels, which are directly correlated with lower expression of the zinc
transporter hZIP1. This study suggests that miR-182 and miR-182 that are co-expressed are also co-regulated. The significance of the differential expression observed for the additional four miRNAs in our microarray study will require further investigation in future studies. Considering the widespread role miRNAs have been shown to play in cancer, it is very likely that more miRNA candidates beyond miR-221 and miR-182, such as those alluded to above, also contribute to progression of prostate cancer.

With respect to potential future studies to decipher the functional role miR-182, a critical first step is to confirm our results in other prostate cancer cell lines. The cell model used in this study was human prostate cancer cell line, LNCaP. An important consideration should be to test the effects of miR-182 ectopic expression in several other established cell lines such as DU145 and PC-3. Although our work has clearly implicated miR-182 as a novel prostate cancer oncogene, mouse models would be great means of investigating the effects of overexpressing miR-182 in an in vivo setting. Future approaches exploring miR-182 function by utilizing genetically engineered mouse models, including spontaneous and implanted xenograft tumors, as well as a comparison of both local and metastatic disease, can further elucidate the role of the miRNA in the progression of prostate cancer. For example, subcutaneous and orthotopic injection of cell lines overexpressing miR-182 and analyzing the rate of tumor growth and metastasis to distant sites would be an ideal strategy to further characterize whether increased miR-182 levels are associated with tumor burden.

In order to gain a full appreciation of the complexity of prostate cancer genome, bona fide oncogenes and tumour suppressors need to be identified and validated, and the signaling pathways that they regulate must be characterized. Recent technological advancement has made examination of the genome more possible than ever before. In recent years, DNA sequencing
technology has transformed the field of cancer genomics (255-257). After three decades of relying on Sanger-based sequencing technologies, next-generation sequencing or “deep” sequencing now allows for the acquisition of tens of gigabases (Gb) of DNA sequence in one experiment. Numerous studies have now utilized next-generation sequencing to analyze diverse aspects of both normal and cancer genomes (258-260). Whole-transcriptome profiling using next-generation sequencing has also allowed for quantification of transcript expression (mRNA and miRNA) (261-263). By using this method, there does not need to be a prior knowledge of the transcriptome under study, and global assessment of the known and unknown transcriptome would eliminate any bias associated with characterization of transcriptome. As the cost associated with the using the technique and analyzing the results becomes more affordable every year, deep sequencing- profiling of prostate cancer genome and transcriptome will definitely become more widespread. Hence, next-generation sequencing-based strategies for analyzing cancer genetics can play a decisive role for a more complete characterization prostate cancer and discovering new biomarkers.
APPENDIX 1: MICRORNA DETECTION IN PROSTATE TUMORS BY QUANTITATIVE REAL-Time PCR (QRT-PCR)

A version of this appendix has been published in Journal of Visualized Experiments as Gordanpour A, Nam RK, Sugar L, Bacopulos S, Seth A. 2012 May 16;(63):e3874. doi: 10.3791/3874.
A1.1. ABSTRACT
Quantitative Real Time polymerase chain reaction (qPCR) is a rapid and sensitive method to investigate the expression levels of various miRNA molecules in tumor samples. Using this method expression of hundreds of different miRNA molecules can be amplified, quantified, and analyzed from the same cDNA template. Methods used to investigate miRNA expression include SYBR green I dye-based as well as Taqman-probe based qPCR. If miRNAs are to be effectively used in the clinical setting, it is imperative that their detection in fresh and/or archived clinical samples be accurate, reproducible, and specific. qPCR has been widely used for validating expression of miRNAs in whole genome analyses such as microarray studies (264). The samples used in this protocol were from patients who underwent radical prostatectomy for clinically localized prostate cancer; however other tissues and cell lines can be substituted in. Prostate specimens were snap-frozen in liquid nitrogen after resection. Clinical variables and follow-up information for each patient were collected for subsequent analysis (238).

The main steps in qPCR analysis of tumors are: Total RNA extraction, cDNA synthesis, and detection of qPCR products using miRNA-specific primers. Total RNA, which includes mRNA, miRNA, and other small RNAs were extracted from specimens using TRIzol reagent. Qiagen’s miScript System was used to synthesize cDNA and perform qPCR (Figure A1.1). Endogenous miRNAs are not polyadenylated, therefore during the reverse transcription process, a poly(A) polymerase polyadenylates the miRNA. The miRNA is used as a template to synthesize cDNA using oligo-dT and Reverse Transcriptase. A universal tag sequence on the 5’ end of oligo-dT primers facilitates the amplification of cDNA in the PCR step. PCR product amplification is detected by the level of fluorescence emitted by SYBR Green, a dye which
intercalates into double stranded DNA. Specific miRNA primers, along with a Universal Primer that binds to the universal tag sequence will amplify specific miRNA sequences.

