Variation and Modulation of microRNAs in Prostate Cancer and Biological Fluids

Sarah Seashols
Virginia Commonwealth University

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Variation and Modulation of microRNAs in Prostate Cancer and Biological Fluids

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry at Virginia Commonwealth University

by
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December 2013
Acknowledgement

A very large group of people have been critical to the success of my doctoral research; it truly has taken a village to support this work. I want to start by thanking my undergraduate research mentor, Dr. Eric Bradley, who mentioned in his Endocrinology class that he was looking for a student researcher for the summer. My work with him, on cloning and sequencing the p450c17alpha gene in Peromyscus leucopus, was the essential experience that got me the position after graduation from William & Mary as a research associate with Dr. Suzanne Barbour at VCU. I will be forever grateful to her for taking a chance on a pregnant recent B.S. graduate, and treating me like a researcher, and not a technician.

As my career developed in the forensic science field, my supervisor Lisa Schiermeier-Wood in the Forensic Biology section at the Virginia Department of Forensic Science, was always supportive of me, even when she knew that it meant that I had to go on to the position in the Forensic Science faculty at VCU. And within the Department, Dr. Michelle Peace and Dr. Tracey Dawson Cruz were crucial in their support for my continued interest in pursuing a doctoral degree. As program director of the Biochemistry Department, Dr. Tomasz Kordula probably had no idea how much trouble I would become in my final semester, and I thank him for always being positive and happy to help me out, even after the third set of paperwork requiring his support.
I want to thank my family, and extended family, from the bottom of my heart. She and my dad have always known of my goal to obtain my Ph.D., have been tremendously supportive, and assisted me in any way that they were able. Bill Williams is always excited to hear of my progress and breakthroughs. Dennis Williams has been such an incredible source of love and support for the past 2 years. My daughters Kaia and Alyssa have ever been their sweet, understanding selves with me, enduring my working late, writing when I should be spending time with them, and the occasional emotional meltdown that occurs from trying to be everything all at once.

Dr. William Budd, Danielle Weaver, and Francy Nogales assisted with various portions of my projects. Finally, I want to thank Dr. Zendra Zehner and Dr. Walter (Mike) Holmes from the bottom of my heart for taking me on and supporting me through the past 3 years. I’m sure that Zendra knew that I would be a troublesome case when I told her that I wanted to perform research with her on microRNAs, but I was still planning on teaching in Forensic Science as well. She has been ever gracious and patient with me, and supportive not only of my research, but emotionally as well, given the huge personal changes that occurred over the past 3 years. I only hope that I have paid her back in kind, and will continue to do so through our ongoing collaborative research projects.
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Abbreviations and symbols

µg  microgram
µL  microliter
µm  micron
aatf  anti-apoptosis transcription factor
Ago  Argonaute
AKT1  Rac protein kinase alpha (v-akt murine thymoma viral oncogene homolog 1)
ALL  acute lymphoblastic leukemia
AML  acute myeloid leukemia
ApoB  Apolipoprotein B
AR  Androgen Receptor
BAD  Bcl-2-associated death promoter
Bax  Bcl-2-associated X protein
BCL2  B-Cell CLL/Lymphoma 2
BCL6  B-Cell CLL/Lymphoma 6
BDNF  Brain-derived neuregulin factor
BPH  benign prostatic hyperplasia
BRCA1  breast cancer type 1 susceptibility protein
BSP  bone sialoprotein
CaP  Prostate Cancer
CD  cluster of differentiation (see CD34, CD44, CD117, etc)
CD117  see cKit
CDH1   e-cadherin
CDKN1  cyclin-dependent kinase inhibitor 1 (see CDKN1A, CDKN1B, CDKN1C,p21)
cDNA    complementary DNA
Che-1   see AATF
c-KIT   Mast/Stem cell growth factor receptor (V-Kit Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene Homolog)
CLL     chronic lymphocytic leukemia
COL1A1  collagen, type I, alpha 1
Ct      Cycle Threshold
CXCR4  C-X-C chemokine receptor type 4 (fusin or CD184)
dCt     change in cycle threshold
DDIT4  HIF-1 responsive protein (DNA damage inducible transcript 4)
DGCR8  DiGeorge Syndrome Critical Region Gene 8
DNA    deoxyribonucleic acid
DOD    Department of Defense
DU145  metastatic prostate cancer cell line
E2F    E2F transcription factor
ea     horse
EDTA   Ethylenediaminetetraacetic acid
EGF    epidermal growth factor
EMT    epithelial to mesenchyal transition
ErbB2 & 3  V-Erb-B2 Erythroblastic Leukemia Viral Oncogene Homolog
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ERG</td>
<td>v-ets erythroblastosis virus E26 oncogene homolog</td>
</tr>
<tr>
<td>ETS-1</td>
<td>v-ets erythroblastosis virus E26 oncogene homolog 1 (Avian)</td>
</tr>
<tr>
<td>F6</td>
<td>poorly tumorigenic prostate cell line (rescued M12 via chr19)</td>
</tr>
<tr>
<td>FFPE</td>
<td>formalin-fixed paraffin embedded</td>
</tr>
<tr>
<td>FGA</td>
<td>fibrinogen alpha chain</td>
</tr>
<tr>
<td>FGB</td>
<td>fibrinogen beta chain</td>
</tr>
<tr>
<td>FOXO1</td>
<td>Forkhead box 01</td>
</tr>
<tr>
<td>FSCN1</td>
<td>fascin homolog 1, actin-bundling protein</td>
</tr>
<tr>
<td>G1</td>
<td>phase of cell cycle</td>
</tr>
<tr>
<td>G2/M</td>
<td>check point of mitotic cell cycle</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GIST</td>
<td>gastrointestinal stromal tumours</td>
</tr>
<tr>
<td>Grb2</td>
<td>Growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>GS</td>
<td>gleason scoring</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen Synthase Kinase 3</td>
</tr>
<tr>
<td>GSTP1</td>
<td>glutathione S-transferase pi 1</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>H2O</td>
<td>water</td>
</tr>
<tr>
<td>HCN2</td>
<td>hyperpolarization activated cyclic nucleotide-gated potassium channel 2</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
</tr>
<tr>
<td>HOXA9</td>
<td>Homeobox A9</td>
</tr>
<tr>
<td>HOXC6</td>
<td>Homeobox C6</td>
</tr>
</tbody>
</table>
HRP  Horseradish Peroxidase
hsa  homo sapiens (human)
IGF2  insulin-like growth factor 2 (somatomedin A)
IgG  Immunoglobulin G
ING1  Inhibitor of growth family, Member 1
IRB  Institutional Review Board
JAK  Janus Kinase
JNK  c-Jun NH(2)-terminal kinase
KCNA3  potassium voltage-gated channel, shaker-related subfamily, member 3
K-Ras  V-Ki-Ras2 Kirsten Rat Sarcoma Viral Oncogene Homolog
laf  elephant
LASP1  LIM and SH3 protein 1
LCM  laser-captured microdissection
LNA  locked-nucleic acid
LNC RNA  long non-coding RNA
LnCaP  series of hormone-progressive prostate cancer cell lines
M12  metastatic prostate cancer cell line
M2182  moderately metastatic prostate cancer cell line
MALAT-1  Metastasis Associated Lung Adenocarcinoma Transcript 1
MAPK  mitogen-activated protein kinase
mcs  multiple cloning site
MET  Hepatocyte growth factor receptor (met proto-oncogene)
mfe  minimum free energy
miR, microRNA
miRNA
mL milliliter
MMCT Microcell-mediated chromosome transfer
mml rhesus monkey
MMP matrix metalloproteinase
mmu mouse
mRNA messenger RNA
MS marrow-stromal
MVB multivesicular body
MyB v-myb avian myeloblastosis viral oncogene homolog
Myc V-myc myelocytomatosis viral oncogene homolog
MycN V-myc myelocytomatosis viral oncogene homolog, Neuroblastoma derived
NDUFA4 NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4
NF-kB nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NMES-1 non-mucosal esophageal protein 1
NPC nasopharyngeal carcinoma
NPM1 nucleophosmin 1
OC Osteocalcin
oga bushbaby
OPN Osteopontin
OPN Secreted phosphoprotein 1 (Osteopontin)
p (protein) phosphorylated version of (protein)
p21 WAF1, cyclin-dependent kinase inhibitor 1 or CDK-interacting protein 1
p27 26S proteasome non-ATPase regulatory subunit 9
p53 tumor protein 53
P69 benign epithelial prostate cell line
PBS phosphate-buffered saline
PC3 metastatic prostate cancer cell line
PCR polymerase chain reaction
PDGF platelet derived growth factor
PI3K Phosphoinositide 3-kinase
PLAG1 pleiomorphic adenoma gene 1
PLC phospholipase C
POLR3F polymerase (RNA) III (DNA directed) polypeptide F
PROX1 Prospero Homeobox 1
PSA prostate-specific antigen
PTBP2 polypyrimidine tract binding protein 2
PTEN Phosphatase and tensin homolog
ptr chimpanzee
qPCR quantitative polymerase chain reaction
Rb Retinoblastoma protein
REST RE1-Silencing Transcription Factor
RISC RNA-Induced Silencing Complex
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNU</td>
<td>Small Nucleolar RNA (also SNORD)</td>
</tr>
<tr>
<td>RPMA</td>
<td>Reverse-phase Protein Microarray</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>reverse-transcription quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>SCARA3</td>
<td>scavenger receptor class A, member 3</td>
</tr>
<tr>
<td>SCF</td>
<td>stem cell factor</td>
</tr>
<tr>
<td>SCFR</td>
<td>Stem Cell Factor Receptor (see cKit)</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering ribonucleic acid</td>
</tr>
<tr>
<td>SNP</td>
<td>single-nucleotide polymorphism</td>
</tr>
<tr>
<td>socs5</td>
<td>Suppressor of cytokine signaling 5</td>
</tr>
<tr>
<td>SOS</td>
<td>Son of Sevenless protein</td>
</tr>
<tr>
<td>Sp1</td>
<td>specificity protein 1 transcription factor</td>
</tr>
<tr>
<td>SRC</td>
<td>Proto-oncogene tyrosine-protein kinase</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducers and Activators of Transcription</td>
</tr>
<tr>
<td>STR</td>
<td>short tandem repeat</td>
</tr>
<tr>
<td>SV40T</td>
<td>simian virus 40 large T antigen</td>
</tr>
<tr>
<td>TAGLN2</td>
<td>transgelin 2</td>
</tr>
<tr>
<td>tbe</td>
<td>tree shrew</td>
</tr>
<tr>
<td>TBK1</td>
<td>NF-κB activating kinase (Tank-binding kinase 1)</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered Saline with Tween-20</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TMB</td>
<td>tetramethylbenzidine test for blood detection</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor-alpha</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VCU</td>
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</tr>
<tr>
<td>vegf</td>
<td>vascular endothelial growth factor</td>
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<td>WMPY1</td>
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<td>ZFP3</td>
<td>zinc finger protein 3 homolog (mouse)</td>
</tr>
<tr>
<td>ZNF148</td>
<td>Zinc finger protein 148</td>
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Abstract

VARIATION AND MODULATION OF MICRORNAS IN PROSTATE CANCER AND BIOLOGICAL FLUIDS

By Sarah Joy Seashols, Ph.D.

A dissertation) submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry at Virginia Commonwealth University.

Virginia Commonwealth University, 2013

Major Director: Zendra Elizabeth Zehner, PhD.  Professor, Department of Biochemistry & Molecular Biology

Prostate cancer is the second-most diagnosed and fatal carcinoma for males in the United States, and better diagnostic markers and potential therapies are needed. microRNAs are small, single-stranded RNA molecules that affect protein expression at the translational level, and dysregulation can dramatically affect cell metabolism. Comparison of 736 microRNA expression levels between the poorly metastatic SV40T immortalized prostate epithelial cell line P69 to its highly tumorigenic and metastatic subline M12 identified 231 miRs that were overexpressed and 150 miRs that showed loss of expression in the M12 cell line. Further evaluation of fourteen identified miRs was accomplished using other prostate cell lines as well as laser-capture microdissected prostate samples. Inhibition of miR-147b was found to affect proliferative, migratory and invasive capabilities of M12 cells, and reduced tumour
growth in nude athymic mice. AATF, an activator of the cell-cycle inhibitor p21, was identified as a target. Overexpression of miR-9 was found to affect the epithelial to mesenchymal transition through suppression of e-cadherin, a protein characterized as lost in EMT, as well as suppression of SOCS5, an attenuator of JAK-STAT signaling. Inhibition of miR-9 resulted in reduction of migratory and invasive potential, and significant reduction of tumorigenesis and metastases in male nude athymic mice.

miR-17-3p was previously identified as down-regulated in prostate cancer and loss of miR-17-3p shown to cause vimentin transcriptional activation. Reverse phase microarray analysis (RPMA) identified c-KIT as a potential second mRNA target for miR-17-3p. miR-17-3p was shown to modulate not only protein levels, but also messenger RNA levels of c-KIT. Four miR-17-3p binding sites in the c-KIT mRNA were identified. Thus, a number of microRNAs involved in prostate cancer were identified, and their targets found to be highly relevant to tumour progression and could potentially be used as targets for therapy or diagnostics.

Stability of microRNAs in forensically relevant biological fluids was evaluated through heat treatment, ultraviolet radiation, and chemical treatment. The dried body fluids showed some susceptibility to harsh treatment, but in most cases microRNAs were still detectable in the samples. microRNAs could represent a highly stable species for body fluid identification methods in forensic science.
I. The prostate and incidence of cancer

The prostate is an exocrine organ located at the bladder-urethral junction that serves to secrete an alkaline fluid that makes up 50-70% of seminal fluid. This fluid is composed of nutrients and other compounds that facilitate spermatozoa activation, survival and genomic integrity\(^1\). As a ductal gland, the prostate consists of multiple lobes in which epithelial cells secrete their expressed products into glandular lumens, and are compressed by the pelvic floor muscles during ejaculation to secrete the prostatic fluid along with spermatozoa stored in the ejaculatory ducts into the urethra.

Prostate cancer (CaP) is the most common cancer for men in the United States other than skin cancer, and the second leading cause of cancer deaths in the US, with over 29,000 fatalities each year\(^2\). While two-thirds of the prostate cancers diagnosed are indolent, wherein the tumour grows so slowly that the diagnosed male will likely die of other causes before the carcinoma develops to a dangerous level, the other one-third of diagnosed prostatic neoplasias are highly aggressive and metastasize readily to bone and brain\(^3,4\).

Diagnosis of Prostate Cancer

The current standard for diagnosis of a potential prostate cancer diagnosis is use of the prostate-specific antigen (PSA) test as a screening test, followed by manual examination and ultrasound-guided transrectal biopsy\(^5\). PSA is a protein that is produced in the prostate, and is normally found in high levels in seminal fluid, with lower levels in male urine, and also in ultra-low levels in breastmilk, amniotic fluid, cerebrospinal fluid, and other minor fluids\(^6\). Identification of PSA in the bloodstream has
been shown to be indicative of abnormal prostate growth\textsuperscript{2,3,6-8}. Mechanistically, PSA leaks into the bloodstream as the tumour both increases PSA production, and perturbs the glandular form of the prostate, resulting in leakage of the overexpressed PSA into the bloodstream\textsuperscript{9}.

Before the implementation of PSA testing in the US in 1979, prostate cancer diagnosis was made via a prostate examination only. Diagnosis using physical examination often was only positive after the tumour was well-advanced, and thus prostate cancer was often progressed to the point that chemotherapeutics were not significantly effective. PSA level analysis was first used for monitoring therapeutic outcomes\textsuperscript{10}. However, further research showed that it could also be used for diagnosis, and upon implementation of PSA screening, death rates for prostate cancer have dropped 4% per year.

A blood PSA level of 4.0 ng/mL is considered to be abnormal in the United States. PSA screening is not recommended for men of limited life expectancy, which is typically classified as men 75 years or older, due to the nature of the very common indolent form of prostate cancer\textsuperscript{8}. In a 5-year DOD study, of 295,645 men included in the analysis, 8.5% of the men evaluated had an abnormally high PSA level. Of the 67% who did not have a biopsy, 0.1% died of prostate cancer. Of the 33% that had a biopsy performed, 5220 (62.8%) were diagnosed with high-risk prostate cancer and were treated. 3.5% of the 1161 diagnosed with low-risk CaP died from prostate cancer, an average death rate for cohorts with or without treatment\textsuperscript{8}. Thus, this and multiple additional studies have shown that the intervention rate is well above that which is necessary\textsuperscript{4}.
Interventions tend to have drastic consequences for the diagnosed male; radical prostatectomies, cryosurgery, and androgen ablation therapy significantly affect patient quality of life through high levels of incontinence, psychological, and sexual side effects\(^3\). Thus, the identification of new screening practices is critical to diagnosing not only prostate cancer, but also differentiating between the aggressive and indolent forms of the disease.

*Transmission of genetic predisposition for prostate cancer*

While high incidences of prostate cancer are common in those of Northern European and African American descent, native Africans do not suffer such high incidences, although it could also be underestimated due to lack of surveillance\(^7\). Additional evidence indicates that the susceptibility is Northern European in origin, specifically of Swedish and/or Scandinavian origin, and follows the historical migration routes of the Scandinavian people, with a higher incidence on the coasts of continents. The high incidence seen in African Americans as compared to their native African counterparts is most likely due to low levels of Northern European inter-breeding\(^7\), but diet could also have an additive effect\(^11\). Likewise, men of Asian descent do not develop malignancies at high rates\(^7\).

As may be expected from the observed ethnic disparities, monozygotic twin studies have shown that inheritance of the predisposition for prostate cancer is real\(^12\). Additionally, evaluation of paternal and maternal histories show similar inheritance patterns, which indicates autosomal transmission\(^4,13,14\). Recent genome-wide association studies (GWAS) have identified forty-six SNPs that show enhanced
susceptibility to CaP; studies are currently underway to evaluate both diagnostic possibilities as well as treatment protocols based on those loci\textsuperscript{13}.

\textit{Prostate Cancer tumorigenesis & histopathology}

Prostate cancer, like breast cancer, is the tumorigenesis of a glandular tissue. As such, the carcinoma begins in the ducts of the gland, with small lesions gradually building up over time and collecting additional mutations that allow the tumour to progress to the invasive stage. At this point, the neoplastic cells are able to disrupt the periglandular basement membrane and migrate into the surrounding stromal tissue\textsuperscript{15}. Tumour progression is scored using the Gleason scoring model, wherein the average and maximum observed tumour grade are evaluated by a pathologist (Figure 1-1)\textsuperscript{16}. 

Figure 1-1: Schematic diagram of histological progression of Prostate Cancer using Gleason scoring. In the progression of CaP, (GS-1) normal prostate epithelium begins to become hyperplastic (GS-2), and progression into a tumour with smooth round edges (GS-3). As the tumour becomes invasive, the edges of the tumour becomes ragged and glandular fusion is observed (GS-4). A GS-5 score is made when the tumour has progressed to the point that the tumour core has necrosed, due to the need for oxygen and blood flow (Adapted from Epstein et al.16).
Treatment of Prostate Cancer

Treatment of newly diagnosed prostate cancer typically involves a multi-modal approach. Tumour removal can be accomplished through radical prostatectomy or cryosurgery, which entails laparoscopic liquid nitrogen ablation of tumour cells. When necessary, pelvic lymphadenectomy is performed as well to determine the invasiveness of the carcinoma. Surgical removal is then usually followed by radiation therapy using external beam therapy, brachytherapy, and/or proton beam therapy. Traditional chemotherapeutics and diet modification are also commonly implemented.

Treatment of early prostate cancer also typically involves attacking the hormone-dependent nature of the early tumour. As it is androgen-dependent for growth, early success in tumour treatment typically involves hormonal ablation through the use of androgen chelating agents, or even as extreme as surgical castration. However, this treatment is only effective in the short-term, as the neoplasias that survive then become hormone-independent, and can metastasize readily throughout the body. At that point, treatment options are limited, and the cancer is usually terminal. Prostate cancer cells exhibiting androgen insensitivity still have active androgen receptor (AR) pathways. Thus, recent work has shown that androgen-independence of the tumour cell has been shown to be not so much “independence” of androgens per se, but a bypass of the need for external androgen activation of those same pathways.
II. Tumour progression through the epithelial to mesenchymal transition, angiogenesis, and metastasis

All epithelial tumours that eventually metastasize go through the epithelial to mesenchymal transition (EMT), wherein the cells develop a phenotype that is no longer polar, stationary and adherent, and assume a mesenchymal phenotype, which is motile. This change allows for invasion through the basement membrane into the lymphatic system, and eventually the bloodstream. The circulating tumour cells can then exit the bloodstream, and undergo the reverse phenomenon, mesenchymal to epithelial transition (MET), for clonal growth at the metastatic site. The EMT transition is marked by changes in cytoskeletal protein expression and matrix metalloproteinase induction.

Changes in cytoskeletal protein expression

Changes in cytoskeleton protein expression are required for EMT, and canonically used as a marker of the EMT state of the cancer cell. The early tumour epithelial cell must suppress e-cadherin and develop a concomitant increase in vimentin expression. E-cadherin is a transmembrane adherens protein that anchors epithelial cells to each other in a calcium-dependent manner, and ultimately through interaction cytoplasmically with β-catenin, to the actin cytoskeleton. Loss of e-cadherin results in dissociation from the epithelial sheet, and allows for extravasation through the basement membrane. Likewise, increased expression of vimentin, an intermediate filament protein that imparts stability to the motile cell, is a canonical marker of the mesenchymal cell and has been shown to be expressed in poorly differentiated prostate cancer tumours. Additionally, siRNA inhibition of vimentin expression has been shown to significantly impact motility of PC-3 prostate cancer cells, although the
complete mechanism is unknown\textsuperscript{26}. Matrix metalloproteinase enzymes are shown to cleave the cadherins (E-, N-, and P-), contributing to release of cell-cell contact, increased cell migration, and loss of epithelial cell polarization\textsuperscript{27-29}.