The miScript Primer Assays are available for over a thousand human-specific miRNAs, and hundreds of murine-specific miRNAs. Relative quantification method was used here to quantify the expression of miRNAs. To correct for variability amongst different samples, expression levels of a target miRNA is normalized to the expression levels of a reference gene. The choice of a gene on which to normalize the expression of targets is critical in relative quantification method of analysis. Examples of reference genes typically used in this capacity are the small RNAs RNU6B, RNU44, and RNU48 as they are considered to be stably expressed across most samples. In this protocol, RNU6B is used as the reference gene.
Figure A1.1- Steps in qRT-PCR
A1.2. PROTOCOL

Prostate sample collection

- Collect the prostate samples at the time of prostatectomy. The specimen is oriented using anatomic landmarks. The prostate and seminal vesicles are painted as follows: right side green, left side blue.

- A random transverse midsection of the prostate is taken perpendicular to the rectal surface, frozen in liquid nitrogen, and stored at -80°C (265).

- Banked slices of specimens are photocopied, oriented (anterior, posterior, right and left), quadrisected. Sections are cut using the Cryostat.

- Sections are stained with H&E and reviewed by a pathologist to determine and delineate tumor versus normal areas on the stained slides and a corresponding image. The marked areas are used as a guide to indicate areas from which to extract the tumor tissue from which RNA will be extracted in the subsequent steps.
Figure A1.2- Prostate Sample preparation
Isolating total RNA, including miRNA, from samples

- Place frozen prostate samples on dry ice and referring to the delineated photocopy, cut out a small portion of the prostate tumor (between 50 to 100 mg).
- Homogenize the prostate tumor tissue in 1 mL of TRIzol Reagent. The quantities in the following steps are based on use of 1 mL of TRIzol Reagent.

Note: Here we have used TRIzol Reagent for extracting RNA, however other kits that isolate small RNA-containing total RNA can also be used.

- Incubate the homogenized samples for 5 minutes at room temperature.
- Add 0.2 mL of chloroform to the samples and shake vigorously for 15 seconds. Incubate samples for 3 minutes at room temperature, then centrifuge at 12,000 x g for 15 minutes at 4°C.
- Transfer the colorless upper aqueous phase to fresh tubes, and add 0.5 mL of isopropyl alcohol. Incubate samples for 10 minutes at room temperature, then centrifuge at 12,000 x g for 10 minutes at 4°C.
- Carefully aspirate the supernatant without disturbing the pellet containing the RNA. Wash the RNA pellet with 1 mL of 75% ethanol. Vortex the sample and re-sediment by centrifugation for 5 minutes at 7,500 x g at 4°C.
- Carefully aspirate the supernatant and dry the RNA pellet for 5-10 minutes, making sure the RNA pellet is not completely dry. Re-dissolve in Nuclease-free water appropriate to pellet size. Measure the concentration of RNA using the NanoDrop 1000 spectrophotometer (measure absorbance at 260 nm and 280 nm).
- Check the quality and integrity of the RNA samples using Agilent Bioanalyzer.
Reverse transcription of RNA

- Reverse transcription of RNA was performed using miScript Reverse Transcription Kit according to manufacturer’s instructions (Qiagen). This kit includes a reverse transcriptase and a poly(A) polymerase. The miScript RT Buffer includes Mg^{2+}, dNTPs, oligo-dT primers, and random primers.

- Use between 10 pg and 1 μg of RNA to synthesize cDNA. If using more than 1 μg of RNA, scale up the reaction linearly to the appropriate volume.

- Prepare a master mix that contains 5X miScript RT Buffer (4 μl), miScript Reverse Transcription Mix (1 μl), and RNase-free water to bring reactions to final volume of 20 μl. Also include template RNA (up to 1 μg) in the master mix.

- Incubate the samples for 60 minutes at 37°C followed immediately by incubation for 5 minutes at 95°C. This step can be performed in a PCR machine, heating block, or water bath. Thermocyclers are the most suitable and accurate method. Store the cDNA on ice for short term, and -20 °C for long term storage.

Generating a standard curve

- Prior to experiment with target miRNAs, a standard curve is generated by using cDNAs of known concentrations against their crossing points (CP) (Fig A1.3).

- Prepare a series of dilutions of 2-fold, 10-fold, 50-fold, 250-fold, and 1250-fold the original cDNA of a sample that is known to have a substantial expression of your gene of interest.

- Run the PCR as specified in Section 5 “Real-time PCR for detection of miRNA”, with the modification that cDNA is in serial dilutions not a static 40x dilution.

- Perform analysis using RelQuant software (Roche) to generate your standard curve.
Note: a new standard curve must be generated for each gene of interest.
Figure A1.3- Generating a standard curve
Real-time PCR for detection of miRNA

- Real time PCR for miRNAs was performed using miScript SYBR Green PCR Kit and miScript Primer Assay according to manufacturer’s instructions (Qiagen). Prepare a master mix containing 2x QuantiTect SYBR Green PCR Master Mix, 10x miScript Universal Primer, 10x miScript Primer Assay, and RNase-free water. Prepare a master mix for a 20 μl volume reaction.