\textit{Angiogenesis of the prostate tumour}

A microtumour can develop to the size of 2-3 mm with no requirement for angiogenesis, as that is the diffusion limit of oxygen and nutrients from surrounding capillaries. As the tumour grows larger, the core of the tumour necroses (Figure 1-1), and the surrounding tumour cells adapt to induce angiogenesis. Key proteins induced in the angiogenic process include vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), platelet derived growth factor (PDGF), and tumor necrosis factor alpha (TNF-\alpha) (reviewed in Amankwah et al.\textsuperscript{30}), all of which act to stimulate the proliferation and migration of endothelial cells in microvasculature formation\textsuperscript{31}.

\textit{Metastases in Prostate Cancer}

Metastases in CaP typically travel through the lymphatic system first to lymph nodes, then move into the bloodstream and home to bone and brain. Bone metastases commonly occur in the vertebrae, sternum, pelvic bone, ribs, and femurs\textsuperscript{32}. During the malignant transformation of prostate cells to metastatic neoplasias, it has been commonly observed that the cells switch from an epithelial to a osteomimetic, or bone-like expression, with initiated expression of bone matrix noncollagenous proteins such as osteopontin (OPN), Osteocalcin (OC), and bone sialoproetin (BSP). These proteins are essential in that OPN assists in osteoclast adhesion, BSP stimulates osteoblast differentiation, and OC is secreted as a chemoattractant for osteoclast remodeling. This
change in expression has been postulated to allow for integration of the neoplasia into the bone.

III. microRNAs and their role in cancer progression

MicroRNAs are a class of small RNAs that were described in C. Elegans in 1993, and have since emerged as major regulators of protein levels through attenuation of translation at the ribosome. MicroRNAs, or miRs, are 19-22 nucleotide single-stranded RNA sequences that are guided by a protein complex to their mRNA targets, typically in the 3’-UTR of the mRNA. miRs are present in all animals and plants, as well as chromalveolata, slime molds, viruses, and yeast. Over 2578 mature microRNAs have been described in humans. microRNA genes are found throughout the genome, and can be alone, part of another gene within or without exonic regions, or in a multicistron of multiple microRNAs in tandem.

miR transcription and processing

miRNA transcription is often driven by standard transcription factor activation, including c-Myc, Sp1, Ets-1, STAT1α, and NF-kB, and most miRs are transcribed by RNA Polymerase II. The immature miRNA anneals onto itself in the form of a hairpin loop, whether transcribed solo, within a gene, or in a polycistron. The hairpin loop is cleaved from the surrounding message within the nucleus by the RNase III enzyme Drosha microprocessor complex, which requires the DGCR8 protein as a cofactor. The resultant 65-100 nt hairpin pre-miRNA is then exported from the nucleus via Exportin V, which recognizes the double-stranded stem and short single-
Figure 1-2: Biogenesis of microRNAs. The microRNA is transcribed by Pol II, and the pre-miRNA is excised from the transcript by Drosha. Export from the nucleus is active and assisted by Exportin V, and upon exit into the cytoplasm, the pre-miRNA is bound and cleaved into the mature single-stranded form by the DICER complex. The RISC complex then guides the mature miR to its target mRNA for translational suppression and cleavage in some cases. (Adapted from Davis-Dusenbery et al.\textsuperscript{46})
stranded overhang created by Drosha cleavage\textsuperscript{49,50}, and further processed into a mature, 19-21 nucleotide single strand in one of two ways (Figure 1-2). Typical processing occurs by Dicer, an RNase type III protein, along with its cofactors, but in some cases, Argonaute 2 (Ago2) itself has been shown to perform cleavage without need for Dicer\textsuperscript{46,51}. At this point, the selected strand of the pre-miR hairpin is retained in the RNA-Induced Silencing Complex (RISC) and the passenger strand is ejected from the complex. Both strands of the immature microRNA can be active in mRNA translation suppression. Strand selection of the Ago protein that receives the mature miR has not yet been resolved, although it appears that while some miRs can be processed and used for repression using all Ago proteins, some miRs are Ago-specific\textsuperscript{51}.

The RISC is a complex that is primarily composed of Argonaute, an RNA-binding protein, along with the mature miR strand and several co-factor proteins\textsuperscript{50,52,53}. There are four Argonaute proteins that are capable of forming the RISC and interacting with microRNAs for translational repression of targets.

\textit{miR action}

The RISC enfolds around the mature microRNA such that the single-stranded RNA is bound within the protein, with both 5’ and 3’ ends protected from RNase degradation, and nucleobases 2-9 on the 5’-end of the miR in optimal position for binding to complementary sequences\textsuperscript{54}. The RISC complex then travels along the mRNA, the miR binds to the complementary portion, and this
Figure 1-3: Mechanism of action on mRNA targets of the microRNA-RISC complex. Most microRNA binding events result in binding to the mRNA target and translational suppression. However, in the presence of Argonaute 2, which has been shown to have Slicer activity, a perfect seed-region match (nt 2-9 of the mature microRNA) to a complementary sequence of the mRNA results in message cleavage and subsequent degradation.
binding can cause translational repression, or when the seed-mRNA match is exact and Ago2 is present, cleavage of the mRNA and subsequent degradation⁴⁴,⁵¹,⁵⁵ (Figure 1-3).

**Secreted miRs**

microRNAs can be secreted from the cell for signaling in a paracrine, endocrine or autocrine manner. microRNAs can be bound in microvesicles, in protein complexes, or encapsulated in HDL particles in association with ApoB⁵⁶-⁵⁹. The miRs present in vesicles appear to be miRs secreted for a purpose such as communication, as the relative abundance levels of miRs found in exosomes of a particular cell type are not representative of the miR abundance levels found in those cells⁵⁹. Indeed, cell-cell communication and resultant changes in protein expression of receiving cells have been shown in recent reports⁴⁴.

microRNAs that have been shown to be found in vesicles fuse with the plasma membrane through the multivesicular bodies (MVBs). They display the same general characteristics observed in other secreted vesicles, with similar size and shape, as well as the presence of CD63 antigens when secreted by dendritic, B and T cells⁵⁹. miRs contained in vesicles and released into the extracellular environment have been shown to be resistant to RNase as well as trypsin, indicating that the miRs are neither free-floating in media, nor anchored on the outside of the vesicle.⁵⁹ miRs have also been found to be protected from RNase digestion while associated with free protein complexes, including nucleophosmin 1 (NPM1)⁵⁷.

*miRs as tumour suppressors and oncomiRs*
microRNAs can bind either perfectly or imperfectly to an mRNA target, thus making it possible for one miR to attenuate the translation of tens to hundreds of different targets. Additionally, miRs have been shown to impact all aspects of the proteome, from cell proliferation and apoptosis to mitochondrial and metabolic processes, to cytoskeleton and secreted products. Thus, microRNAs have recently been subject to intense scrutiny as modulators of protein levels in cancer, as they are increasingly being shown to influence carcinogenesis and tumour progression. Since the target protein level has an inverse relationship to its regulating microRNA, an overexpressed microRNA is known as an oncomiR, and causes excessive suppression of its tumour suppressor target mRNA. A tumour suppressor microRNA is one that has reduced or lost expression, and thus an oncogene that it should regulate is translated and expressed in an unchecked manner.

IV. The laboratory’s research objectives and models in use

The objective of our laboratory’s research is to identify potential biomarkers or therapeutic targets for prostate cancer. We use a combination of in-vitro, in-vivo, and human prostate cancer samples for validation and verification of our findings.

The P69 cell line is the foundation for a unique progression model for prostate cancer. The cell line was created from human non-neoplastic prostate epithelial cells isolated from a transurethral resection of a 63-year old African American male. The cells were isolated from the extracellular matrix using collagenase and then immortalized by transfection with the SV40 large T antigen gene. They retain epithelial, and specifically basal epithelial cytokeratin markers (basal identification...
unpublished data from ZEZ), and are poorly tumorigenic (2/18 mice injected subcutaneously developed tumours after a long latent period, no metastases).

Metastatic sublines were created by injecting P69 cells subcutaneously into nude athymic mice. Tumours that developed were excised, analyzed for histopathological origin, and placed into culture. They were then grown up and subsequently injected subcutaneously into additional nude athymic mice. This cycle was repeated two additional times. Each successive injection resulted in faster and more frequent tumorigenesis\textsuperscript{61,62}. The M2182 cell line was derived from a tumour derived from the second series of injections, and the M12 cell line, a highly tumorigenic, highly metastatic cell line\textsuperscript{63}, was derived from the tumour excised from a mouse from the third series of injections. The M12 cell line produces metastases in lung and diaphragm when injected subcutaneously, intraperitoneally, and intraprostatically\textsuperscript{60}. Thus, the P69, M2182, and M12 cell lines represent a progressively tumorigenic model of prostate cancer (Figure 1-4).
Figure 1-4: Generation of metastatic sublines from the poorly tumorigenic normal prostate epithelial SV40T immortalized P69 cell line. Injection of P69 cells resulted in tumours in only 2 of 18 mice after a long latent period. An excised tumour was then placed into culture, and the subsequent cells injected into a second nude athymic mice.

This process was repeated one more time, resulting in the highly metastatic and tumorigenic M12 cell line, which develops tumours in 100% of mice within 12 days, and produces metastatic sites in the diaphragm and lung.
Cytogenetic analysis of the M12 cell line revealed deletion of the p-arm and a portion of the proximal arm of chromosome 19 (19q13.1 → 19pter), as well as loss of the Y-chromosome\textsuperscript{60}. Serendipitously, identification of this deletion sparked the laboratory's initial interest in microRNAs as chromosome 19 contains a large cluster of microRNAs known to be crucial to development. Additionally, the miR-17-92 cluster on chromosome 13 is known to be highly regulated, activated by c-Myc, and dysregulation has been shown to be associated with cancer\textsuperscript{42,64,65}. Eight of the 21 miRs known to translationally modulate p21, a cell-cycle inhibitor protein and known effector of multiple tumour suppressor pathways, are found in the chromosome 13 miRNA cluster\textsuperscript{37}. Chromosome 19 was reintroduced into the M12 cell lines using microcell-mediated chromosome transfer (MMCT), and injection of this F6 cell line showed that reintroduction of chromosome 19 resulted in poor tumorigenesis, loss of metastatic potential, and increased adhesion of the resultant cells\textsuperscript{66}.

The P69, M12, M2182, and F6 lines are pseudodiploid, which makes the model appropriate to study early pre-aneuplodic prostatic carcinomas. In contrast, the canonical prostate cell lines PC-3, DU145, and LnCaP cell lines were derived from metastatic sites (reviewed in Chapter 3), and are known to have high chromosomal numbers\textsuperscript{60,61}, an observation commonly made of late stage carcinomas.

*Dysregulated miRs identified through the P69-M12 progressive model*

Previous work in the laboratory has shown that miR-17-3p, a microRNA found in the miR-17-92 cluster on chromosome 19, is downregulated in the M12 cells as compared to the P69 cell line. This loss of expression was shown to have a positive
effect on vimentin expression through direct targeting by miR-17-3p on vimentin. Thus, loss of miR-17-3p resulted in an increased level of vimentin, which was then shown to be reversed upon stable transformation with miR-17-3p, along with reduced tumorigenicity (Figure 1-5)\textsuperscript{25}.

Recent work has focused on miR-125b as a tumour suppressor miR that was lost in M12 cells as compared to P69. Analysis of benign and tumour tissue captured through laser-captured microdissection of prostate biopsies showed a similar pattern. Restoration of miR-125 through stable transformation resulted in reduced migratory and invasive potential of M12 cells (Figure 1-6)\textsuperscript{67}. miR-125b has been well characterized as a suppressor of ERBB2 & 3, receptor tyrosine kinase hormone receptors known to be upregulated in some carcinomas\textsuperscript{68-71}.
Figure 1-5: miR-17-3p directly binds and modulates vimentin mRNA, an intermediate filament key to the EMT transition. A: Vimentin mRNA expression is increased through the P69 progression line, and B: miR-17-3p is decreased concomitantly. C: miR-17-3p directly binds to a portion of the vimentin 3’-UTR, and D: mutation of the 3’-UTR binding site results in loss of vimentin suppression. From Zhang et al.25.
Figure 1-6: miR-125b is downregulated in prostate cancer, which allows for activated transcription of ERBB2/3 and activation of the AKT pathway. A: miR-125b was shown to be down-regulated in M12 cells as compared to P69 cells, as well as in prostate cancer tumour tissue captured via laser capture microdissection. B: Restoration of miR-125b resulted in reduced migratory and invasive capability of M12 cells C: through postulated inhibition of the AKT pathway. From Budd et al\textsuperscript{67}. 

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**Figure 3-5: Model of miRNA dysregulation of PI3K/AKT pathway**

- **miR-125b**
- **ERBB2/ERBB3**
- **miR-22**
- **PI3K**
- **PTEN**
- **AKT**
- **Increased proliferation and decreased apoptosis**

---

**A**

![Histogram showing miRNA expression levels](image)

**B**

![Bar graph showing invasion levels](image)

---

**C**

Diagram illustrating the dysregulation of PI3K/AKT pathway by miR-125b.
V. Scope of this thesis

Identification of dysregulated microRNAs in prostate cancer and their target mRNAs

Our laboratory is focused on identifying microRNAs that are modulated in cancer along with their mRNA targets, thus providing not only a microRNA, but to further elucidate the modified pathway and its contribution to progression of the prostate carcinoma. The work in Chapter 3 details a panel-wide analysis of dysregulated miRs in the M12 cell line, and confirmation of oncomiR or tumour suppressor expression through additional prostate cell line analysis along with human prostate samples.

Chapters 2, 4, and 5 investigate specific miRs shown to be modulated in prostate cancer through the multifaceted analysis such as used in Chapter 3. c-KIT, an RTK growth factor receptor, is identified and confirmed as an additional target of miR-17-3p; thus loss of that miR results in increased levels of c-KIT and activation of proliferative pathways. Chapter 4 focused on the identification of miR-9 as an oncomiR, and evaluates proven targets e-cadherin, NF-kB, and SOCS5 for modulation in the prostate cancer model. Chapter 5 identifies miR-147b as a novel oncomiR in cancer, and specifically evaluates modulation and consequent impact on tumour growth and invasive capabilities. AATF is identified as a novel target for miR-147b through protein and mRNA expression analysis.

This work culminates in an analysis that, as expected, illustrates that no one microRNA is responsible for the progression of a prostate tumour. Rather, the compilation of the work of this laboratory has identified multiple microRNAs that each
contributes to the progression of the tumour, and their individual contributions result in an overwhelming activation of proliferative pathways and repression of terminal ones.

*Stability of microRNAs in forensically relevant biological fluids*

Dr. Zehner’s and my work began with a question of whether microRNAs could be used for body fluid identification in forensic samples. As a forensic scientist and a researcher, body fluid identification has long been my passion, and being well familiar with the limitations of the currently accepted and researched techniques, she and I were curious as to whether her interest, microRNAs, could be used in a forensic context. This collaboration began at the cusp of the exploratory literature of microRNAs in the forensic biology field, and thus our interest in using miRs as a potential molecular method of body fluid identification must first be tested through an evaluation of the stability of miRs in forensically relevant body fluids. Thus, while wildly different from the rest of the thesis, this stability evaluation also holds a place in our research, and answers some unresolved questions in the forensic science field about the utility of miRs for forensic science.
Chapter 2

Identification of c-KIT as a novel target for miR-17-3p in prostate cancer
Our laboratory uses the previously described SV40T immortalized human prostate cancer cell line progression model, initiated with P69. The P69 line is analogous to and derived from normal prostatic tissue and is poorly tumorigenic in nude athymic mice, whereas the subline M12 is highly tumorigenic and highly metastatic. This unique model in prostate cancer research permits the identification of additional players required for the progression of prostate tissue from benign to tumour. Thus, both microRNAs and their protein targets that are in fact driving the change in neoplastic potential between the two cell lines can be elucidated.

Previous work had identified vimentin mRNA as differentially expressed between the P69 and M12 cell lines\(^{26}\) (Figure 2-1a), in correlation with the change in EMT between the related cell lines and acquired invasive potential of the M12 subline. Investigation into the source of this differential expression revealed a highly conserved 3'-UTR with a binding site for miR-17-3p in the human vimentin mRNA. Evaluation of miR-17-3p levels in the P69 to M12 progression model showed a gradual loss of miR-17-3p (Figure 2-1b), with a concomitant increase in vimentin expression. Restored expression of miR-17-3p in the M12 cell line by stable transformation resulted in a five-fold increase in miR-17-3p expression, causing reduced tumour growth in subcutaneous injection in mice and reversion of vimentin message and protein expression (Figure 2-1c).
Figure 2-1: miR-17-3p is lost in the P69-M12 progression model, which affects vimentin expression. A: Vimentin mRNA levels increase through the P69 to M12 progression model. B: The increase in vimentin is inversely correlated to a loss of miR-17-3p in this progression. C: Stable reintroduction of miR-17-3p results in suppression of vimentin (from Zhang et al\textsuperscript{25}).
As part of the miR-17-92 cluster on chromosome 13q31.3, miR-17 (5p and 3p), as well as the other members of the cluster (miRs-18a, -19a&b, -20a, and -92a) are all transcribed by Pol II in the same polycistronic RNA, cleaved to individual precursor miRs, and exported and processed to mature miRNAs in the cytoplasm. Transcription of the cluster is activated through c-Myc and the protooncogene Pim-1, and thus the miR-17-92 cluster is commonly described as overexpressed in cancer. miR-17-3p is not well researched, while the other strand, miR-17-5p, has been well-established as an oncomiR. Thus, it is postulated that during tumorigenesis, activation of the miR-17-92 cluster results in overexpression of certain members of the cluster, with strand selection for miR-17-5p and concomitant loss of miR-17-3p. This could result in not only loss of tumour suppressor protein expression by miR-17-5p, but also loss of regulation of oncogenes by miR-17-3p.

In an effort to identify additional proteins that may be modulated through the loss and subsequent re-expression of miR-17-3p in M12 cells, a collaboration was formed with Dr. Emanuel Petricoin, of George Mason University, who along with Dr. Lance Liotta, first described the Reverse phase Protein Microarray, or RPMA. The RPMA is a method in which a cell or tissue lysate, containing the entire proteome of that sample, is spotted at a concentration of 250 µM onto glass-backed nitrocellulose slides, and then fluorescently tagged antibodies are washed across the slides. This allows for rapid analysis of hundreds of individual samples, with demonstrated use in therapeutic proteomics. Spotting the same lysate onto hundreds of slides can make for rapid immunohistochemical analysis of a large set of proteins that could be implicated in tumorigenesis. RPMA analysis and comparison between the P69, M12, and M12 cells
+ miR-17-3p identified the phosphorylated version of the V-Kit Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene Homolog, also known as c-KIT, CD117, and/or Stem Cell Factor Receptor (SCFR), as differentially phosphorylated between the M12 and P69 cell lines\textsuperscript{67}. In fact, c-KIT was the most dramatic differentially expressed protein between the two cell lines, warranting further investigation (Table 2-1).

Interestingly, reintroduction of miR-17-3p to the M12 cell line also resulted in total ablation of the p-c-KIT signal in the proteomics analysis (Figure 2-2). Phospho-c-KIT levels were undetectable in the benign prostatic cell line P69, and highly detectable in the tumorigenic and metastatic M12 cell line. Reintroduction of miR-17-3p resulted in total loss of the p-c-KIT signal.

Given these dramatic changes in c-KIT levels between the P69 and M12 cell lines with complete ablation in the M12 + miR-17-3p transformant, it was important to determine the mechanism of c-KIT modulation, as well as investigating which signaling pathway(s) were subject to the tumour suppressor activity of miR-17-3p.
Table 2-1 – Significant differences in key proteins in M12 versus P69 cell lines as determined by RPMA proteomic analysis. $p =$ phospho-specific antibody

<table>
<thead>
<tr>
<th>Protein</th>
<th>Fold Change (M12 to P69)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-c-KIT</td>
<td>700</td>
</tr>
<tr>
<td>p-ErbB3</td>
<td>2</td>
</tr>
<tr>
<td>p-ErbB2</td>
<td>1.8</td>
</tr>
<tr>
<td>p-Androgen Receptor</td>
<td>1.7</td>
</tr>
<tr>
<td>p-BAD</td>
<td>1.7</td>
</tr>
<tr>
<td>Caspase 7</td>
<td>1.5</td>
</tr>
<tr>
<td>p-PI3 kinase</td>
<td>1.4</td>
</tr>
<tr>
<td>p-GSK3A/B</td>
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</tr>
<tr>
<td>BCL2</td>
<td>1.2</td>
</tr>
<tr>
<td>p-p27</td>
<td>0.7</td>
</tr>
<tr>
<td>Cyclin B1</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*adapted from Budd dissertation^67
Figure 2-2: Re-Introduction of miR-17-3p into M12 cells ablates p-c-KIT levels.

RPMA analysis was conducted on 3 sequential passages of each cell line. Serial dilutions of each replicate were probed for phospho-c-KIT, and expression normalized against the global protein expression mean.
c-KIT is a 145 kDa transmembrane receptor tyrosine kinase, identified immunologically as CD117. It is typically only expressed in high levels in hematopoietic stem cells, mast cells, melanocytic cells, germ cells, and interstitial cells of Cajal, and functions to promote cell proliferation and survival through interaction with its ligand stem cell factor (SCF). SCF can be either membrane-bound for an autocrine function, or secreted from the cell for a paracrine effect. Activation of c-KIT through ligand binding by SCF induces homodimerization, intermolecular tyrosine phosphorylation, and immediate internalization and rapid degradation via the ubiquitination pathway.

Upon binding and internalization, c-KIT protein is very slow to recover to previous levels, on the order of >24 hours. Levels are also heavily influenced by translational and transcriptional repression, further confirming a short half-life for the cell-surface protein.

The c-KIT gene is located in the white spotting locus 4q11-12, and has also been associated with piebaldism in both human and mouse. Transcriptional activation is accomplished through a variety of common transcription factors, including multiple Sp1, Ets, AP-2, and MyB binding sites. The signal transduction pathways activated by c-KIT include MAPKinase, PI3-Kinase, JAK/STAT, Src, and phospholipase C pathways (Figure 2-3, reviewed in Reber et al., Lennartsson et al.). As the majority of these pathways are pro-survival and/or pro-proliferation, activation of c-KIT in neoplasias is unsurprising, and consequently it is the target of several chemotherapeutic approaches.
Figure 2-3: c-KIT activation induces signal transduction in a variety of proliferative pathways (adapted from Reber et al.77)
Mutation causing a constitutively active c-KIT results in uncontrolled proliferation and has been found to be a primary tumorigenic cause of gastrointestinal stromal tumours (GIST)\textsuperscript{82}. This mutation is typically a ligand-independent RTK activity, resulting in constant stimulation of downstream proliferative pathways.

In normal prostatic tissue, c-KIT expression historically has been found only in mast cells\textsuperscript{83}, but recent studies have shown increasing c-KIT expression as tumour progression increases, with c-KIT positive tumours leading to disease relapse and bone metastasis\textsuperscript{76,84}.

In this study, we evaluated the potential of miR-17-3p as a modulator of c-KIT expression in a prostate cancer model. Proteomic analyses showed that c-KIT expression, while absent in the poorly-tumorigenic P69 cell line, was highly expressed in the M12 cell line, and reintroduction of miR-17-3p to the same cell line resulted in complete loss of c-KIT signal and reduced tumorigenesis and metastases.

*Project Aims:*

1. Confirm c-KIT modulation between the P69, M12, and M12 with stably expressed miR-17-3p cell lines through mRNA and protein expression analysis.
2. Identify miR-17-3p binding sites within the c-KIT message, and determine if they contribute to message suppression.
METHODS AND MATERIALS

Cell Culture

Adherent cells were cultured in 75 cm\(^2\) flasks using RPMI 1640 supplemented with L-Glutamine (Caisson Labs, North Logan, UT), 5% fetal bovine serum, 5 µg/mL insulin, 5 µg/mL transferrin, 5 µg/mL selenium (ITS, Collaborative Research, Bedford, MA). Gentamycin (0.05 mg/mL) was used to prevent bacterial contamination. M12 cells previously stably transformed with pSIREN:miR-17-3p vector (Clontech Laboratories, Inc., Mountain View, CA) were maintained using puromycin\(^{25}\) (100 ng/mL). Cells were passaged when 50-65% confluent, and maintained for no longer than 20 passages. Passaging was accomplished by incubating with 0.25% Trypsin-EDTA (Gibco-Life Technologies, Carlsbad, CA) for 5 minutes at 37°C, after which the trypsin was inactivated by washing the cells in serum-containing media. The cells were then pelleted through centrifugation at 1500xg for 5 minutes and either passaged, plated, or pellets preserved. Cell pellets for analysis were produced by washing the pellet in 10 mL PBS, centrifugation at 1500xg for 5 minutes, removal of the supernatant, and flash freezing in liquid nitrogen. Pellets were stored at -80°C for at least 24 hours.

RNA Isolation

RNA was isolated from cell pellets using the miRVana™ RNA isolation method according to the manufacturer's protocol (Ambion-Life Technologies, Carlsbad, CA), eluted in 50 µL of Elution Buffer, and stored at -80°C. RNA concentration was estimated using either the Smart Spec™ 3000 Spectrophotometer (Bio-Rad
Laboratories, Hercules, CA) or the NanoDrop ND-2000 Spectrophotometer (Thermo-Fisher Scientific, Inc., Waltham, MA).

**Messenger RNA Analysis**

Messenger RNA was quantified using reverse transcription-quantitative PCR (RT-qPCR). Reverse transcription was accomplished using the iScript™ cDNA Synthesis kit (Bio-Rad Laboratories). Briefly, 50 ng of RNA was mixed with 4 µL of the 5X iScript reaction mix, 1 µL of the iScript Reverse Transcriptase, and the volume brought to 20 µL with nuclease-free water. The reaction mixture was incubated for 5 minutes at 25°C, followed by 30 minutes at 42°C, and heat inactivation for 5 minutes at 85°C. Reactions were stored at -20°C until analysis. The qPCR reactions were prepared by adding 3 µL of the prepared cDNA, 10 µM primer pairs for the relevant mRNA target (GAPDH: Forward: 5’-ACCACAGTCCATGCCATCAC; Reverse: 5’-TCCACCACCTGTGTGCTGTA. c-KIT: Forward: 5’-ATGAGAGGCGCTCGGCGC; Reverse: 5’-AGCTTGGGAGGATCTCTCAAC), 1X FastStart Universal SYBR Green Master Mix (Roche Diagnostics, Indianapolis, IN), and brought to 20 µL volume with nuclease-free water. qPCR was conducted in an Applied Biosystems 7300 real-time PCR instrument (Life Technologies) using the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 55°C for 35 seconds, and 68°C for 35 seconds. Data was analyzed using SDS software v1.3.1 (Life Technologies), using automatic threshold and baseline settings. Each mRNA evaluated was analyzed in triplicate using a minimum of three separate cell passage RNA extractions. GAPDH was used as a normalization control, and relative expression was calculated using the comparative Cₜ method.⁸⁵
**Western Blotting**

Cultured cell pellets were lysed in 4% sodium dodecyl sulfate (SDS) in phosphate-buffered saline (PBS) with 1X PhosSTOP phosphatase inhibitor (Roche Diagnostics) and COMplete protease inhibitor (Roche Diagnostics), followed by sonication for five minutes at 4°C. Protein concentration was calculated using the Dc Protein Assay (Bio-Rad Laboratories), and absorbance read at 750 nm on the Smart Spec™ 3000 Spectrophotometer (Bio-Rad Laboratories).

For western blotting, equal quantities of cell lysate (30-40 µg) were loaded onto a Novex® 4-12% Tris-Glycine SDS polyacrylamide gel (Life Technologies), under denaturing conditions using a loading buffer with 2% SDS, 5% β-Mercuriethanol, 20% glycerol and 0.004% bromophenol blue in 0.125M Tris-HCl, and electrophoresed in a 25 mM Tris, 190 mM glycine, 0.1% SDS running buffer for approximately 90 minutes at 140 volts. The samples were transferred onto a Trans-Blot® nitrocellulose membrane (Bio-Rad Laboratories) for 90 minutes at 200 mA and 4°C in a 48 mM Tris, 39 mM glycine, 0.04% SDS transfer buffer. Nonspecific binding was prevented by washing the blot for 30 minutes at room temperature in 5% nonfat dry milk in Tris-buffered saline with 0.05% Tween-20 (TBST). The blot was rinsed briefly in TBST, and incubated overnight (14-20 hours) at 4°C in primary IgG antibody in 5% BSA in TBST. Primary antibodies used were 1:1000 α-β-actin (C4) produced in mouse (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA) and 1:500 α-CD117 produced in rabbit (Dako, Glostrup, Denmark). After incubation in primary antibody, the blot was washed with TBST four times for 5 minutes, followed by incubation in secondary antibody (1:1000 α-mouse IgG-HRP produced in goat (Santa Cruz Biotechnologies) and/or 1:1000 α-rabbit IgG-HRP
produced in goat (Cell Signalling Technology, Danvers, MA), for 1 hour at room temperature. The blot was again washed with TBST four times for 5 minutes, and luminol reaction developed using the Western Lightning® chemiluminescent reagents (Perkin Elmer, Boston, MA). Western blots were exposed and bands quantified using the ODYSSEY® Fc Imaging System (LI-COR Biosciences, Lincoln, NE).

**Cloning of 3’-UTR constructs**

A 612 base pair portion of the 3’-UTR of c-KIT containing both potential miR-17-3p binding sites was amplified from a cDNA plasmid containing the c-KIT 3’-UTR (gift from Dr. Jeff Krystal, Veteran’s Administration Hospital), and inserted into the XbaI restriction site in the MCS downstream from the luciferase gene in the pmiR-Glo vector (Promega Corporation, Madison, WI) using standard cloning techniques. This cloning reaction resulted in not only the desired insert in the correct orientation, but also 2 tandem copies of the 612 bp fragment.

Wild type and mutated fragments of the c-KIT 3’-UTR were synthesized through Invitrogen for oriented cloning into pmiR-Glo. The Wild type fragments were 63 and 64 nucleotides long (sites 2 and 1, respectively), and mutations at the seed region (Table 2-2) were synthesized as indicated. Failure of these mutations to bind miR-17-3p was verified through RNAHybrid\(^\text{86,87}\).
**Table 2-2: c-KIT 3'-UTR fragment constructs.** miR-17-3p binding site is highlighted in yellow, the seed region is highlighted in green, and mutated nucleotides are highlighted in red.

<table>
<thead>
<tr>
<th>Clone ID</th>
<th>Size</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site-1 WT</td>
<td>64</td>
<td>ATGATTGCTGGATTGATTTGATGCTGTTT<strong>G</strong>ACAAAGTTACTGATT<strong>C</strong>ACTGCATGGCTCCACAGGAGTGGGAAA</td>
</tr>
<tr>
<td>Site-1 Mut</td>
<td>64</td>
<td>ATGATTGCTGGATTGATTTGATGCTGTTT<strong>G</strong>ACAAAGTTACTGATT<strong>C</strong>GGACAATGGCTCCACAGGAGTGGGAAA</td>
</tr>
<tr>
<td>Site-2 WT</td>
<td>63</td>
<td>GTACAGCAAATAAAATATAGGTTTAGGCTTCTCTCT<strong>T</strong>CCAGGCAATGTCTGGACACCGGGGCCAGT</td>
</tr>
<tr>
<td>Site-2 Mut</td>
<td>63</td>
<td>GTACAGCAAATAAAATATAGGTTTAGGCTTCTCTCT<strong>T</strong>AGTGACGTGTGTCTGGACACCGGGGCCAGT</td>
</tr>
</tbody>
</table>
**Transfection & Luciferase Activity Analysis**

Transient transfections of the 3'-UTR fragments cloned into a firefly luciferase/renilla reporter vector were performed using TransIT®-LT1 Transfection Reagent (Mirus BIO LLC, Madison, WI, USA). Briefly, cells were plated to achieve 50% density in appropriate serum-containing media after 24 hours (1x10⁵ cells/well for a 6-well plate, or 2.5x10⁴ cells/well for a 24-well plate). The cells were allowed to adhere for 24 hours, rinsed with 0.5 mL PBS, and media replaced (2 mL for 6-well plate, 0.5 mL for 24-well plate). For 6-well plate transfections, 2.5 µg of the cloned 3'-UTR:vector was mixed with 250 µL of RPMI media with no serum or additives plus 7.5 µL of TransIT®-LT1 reagent at room temperature for 15 minutes. For 24-well plate transfections, 0.5 µg of the cloned UTR:vector was mixed with 50 µL of RPMI media with no serum or additives and 2.0 µL of TransIT®-LT1 reagent at room temperature for 15 minutes. The solution was added dropwise to each well, and gently rocked back and forth. The transfections were incubated for 24 hours under standard cell culture conditions.

Cell lysis and luciferase assays were conducted using the Dual-Luciferase Reporter Assay System (Promega Corporation). The transfection media in each well was suctioned off, wells rinsed in PBS, and 1X Passive Lysis Buffer (Promega Corporation) (500 µL – 6 well plates; 100 µL – 24 well plates) was pipetted into each well. The samples were rocked for 15 minutes at room temperature, and the lysate transferred to 1.5 mL microcentrifuge tubes and frozen at -20°C until analysis. Luciferase and Renilla measurements were taken in a GloMax® 20/20 luminometer (Promega Corporation). Lysate (20 µL) was added to a new 1.5 mL microcentrifuge tube, and 30 µL of firefly Luciferase Assay Reagent II added to the lysate and luciferase
activity measured at a wavelength of 560 nm. Subsequently, 30 µL of Stop & Glo was added, the solution mixed, and renilla luciferase activity was measured at the 480 nm wavelength.

Data Analysis

Statistical analyses were conducted in the Microsoft® Excel software platform, using the independent Student's t-test, assuming equal variance between the two groups. Statistical significance was defined as p < 0.05.

RESULTS

Western blot analysis of c-KIT protein in cell lysates corresponded with the RPMA analysis data, showing that while c-KIT was minimally detectable in P69 cells, it was highly expressed in the metastatic M12 cell line, and introduction of miR-17-3p resulted in total loss of the c-KIT protein expression (Figure 2-4). This demonstrates that the protein levels themselves are dramatically changing, and not just the active phosphorylated quantity.
Figure 2-4: c-KIT protein mRNA expression and protein levels are significantly reduced when miR-17-3p levels are high. A: messenger RNA levels of c-KIT are significantly reduced in P69 cells as compared to the M12 line ($p < 0.0001$), with further reduction when miR-17-3p is overexpressed in M12 cells ($p < 0.05$). Data is the average of 3 independent experiments. B: Western blot analysis and C: Densitometry of c-KIT protein expression in the P69, M12, and M12 overexpressing miR-17-3p. Blot is representative of 3 independent experiments; quantitation is compiled data from the 3 experiments ($p < 0.05$ for P69 to M12, and M12 to M12+miR-17-3p c-KIT).
Review of the major microRNA-Target prediction software platforms (DIANA\textsuperscript{88}, MiRDB\textsuperscript{89}, TargetMiner\textsuperscript{90}, RNA22\textsuperscript{91}) showed that only RNA22 and TargetMiner included targeting of miR-17-3p to c-KIT in a list of the top 40 candidate target genes for miR-17-3p. TargetScan predicted binding, but at a lower confidence level. Analysis of the c-KIT 3’-UTR using TargetScan revealed multiple highly conserved potential candidate sites where miR-17-3p might bind (Figure 2-5).\textsuperscript{92-94} Analysis of those sites using RNAHybrid yielded the top two candidates, based on the best binding of the microRNA seed, with large, equal predicted free energy values (Figure 2-6).\textsuperscript{86,87}

To validate the predicted binding of miR-17-3p to the c-KIT 3’-UTR, a 612 base pair portion of the 3’-UTR of c-KIT containing both potential binding sites was amplified and inserted downstream of the firefly luciferase gene in the Dual-Luciferase miRNA Target Expression vector pmiR-Glo\textsuperscript{®} (Promega Corporation). The cloning preparation resulted in not only a pmiR-Glo\textsuperscript{®} vector with a single copy of the c-KIT 3’-UTR, but also a second clone with two tandem copies inserted downstream of the luciferase gene. Comparison of luciferase activity (relative to Renilla luciferase activity) showed that expression was suppressed with a single copy of the cloned portion of the 3’-UTR, and that 2 tandem copies of that portion suppressed activity even further in M12 cells. Evaluation in the M12 + miR-17-3p overexpressing cell line showed even greater effect, substantiating that miR-17-3p is directly responsible for at least a portion of suppression of c-KIT expression (Figure 2-7).
Figure 2-5: In-silico evaluation of the top two miR-17-3p binding sites in the c-KIT 3'-UTR region show high conservation. Nucleotides 959-1075 of the 3'-UTR region of c-KIT show two potential binding sites for miR-17-3p. Red and brown fonts outline entire miR-binding region, and red and brown boxes denote the miR seed-pairing region wherein canonical miR binding is best. (hsa: homo sapiens ptr: chimpanzee mml: rhesus monkey oga: bushbaby tbe: tree shrew mmu: mouse laf: elephant eca: horse)
Figure 2-6: Predicted binding of miR-17-3p to 2 c-KIT 3’-UTR region binding sites.

The two binding sites with highest free energy are within 100 nucleotides of each other. Green is the sequence of miR-17-3p, and red is the complementary region of the 3’-UTR of c-KIT. (Adapted from RNAHybrid\textsuperscript{86,87}).
Figure 2-7: c-KIT expression is suppressed by miR-17-3p. M12 cells and M12 cells stably transformed with a vector expressing miR-17-3p were transiently transfected with a luciferase reporter construct containing a single or double tandem copy of a portion of the c-KIT 3'-UTR. Firefly luciferase expression is reported normalized to renilla luciferase activity and relative to empty vector expression. Results are the mean of 3 independent experiments, each performed in triplicate.
Subcloning efforts to isolate and mutate both miR-17-3p binding sites individually through synthesized 3’-UTR fragments revealed through luciferase 3’-UTR constructs that both binding sites have a minor but significant impact on c-KIT expression, suggesting that the additive effect of the two could allow for significant repression of translation (Figure 2-8).

c-KIT expression affects many downstream pathways, including the AKT, ERK, and JAK/STAT pathways. Through RPMA analysis, we found that neither p-AKT, p-STAT3, nor p-ERK were significantly affected by the addition of miR-17-3p into the M12 cell line (Figure 2-9).
Figure 2-8: Suppression of each binding site in the c-KIT 3’-UTR contributes to mRNA translation suppression. M12 cells expressing miR-17-3p were transiently transfected with wild type (WT) or mutated (MUT) sequence of each of the two binding sites for miR-17-3p within a 612 bp portion of the c-KIT 3’-UTR. Firefly luciferase expression is reported normalized to renilla luciferase activity and relative to mutant expression. Results are the mean of 2 independent experiments, each performed in triplicate.
Figure 2-9: miR-17-3p does not significantly affect downstream levels of c-KIT pathways. RPMA analysis was conducted on 3 sequential passages of each cell line. Serial dilutions of each replicate were probed for the phosphorylated antibodies shown. Data is represented as average expression normalized by the global phosphoprotein mean and relative to M12 cell line expression levels.
DISCUSSION

c-KIT is a oncoprotein that is well-characterized in a variety of cancers including breast, gastrointestinal, non small-cell lung carcinomas, and oral squamous cell carcinomas\textsuperscript{82,95-97}. While initial reports in prostate cancer were conflicting in their determination of c-KIT expression, the current literature indicates that c-KIT expression is found in more aggressive, metastatic prostatic neoplasias, but not in early tumours, and has some role in promoting establishment of bone metastases\textsuperscript{84}.

In this report, previous RPMA analysis work that identified c-KIT as a modulated protein between the P69 and M12 cell lines was confirmed, showing consequent loss of expression in the M12 cells transformed with a miR-17-3p expression construct\textsuperscript{67}. Western blot analysis showed that while only slightly detectable in the P69 cell line, c-KIT was highly expressed in the M12 cells, and subsequently lost in the M12 cells overexpressing miR-17-3p. This analysis provided not only confirmation of differences as seen with RPMA, but also showed that protein levels themselves are changing, and not just the amount of active, phosphorylated c-KIT protein as indicated by the RPMA analysis. Interestingly, messenger RNA levels of c-KIT were also modulated by miR-17-3p. As miR binding to the 3'-UTR of a target mRNA typically results in translational repression, and only results in cleavage when the seed pairing is a perfect match and in complex with Argonaute 2\textsuperscript{34,51,55}, mRNA modulation indicates at least one perfectly matching seed region and cleavage of the message by Argonaute 2.

Subsequently, two strong potential binding sites for miR-17-3p in the 3'-UTR of c-KIT were identified, and through reporter luciferase assays determined that:
1. The 3' UTR is bound and translation suppressed by miR-17-3p.

2. Comparison of reporter activity using wild type and mutated potential binding sites showed that both had modest suppressive activity. Thus, we suggest that the additive effect of the two miR-17-3p binding sites contributes to the overall suppression of translation for the c-KIT protein in our model cell lines.

There is some concern that even the additive effects of the two identified miR-17-3p binding sites in the 3' UTR may not be sufficient to completely ablate protein levels in the P69 and M12+miR-17-3p cell lines. Further analysis of the c-KIT gene identified two additional potential binding sites for miR-17-3p within the c-KIT gene. These potential sites were not previously identified because they are located within the coding regions of the message itself, and not the 3' UTR, which is canonically the location for miR-target binding. However, there is a growing body of evidence that miRs can bind and suppress translation of their target mRNAs not only in the 3' UTR, but also within the 5' UTR and coding regions of the messenger RNA\(^98,99\). One example in particular is interesting and similar to our c-KIT evaluation – miR-34a has been proven to target Diacylglycerol Kinase \(\zeta\) in T-cells through binding sites in both the 3' UTR and the coding region\(^99\).

The binding for the two sites within the cKIT mRNA is particularly strong, and are the only two potential sites in c-KIT that are stronger in free energy than the two 3' UTR sites that were identified and modulated here (Figures 2-10 A&B). Binding site CD-A is a strong match, with full complementarity in the 3-7 nt seed region of miR-17-3p, and interestingly, the binding begins at nucleotide 1 in the c-KIT messenger RNA, even prior to the signal peptide sequence. Binding site CD-B is found in exon 13, which is part of
Figure 2-10: Additional potential binding sites for c-KIT can be found in regions other than the 3'-UTR. CD-A is found at the 5’ end of the message, starting at nucleotide 1. CD-B is found in exon 13 of the c-KIT message. Green is the sequence of miR-17-3p, and red is the complementary region of the c-KIT mRNA. (Adapted from RNAHybrid86,87).
the intracellular domain of the protein\textsuperscript{77} and is found in 3 of the 6 alternative splicing
forms of the c-KIT protein.

As an important primary signal transduction initiator, c-KIT is naturally heavily
regulated, not only at the transcriptional level, but growing evidence indicates that
multiple microRNAs are important for regulation at the translational level. miR-221 has
been shown to regulate c-KIT levels in erythroleukemic cells\textsuperscript{100}, but was within the
normal range in our evaluations and thus was not pursued. MiR-34a has recently been
shown to directly target c-KIT levels in a colorectal cancer cell line, with two binding
sites in the 3'-UTR\textsuperscript{101}. miR-34a was evaluated by other work in the laboratory and
shown to be an oncomiR in both the cell line progression model as well as in patient
LCM analyses\textsuperscript{67}. An interesting observation from the compilation of unpublished data
show that while miR-34a is dramatically upregulated in the P69 cells as compared to the
M12, this seems to have no effect on the c-KIT levels. It may be that miR-34a is
involved in regulation of other tumour suppressor mRNAs, and the c-KIT message is not
efficiently targeted in the prostate cancer system.