- The Primer Assay is specific to the miRNA of interest. To reconstitute 10x miScript Primer Assay, centrifuge the vial briefly, and add 550 μl TE buffer, pH 8.0. Vortex the vial briefly to mix, aliquot primers to smaller volumes, and store at -20°C. Two primers are required: primers for the target gene and the reference gene. RNU6B is used as the reference gene.

- Dilute the cDNA 40x and store extra aliquots at -20°C.

- cDNA serves as the template for the PCR. Use 2 μl of 40x diluted cDNA and dispense to the 20 μl light cycler capillaries (Roche).

- Add 18 μl of the master mix to each capillary, and centrifuge using a capillary adapter.

- Place the capillaries in a capillary based Real-Time cycler, such as LightCycler ® 3.5 Real-Time PCR System with a 32-capillary carousel format.

- Run the PCR cycling program as follows:

  To activate HotStarTaq Polymerase that is in the 2x QuantiTect SYBR Green PCR Master Mix, pre-incubate at 95°C for 15 minutes.

  Followed by 50 cycles of:

  Denaturation, 15 s, 94°C;
  
  Annealing, 30 s, 55°C;
  
  Extension, 30 s, 70°C.
• Select a sample to be the calibrator, and set its normalized target amount to 1. Compare the relative expression of the miRNA in all the other samples to the calibrator.

Note: Within a study, the same calibrating sample should be used to maintain consistency of results.

Analyzing data
• Amplification curves for the PCR reactions are depicted graphically and numerically by Molecular Biochemicals LightCycler Software version 3.5 (Roche) (Fig A1.4). Quantify reactions in the “Quantification” tab, and export the data to a text file.

• Import the data to the RelQuant analysis software (Roche) to generate quantification results. Import separate files for target gene, reference gene, and standard curve data.

• Specify the position of calibrator for both target and reference gene. Also specify positions of the samples. Data is expressed as the target to reference ratio of different samples divided by the target to reference ratio of the calibrator. The standard curve previously generated for a particular miRNA and housekeeping gene is used as a reference standard for extrapolating quantitative data for miRNA targets of unknown concentrations.

• Three replicates of samples are analyzed as a group and mean concentrations and standard deviations of the triplicate is calculated. If one of the triplicates is inconsistent with the rest of the set, it will be excluded by the program (Fig A1.5).
Figure A1.4 - Amplification curve for PCR reaction
<table>
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<tr>
<th>Nr</th>
<th>Sample information</th>
<th>Crossing point</th>
<th>CP median</th>
<th>Delta CP median</th>
<th>Ratio conc.</th>
<th>Normalized ratio</th>
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<tr>
<td>1</td>
<td>PC3-98</td>
<td>25.94</td>
<td>26.29</td>
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<td>1.10</td>
<td>1.00</td>
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<td>2</td>
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</tr>
<tr>
<td>3</td>
<td>Repli. of PC3-98</td>
<td>26.29</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>pc3-u6</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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</tr>
<tr>
<td>3</td>
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<tr>
<td>4</td>
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</table>

**Figure A1.5-** Data analysis for PCR reaction
A1.3. DISCUSSION

Aberrant expressions of some miRNAs have been consistently found in prostate tumors when compared to normal tissue (235), and some of these miRNAs have been named as potential novel therapeutic agents against prostate cancer (252). Hence the aberrant expression levels of miRNAs can be useful diagnostic and/or prognostic biomarkers. The Real-Time qPCR methodology presented here provides an assay for accurate quantification of miRNA levels in prostate tumor tissues. The miScript PCR system used can detect single nucleotide differences between mature miRNAs. The miScript miRNA qPCR Assays, however, are not intended for detection of stem loop precursor miRNAs, for which different miScript Precursor Assays are available.

The reliability of this technique depends on the quality of the input RNA, therefore concentration, integrity, and purity of RNA should be tested prior to Real-Time PCR. Moreover, ribonucleases are very stable and readily degrade RNA, thus extra caution should be taken in handling of the RNA. All reactions should be set up on ice to minimize RNA degradation. RNase inhibitors can also be added to the reaction prior to reverse transcription. Gloves should be frequently changed, while sterile and disposable plasticware must be used throughout the procedure. If there is no PCR product or the amplification curve is detected late in Real-Time PCR, try increasing the number of PCR cycles, and make sure that the cycling program includes activation of HotStarTaq DNA polymerase for 15 minutes at 95°C. Low amplification might also be due to inadequate starting cDNA template, therefore try to increase the amount of cDNA. Late amplification can also represent a false positive.
REFERENCES


(74) Lytle JR, Yario TA, Steitz JA. Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR. Proc Natl Acad Sci U S A 2007 Jun 5;104(23):9667-72.


Cai X, Hagedorn CH, Cullen BR. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. RNA 2004 Dec;10(12):1957-66.