A particularly interesting microRNA:c-KIT interaction case is that of miR-494.
This miR was proven to downregulate c-KIT expression levels in gastrointestinal stromal
tumour cells (GIST) through multiple binding targets within the c-KIT 3'-UTR\textsuperscript{82}. Proliferation was affected via the c-KIT downstream pathways. Interestingly, in the
panel analysis (Chapter 3), miR-494 was undetected in both the P69 and M12 cell lines
by RT-qPCR analyses, and thus is not a likely player in c-KIT modulation in prostate
cancer. Thus, this discovery of miR-17-3p’s regulation of c-KIT presents an interesting
parallel case to the miR-494 in the GIST report, wherein different microRNAs through
tissue-specific expression fulfill a similar tumour suppressing role. Loss of expression in their respective tissues results in uncontrolled growth through loss of suppression of an oncogene.

c-KIT positive cancers from a variety of origins are commonly treated with Imatinib and other growth factor receptor chemotherapeutics. Imatinib directly blocks c-KIT activation at the ATP-binding pocket. However, constitutively activated mutations for c-KIT are well known, and blocking therapeutics are not effective for those mutants. A method that blocks translation of the mutated protein could be a very effective therapeutic tool. Given that c-KIT has been so well-established as a rapidly cycling protein, its translational repression could have an almost immediate effect on the reception of growth signals and downstream proliferative signaling to tumour cells resulting in the ultimate attenuation of growth and cell cycling, as was shown in the study evaluating miR-494 inhibition. A combination of miRs shown to target the c-KIT mRNA, but without additional known pro-growth effects (such as miR-34a) could potentially work to attenuate the growth and chemotaxis of neoplasias through suppression of the initiator of those signal transduction processes.
Chapter 3:

Identification of modulated microRNAs through a sequential analysis of prostate cancer cell lines and patient samples
Most researchers approach miR discovery in a single approach. Some have a protein or pathway that encompasses their entire research, while others are interested in identifying microRNAs on a global scale that affect diseases and conditions such as cancer. While our laboratory initially began working on microRNAs through the discovery of miR-17-3p as an attenuator of vimentin protein levels, we have continued to work in microRNA discovery in prostate cancer in a search for additional microRNAs that could be serving as oncomiRs or tumour suppressors. And while we begin this research on a global scale, we are interested in not only identifying those miRs, but determining their mechanism of action through target mRNA identification and connection with a signaling pathway that progresses the carcinoma.

The previously described progression cell lines P69 and M12 are a unique model for prostate cancer, in that P69, having been immortalized from a human non-neoplastic prostate epithelium section\textsuperscript{60,61}, is poorly tumorigenic and non-metastatic in nature. In contrast, the M12 cell line, which was derived from 3 sequential injections into nude athymic mice, is highly tumorigenic and highly metastatic. This set of cell lines serves as an excellent model for prostate cancer research, in that the M12 cells are derived from the P69 cells and therefore share a common basic genetic complement.

As the M12 and P69 cell lines are not readily available to other researchers, several cell lines are commonly used as models for prostate cancer (Table 3-1). The PC3 cell line is a line that was derived from a vertebral bone metastasis in a 62-year old male, and is androgen-insensitive\textsuperscript{102}. It no longer expresses PSA or the androgen receptor\textsuperscript{103} and is highly tumorigenic and metastatic, and thus is commonly used to evaluate prostate cancer therapeutic targets for advanced cancers. The other canonical
prostate cell line is the Dunnings-145 (DU-145), which was derived from the brain of a 69-year old white male with both CaP and lymphocytic leukemia, is only moderately metastatic but also androgen-insensitive\textsuperscript{103,104}. DU145, like PC3, also does not express PSA or the androgen receptor. A non-tumorigenic cell line known as BPH-01 is a SV40T immortalized line derived from a human benign prostatic hypertrophic (BPH) biopsy\textsuperscript{23,105}. While there is some disagreement among researchers as to whether BPH always ultimately develops into neoplasia in\textit{vivo}, in\textit{vitro}, the BPH-01 cell line has only been shown to form adenocarcinomas when injected with carcinoma-associated fibroblasts\textsuperscript{24}. WMPY-1 is a stromal myofibroblast line that is sometimes referred to in CaP research, but since it is not endothelial in nature, it is not as relevant as the other lines are to the progression of CaP\textsuperscript{105}.

The only publicly available progression model for prostate cancer is the LNCaP series of cell lines. The original LNCaP cell line was derived in 1977 from a metastatic supraclavicular lymph node in a 50-year-old male\textsuperscript{106,107}. It is unique from the other cell line models in that tumour development is androgen-sensitive. The C4 and C5 sublines were derived through the injection of the LNCaP cells into male nude athymic mice, followed by subsequent castration after 8 weeks\textsuperscript{108}. Tumours that were maintained after castration exhibited hormone independence, and thus formed the basis of the C4 and C5 sublines. Subsequent reinjection of the C4 cells into nude athymic mice in combination with human marrow stromal (MS) fibroblasts generated an even more androgen-refractory cell line designated LNCaP-C4-2\textsuperscript{108}. Interestingly, the C4, C5, and C4-2 sublines have lost the Y-chromosome, continue to express PSA and have a lower
Table 3-1: Cell line models used in prostate cancer research

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Metastatic Potential</th>
<th>Androgen Sensitivity</th>
</tr>
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<tr>
<td><strong>Stand-Alone Cell Lines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC3</td>
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</tr>
<tr>
<td>DU-145</td>
<td>Moderate</td>
<td>No</td>
</tr>
<tr>
<td>BPH-01</td>
<td>None – benign hyperplastic epithelium</td>
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</tr>
<tr>
<td><strong>Progressive Models</strong></td>
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<tr>
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<tr>
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</tr>
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<td>LNCaP-C4-2</td>
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<td>No</td>
</tr>
</tbody>
</table>
level of the androgen receptor, all trademarks of hormone-refractory CaP in humans\textsuperscript{109}. Thus, the LNCaP progression set of cell lines is a well-established progression model for studying prostate cancer. Additionally, the series is an accurate model for metastatic potential, as the sublines form osteoblastic lesions\textsuperscript{32}, the canonical mechanism for human prostate cancer metastases to bone, in contrast to the PC3 and DU-145 lines, which form osteolytic lesions similar to the bone lesions seen in breast and other cancers\textsuperscript{105}.

While each of the described cell lines can act as good models for prostate cancer, any one cell line alone should not be used as a discovery tool for identifying novel modulated species, including microRNAs. Any balanced discovery should include not only the easily obtainable cell lines, but also patient samples. In this way, confirmation of the preliminary results obtained from the cell-line work can be confirmed as also occurring in the prostate cancer patient, and thus will be more relevant not only in identifying new markers, but also in developing new therapeutics against prostate cancer. In this study, we proposed to identify microRNAs that have been modulated in prostate cancer through a progressive, sequential analysis that begins at the global miRNA level, proceeds through single-miR confirmatory analysis, followed by evaluation of miR expression in multiple additional prostate cancer cell lines and finally analysis of modulated microRNAs through laser-capture microdissection (LCM) of benign, stromal, and tumour tissue from human patient biopsies.
Project Aims:

1. Identify miRs that are dysregulated in prostate cancer through RT-qPCR analysis of the P69 and M12 cell lines of a human panel of over 700 miRNAs. This will serve to identify microRNAs as potential oncomiRs when overexpressed in M12 vs. P69, and as potential tumour suppressors when lost in M12 vs. P69.

2. Confirm miRs identified in Aim 1 through single-miR RT-qPCR analysis in the entire P69 cell line progression model. Evaluate any differences between results seen with panels as compared to the single-miR analysis.

3. Evaluate those modulated miRs that continue to act as oncomiRs and tumour suppressors through analysis of additional prostate cell lines. This will serve to narrow the field to those miRs that are more indicative of CaP through gain or loss of expression.

4. Using CaP patient biopsies, perform laser-capture microdissection in order to separate benign from tumour tissue within the same biopsy slice, and compare miR expression between benign and tumour, thus confirming (or eliminating) the proposed miRs as modulated in prostate carcinomas.

METHODS & MATERIALS

Cell Culture

P69, LNCaP and sublines, BPH-01, PC-3, and DU-145 cells were previously cultured and pellets stored at -80°C until RNA isolation. Adherent M12 cells were cultured in 75 cm² flasks using RPMI 1640 supplemented with L-Glutamine (Caisson Labs, North
Logan, UT), 5% fetal bovine serum, 5 µg/mL insulin, 5 µg/mL transferrin, 5 µg/mL selenium (ITS, Collaborative Research, Bedford, MA). Gentamycin (0.05 mg/mL) was used to prevent bacterial contamination. Cells were passaged when 50-65% confluent, and maintained for no longer than 20 passages. Passaging was accomplished by incubating with 0.25% Trypsin-EDTA (Gibco-Life Technologies, Carlsbad, CA) for 5 minutes at 37°C, after which the trypsin was inactivated by washing the cells in serum-containing media. The cells were then pelleted through centrifugation at 1500xg for 5 minutes and either passaged, plated, or pellets preserved. Cell pellets for analysis were produced by washing the pellet in PBS, centrifugation at 1500xg for 5 minutes, removal of the supernatant, and flash freezing in liquid nitrogen. Pellets were stored at -20°C or -80°C for at least 24 hours.

**RNA Isolation**

RNA was isolated from cell pellets using the miRVana™ RNA isolation method according to the manufacturer’s protocol (Ambion-Life Technologies, Carlsbad, CA), eluted in 50 µL of Elution Buffer, and stored at -80°C. RNA concentration was estimated using either the Smart Spec™ 3000 Spectrophotometer (Bio-Rad Laboratories, Hercules, CA) or the NanoDrop ND-2000 Spectrophotometer (Thermo-Fisher Scientific, Inc., Waltham, MA).

**Laser-Capture Microdissection**

Frozen radical prostatectomy samples were obtained from VCU’s Tissue and Data Acquisition and Analysis Core, which obtained all samples following approved institutional review board (IRB) protocols. Each sample was reviewed and scored by a
board certified pathologist with expertise in prostate cancer diagnosis. 8 μm tissue slices from the biopsy cores were placed on uncharged glass slides, and stained with hematoxylin and eosin (H&E) using a standard protocol. Laser capture microdissection (LCM) was performed using the Arcturus Veritas™ laser capture microdissection system (Life Technologies). Each tissue type (benign, stroma, venous, tumour) was separately captured onto CapSure® Macro LCM caps (LifeTechnologies). At least ten slides were captured for each patient included in the study.

**RNA extraction of LCM samples**

Total RNA was isolated from LCM caps using the ARCTURUS® PicoPure® RNA Isolation Kit (Life Technologies), following the manufacturers protocol. Briefly, the LCM caps were incubated for 30 minutes at 42°C in 50μl of extraction buffer. This extract was then stored at -20°C until similar cell extracts from successive slides were pooled into a single tube and mixed with 1 volume of 70% ethanol. The precipitated RNA solution was loaded onto a MiraCol™ column, centrifuged, washed, and total RNA eluted in 11 μL. RNA quality and quantity were estimated using an Agilent RNA 6000 Pico chip with the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) using the manufacturer’s recommendations.

**Locked-Nucleic Acid miR Panel Analysis**

M12 and P69 RNA (50 ng) were subjected to the miRCURY LNA™ Universal RT microRNA PCR reaction (Exiqon A/S, Denmark) according to the manufacturer’s recommended protocol. Briefly, 4 μL of 5X Reaction buffer and 2 μL of reverse transcriptase enzyme mix were combined with appropriately diluted RNA and brought to
20 µL volume with RNase-free H₂O. The RNA was reverse transcribed for 60 minutes at 42°C, followed by heat inactivation for 5 minutes at 95°C. The cDNA was diluted and aliquotted across Exiqon miRCURY LNA™ Universal RT microRNA PCR Panels I and II (Version 2.1.M) 384-well plates according to protocol, and qPCR was undertaken in an Applied Biosystems 7900HT fast real-time PCR instrument (Life Technologies, Carlsbad, CA) using recommended cycling conditions. Threshold and baseline settings were set according to protocol recommendations. The data was corrected for interplate variability using on-plate calibrators, and normalized against the global mean using Exiqon GenEx software. Expression changes were calculated in Microsoft Excel® using the \(2^{ΔΔT}\) method and recorded as M12 expression relative to P69.

\textit{RT-qPCR (Exiqon)}

Single-miR RT-qPCR analysis was conducted using the LNA platform as well. RNA (40 or 50 ng (LCM and cell lines, respectively)) was reverse transcribed using the miRCURY LNA™ Universal reverse transcription reaction as described for the Panel analysis. The cDNA was diluted 1:10 and qPCR mix prepared by adding 5 µL of 2X Sybr® Green PCR master mix (Exiqon A/S), 1 µL of appropriate primer mix, and 4 µL of diluted cDNA. qPCR was undertaken in an Applied Biosystems 7300 real-time PCR instrument (Life Technologies) using recommended cycling conditions. Data was analyzed using SDS software v1.3.1 (Life Technologies). Threshold and baseline settings were set according to protocol recommendations, with baseline correction between cycles 3 and 12 and an automatic threshold. Each miR was analyzed against a cell line with a minimum of one biological and three technical replicates.
RESULTS

Microarray Panel Analysis of M12 and P69 cell lines

Locked-nucleic acid (LNA) RT-qPCR panel analysis of the M12 versus the P69 cell line was the starting point in a search for microRNAs that are truly modulated, not only in cell-line models, but also in human tumours. Analysis of both Panels 1 and 2 allowed for analysis of known human miRs as of 2011. Out of 736 microRNAs assayed, 231 were found to be oncomiRs (≥2 fold increase in M12 vs. P69 cell lines), 150 were found to be tumour suppressors (≤.5 fold decrease in M12 vs. P69 cell lines), with the remaining microRNAs within the normal range (Figure 3-1). Even though the majority of microRNAs remained within the normal range, this still left many potential tumour suppressors and oncomiRs to evaluate (Tables 3-2a and b, respectively).

The choice of which microRNAs to investigate further was accomplished through an evaluation of not only the expression level difference between P69 and M12, but also initial literature reviews and analysis of potential targets using microRNA-target databases including TargetScan, DIANA, and miRDB86-89,92,93,110. Literature searches were conducted in order to determine whether the microRNAs in question had been previously identified as modulated in prostate or any other neoplasias. The microRNAs that were selected for further examination comprised the spectrum of both gain and loss of expression (Table 3-3). While most selected miRs have some demonstrated evidence promoting carcinoma progression, whether in the form of proven targets or previous literature in other neoplasias, in some cases, the expression level difference
Figure 3-1: Analysis of microRNA expression (fold difference in M12 vs. P69 cell lines). Cultured cells were pelleted, RNA isolated, and subjected to Exiqon miRCURY LNA™ microRNA system Human panels I and II (version 2.0). Panel I was performed in duplicate using 20 and 50 ng of RNA; Panel II was performed once, using 50 ng of RNA. Data was normalized using the global mean, and fold difference calculated. Values greater than 1 represent higher expression in M12 as compared to the P69 cell line.
Table 3-2a: Tumour suppressor microRNAs identified through miRcury RT-qPCR panel analysis of M12 vs P69 cell lines panels I and II (Version 2.M). Data is sorted by expression level, and values are fold expression difference in M12 from P69.

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<th>Expression Difference</th>
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Table 3-2b: OncomiR microRNAs identified through miRcurry microarray analysis of M12 vs P69 cell lines panels I and II (Version 2.M). Data is sorted by expression level, and values are fold expression difference in M12 from P69.

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Table 3-3: microRNAs selected for further analysis

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<th>microRNA</th>
<th>Expression difference (M12 vs. P69)</th>
<th>Proven Targets</th>
<th>Reported in cancer:</th>
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<tr>
<td>9</td>
<td>6.68</td>
<td>CDH1, REST, CDKN1A, BCL6, FOXO1, NF-kB1, ETS-1, CD34,</td>
<td>OncomiR in glioblastoma, lung, hepatocellular, prostate, gastric, bladder, mesothelioma, Tumour suppressor in ovarian</td>
</tr>
<tr>
<td>144</td>
<td>0.374</td>
<td>PLAG1, FGA, FGB</td>
<td>OncomiR in lung, prostate, esophageal, nasopharyngeal, Tumour suppressor in bladder, bone, colorectal</td>
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<td>133a</td>
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<td>FSCN1, TAGLN2, GSTP1, COL1A1, LASP1</td>
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<tr>
<td>133b</td>
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<td>FSCN1, PTBP2, ERG, HCN2, MET</td>
<td>Tumour suppressor in bladder, uterine, gastric, prostate</td>
</tr>
<tr>
<td>146a</td>
<td>0.459</td>
<td>---</td>
<td>OncomiR in bone, liver, ovarian, prostate, SNP mutation confers oncomiR in breast, colorectal, ALL, pancreatic, Tumour suppressor in lung</td>
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<tr>
<td>147b</td>
<td>201.4</td>
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<td>Tumour suppressor in rectal</td>
</tr>
<tr>
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<td>MAPK8, MET, CD44, MAPK1, AKT1, MET</td>
<td>OncomiR in colon, gastric, biliary, Tumour suppressor in testicular, bladder, endometrial, bone, liver</td>
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<tr>
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<td>MMP13</td>
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was so compelling that miRs were chosen without any known targets or previous association with cancer.

Analysis of selected miRs using prostate cell lines

The next phase of miR selection was undertaken through single-miR RT-qPCR analysis of the subset of miRs selected for further investigation (Table 3-3). Through this work, we hoped to accomplish a two-fold goal: first of all, to confirm the miRcury microarray data through single-miR analysis of RNA from additional passages of M12 and P69 cell line RNA; secondly, to ascertain whether our initial classification of the selected miRs as tumour suppressors or oncomiRs was genuine through analysis of additional model cell lines. This two-fold approach would allow for elimination of those miRs that did not follow through as initially expected. Narrowing of the candidates could be important, as the quantity of RNA extracted through laser-capture microdissection of patient samples was expected to be low, and the number of miRs that could be analyzed was finite. As was suspected, confirmatory single-miR RT-qPCR analysis of the selected miRs showed correlative results in fold expression differences from P69 to M12 for most, but not all, microRNAs selected (Table 3-4 and Appendix 1:Table 1). While the results were mostly correlative in that oncomiRs typically were overexpressed and tumour suppressors lost as compared to P69 levels, the level of those differences was often highly variable. miRs 622, 221, and 299-3p were found to be so different that they were opposite in expression as originally observed. These findings in particular were repeated and confirmed with at least one additional RNA extraction. The differences may have been due to the normalization parameters, as the panel data was
Table 3-4: Expression differences are typically observed between Panel and single miR “confirmatory” RT-qPCR analysis. RNA was isolated from different passages of M12 and P69 cells than those used for the panel analysis, and subjected to individual probing for selected miRs using LNA RT-qPCR. In both panel and single-miR analysis, data was normalized (against the global mean in the panel, and against RNU48 for single-miR analysis), and the expression level in M12 calculated relative to the P69 cell line. Each sample was analyzed a minimum of three times. 299-5p results appeared correlative, but the RNU48 amplification was atypical. miR-144 was undetected for both P69 and M12 in single-miR analysis.

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<th>133b</th>
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<td>0.344</td>
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normalized to the global mean, while single-miR analyses were normalized to RNU48. This could be expected to result in minor differences, but major discrepancies such as those seen in miRs 622, 221, and 299-3p cannot be attributed to the normalization method.

Each of the selected miRs was then further analyzed using the remainder of the P69 progression model, as well as additional prostate cancer cell lines, in an effort to determine which microRNAs warrant further investigation through modulation and target confirmation. Through analyzing multiple prostate cancer and associated cell lines, we hoped to gain confidence in our chosen miRs prior to using precious patient laser-capture microdissected RNA. Through this analysis, we chose those miRs that were the most consistently expressed among the cell lines (depending on the nature of the cell line), and/or those miRs that continued to intrigue us based on their targets or known roles in carcinogenicity (Figures 3-2, 3-3, and 3-4, Appendix 1-Table 2).
Figure 3-2: Relative Expression of oncomiR microRNAs in P69-M12 progression model. miR levels were normalized to RNU48, and expression level calculated relative to the P69 cell line. Each sample was analyzed in triplicate at minimum.
Figure 3-3: Relative Expression of Tumour suppressor microRNAs in P69-M12 progression model. miR levels were normalized to RNU48, and expression level calculated relative to the P69 cell line. Each sample was analyzed in triplicate at minimum. M12 data for miR-299-5p was not included due to a discrepancy in RNU48.
Figure 3-4: Relative Expression of microRNAs in additional prostate cell lines.

miR-levels were normalized to RNU48, and are expressed as the dC_T (C_T(miR) – C_T(RNU48)). A lower C_T indicates higher expression. Each sample was analyzed in triplicate at minimum.
miR analysis of Laser-Capture Microdissected (LCM) prostate samples

Laser-captured microdissected samples were captured from 5 patient biopsies at varying stage of prostate cancer. The majority of the tumours analyzed were classified as Gleason 3, 4, or 5, and in some cases, it was only possible to isolate not only benign and tumour tissue, but also stroma, lymphocyte, and/or venous tissue. miR LNA RT-qPCR analysis of the 5 sets of patient samples revealed that, as expected, there is considerable variability in the nature of the individual patient sample. A majority of the miRs tested varied wildly from tumour to tumour; however, a few miRs assayed showed consistency with their predicted role compared to the model cell lines (Figure 3-5, Appendix 1: Table 3). In particular, miR-9 and miR-147b were strongly and consistently oncomiRs in tumour samples, and miRs 299-3p and 199a-3p were consistently lower in tumour samples than benign, and thus classified as potential tumour suppressors.
**Figure 3-5:** Relative expression of microRNAs in laser capture microdissected tissue samples. miR levels were normalized to RNU48, and expression level calculated relative to the benign tissue of the same patient. Each sample was analyzed in triplicate.
DISCUSSION

Array analysis

Array analyses of cell lines, while they should not be used as the sole method for identifying miRs that are modulated in neoplasias, can be an excellent launching point for narrowing the potential candidates, provided that the cell line model is accurate to in-vivo processes. In prostate cancer work, some cell lines serve different purposes. For example, the LNCaP series is an excellent model for identifying miRs that could contribute to the pathways required for androgen independence. However, since the LNCaP cell lines require coinjection with MS fibroblast cells for tumorigenesis and metastasis in the later lines, they are not a good model for initial tumour development. The P69/M12 progression model is the best model for human prostate cancer tumorigenesis, and thus we felt that a miR array comparing those cell lines would best catalogue modulated miRs in a manner most faithful to human tumorigenesis. Of the 15 miRs selected for further analysis, 10 of the 15 (67%) maintained their oncomiR or tumour suppressor status in single miR analysis (Table 3-4). Unfortunately, that indicates a relatively high (23%) incorrect read rate for miR analysis using panels. There are a variety of technical reasons as to why panel analysis is more subject to errors or inaccuracies, but one likely reason is that of stochastic effects. Splitting one reverse transcription reaction across 768 wells is a dilution factor far above that used for single-miR analysis, and the sheer chance of an accurate representation of all miRs in the sample getting into each of those wells can be a factor in expression level variation. Because of that concern, we ran technical duplicates of both M12 and P69 for Panel 1. We found that there was high variability between the duplicate runs of Panel 1, and thus
the miRs chosen from Panel 1 were those that were consistently modulated (up or down) in both plates.

Variation between technical duplicates is well-known in miR microarray analysis; a study evaluating and comparing locked-nucleic acid (LNA) arrays to bead-based and Taqman low-density arrays found that the LNA inter-plate reproducibility was higher than the other methods, at approximately 75%\textsuperscript{111}, consistent with the findings presented here. An additional study comparing microarray data to single-miR analysis found anywhere from 0 to 10-fold differences in expression between the two platforms\textsuperscript{112}, similar to differences observed in this study.

\textit{miR 9}

As a well-established miR involved in multiple cancers, miR-9 was not a surprising find. miR-9 was overexpressed in all CaP cell lines analyzed, and in 3 of the 5 patient samples. Overexpression was dramatic, in the thousand-fold range for the DU145 and PC3 cell lines, and a more reasonable overexpression level in M12 and patient samples (2.5-78 fold higher than benign). There are only two reports on miR-9 in prostate cancer, one being a microarray study and the other focused on the Androgen Receptor (AR). The microarray study found that miR-9 was the most dramatic and consistently upregulated miR in prostate cancer, and was expressed as an oncomiR in both low and high-grade regions within the same tumour\textsuperscript{113}.

miR-9 has many well-established protein targets, in particular, e-cadherin, NF-κB, SOCS5 and ETS-1. A modest but consistent decrease in the androgen receptor level has been shown when miR-9 was overexpressed \textit{in-vitro}\textsuperscript{113}, which could allow for
an eventual switch from the early androgen-dependent tumour to a hormone-refractory state.

**miR-144**

miR-144 has been well-characterized as a modulated microRNA in multiple cancers. However, it seems to have different roles depending on tissue type, and as such can be either an oncomiR (lung, esophageal, nasopharyngeal) or tumour suppressor (bladder, bone, colorectal), depending on the cancer type. While our initial panel analysis showed miR-144 as a tumour suppressor (0.37-fold downregulated in M12 vs. P69), it was undetected in both P69 and M12 single-miR analyses, but highly expressed in the M2182 and F6 lines. To further complicate the picture, patient LCM samples showed significant upregulation in tumour compared to benign tissue from the same patient, correlative with a recent microarray study reporting miR-144 as a potential oncomiR for prostate cancer. While the LNA technology used for miR analysis in this study is highly specific for the miR in question, recent work in our laboratory has found other miR RT-qPCR platforms to be more sensitive in detection, and perhaps clarification of the P69 vs. M12 cell line analysis could be further investigated using a different platform. This particular example highlights the need for a thorough and balanced approach to miR discovery, using not only cell lines, but also patient samples. Given the consistency in patient samples, miR-144 could be an oncomiR in prostate cancer and warrants further investigation, although it would not be recommended to use the P69/M12 progression line for miR-144 work.
**miR-221**

miR-221 was highly inconsistent in CaP samples, both in cell line analysis and patient samples. Initial panel analysis showed miR-221 to be slightly tumour suppressive, with an average expression difference of 0.783 in M12 vs. P69 cells. Single-miR analysis showed a 1.38-fold upregulation in the M12 line. Given known error levels in miR panel analysis, this expression difference could be considered to be within the range of error. Analysis of miR-221 in other cell lines and patient samples was likewise inconsistent, the only exception being patient 09-362, who had overexpression of miR-221 in not only tumour, but also stroma and lymphatic tissue (Appendix 1, Table 3). Thus, miR-221 cannot be considered as an indicator miR for prostate carcinoma, but may in fact still be a player in advanced prostate cancer: while several studies report that miR-221 is lost in prostate cancer, others have shown that in highly metastatic cancers, miR-221 is overexpressed in association with high-risk carcinomas\textsuperscript{114} and plays a role in androgen independence\textsuperscript{17}.

**miR-488**

The loss of miR-488 expression was even more impressive in single-miR analysis as compared to the panel analysis (0.039 vs. 0.526-fold drop in expression in M12 vs. P69). Similar expression levels to the metastatic M12 cell line were noted in the PC3, DU145, and other prostate cell lines, which led us to believe that miR-488 could be a substantial tumour suppressor. Unfortunately, it was undetected in both benign and tumour samples of all patients (Appendix 1, Table 3). Exiqon LNA RT-qPCR is highly specific, but not as sensitive as other applications. Thus, evaluation with
a different platform for miR RT-qPCR analysis could yield results. Additionally, a study in 2011 was unable to detect miR-488 in the prostate cell lines (DU145, PC3, LNCaP), but evaluated the miR regardless and found that it is able to affect androgen receptor translation directly. They also found that when they added back miR-488, proliferation and viability decreased, and apoptosis was enhanced\textsuperscript{115}. Unfortunately, the literature shows no correlation of miR-488 with any cancer, and expression is mainly seen in neural development. miR-488 levels have also been shown to be so consistent in lung cancer patient samples when comparing benign to tumour that it was proposed as a potential normalization control\textsuperscript{116}.

\textit{miRs-299-3p & 5p}

miRs 299-3p and 5p were cast via panel analysis as opposite in expression, with miR-299-3p an oncomiR and 299-5p as a tumour suppressor. This possibility was rather intriguing, as it indicated that a shift in strand selection at the miR maturation level. Unfortunately, the high relative level of expression for miR-299-3p did not correlate under single-miR analysis, and multiple replicates of miR-299-3p analyzed in P69 and M12 showed miR-299-3p expression lost in M12 as compared to P69. Similarly, miR-299-3p was lost in PC3 cells as well. Of the five patients tested for miR-299-3p, two had detectable levels in benign, with lost or lower expression of miR-299-3p in tumour samples. The remaining 3 samples were undetected in tumour and benign tissues. Thus, miR-299-3p appears to be consistently acting as a tumour suppressor, in contrast to its originally perceived role as an oncomiR from the panel analysis. There is no literature or proven targets for miR-299-3p, but many potential binding target mRNAs, as predicted by miR prediction platforms\textsuperscript{86,87,89,117}. 
miR-299-5p was identified as a tumour suppressor in the panel analysis, which was confirmed by the single-miR analysis. Expression was similar between M2 and PC3 cells, but the miR was undetected in DU145 cells. Patient analysis found that of the 5 patients tested for miR-299-5p, two showed overexpression, in direct contrast to that observed by the cell line analyses. Three patients had undetected levels of miR-299-5p in both tumour and benign samples. Of the limited literature available for miR-299-5p, it has been shown to act as a tumour suppressor in breast cancer, as loss of miR-299-5p allows relief of suppression for the Osteopontin protein\textsuperscript{118}, which is known to play an important role in inflammation, tissue remodeling, invasion, migration, and angiogenesis\textsuperscript{119}. Osteopontin is also well-characterized as surging in expression during the preosteoblast stage of bone cell development, which could parallel the CaP cell integration in to bone metastases; moreover, increased osteopontin levels have been observed in the LNCaP C4/C5/C4-2 androgen-independent sublines\textsuperscript{32}. Given its bimodal observed role in cell lines and patient samples, it is possible that analysis of miR-299-5p with the LNCaP series could illuminate a potential hormone-dependent switch.

miR-147

There is not a lot of literature regarding the expression of miR-147 (also known as miR-147a) in cancer, but it has been shown to act as both a tumour suppressor and as an oncomiR in different cancers. Only one mRNA target has been proven, VEGFA, which has been shown to stimulate angiogenesis, wound healing and tumour development\textsuperscript{120}. In our analyses, while the panel identified miR-147 as a potential oncomiR (27.9-fold increased expression), single-miR analysis showed the opposite
pattern, with M12 cells expressing 0.138 fold the level of the P69 cells. Because of this inconsistency, we did not analyze the additional cell lines, and only tested one set of patient LCM samples, in which miR-147 levels were undetected in both benign and patient. Consequently, we do not consider miR-147 to have a consistent role in prostate cancer.

**miR-147b**

miR-147b is one of the most consistent and promising potential oncomiRs to develop from this study. Panel and single-miR confirmation showed this miR to be highly upregulated (201 and 16-fold, respectively), and this high expression was duplicated in other prostate cell lines, in a pattern consistent with the neoplastic and metastatic nature of the cell lines tested (Figure 3-3). In particular, the levels of miR-147b in PC3 cells were exceptionally high. Through patient LCM sample analysis, 4 of the 5 patients showed significant upregulation, with an average of 11.1-fold overexpression as compared to paired benign tissue (Appendix 1, Table 3). This miR is relatively uncharacterized; it has not been described in any cancers, and has no known, proven targets, although one study did some preliminary work using luciferase 3’-UTR constructs and showed some modulation for a few target mRNAs. However, the focus was less on proving target binding and consequent effects and more on comparing seed functionality to other miRs; thus, those targets have not been conclusively shown as miR-147b targets. As a relatively unresearched microRNA, miR-147b will require considerable research effort to hammer out its oncogenic mechanism.
**miR-622**

While there is not a lot of literature regarding miR-622 in neoplasias, the literature does agree that miR-622 is upregulated in some rectal cancers, particularly those responsive to specific chemotherapeutics\(^1\) and those associated with Lynch Syndrome\(^2\). However, one report showed that miR-622 acts as a tumour suppressor in gastric cancer, with ING1 as a proven target, a component of the p53 pathway involved in cell cycle arrest and apoptosis\(^3\). While expression of miR-622 in the panel RT-qPCR was not replicated in single-miR analysis, PC3 and DU145 expressions were relatively high, but so was the benign stromal line WMPY-1. Additionally, only 2 of the 5 patients sampled showed upregulation of miR-622; thus, unless additional patients are sampled, it can't be concluded with confidence that miR-622 is an oncomiR in CaP.

**miRs-127-3p and 5p**

miRs 127-3p and 5p expression levels were lower in M12 cells as compared to P69, which was confirmed in single-miR analysis. They were not tested against the additional prostate lines, but were tested against the patient LCM samples. three out of five patients showed loss of expression in miR-127-3p in tumour compared to adjacent benign tissue, as did three out of five for 127-5p (though not the same three patients for both miRs). miR-127 deregulation has been shown in gastric and hepatocellular cancer, specifically through epigenetic methylation silencing\(^4\). This silencing would in fact silence both strands, thus making it more difficult to clarify which is having a more pronounced effect on tumorigenesis or metastatic behavior.
miR-127-3p has at least one proven target, MAPK4, which has already been shown to be pivotal in gastric cancer invasion and metastasis\textsuperscript{124}. miR-127-5p was shown to attenuate MMP13 through 3'-UTR and western analysis, which moderately promotes invasion and migration\textsuperscript{125}. miR-127-5p and -3p certainly warrant further investigation as a potential tumour suppressor set of microRNAs.

\textit{miRs-133a and b}

miR-133a upregulation was consistent in single-miR analysis, but this upregulation did not correlate with the other prostate cell lines tested, and only 2 of the 5 patient samples showed overexpression of miR-133a. While miR-133b yielded a predictable cell line pattern, in that it was overexpressed in the benign hypertrophic cell line BPH-04, as well as in the M12 cells (Appendix 1, Table 1), there was not an appreciable overexpression in either DU145 or PC3 cells. Thus, this miR was not analyzed with the limited cDNA created from the patient LCM samples. Both miRs have been well-studied in other cancers as tumour suppressor miRs, and have several proven and defined targets in the tumour suppression pathway, and thus they are worthy of further research to clarify what is occurring in prostate samples.

\textit{miR-146a}

Several studies have shown miR-146a as an oncomiR in prostate cancer, but one report has shown that overexpression of miR-146a is associated with early stage, androgen-dependent neoplasias, while a loss of function of miR-146a is essential for androgen independent invasion and metastasis\textsuperscript{126}. Single-miR analysis confirmed the panel-identified loss of expression in M12 as compared to the benign P69, and so initial
findings identified miR-146a as a tumour suppressor in cell line analysis. Unexpectedly, only one patient’s tumour had detectable levels of miR-146a. The miR was undetectable in the remaining 4 patient benign and tumour tissues.

**miR-199a-3p**

While miR-199a-3p was initially identified and confirmed as an oncomiR in the P69/M12 progression model, in the 2 patients tested, it consistently showed a ~0.5 fold loss of expression in tumour as compared to benign. The only tissue that it was dramatically overexpressed was the stroma of one patient. miR-199a-3p has been shown to act as both oncomiR and tumour suppressor in various cancers, and has multiple proven targets to promote both natures; additional work may need to be conducted to further evaluate miR-199a-3p’s role towards the progression of CaP, if any.

**Overall Conclusions & Future Directions**

Analysis of a panel of over 700 miRs certainly generates a great deal of information, such that one research laboratory couldn’t begin to track down each of the modulated microRNAs. Based on bioinformatics analysis and literature research, 15 microRNAs were chosen that showed promise through the panel comparison of the highly tumorigenic, metastatic M12s to the non-tumourigenic parental P69 line. This comparison is the first of its kind in prostate cancer cell line research; only the P69/M12 progression can compare a poorly tumorigenic parental line to its metastatic, tumorigenic subline that requires no coinjections to generate tumours in mice. Based on the results of these analyses, a balanced approach including not only cell-line but
also patient sample analysis allowed elimination of several candidates that appeared to be dysregulated. Of the 15 candidate miRs chosen for further analysis, 5 did not correlate with single-miR analysis. Of the 14 miRs analyzed using patient LCM tissues, only 5 (miR-9, miR-147b, miR-299-3p, miR-127-3p and miR-127-5p) showed sufficient consistency between cell-line analyses and between patients to warrant further investigation. Thus, a 33% success rate was achieved based on initially identified dysregulated microRNAs. The majority of the miRs selected for further investigation are, for the most part, not well-described in prostate cancer, and the challenge will be to identify those mRNA targets that lead to tumour progression through either a tumour suppressor or oncomiR role.
Chapter 4

miR-147b is an oncomiR in prostate cancer and suppresses AATF
INTRODUCTION

Previous work identified miR-147b as being up-regulated in not only prostate cancer cell lines (Figure 4-1), but also in prostate cancer biopsy tumour samples as compared to the benign laser-capture microdissected samples (Figure 4-2).

miR-147b is not well-studied, having been neither identified as an oncomiR or tumour suppressor miR, nor has significant work been reported in proving potential mRNA targets. miR-147b has a similar mature sequence to that of miR-147a\textsuperscript{38-41}, differing by only one base in the seed region (miR-147b – UGUGCG, miR-147a -- UGUGUG). While miR-147a has a proven target in vascular endothelial growth factor A (VEGFA), a protein known to be pro-angiogenic and pro-migration, there are no proven targets for miR-147b.

Previously, miR-147b has been labeled as miR-147 in some reports\textsuperscript{127}, and is found on chromosome 15q21.1\textsuperscript{128}, specifically in the 3’-UTR of the normal mucosa of esophagus-specific gene 1 protein (NMES1) (Figure 4-3). Interestingly, NMES1 siRNA does not inhibit mature miR-147 levels in mice\textsuperscript{127}. While there are several targets that are predicted by various prediction software platforms (Table 4-1), none have been proven thus far, and very few show potential as downregulated tumour suppressors.

miR-147b has an identical seed region to that of miR-210 (UGUGCG), and while the two miRs have been shown to have some similarity in target effectiveness, they are not completely duplicative in their target selection (Figure 4-4)\textsuperscript{44}. While miR-210, 147a and 147b have been shown to have some overlapping predicted targets, each also has targets unique to the individual miR. Interestingly, the number of predicted targets for
miR-147a is much higher than the sum of the miR-147b and miR-210 targets. It is assumed that the remainder of the miR sequence has a role in target mRNA selection as well, and thus this finding is not surprising. In our panel analysis of microRNA expression in the P69-M12 cell lines, we found miR-210 to be overexpressed approximately 2-fold in M12 cells as compared to P69. Given that miR-147b showed an almost 17-fold overexpression, it is doing the bulk of the target repression in the M12 model if in fact miR-147b and miR-210 have similar regulatory objectives.
Figure 4-1 (Adapted from Chapter 3): miR-147b levels are upregulated in all prostate cancer cell line models. A: miR-147b levels were measured in P69, M2182, M12, and F6 cells. Data was normalized to RNU48 and expressed relative to P69 using the comparative C_T method. P69 and M12 cells were analyzed using 3 separate extractions (p<0.05). B: miR-147b levels were measured in M12, DU145, and PC3 cells. Data was normalized to RNU48 (dC_T) and expressed as a box plot. The dC_T for the P69 cell line is visualized as the red box.
Figure 4-2 (Adapted from Chapter 3): miR-147b expression is upregulated in 80% of tested patient tumours. miR-147b levels were measured in tumour and benign tissue separated from prostate biopsies using laser-captured microdissection (LCM). Data was normalized to RNU48 and expressed relative to the benign tissue using the comparative Ct method.
**Figure 4-3:** The pre-miR-147b is located in the 3’-UTR of the NMES1 primary transcript on chr15q21.1. NF-κB and STAT1α bind upstream of the pre-miRNA, and activate expression. Mature miR-147b has been shown to develop from cloned primary transcripts of NMES1, as well as being independently transcribed\textsuperscript{127}. Blue represents the untranslated regions (UTRs) of the NMES1 mRNA, shades of green represent the exons, and yellow indicates the relative position of the miR-147b transcript.
**Figure 4-4: Similarities between miRs-147a, -147b, and -210.** A: Table of miR sequences. The seed region is highlighted in green. B: Targetscan analysis of potential targets for miRs 147a, 147b, and 210. While 62 potential targets are in common for all three miRs, each also has unique as well as overlapping mRNA targets. (from Bertero et al.⁴⁴)
It is difficult to tease out early (2012 and previous) literature on the miR-147 family. Previous incarnations of the commonly used microRNA RT-qPCR arrays (Applied Biosystems, Qiagen) had only “miR-147”, with no indication of sequence or A/B specificity. In one instance, miR-147 was designated as such, but the Methods & Materials section confirmed it to be miR-147b. In this report, Toll-like Receptor 2 (TLR2) and Type 1 Interferon (IFN) stimulation in mice induced miR-147b expression through activation and subsequent binding of NF-κB and STAT1α to the miR-147 promoter. Inflammatory cytokine expression was subsequently attenuated through a negative feedback loop\textsuperscript{127}. This same study reported 3 potential NF-κB binding sites in the mouse miR-147 promoter, two of which were shown through luciferase activity assays to be responsive to NF-κB binding.

Given the remarkable consistency in upregulation of miR-147b in our work, not only in prostate cancer cell lines, but also in patient samples, it was important to evaluate how inhibition of miR-147b could impact the tumorigenicity of a prostate cancer cell line model, and to identify potential tumour suppressor mRNA targets for miR-147b.

*Project Aims*

1. Inhibit miR-147b levels in the M12 cell line and evaluate any changes in proliferation, tumorigenicity, or invasive potential.

2. Identify potential targets and pathways for a mechanism of miR-147b action.
Methods and Materials

Cell Culture

Adherent cells were cultured in 75 cm² flasks using RPMI 1640 supplemented with L-Glutamine (Caisson Labs, North Logan, UT), 5% fetal bovine serum, 5 µg/mL insulin, 5 µg/mL transferrin, 5 µg/mL selenium (ITS, Collaborative Research, Bedford, MA). Gentamycin (0.05 mg/mL) was used to prevent bacterial contamination. Cells were passaged when 50-65% confluent, and maintained for no longer than 20 passages. Passaging was accomplished by incubating with 0.25% Trypsin-EDTA (Gibco-Life Technologies, Carlsbad, CA) for 5 minutes at 37°C, after which the trypsin was inactivated by washing the cells in serum-containing media. The cells were then pelleted through centrifugation at 1500xg for 5 minutes and either passaged, plated, or pellets preserved. Cell pellets for analysis were produced by washing the pellet in 10 mL PBS, centrifugation at 1500xg for 5 minutes, removal of the supernatant, and flash freezing in liquid nitrogen. Pellets were stored at -80°C for at least 24 hours.

Transfection

Stable transformations of an early passage of the M12 cell line with a vector containing a miR-147b inhibiting sequence or scrambled control (pEZX-AM03, Geneocopeia Inc., Rockville, MD) were performed using TransIT®-LT1 Transfection Reagent (Mirus BIO LLC, Madison, WI, USA). Briefly, 1.5 x 10⁵ cells were plated in a 25 cm² flask in appropriate serum-containing media for 24 hours. The cells were then rinsed with PBS, and serum-containing media replaced. The plasmid (6.5 µg) was mixed with 650 µL of RPMI media with no serum or additives and 19.5 µL of TransIT®-
LT1 reagent at room temperature for 15-30 minutes. The solution was added dropwise to each flask, and gently rocked back and forth. The transfections were incubated for 48 hours under standard cell culture conditions, and then selected for stable transformants using 200 ng/mL of Puromycin or 300 ng/mL of Hygromycin for two weeks. After the selection die-off, the cells were maintained using Puromycin (100 ng/mL) or Hygromycin (150 ng/mL).

**RNA Isolation**

RNA was isolated from cell pellets using the miRVana™ RNA isolation method according to the manufacturer’s protocol (Ambion-Life Technologies, Carlsbad, CA), eluted in 50 µL of Elution Buffer, and stored at -80°C. RNA concentration was estimated using either the Smart Spec™ 3000 Spectrophotometer (Bio-Rad Laboratories, Hercules, CA) or the NanoDrop ND-2000 Spectrophotometer (Thermo-Fisher Scientific, Inc., Waltham, MA).

**Messenger RNA Analysis**

Messenger RNA was quantified using reverse transcription-quantitative PCR (RT-qPCR). Reverse transcription was accomplished using the iScript™ cDNA Synthesis kit (Bio-Rad Laboratories). Briefly, 50 ng of RNA was mixed with 4 µL of the 5X iScript reaction mix, 1 µL of the iScript Reverse Transcriptase, and the volume brought to 20 µL with nuclease-free water. The reaction mix was incubated for 5 minutes at 25°C, followed by 30 minutes at 42°C, and heat inactivation for 5 minutes at 85°C. Reactions were stored at -20°C until analysis. The qPCR reactions were prepared by adding 3 µL of the prepared cDNA, 10 µM primer pairs for the relevant
mRNA target (GAPDH: Forward: 5’-ACCACAGTCCATGCCATCAC; Reverse: 5’-TCCACCACCTGTTGCTGTA. AATF: Forward: 5’-GACACGGACAAAGGTATTGCG; Reverse: 5’-CCGGTGTTTTTGAGCGAGTGG), 1X FastStart Universal SYBR Green Master Mix (Roche Diagnostics, Indianapolis, IN), and brought to 20 µL volume with nuclease-free water. qPCR was conducted in an Applied Biosystems 7300 real-time PCR instrument (Life Technologies) using the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 55°C for 35 seconds, and 68°C for 35 seconds. Data was analyzed using SDS software v1.3.1 (Life Technologies), using automatic threshold and baseline settings. Each mRNA evaluated was analyzed in triplicate using a minimum of three separate cell passage RNA extractions. GAPDH was used as a normalization control, and relative expression was calculated using the comparative C\text{t} method.\textsuperscript{85}

**Western Blotting**

Cultured cell pellets were lysed in 4% sodium dodecyl sulfate (SDS) in phosphate-buffered saline (PBS) with 1X PhosSTOP phosphatase inhibitor (Roche Diagnostics) and COMplete protease inhibitor (Roche Diagnostics), followed by sonication for five minutes at 4°C. Protein concentration was calculated using the Dc Protein Assay (Bio-Rad Laboratories), and absorbance read at 750 nm on the Smart Spec\textsuperscript{TM} 3000 Spectrophotometer (Bio-Rad Laboratories).

For western blotting, equal quantities of cell lysate (30-40 µg) were loaded onto a Novex\textregistered 4-12% Tris-Glycine SDS polyacrylamide gel (Life Technologies), under denaturing conditions using a loading buffer with 2% SDS, 5% β-Mercaptoethanol, 20%
glycerol and 0.004% bromophenol blue in 0.125M Tris-HCl, and electrophoresed in a 25 mM Tris, 190 mM glycine, 0.1% SDS running buffer for approximately 90 minutes at 140 volts. The samples were transferred onto a Trans-Blot® nitrocellulose membrane (Bio-Rad Laboratories) for 90 minutes at 200 mA and 4°C in a 48 mM Tris, 39 mM glycine, 0.04% SDS transfer buffer. Nonspecific binding was prevented by washing the blot for 30 minutes at room temperature in 5% nonfat dry milk in Tris-buffered saline with 0.05% Tween-20 (TBST). The blot was rinsed briefly in TBST, and incubated overnight (14-20 hours) at 4°C in primary IgG antibody in 5% BSA in TBST. Primary antibodies used were 1:1000 α-β-actin (C4) produced in mouse (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA), 1:100 α-NF-kB produced in rabbit (Santa Cruz Biotechnologies, Inc) and 1:500 α-AATF produced in mouse (Abnova, Tapiei, Taiwan). After incubation in primary antibody, the blot was washed with TBST four times for 5 minutes, followed by incubation in secondary antibody (1:1000 α-mouse IgG-HRP produced in goat (Santa Cruz Biotechnologies)) for 1 hour at room temperature. The blot was again washed with TBST four times for 5 minutes, and luminol reaction developed using the Western Lightning® chemiluminescent reagents (Perkin Elmer, Boston, MA). Western blots were exposed and bands quantified using the ODYSSEY® Fc Imaging System (LI-COR Biosciences, Lincoln, NE).

Proliferation Assay

M12 and M12 cells transformed with the miR-147b inhibitor plasmid (5,000 cells) were plated into each well of a 24-well plate in triplicate. The adherent cells were released by incubating with 0.25% Trypsin-EDTA (Gibco-Life Technologies) for 5 minutes at 37°C, after which the trypsin was inactivated by washing the cells in serum-
containing media. The cells were then pelleted through centrifugation at 1500xg for 5 minutes and the pellets resuspended in 1 mL of PBS, and counted using a Coulter Counter® Analyzer (Beckman Coulter, Inc., Brea, CA) or Vi-Cell™ XR Cell Viability Analyzer (Beckman Coulter, Inc) using a trypan blue solution. Cells were >90% live when analyzed in this fashion.

**Migration and Invasion Assays**

For evaluation of migratory capabilities, M12 cells stably transformed with either a scrambled control or miR-147b inhibitor were diluted in serum-free RPMI 1640 medium and 5 x 10^4 cells added to a ThinCert™ TC membrane support insert (Greiner Bio-one BVBA/SPRL, Belgium) placed in the well of a 24-well plate. For invasion assays, 30 µL of a 10% solution of Cultrex® Basement Membrane Extract (Growth Factor Reduced), (R&D Systems®, Minneapolis, MN) in RPMI 1640 was pipetted onto the membrane support insert, and incubated at 37°C for 1 hour prior to adding 1.25 x 10^6 cells to the upper chamber. For both assays, 1 mL of RPMI 1640 medium containing 5% FBS and 10 ng/mL EGF was pipetted into the lower chamber of the well as the chemoattractant. The unit was incubated for 24 hours. Media was aspirated and cells were fixed by adding 0.025% of gluteraldehyde in ddH₂O to the bottom chamber for 20 minutes, followed by staining with 0.1% leucocrystal violet in 10% EtOH and PBS for a minimum of 30 minutes. The membranes were excised and mounted to a microscope slide using Permoun® (Fisher Scientific, Waltham, MA). Cell counts representing migratory or invasive cells were performed in 5 random fields for each replicate, averaged, and expressed as relative to the control. Data was the result of a minimum of 3 independent experiments, each performed in triplicate.
In-Vivo studies

M12 and M12 cells stably transformed with the miR-147b inhibitor plasmid (1x10^6) were subcutaneously (SC) injected into 10 (5 for each treatment) male athymic nude mice (Harlan Laboratories, Inc., Indianapolis, IN) to assess tumorigenicity. Tumour growth was monitored with caliper measurement and tumour volume calculated as length x width^2/2 (mm^3) over a period of 45 days until sacrifice. All experiments were conducted under a protocol approved by the Institution Animal Care and Use Committee of Virginia Commonwealth University (VCU).

Data Analysis

Data were presented as the mean ± standard deviation. Statistical analyses were conducted in the Microsoft® Excel software platform, using the independent Student's t-test, assuming equal variance between the two groups. Statistical significance was defined as p ≤ 0.05.

Results

Inhibition of miR-147b in the M12 cells had significant effects on migratory and invasive potential of the highly metastatic cell line (p <0.001 for both assays) (Figure 4-5). Additionally, stable transfection with a miR-147b inhibitor inhibited proliferation in M12 cells by an average of 58.5% (p <0.03) (Figure 4-6). This inhibition is uncommon in our experience in this cell line progression model, as the cells have been immortalized via SV40T, which is known to bind to the Rb protein and prevent the Rb-mediated cell-cycle stop at G1(129) (reviewed in Dick et al.130). Thus, we would predict that a target of miR-147b would be a tumour suppressor protein involved in cell cycle
progression. Inhibition of miR-147b results in loss of translation suppression of that tumour suppressor, which can then act on the cell cycle as a stop signal, and consequently, cell doubling times are reduced.

Cells (1x10⁶) were injected subcutaneously into the flanks of nude athymic mice and monitored for tumour volume for up to 47 days after injection. M12 cells with the miR-147b inhibitor showed a significantly lower level of tumour growth (p=.085) (Figure 4-7). All mice (with the exception of one M12 injected mouse) developed tumours.
Figure 4-5: Inhibition of miR-147b significantly reduces migratory and invasive potential. M12 cells were stably transformed with scrambled control or miR-147b inhibitor, plated on a ThinCert transwell membrane (with basement membrane added for invasion assay) and assessed for (A) migratory (p < 0.001) and (B) invasive potential (p < 0.001). Data is the mean of 3 independent experiments, each performed in triplicate.
Figure 4-6: Cell proliferation is impacted by miR-147b inhibition. Inhibition resulted in a 58.5% reduction in proliferation (p < 0.03). Results are representative of 4 independent experiments, each performed in triplicate.
Figure 4-7: Tumour growth is significantly reduced upon miR-147b inhibition. Subcutaneous injections of the respective cell lines into the flank of nude athymic mice. Tumour reduction was consistently observed ($p = .085$). Results are reported as the average ratio of tumour volume to the final average M12 tumour volume. N=5 mice for each treatment.
Given our observations of the difference in oncogenic potential when miR-147b is inhibited, we felt confident that this miR has at least one significant target that would prevent tumour progression when released from suppression. We therefore focused our efforts on identifying potential targets for activation.

NF-κB is one of the potential activators of miR-147b transcription and thus we were interested in determining if it was upregulated, and the consequent cause of the observed increase in miR-147b levels. However, western blot analysis did not indicate a change in NF-κB levels (Figure 4-8). Thus, we do not view it as the source of the miR-147b upregulation.
Figure 4-8: NF-kB levels are not increased in M12 cells, and thus likely not contributing to increased miR-147b expression. Western blot analysis of NF-kB and β-Actin as loading control. Blot is representative of 2 independent experiments.
As there are no absolutely proven targets for miR-147b, a search for potential targets is literally a fishing expedition. While the majority of miR binding events occur in the 3'-UTR of the messenger RNA, a growing body of literature has shown that miRs can bind and modulate mRNA translation throughout the message, including the 5'-UTR and coding regions\textsuperscript{98,99}. This certainly complicates the search, as the majority of miR-target prediction software platforms search the 3'-UTRs of genes alone. We first turned to miR-prediction software platforms for identification of potential targets. Review of the most commonly identified, highest confidence mRNA targets using TargetScan\textsuperscript{92-94,110}, TargetMiner\textsuperscript{90}, miRDB\textsuperscript{86,87,89,117}, and Diana\textsuperscript{88} resulted in a large list. Upon identifying targets, we reviewed the seed-pairing potential between miR and mRNA, and performed a literature search to determine each protein’s known role, possible correlations to malignancy. The majority of proteins identified as having high seed-pairing capability to miR-147b were either not previously shown to be modulated in cancers, or were in fact being upregulated in tumours and thus would not likely be active targets for translation suppression by miR-147b in the prostate. The only protein identified that had a known tumour-suppressive role in cancers was AATF, or apoptosis-antagonizing transcription factor.
Table 4-1: Top predicted targets for miR-147b by bioinformatic analysis.

Evaluation of the top potential mRNA candidates from 3 different miRNA target mining software programs, DIANA, miRDB, and TargetScanHuman, release 6.2, revealed that with the exception of AATF, none of the top targets have known tumour suppressive function.

<table>
<thead>
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<th>Protein</th>
<th>Abbreviation</th>
<th>Function</th>
<th>Role in Carcinogenicity?</th>
<th>Predicted by</th>
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<td>NDUFA4</td>
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<td>Unknown</td>
<td>DIANA, miRDB, TargetScan</td>
</tr>
<tr>
<td>scavenger receptor class A, member 3</td>
<td>SCARA3</td>
<td>macrophage scavenger receptor-like protein which deplete reactive oxygen species; induced by oxidative stress.</td>
<td>mRNA up in ovarian tumours compared to breast</td>
<td>DIANA</td>
</tr>
<tr>
<td>insulin-like growth factor 2 (somatomedin A)</td>
<td>IGF2</td>
<td>insulin family of growth factors, the predominant growth factor in adults.</td>
<td>Tumour progression through initiation of steroidogenesis to breast</td>
<td>DIANA</td>
</tr>
<tr>
<td>apoptosis antagonizing transcription factor polymerase (RNA) III (DNA directed) polypeptide F</td>
<td>POLR3F</td>
<td>Subunit of RNA Polymerase III, transcribes ribosomal and tRNA genes</td>
<td>High levels in many cancers through loss of epigenetic control, including prostate</td>
<td>DIANA, miRDB</td>
</tr>
<tr>
<td>zinc finger protein 3 homolog (mouse)</td>
<td>ZFP3</td>
<td>May be involved in transcriptional regulation</td>
<td>unknown</td>
<td>miRDB</td>
</tr>
<tr>
<td>potassium voltage-gated channel, shaker-related subfamily, member 3</td>
<td>KCNA3</td>
<td>Voltage-gated potassium channel, essential in T-Cell proliferation and activation</td>
<td>Upregulated in rat prostate cancer cell lines</td>
<td>miRDB</td>
</tr>
<tr>
<td>Homeobox C6</td>
<td>HOXC6</td>
<td>Transcription factor involved in development</td>
<td>Upregulated in gastric cancer Deregulated in head and neck squamous cell carcinoma Upregulated in prostate cancer</td>
<td>TargetScan</td>
</tr>
<tr>
<td>Zinc finger protein 148</td>
<td>ZNF148</td>
<td>Transcriptional effector involved in cell growth and death</td>
<td>Upregulated in colorectal cancer Effect of Vimentin</td>
<td>TargetScan</td>
</tr>
<tr>
<td>Brain-derived neurotrophic factor</td>
<td>BDNF</td>
<td>Nerve growth factor involved in striatal neurons</td>
<td>Decreased in colorectal cancer pt serum Elevated in gastric cancer High expression associated with poor prognosis in lung cancer</td>
<td>TargetScan</td>
</tr>
<tr>
<td>Homeobox A9</td>
<td>HOXA9</td>
<td>Transcription factor involved in morphogenesis and differentiation</td>
<td>Elevated in ovarian, prostate, meningioma, oral cancer Decreased in bladder cancer</td>
<td>TargetScan</td>
</tr>
</tbody>
</table>
The Apoptosis-Antagonizing Transcription Factor (AATF) was first isolated in rats, and subsequently in humans during a search for TGF-β regulated genes involved in the differentiation of intestinal crypt cells\textsuperscript{135}. It is located on chromosome 17q11.2-q12, and is also known as Che-1\textsuperscript{136,137}. The protein contains 560 amino acids, a nuclear localization signal, three nuclear hormone receptor LXXLL binding motifs, a leucine zipper motif, and several phosphorylation sites for different kinases\textsuperscript{135,137}. AATF is expressed in every tissue, but expression is enriched in brain, heart, thymus, kidney, and placenta\textsuperscript{135}. Interestingly, AATF shares homology with SV40 large T antigen as well as with E. coli ς-factor 70, and interacts with the Rb protein in a similar fashion to SV40T\textsuperscript{138}. As an RNA Polymerase-II binding protein, AATF has been shown to have a key role in cellular processes including DNA damage response, cell-cycle control, chromatin remodeling, and apoptosis\textsuperscript{139}.

Evaluation of AATF message and protein expression was consistent with it being regulated by miR-147b (Figure 4-9). Western blot analysis indicated that while low in abundance, AATF is certainly detectable in P69 and the M12 cells treated with miR-147b inhibitor. However, the AATF signal was not visible in the M12 cell lysates. Additionally, RT-qPCR analysis showed that AATF messenger RNA levels are also being modulated by miR-147b. As in our evaluation of the c-KIT message (Chapter 3), AATF mRNA must be cleaved by the miR-147b/Argonaute 2 complex in order for messenger RNA levels to be changed between the cell lines. While there could be and is a considerable level of complexity between the P69 and M12 cells (Chapter 2), modulation between the M12 and M12 treated with miR-147b inhibitor could only be caused by miR-147b cleavage.
Figure 4-9: messenger RNA and protein levels are returned to P69 levels by miR-147b inhibition. AATF expression in the P69, M12, and M12 stably transformed with vector expressing miR-147b inhibitor. A: Western blot analysis and B: quantitation. Blot and quantitation are representative of 2 independent experiments, normalized to β-Actin and reported as relative to the M12 cell line. C: messenger RNA relative quantitation shows that mRNA levels are impacted by miR-147b, and inhibition reverts levels to P69 levels (p<0.03 for P69 and miR-147b inhibited lines vs. M12). mRNA is normalized to GAPDH and reported as relative to the M12 cell line. Results are compiled data from two biological replicates, each performed in triplicate.
The AATF 3’-UTR is very short, approximately 207 nucleotides long. Analysis of the AATF 3’-UTR using TargetScan and RNAHybrid revealed no sites in the 3’-UTR where miR-147b might bind. Analysis of the entire messenger RNA using RNAHybrid and Diana identified six excellent potential matches, all with free energy values below -22 kcal/mol (Table 4-2).
Table 4-2: Top potential binding sites for miR-147b are within the coding regions of the mRNA. Binding sites are ordered by descending free energy values. Adapted from RNAHybrid\textsuperscript{86,87} analysis of AATF mRNA (NM_012138.3).

<table>
<thead>
<tr>
<th>Position</th>
<th>Target</th>
<th>MiRNA</th>
<th>mfe (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>665 (Exon 1)</td>
<td>5' G CAC AAA A 3' AGCAGAAGC UCUUGCA AC UCGUCUUCCG AGGCGU UG</td>
<td>3' A UAA G 5'</td>
<td>-30.7</td>
</tr>
<tr>
<td>190 (5'-UTR)</td>
<td>5' G C G G U G G G G 3' GCC G G G U C C G C A</td>
<td>3' A U U U GU AA G 5'</td>
<td>-29.2</td>
</tr>
<tr>
<td>105 (5'-UTR)</td>
<td>5' G G U G G C C C C 3' AGC G G U C</td>
<td>C C G C G C G A C G C C</td>
<td>3' A U U U AA G 5'</td>
</tr>
<tr>
<td>58 (5'-UTR)</td>
<td>5' C C C C C C C C C C C C C C C C C C C C C C</td>
<td>GCAGG G C C C G C G C</td>
<td>3' AU U U U AAA G 5'</td>
</tr>
</tbody>
</table>
Discussion

In this report, we sought to confirm miR-147b’s previously identified status as an oncomiR, which was based not only on analysis of the poorly-tumorigenic P69 and its highly metastatic subline M12, but also other well-established prostate cancer cell lines, and finally through confirmation using human biopsy samples retrieved by laser-capture microdissection. Inhibition of miR-147b in M12 cells resulted in markedly reduced cell proliferation, and reduced migratory and invasive potential. Tumour growth in-vivo was also reduced in male nude athymic mice when miR-147b was inhibited.

We also hoped to identify potential proteins whose mRNAs were targets of miR-147b, thus elucidating a mechanism for the observed oncogenic effects. Through a combination of bioinformatics analyses and literature reviews of potential targets, we identified AATF as a strong candidate for miR-147b targeting. AATF appears to act either as an oncogene or tumour suppressor, and its action is strongly dependent on the tumour origin. It is known to interact with subunit 11 of RNA Polymerase II, and repress growth arrest by activation of p21, a cyclin-dependent kinase inhibitor\textsuperscript{138,140}. The same study showed that a significant amount of AATF is complexed with Rb protein, and competes with Rb-recruited HDAC1 for binding, which can lead to dysregulation of proliferation targets\textsuperscript{140}.

AATF also has a known role as an oncogene. In breast cancer and leukemia, AATF is known to be upregulated, likely through the c-Myc response element in its promoter\textsuperscript{141}. RNA interference with AATF message results in decreased cell proliferation and induction of apoptosis through down-regulation of Bcl2 and up-
regulation of Bax. AATF is also known to interact with the tumour suppressor Rb, releasing repression on E2F-mediated transcription, and is implicated in co-activation of the androgen receptor. Some research has shown that AATF is phosphorylated and stabilized upon DNA damage, and in a p53-dependent manner, AATF works to modulate the G2/M checkpoint.

The low levels observed in the prostate cell lines here described are consistent with observed down-regulation in kidney and colon carcinomas. In-vitro, overexpression of AATF in human colon carcinoma cells caused cell accumulation at the G1 checkpoint, and directly stimulated p21 transcription in a p53-independent manner. Such a block would be consistent with our observations of reduced cell proliferation when miR-147b was inhibited (Figure 4-5), and AATF consequently upregulated. This results in competitive binding over HDAC1 for the SP1 binding sites of p21, and therefore transcription of the tumour suppressor (Figure 4-10). Interestingly, NMES-1, the gene in which miR-147b is located, has been shown to be downregulated in colon and esophageal carcinoma tumour tissues. If miR-147b levels were found to be upregulated in these tissues as well, it would indicate that miR-147b is being selectively overexpressed, as compared to being overexpressed during the overexpression of NMES1.
A: Benign State

B: Tumour State

Figure 4-10: Proposed mechanism of miR-147b action on cell cycle progression.

A: In the benign state, miR-147b is not well-expressed, and thus AATF protein is produced and can interact with the SP1 binding sites on the p21 protein, outcompeting HDAC1. B: In the neoplastic cell, miR-147b is overexpressed in prostate, colon, and kidney carcinomas, and acts to cleave and reduce AATF message, thus allowing for deacetylation of the p21 gene and consequently less transcription, which leads to loss of the tumour suppressor protein and an increase in cell cycle progression.
Our results show a significant effect of miR-147b on AATF through mRNA and western blot analysis of protein levels. Thus, there is certainly a tie between miR-147b and AATF. In order to prove that this interaction is a direct effect by miR-147b and not suppression through an indirect manner, cloning wild type and mutated versions of each of the seven potential binding sites of the miR-147b mRNA into 3’-UTR luciferase constructs will need to be undertaken. This final group of experiments will elucidate the manner of action of miR-147b on the AATF protein levels.
Chapter 5:

miR-9 acts as an oncomiR in prostate cancer by regulation of e-cadherin and SOCS5 through multiple pathways that drive towards tumour progression and metastasis
Previous work identified miR-9 as up-regulated in not only prostate cancer cell lines (Chapter 3), (Figure 5-1), but also in 3 out of 4 of prostate cancer biopsy tumour samples as compared to the benign laser-capture microdissected tissue (Figure 5-2). The consistency and high expression of miR-9 in the majority of samples analyzed led to an investigation of miR-9’s mode of action in prostate cancer.

miR-9 has been well characterized in neural tissue development (reviewed in Yuva-Aydemir et al.146), but is detectable in a variety of human tissues. miR-9 is a family of three identical sequences (ucuuugguuaucuagcuguauga). These 3 homologues are designated as miR-9-1, 9-2, and 9-3. miR-9-1 is located within an open reading frame on chr 1q22, miR-9-2 is located within the reading frame of a LNC-RNA on chr 5q14.3, and miR-9-3 is located on chr15.26.1. The miR-9-3 locus has Myc and MycN binding sites that have been shown to activate miR-9 expression43. In at least one instance, miR-9 has been upregulated through action of Prospero Homeobox 1 (PROX1)29.

While hundreds of putative miR-9 targets have been identified through a variety of miR-target prediction programs, only six have been definitely proven as being directly targeted by miR-9; i.e., that miR-9 binds to a portion of the mRNA, and that binding affects protein levels (Table 5-1).
Figure 5-1 (Adapted from Chapter 3): miR-9 levels are upregulated in all prostate cancer cell line models. A: miR-9 levels were measured in P69, M12, M2182, and F6 cells. Data was normalized to RNU48 and expressed relative to P69 using the comparative C_T method. B: miR-9 levels were measured in M12, DU145, WMPY1 and PC3 cells. Data was normalized to RNU48 (dC_T) and expressed as a box plot. The dC_T for the P69 cell line is visualized as the red box.
Figure 5-2 (adapted from Chapter 3): miR-9 expression is upregulated in 60% of tested patient tumours. miR-9 levels were measured in tumour and benign tissue separated from prostate biopsies using laser-captured microdissection (LCM). Data was normalized to RNU48 and expressed relative to the benign tissue using comparative CT method. miR-9 was undetected in both benign and tumour of Pt 09-225.
**Table 5-1: Proven direct targets of miR-9.** The targets listed have been proven through both luciferase assay analysis and mutation of the 3’-UTR, as well as at least one additional analytical technique (western blot of protein level, microarray, etc.).

<table>
<thead>
<tr>
<th>mRNA target</th>
<th>Shown in</th>
</tr>
</thead>
<tbody>
<tr>
<td>e-cadherin</td>
<td>Breast cancer</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Metastatic melanoma</td>
</tr>
<tr>
<td>CXCR4</td>
<td>Nasopharyngeal carcinoma</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Ovarian Cancer</td>
</tr>
<tr>
<td>Stathmin</td>
<td>Human embryonic stem cells(^{147})</td>
</tr>
<tr>
<td>SOCS5</td>
<td>Non-small cell lung cancer and melanoma cell lines</td>
</tr>
</tbody>
</table>
E-cadherin has been described as a direct target for miR-9 in multiple instances, including colon, hepatocellular, and breast cancer\textsuperscript{20,148,149}. E-cadherin (CDH1) is a transmembrane glycoprotein that forms adherens junctions between adjacent epithelial cells. The cytoplasmic tail associates with intracellular proteins, ultimately linking the adherens junctions to the actin cytoskeleton\textsuperscript{24,150,151}. The extracellular domain of e-cadherin interacts with e-cadherin from neighboring cells to form adherent sheets of epithelial cells\textsuperscript{20,152}.

NF-kB is also known to be a direct target of miR-9. While NF-kB is known to be activated in an inflammatory response and early tumour apoptosis, ultimately many neoplasias show constitutive activation of NF-kB and transcription of pro-survival gene products\textsuperscript{153,154}. NF-kB activity as related to miR-9 appears to occur in the cancers where miR-9 expression is lost, and NF-kB is consequently upregulated\textsuperscript{155,156}. Many of the cancers describing miR-9 overexpression do not note a change in NF-kB levels, and thus competitive binding for miR-9 or promoter elements in NF-kB may be the cause for the lack of observed change in miR-9 overexpressing carcinomas.

Suppressor of Cytokine Signalling, or SOCS5, are a family of proteins that work to inhibit downstream JAK/STAT signaling through inhibition of STAT phosphorylation by multiple actions. Some SOCS proteins bind and inhibit JAK kinases, while others compete with STATs for phosphotyrosine binding sites on cytokine receptors\textsuperscript{157}. SOCS proteins also interact with the ubiquitination machinery through a motif called the SOCS box to ubiquitinate the signaling intermediates and thus reduce signal transduction\textsuperscript{158}.
Given the consistent upregulation of miR-9 in prostate cancer cell lines and patient samples as described in Chapter 3, it was important to evaluate how inhibition of miR-9 could impact the tumorigenicity of a prostate cancer cell line model, and to evaluate known targets of miR-9 as affecting tumour progression in prostate cancer.

Project Aims

3. Inhibit miR-9 levels in the M12 cell line and evaluate any changes in proliferation, tumorigenicity, or invasive potential.

4. Evaluate potential target mRNAs in prostate cancer; confirm the mode of action of miR-9 as inhibiting e-cadherin and SOCS5 protein expression, thus promoting the EMT transition and angiogenesis through multiple pathways.

Methods and Materials

Cell Culture

Adherent cells were cultured in 75 cm² flasks using RPMI 1640 supplemented with L-Glutamine (Caisson Labs, North Logan, UT), 5% fetal bovine serum, 5 µg/mL insulin, 5 µg/mL transferrin, 5 µg/mL selenium (ITS, Collaborative Research, Bedford, MA). Gentamycin (0.05 mg/mL) was used to prevent bacterial contamination. Cells were passaged when 50-65% confluent, and maintained for no longer than 20 passages. Passaging was accomplished by incubating with 0.25% Trypsin-EDTA (Gibco-Life Technologies, Carlsbad, CA) for 5 minutes at 37°C, after which the trypsin was inactivated by washing the cells in serum-containing media. The cells were then pelleted through centrifugation at 1500xg for 5 minutes and either passaged, plated, or
pellets preserved. Cell pellets for analysis were produced by washing the pellet in 10 mL PBS, centrifugation at 1500xg for 5 minutes, removal of the supernatant, and flash freezing in liquid nitrogen. Pellets were stored at -80°C for at least 24 hours.

**Transfection**

Stable transformations of an early passage of the M12 cell line with a vector containing a miR-9 inhibiting sequence or scrambled control (pEZX-AM03, Geneocopeia Inc., Rockville, MD) were performed using TransIT®-LT1 Transfection Reagent (Mirus BIO LLC, Madison, WI, USA). Briefly, 1.5 x 10^5 cells were plated in a 25 cm² flask in appropriate serum-containing media for 24 hours. The cells were then rinsed with PBS, and serum-containing media replaced. The plasmid (6.5 µg) was mixed with 650 µL of RPMI media with no serum or additives and 19.5 µL of TransIT®-LT1 reagent at room temperature for 15-30 minutes. The solution was added dropwise to each flask, and gently rocked back and forth. The transfections were incubated for 48 hours under standard cell culture conditions, and then selected for stable transformants using 200 ng/mL of Puromycin or 300 ng/mL of Hygromycin for two weeks. After the selection die-off, the cells were maintained using Puromycin (100 ng/mL) or Hygromycin (150 ng/mL).

**RNA Isolation**

RNA was isolated from cell pellets using the miRVana™ RNA isolation method according to the manufacturer's protocol (Ambion-Life Technologies, Carlsbad, CA), eluted in 50 µL of Elution Buffer, and stored at -80°C. RNA concentration was estimated using either the Smart Spec™ 3000 Spectrophotometer (Bio-Rad
Laboratories, Hercules, CA) or the NanoDrop ND-2000 Spectrophotometer (Thermo-Fisher Scientific, Inc., Waltham, MA).

**Messenger RNA Analysis**

Messenger RNA was quantified using reverse transcription-quantitative PCR (RT-qPCR). Reverse transcription was accomplished using the iScript™ cDNA Synthesis kit (Bio-Rad Laboratories). Briefly, 50 ng of RNA was mixed with 4 µL of the 5X iScript reaction mix, 1 µL of the iScript Reverse Transcriptase, and the volume brought to 20 µL with nuclease-free water. The reaction mix was incubated for 5 minutes at 25°C, followed by 30 minutes at 42°C, and heat inactivation for 5 minutes at 85°C. Reactions were stored at -20°C until analysis. The qPCR reactions were prepared by adding 3 µL of the prepared cDNA, 10 µM primer pairs for the relevant mRNA target (GAPDH: Forward: 5’-ACCACAGTCCATGCCATCAC; Reverse: 5’-TCCACCACCTGTTGCTGTA. E-Cadherin: Forward: 5’-GGTGCTCTTCCAGGAACCTC; Reverse: 5’-GAAACTCTCTCGGTCCAGCC. SOCS5: Forward: 5’-CCTCCTTCGCGCCTTCACCTA; Reverse: 5’-TATAAAATCGTGACCAATAGCAGGC), 1X FastStart Universal SYBR Green Master Mix (Roche Diagnostics, Indianapolis, IN), and brought to 20 µL volume with nuclease-free water. qPCR was conducted in an Applied Biosystems 7300 real-time PCR instrument (Life Technologies) using the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 55°C for 35 seconds, and 68°C for 35 seconds. Data was analyzed using SDS software v1.3.1 (Life Technologies), using automatic threshold and baseline settings. Each mRNA evaluated was analyzed in triplicate using a minimum of three separate cell passage RNA
extractions. GAPDH was used as a normalization control, and relative expression was calculated using the comparative C_{T} method^{12}.

**Western Blotting**

Cultured cell pellets were lysed in 4% sodium dodecyl sulfate (SDS) in phosphate-buffered saline (PBS) with 1X PhosSTOP phosphatase inhibitor (Roche Diagnostics) and COMplete protease inhibitor (Roche Diagnostics), followed by sonication for five minutes at 4°C. Protein concentration was calculated using the Dc Protein Assay (Bio-Rad Laboratories), and absorbance read at 750 nm on the Smart Spec™ 3000 Spectrophotometer (Bio-Rad Laboratories).

For western blotting, equal quantities of cell lysate (30-40 µg) were loaded onto a Novex® 4-12% Tris-Glycine SDS polyacrylamide gel (Life Technologies), under denaturing conditions using a loading buffer with 2% SDS, 5% β-Mercaptoethanol, 20% glycerol and 0.004% bromophenol blue in 0.125M Tris-HCl, and electrophoresed in a 25 mM Tris, 190 nM glycine, 0.1% SDS running buffer for approximately 90 minutes at 140 volts. The samples were transferred onto a Trans-Blot® nitrocellulose membrane (Bio-Rad Laboratories) for 90 minutes at 200 mA and 4°C in a 48 mM Tris, 39 mM glycine, 0.04% SDS transfer buffer. Nonspecific binding was prevented by washing the blot for 30 minutes at room temperature in 5% nonfat dry milk in Tris-buffered saline with 0.05% Tween-20 (TBST). The blot was rinsed briefly in TBST, and incubated overnight (14-20 hours) at 4°C in primary IgG antibody in 5% BSA in TBST. Primary antibodies used were 1:1000 α-β-actin (C4) produced in mouse (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA), 1:2500 α-e-cadherin (CDH1) produced in mouse (Sigma Aldrich, St.
Louis, MO), 1:100 α-NF-kB produced in rabbit (Santa Cruz Biotechnologies, Inc), and 1:500 α-SOCS5 (M-300) produced in rabbit (Santa Cruz Biotechnologies). After incubation in primary antibody, the blot was washed with TBST four times for 5 minutes, followed by incubation in secondary antibody (1:1000 α-mouse IgG-HRP produced in goat (Santa Cruz Biotechnologies) and/or 1:1000 α-rabbit IgG-HRP produced in goat (Cell Signalling Technology, Danvers, MA), for 1 hour at room temperature. The blot was again washed with TBST four times for 5 minutes, and luminol reaction developed using the Western Lightning® chemiluminescent reagents (Perkin Elmer, Boston, MA). Western blots were exposed and bands quantified using the ODYSSEY® Fc Imaging System (LI-COR Biosciences, Lincoln, NE).

**Proliferation Assay**

M12 and M12 cells transformed with the miR-9 inhibitor plasmid were plated (5,000 cells) onto a 24-well plate in triplicate. The adherent cells were released by incubating with 0.25% Trypsin-EDTA (Gibco-Life Technologies) for 5 minutes at 37°C, after which the trypsin was inactivated by washing the cells in serum-containing media. The cells were then pelleted through centrifugation at 1500xg for 5 minutes and the pellets resuspended in 1 mL of PBS, and counted using a Coulter Counter® Analyzer (Beckman Coulter, Inc., Brea, CA) or Vi-Cell™ XR Cell Viability Analyzer (Beckman Coulter, Inc) using a trypan blue solution. Cells were >90% live when analyzed in this fashion.

**Migration and Invasion Assays**
For evaluation of migratory capabilities, M12 cells stably transformed with either a scrambled control or miR-9 inhibitor were diluted in serum-free RPMI 1640 medium and 5 x 10^4 cells added to a ThinCert™ TC membrane support insert (Greiner Bio-one BVBA/SPRL, Belgium) placed in the well of a 24-well plate. For invasion assays, 30 µL of a 10% solution of Cultrex® Basement Membrane Extract (Growth Factor Reduced), (R&D Systems®, Minneapolis, MN) in RPMI 1640 was pipetted onto the membrane support insert, and incubated at 37°C for 1 hour prior to adding 1.25 x 10^6 cells to the upper chamber. For both assays, 1 mL of RPMI 1640 medium containing 5% FBS and 10 ng/mL EGF was pipetted into the lower chamber of the well as the chemoattractant. The unit was incubated for 24 hours. Media was aspirated and cells were fixed by adding 0.025% of gluteraldehyde in ddH₂O to the bottom chamber for 20 minutes, followed by staining with 0.1% leucocrystal violet in 10% EtOH and PBS for a minimum of 30 minutes. The membranes were excised and mounted to a microscope slide using Permount® (Fisher Scientific, Waltham, MA). Cell counts representing migratory or invasive cells were performed in 5 random fields for each replicate, averaged, and expressed as relative to the control. Data was the result of a minimum of 3 independent experiments, each performed in triplicate.

In-Vivo studies

M12 and M12 cells transformed with the miR-9 inhibitor plasmid (1x10^6 cells) were subcutaneously (SC) injected into 10 (5 for each treatment) male nude athymic mice (Harlan Laboratories, Inc., Indianapolis, IN) to assess tumorigenicity. Tumour growth was monitored with caliper measurement and tumour volume calculated as length x width^2/2 (mm^3) over a period of 45 days until sacrifice. To assess metastatic
potential, three male athymic nude mice were orthotopically injected with (1x10⁶ cells) of M12 or M12 cells transformed with the miR-9 inhibitor plasmid. The mice were sacrificed at 76 days, and peritoneal metastatic sites were counted. All experiments were conducted under a protocol approved by the Institution Animal Care and Use Committee of Virginia Commonwealth University (VCU).

3’-UTR constructs

A portion of the 3’-UTR of e-cadherin was previously cloned into pmiR-Report, the seed region subsequently mutagenized⁸ and both clones were obtained through Addgene (Plasmids 25038 and 25039; http://www.addgene.org) (Table 5-2).
Table 5-2: E-Cadherin 3'-UTR fragment constructs. miR-9 binding site is highlighted in yellow, the seed region is highlighted in green, and mutated nucleotides are highlighted in red.

<table>
<thead>
<tr>
<th>Clone ID</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT</strong></td>
<td>CAGGACTTAGAATAGTCCT<strong>AAAGTGCTGCAG</strong>CAAAAGACAGAGCGGAACTATGAAAAATGGGCTTA</td>
</tr>
<tr>
<td>Mutated binding site</td>
<td>CAGGACTTAGAATAGTCCT<strong>AAAGTGCTGCAG</strong>TTTC<strong>CA</strong>GAGCGGAACTATGAAAAATGGGCTTA</td>
</tr>
</tbody>
</table>
Transfection & Luciferase Activity Analysis

Transient co-transfections of the 3'-UTR fragments cloned into a luciferase reporter vector along with a renilla luciferase vector for normalization were performed using TransIT®-LT1 Transfection Reagent (Mirus BIO LLC, Madison, WI, USA). Briefly, cells were plated such that they would reach approximately 50% density in appropriate serum-containing media after 24 hours (2.5x10^4 cells/well in a 24-well plate). The cells were allowed to adhere for 24 hours, rinsed with 0.5 mL PBS, and serum-containing media replaced. 0.5 µg of the cloned UTR:vector was mixed with 50 µL of RPMI media and 1 µg of Renilla plasmid with no serum or additives and 1.5 µL of TransIT®-LT1 reagent at room temperature for 15 minutes. The solution was added dropwise to each well, and gently rocked back and forth. The transfections were incubated for 24 hours under standard cell culture conditions.

Cell lysis and luciferase assays were conducted using the Dual-Luciferase Reporter Assay System (Promega Corporation). The transfection media in each well was suctioned off, wells rinsed in PBS, and 100 µL of 1X Passive Lysis Buffer (Promega Corporation) was pipetted into each well. The samples were rocked for 15 minutes at room temperature, and the lysate transferred to 1.5 mL microcentrifuge tubes and frozen at -20°C until analysis. Luciferase and Renilla measurements were taken in a GloMax® 20/20 luminometer (Promega Corporation). Lysate (20 µL) was added to a 1.5 mL microcentrifuge tube, and 30 µL of firefly Luciferase Assay Reagent II added to the lysate and luciferase activity measured at a wavelength of 560 nm. Subsequently, 30 µL of Stop & Glo was added, the solution mixed, and renilla luciferase activity was measured at the 480 nm wavelength.
Data Analysis

Data were presented as the mean ± standard deviation. Statistical analyses were conducted in the Microsoft® Excel software platform, using the independent Student's t-test, assuming equal variance between the two groups. Statistical significance was defined as $p \leq 0.05$.

Results

Evaluation of miR-9’s effect on the tumorigenic potential of the M12 cells was performed through stable expression of a specific miR-9 inhibitor. Inhibition of miR-9 had significant effects on migratory and invasive potential of the highly metastatic cell line as compared to the scrambled control ($p < 0.0001$ for both assays) (Figure 5-3); however, miR-9 inhibition did not affect cell proliferation rates (data not shown). This is not surprising, given that the M12 cell line is immortalized with the SV40T antigen, which binds Rb, a tumour suppressor protein that halts cell cycle progression$^{61,130}$.

M12 or M12 + miR-9 inhibitor cells ($1 \times 10^6$ cells) were subcutaneously injected into the flanks of nude athymic mice and monitored for tumour volume for up to 47 days. M12 cells with the miR-9 inhibitor showed a significantly lower level of tumour growth ($p < 0.0001$) (Figure 5-4). Four of the five M12 injected mice developed tumours, but only two of the five M12+miR-9 inhibited mice developed tumours, which grew at a slower rate, resulting in a smaller tumour volume. Intraprostatic injection resulted in 7 metastatic sites for M12-injected mice, while neither of the M12+miR-9 inhibited mice had any observed metastatic sites by 76 days after injection.
Figure 5-3: Inhibition of miR-9 significantly reduces migratory and invasive potential. M12 cells were stably transformed with scrambled control or miR-9 inhibitor, plated on a ThinCert transwell membrane (with basement membrane added for invasion assay) and assessed for (A) migratory (p < 0.0001) and (B) invasive potential (p < 0.0001). Data is the mean of 3 independent experiments, each performed in triplicate.
Figure 5-4: Tumour growth and metastasis is reduced upon miR-9 inhibition. A: Subcutaneous injections of the respective cell lines into the flank of nude athymic mice. Tumour reduction was significantly reduced in mice injected with M12 cells stably expressing the miR-9 inhibitor ($p < .0001$). Results are reported as the average percent of tumour volume to the final average M12 tumour volume. N=5 mice for each treatment. B: Intraprostatic (IP) injection into nude athymic mice resulted in 0 metastatic lesions when miR-9 was inhibited, as compared to 7 lesions in the mouse injected with M12 cells alone.

<table>
<thead>
<tr>
<th>B</th>
<th>IP injected mice</th>
<th>Metastatic Lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>M12 (n=1)</td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>M12+miR-9 Inh (n=2)</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>
Given the strong evidence for miR-9's contribution to oncogenic potential in the inhibition studies, we felt confident that miR-9 must have at least one significant target that would prevent tumour progression when released from suppression. We therefore focused our efforts on evaluating miR-9's proven targets in prostate cancer using the P69 and M12 progression model.

As a known effector of the epithelial to mesenchymal transition, we were first interested in evaluating e-cadherin, one of the proven targets for miR-9 (Table 5-1). In the early tumour cell's transition from an affixed epithelial cell to a motile phenotype, the transition in expression from e-cadherin to vimentin is well known and described22,24,43,152. Previous work in the P69 and M12 cell lines showed that vimentin is upregulated in the M12 cell line as compared to P69, and that vimentin upregulation is caused in part by miR-17-3p loss in the M12 cell line (Figure 2-1)25.

Evaluation of E-cadherin message and protein expression was consistent with being regulated by miR-9 (Figure 5-5). Western blot analysis indicated that e-cadherin is highly expressed in P69 cells, and considerably lost in the M12 cells. Inhibition of miR-9 results in a corresponding increase in CDH1 levels. Additionally, RT-qPCR analysis showed that CDH1 messenger RNA levels are also significantly modulated by miR-9 (p < 0.03). As in our evaluation of the c-KIT message (Chapter 3), CDH1 messenger RNA must be cleaved by the miR-9/Argonaute 2 complex in order for mRNA levels to be changed between the cell lines. While there could be and is a considerable level of complexity between the P69 and M12 cells (Chapter 2), modulation of CDH1 message between the M12 and M12 + miR-9 inhibitor could be caused by miR-9 cleavage.
Figure 5-5: messenger RNA and protein levels of CDH1 are increased upon miR-9 inhibition. CDH1 expression in the P69, M12, and M12 stably transformed with vector expressing miR-9 inhibitor. A: Western blot analysis and B: quantitation. Blot and quantitation are representative of 5 independent experiments, normalized to β-Actin and reported as relative to the M12 cell line. C: messenger RNA relative quantitation shows that mRNA levels are impacted by miR-9, and miR-9 inhibition relieves messenger RNA and protein levels (p<0.03 for P69 and miR-9 inhibited lines vs. M12). mRNA is normalized to GAPDH and reported as relative to the M12 cell line. Results are compiled data from two biological replicates, each performed in triplicate.
One miR-9 binding site in e-cadherin was identified through an RNAHybrid analysis of the entire mRNA. This site, found in the 3’-UTR, has been proven to be directly bound by miR-9 thereby impacting translation\textsuperscript{21,43}. (Figure 5-6). Interestingly, based on a TargetScan\textsuperscript{92-94,110} analysis, miR-9 is the only conserved microRNA that binds to e-cadherin’s 3’-UTR. Transient transfections of luciferase constructs containing the CDH1 3’-UTR wild type or mutated seed region target (Table 5-2) resulted in loss of suppression as observed through luciferase activity (Figure 5-7).

As another proven target of miR-9, NF-kB is typically only shown as modulated when miR-9 suppression is lost, as is seen in carcinomas that show a loss of miR-9 expression\textsuperscript{153-156,159}. In these instances, relief of suppression of NF-kB results in initiation of transcription of a variety of pro-oncogenic and angiogenic genes. However, a concomitant decrease in NF-kB is not observed in the cancers in which miR-9 is overexpressed. Similarly, no difference between NF-kB levels were observed between the M12 and M12+miR-9 inhibited cells (Figure 5-8). Thus, control of NF-kB was not pursued further.
**Figure 5-6: Proven binding site for miR-9 in e-cadherin.** Adapted from RNAHybrid\textsuperscript{86,87} analysis of CDH1 mRNA (NM_004360.3). Green is the sequence of miR-9, and red is the complementary region of the 3’-UTR of CDH1.
Figure 5-7: e-cadherin expression is suppressed by miR-9. M12 cells were transiently transfected with a firefly luciferase reporter construct containing a portion of the CDH1 3’-UTR, with the wild type or mutated miR-9 binding seed region (Table 5-3) along with a renilla luciferase plasmid. Firefly luciferase expression is reported as normalized to renilla luciferase activity and relative to mutated seed region expression. Results are the mean of 2 independent experiments, each performed in triplicate. (p <0.01)
Figure 5-8: NF-kB levels are not modulated by miR-9. Western blot analysis shows that NF-kB levels do not change significantly between M12 or M12 cells plus miR-9 inhibitor. B-Actin included as a loading control. Blot is representative of 3 independent experiments.
As a negative regulator of EGFR and JAK signaling pathway, SOCS5, recently identified as a direct target of miR-9, could attenuate signal transduction and ultimately transcription of a variety of pro-oncogenic, pro-angiogenic genes\textsuperscript{157,180}. Evaluation of messenger RNA showed no significant difference in levels between P69, M12, and M12 cells transfected with miR-9 inhibitor; however, western blot analysis indicated a strong difference in SOCS5 protein levels between the different cell types (Figure 5-9). Messenger RNA levels do not always change in targets, as perfect seed matches to the miR and Argonaute 2 are required for cleavage of the message\textsuperscript{55}.

An evaluation of the SOCS5 mRNA sequence in RNAHybrid against the miR-9 sequence revealed four potential binding sites for miR-9, two of which are located in the 3'-UTR (one proven\textsuperscript{165}), and two located within coding regions of the mRNA (Table 5-3), all with significant free energy values.
Figure 5-9: While protein levels of SOCS5 are increased upon miR-9 inhibition, messenger RNA levels are not significantly different. SOCS5 expression in the P69, M12, and M12 stably transformed with vector expressing miR-9 inhibitor. A: Western blot analysis and B: quantitation show that miR-9 inhibition results in increased levels of SOCS5. Blot and quantitation are representative of 6 independent experiments, normalized to β-Actin and reported as relative to the M12 cell line. C: messenger RNA relative quantitation shows that mRNA levels are not significantly impacted by miR-9. mRNA is normalized to GAPDH and reported as relative to the M12 cell line. Results are compiled data from three biological replicates, each performed in triplicate.
Table 5-3: Potential and proven binding sites for miR-9 in SOCS5. Adapted from RNAHybrid\textsuperscript{13,14} analysis of SOCS5 mRNA (NM_014011.4). Green is the sequence of miR-9, and red is the complementary region of the 3'-UTR of SOCS5. Two of the potential binding sites are within the 3'-UTR, one of which (shaded in aqua) has been proven through luciferase 3'-UTR assays and western blot analysis of SOCS5\textsuperscript{15}.

<table>
<thead>
<tr>
<th>Position</th>
<th>Target Sequence</th>
<th>miRNA Sequence</th>
<th>mfe (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1180</td>
<td>A GUC U C</td>
<td>AGUA A U</td>
<td>-23.6</td>
</tr>
<tr>
<td></td>
<td>GCAGC AGAUA UGGAGA</td>
<td>UGUCG UCUAU GGUUUC</td>
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<tr>
<td>3101 (UTR)</td>
<td>C UU C</td>
<td>AGU CUAU U</td>
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<td></td>
<td>UAUGGCUA GCCAAGG</td>
<td>AUGUCGAU UGGUUUC</td>
<td></td>
</tr>
<tr>
<td>3696 (UTR)</td>
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<td>AG GAUCU</td>
<td>-20.1</td>
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<td>AUGUAG UAACAAAGA</td>
<td>AUGUC AUUGGUUUC</td>
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<td>G U U A</td>
<td>AGUA UAU</td>
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<tr>
<td></td>
<td>GCGGC UAG AUUGAAGA</td>
<td>UGUCG AUC UGGUUUC</td>
<td></td>
</tr>
</tbody>
</table>

mfe: minimum free energy
Discussion

In this report, we sought to confirm miR-9’s previously identified status as an oncomiR, which was based on not only a comparison between the poorly-tumorigenic P69 and its highly metastatic subline M12, but also other well-established prostate cancer cell lines, and finally through confirmation using human biopsy samples through laser-capture microdissection. Inhibition of miR-9 in M12 cells resulted in significantly reduced migratory and invasive potential in-vitro, as well as reduced tumour growth in-vivo with no metastatic sites observed in nude athymic mice. Evaluation of the expression signatures of known miR-9 targets indicated that while NF-kB is not modulated in this model, e-cadherin and SOCS5 are both regulated by miR-9, resulting in increased levels when miR-9 is inhibited in M12 cells. Luciferase constructs containing the e-cadherin 3'-UTR showed that miR-9 suppression of the mRNA is effective in the prostate cancer model.

E-Cadherin message and protein levels are suppressed by miR-9

Dysregulation of miR-9, although initially assumed as being overexpressed in carcinoma in general, has been shown through recent literature to be cancer type-specific. In breast cancer, neuroblastoma and prostate cancer, miR-9 is observed to be up-regulated, whereas it is downregulated in metastatic melanomas. In these cell types, miR-9 suppresses expression of NF-kB, which results in increased levels of Snail1 and subsequent activation of e-cadherin. Interestingly, higher levels of miR-9 have been associated with a better outcome in ovarian cancer, where it was shown to directly target BRCA1.
The difference in miR-9 expression by cancer type could be due to promoter activity of miR-9, whether through PROX1, Snail1, or hypermethylation. miR-9 is also down-regulated in nasopharyngeal carcinoma (NPC), where it is implicated in the inflammatory response, specifically for Interferon-induced genes\textsuperscript{161}. In this case, hypermethylation of the miR-9 promoter results in reduced transcription, resulting in loss of CXCR suppression, a chemokine receptor that has been shown to be expressed in a number of different tumour types\textsuperscript{162}. Recently, a second report showing the direct effect of miR-9 on CXCR identified that this loss of suppression resulted in the accumulation of β-catenin through the Wnt pathway\textsuperscript{22,163}, and the subsequent transcriptional activation of JAK/STAT proliferative pathways\textsuperscript{164}.

E-cadherin is known to sequester β-catenin, which also works to assist in dynamically connecting the adherens junctions to the cytoskeleton\textsuperscript{21,22,151}. Loss of e-cadherin through miR-9 suppression would free β-catenin from the adherens complex, permitting its movement into the nucleus, where it can interact with the zinc finger transcriptional factors of the Tcf/Lef family to activate transcription of pro-metastatic, pro-angiogenic genes including VEGFA, Siamois, c-Myc and cyclin D1\textsuperscript{22,43} (Figure 5-10). Loss of e-cadherin has also been shown to reduce phosphorylation of β-catenin, which results in increased stability by blocking movement to the proteasome for degradation\textsuperscript{152}.
Figure 5-10: miR-9’s mode of action on tumour progression through e-cadherin.

In the presence of miR-9 (upper panel), e-cadherin message is cleaved and suppressed, resulting in less protein production. This allows for accumulation of β-catenin (green “L”), which can then diffuse into the nucleus, activate transcription factors and drive transcription of pro-survival, pro-proliferation genes including c-Myc and Cyclin D1. c-Myc then initiates additional miR-9 message through a positive feedback signal. In the noncancerous tissue (lower panel), e-cadherin is produced and sequesters β-catenin, preventing activation of downstream transcriptional events.
**SOCS5 protein levels are suppressed by miR-9**

As a recently identified direct target for miR-9, loss of the tumour suppressor SOCS5 results in increased and prolonged activation of JAK/STAT pathways. The full function and target RTKs and JAK/STATS for SOCS5 is still unknown. However, it shares strong homology with the other members of the SOCS family, which are known to interfere with both JAK kinase and STAT protein activity, as well as promote their degradation through ubiquitination\(^{157,158}\). In the original report identifying miR-9 as a regulator for SOCS5 when overexpressed, SOCS5 expression was shown to be downregulated concomitant with increased JAK1, STAT1 and STAT3 phosphorylation levels\(^{165}\). SOCS transcription is also known to be part of a negative feedback loop, in that STAT binding sites activate SOCS5 transcription for ultimate suppression of signal transduction\(^{157,158}\). Even though the JAK/STAT pathway has been shown to be activated in M12 cells\(^{60,61,67}\), the negative feedback loop that would ultimately attenuate JAK/STAT signaling is being prevented through suppression of SOCS5, as we have shown that both SOCS5 mRNA and protein levels are reduced in M12 cells compared to the parental P69 (Figure 5-11).

**Conclusions**

This report has confirmed our previous work identifying miR-9 as an oncomiR in prostate cancer. The action of miR-9 is multifaceted; through suppression of miR-9, the e-cadherin that makes up the cell-cell interactions characteristic of an epithelial lineage is suppressed; thus, promotion from a stationary, embedded cell to a more motile phenotype is stimulated, as is readily observed in the EMT transition for a variety of
Figure 5-11: miR-9 overexpression inhibits SOCS5, causing increased signal transduction through the JAK/STAT pathways. In the presence of miR-9 (left), SOCS5 message is cleaved and translation suppressed, resulting in less protein production. This allows phosphorylation and signal transduction, resulting in p-STAT transcriptional activation of pro-survival, proliferation, and invasion/mestatasis oncogenes. In the normal tissue without miR-9 overexpression (right), SOCS5 is produced and reduces or blocks phosphorylation of JAK kinase and STAT while also promoting ubiquitination, thus attenuating the JAK/STAT signaling cascade, and lowering activation of transcriptional events.
cancers. Loss of e-cadherin also results in release and stabilization of β-catenin, which can then move to the nucleus and activate transcription of VEGFA, c-Myc, Cyclin D1, and other pro-angiogenesis, proliferative genes. Given that c-Myc is a known activator of miR-9-3, this too represents a feedback loop that could further induce even more miR-9 expression. Finally, action of miR-9 on SOCS5 results in loss of attenuation of the JAK/STAT pathways, which ultimately transduces growth signals to promote cell survival and proliferation. This is the first report to show a multi-targeted mode of action of miR-9 in a cancer. Moreover, results observed in-vivo indicate that both tumour growth and metastases are severely impacted by miR-9 inhibition (Figure 5-4). For those carcinomas in which miR-9 is overexpressed, inhibition of miR-9 could be a very effective therapeutic target, both in early neoplasias to prevent the EMT transition, but also in advanced, aggressive cancers to reduce proliferation and combat metastasis.
Chapter 6:

Summary and Future Directions
Dysregulation of microRNAs to drive tumour progression and metastasis is receiving a great deal of attention, as canonical pathways are seen to be regulated at key protein nodes through miR suppression. This can result in either suppression of a tumour suppressor protein through overexpression of the miR (oncomiR), or activation of a previously suppressed oncogene through loss of a tumour suppressor miR.

The prostate cancer cell line progression model developed by Dr. Joy Ware is valuable for identifying modulated miRs and downstream pathways in prostate cancer, as the normal epithelial SV40T immortalized P69 cell line is poorly tumorigenic and not metastatic. The M12 subline, produced by three subsequent injections of tumour cells into nude athymic mice, is highly tumorigenic and metastatic in nature. These two cell lines constitute a unique model in prostate cancer research, in that they have a common genetic background, and presumably selection of the M12 subline mimics a human tumour progression pathway.

Using the P69 and M12 progression model, our laboratory has been able to identify a variety of dysregulated microRNAs and their corresponding target mRNAs, and identify key impacted pathways that progress the neoplasia. These findings have been confirmed through in-vitro modulation of the miRs, showing changes in tumorigenesis, for the most part, metastases, and migratory capabilities, but also through in-vivo work in mice. Moreover, their dysregulation was confirmed in analysis of prostate cancer biopsies.

In this dissertation, we performed a global miR analysis to identify dysregulated miRs between the P69 and M12 cell line, and then confirmed those expression differences in other well-used prostate cancer cell lines as well as laser-capture
microdissected samples from prostate biopsies (Chapter 3). miRs 9 and 147b were identified for further analysis based on their consistently high level of expression.

miR-147b was identified as a potential oncomiR through this work. Inhibition of miR-147b was found to affect not only proliferative, migratory and invasive capabilities of M12 cells, but also reduced tumour growth in male nude athymic mice. AATF was identified as a potential target through western blot and mRNA analysis, along with identification of potential miR-147b binding sites within the mRNA. AATF has been well-established as being involved in cell-cycle progression\textsuperscript{139,143}. Its loss has previously been shown to activate p21 transcription, which is a known inhibitor of cell-cycle progression\textsuperscript{136}.

Similarly, miR-9 was found to be overexpressed in prostate samples and to function as an oncomiR by affecting the epithelial to mesenchymal transition through suppression of e-cadherin. As e-cadherin assists in maintaining the integrity of the epithelial cell to cell contact, loss of e-cadherin would result in destabilization of the cell from the rest of the tissue. Loss of e-cadherin results in increased levels of free β-catenin. β-catenin can then translocate to the nucleus and assist in activation of transcription of proliferative genes, including Cyclin D1 and c-Myc\textsuperscript{21,22,43} (Chapter 5). Inhibition of miR-9 resulted in not only reduction of migratory and invasive potential, but significant reduction of tumorigenesis and metastases, as evaluated through \textit{in-vivo} work with male nude athymic mice.

Building on previous work on miR-17-3p, reverse phase microarray analysis (RPMA) comparing the parental P69 and M12 subline showed a dramatic increase in c-
Table 6-1: dysregulated microRNAs identified in prostate cancer through the P69-M12 progression model

<table>
<thead>
<tr>
<th>microRNA</th>
<th>Target Protein/mRNA(s)</th>
<th>Pathways implicated</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-17-3p&lt;sup&gt;25&lt;/sup&gt;</td>
<td>Vimentin, c-KIT</td>
<td>EMT transition, AKT, ERK, JAK/STAT</td>
</tr>
<tr>
<td>miR-125b&lt;sup&gt;67&lt;/sup&gt;</td>
<td>ErbB2 &amp; 3</td>
<td>AKT</td>
</tr>
<tr>
<td>miR-147b</td>
<td>AATF</td>
<td>Cell Cycle progression, p21</td>
</tr>
<tr>
<td>miR-9</td>
<td>E-cadherin, SOCS5</td>
<td>EMT transition, cell cycle, JAK/STAT, β-catenin, c-Myc</td>
</tr>
</tbody>
</table>
Figure 6-1: Proposed mechanism for a multi-modal effect of dysregulated miR effects on tumour progression. Overexpression of miR-9 results in loss of SOCS5, an attenuator of JAK-STAT signal transduction, and of e-cadherin, an adhesion protein that maintains epithelial sheet integrity, as well as sequestration of β-catenin, a transcriptional activator of c-Myc and Cyclin D1. Likewise, overexpression of miR-147b results in the loss of AATF, and subsequent loss of p21 transcription, an inhibitor of CDK/Cyclin activated cell cycle progression. Loss of miRs-17-3p and 125b both result in increased transcription and levels of RTK growth factor receptors c-KIT and ERBB2/ERBB3, which increases pro-proliferative and survival signal transduction. Loss of miR-17-3p also promotes the EMT transition through relief of vimentin transcriptional suppression, an intermediate filament characteristic of the mesenchymal phenotype.
KIT in the M12 cells\textsuperscript{67}. This increase in c-KIT, a stem cell growth factor receptor tyrosine kinase, was ablated in the M12 cells expressing miR-17-3p. We identified two binding sites in c-KIT’s 3’-UTR that we confirmed as being directly targeted by miR-17-3p (Chapter 2). Two additional binding sites, one in the 5’-UTR and the other in an exon, were identified for further analysis.

The summation of this laboratory’s work in identifying modulated microRNAs in prostate cancer has progressed such that four microRNAs have been identified as contributing to the progression of the prostate tumour (Table 6-1). Evaluation of the impacted pathways show that each is vitally connected to key modulators and/or pathways known to affect tumour progression or cell proliferation. While admittedly some miRs (miR-9) were chosen based on their known targets and connectivity and therefore some bias may be in place there, others (miR-147b) were chosen based on their high (or low) consistent expression, with little to no foreknowledge of their target proteins.

Taken together, we know that through both modeling and patient samples, prostate cancer has a reduced expression of miR-17-3p\textsuperscript{25} and miR-125b\textsuperscript{67}. Loss of miR-17-3p causes relief of suppression for vimentin and c-KIT, with a concomitant increase in EMT transition, and activation of growth signal transduction pathways, thereby enhancing subsequent transcription of proliferation/survival genes (Figure 6-1). On the other hand, miRs 147b and 9 are highly upregulated, each also contributing to the EMT transition as well as growth and proliferation signaling pathways. Consequently, while each miR tends to be evaluated on a case-by-case basis, it is the accumulation of all of these dysregulations that contribute to tumour progression and
the transition to an invasive phenotype in prostate cancer. Thus, the sum of the parts working synergistically is greater than each component working alone.
Chapter 7:

An evaluation of the stability of microRNAs in forensically relevant biological fluids
While forensic DNA analysis has reached a level of maturity in the Forensic Science field with regards to the sophistication of the techniques and confidence in the results, the equally important question of body fluid identification has lagged behind, and could still be considered to be in a primitive state. Current crime scene and in-laboratory methods utilize detection methods that exploit the properties of each biological fluid (e.g. phenolphthalin or TMB testing for blood, amylase detection for saliva, and urease tests for urine), but validated identifying techniques are largely limited to microscopic methods (i.e. identification of spermatozoa) or immunological methods, as seen in the widely used immunochromatographic commercial tests for blood, semen, and other biological fluids. Thus, while there is widespread confidence in the DNA profile generated, there is often significantly less assurance in the identity of the body fluid that the DNA profile was developed from. It is common during trials for attorneys to categorically accept the STR analysis, but probe the forensic scientist on the source of the DNA that generated the profile. Because of this dichotomy, significant efforts have been made over the past ten years in order to develop forensic serological techniques of a more discriminatory nature. There are three main areas that can be exploited for molecular methods for body fluid identification: the genome, the transcriptome, and the proteome. Each has been subject to a great deal of research.

Recently, there has been some work in the forensic science field in regards to exploring microRNAs (miRs) for a molecular-based, forensic body fluid identification method. MiRs are small structures that are transcribed as larger precursors (60-100 nts) that immediately form a stem-loop structure with incomplete base-pairing and flanking nucleotides\(^{34}\). Processing of the immature miR is accomplished through
excision of the loop, resulting in a mature miR of 19-23 nucleotides long. There are no known postprocessing modifications, and thus miRs are simpler, and potentially less problematic for detection than proteins and mRNAs. MicroRNAs show distinct promise for forensic body fluid identification on several grounds. There is significant literature that some miRs are differentially expressed, and in fact are involved in embryonic stem cell development and tissue differentiation\textsuperscript{35,166,167}. They are found in extracellular fluids\textsuperscript{166}, and thus the discovery and application of unique miRs to forensically relevant body fluids is a distinct possibility.

Because of their small size and lack of a poly-A tail, miRs are inherently less susceptible to degradation than mRNA. Additionally, miRs are very hardy, and can be recovered from highly compromised samples including formalin-fixed paraffin embedded (FFPE) tissue\textsuperscript{25}. In serum, miRs have been shown to survive harsh conditions such as boiling, low or high pH, cycles of freeze-thaw, and extended storage\textsuperscript{56}. In the clinical arena, miRs have been shown in urine, and potential markers for bladder cancer have been evaluated\textsuperscript{168,169}. A recent study evaluated liquid semen, kept at room temperature for up to seven days, and frozen and thawed up to eight times, and found minimal differences in levels of three miRs evaluated\textsuperscript{168}. Urine stability was evaluated in up to four consecutive freeze-thaw cycles, and no significant difference was found in mIR-1 and miR-16 levels\textsuperscript{170}.

There is considerable evidence that many miRs are encapsulated in an exosome, which, depending on the microRNA and the secretion process, could be membrane- or protein-based\textsuperscript{58,171}. Because of this, recent studies have shown that samples can even be treated with RNase enzymes and the encapsulated miRs are still
detectable. This implies a high degree of stability of the species, and therefore, a very good possibility that if body-fluid specific miRs are found and described, a very robust miR panel for forensic body fluid identification could be developed.

Recently published studies examining miRs for forensic body fluid ID purposes utilized microarray screens and/or RT-qPCR, with varying results. A panel that can discriminate between blood, semen, saliva, vaginal secretions, and menstrual blood has already been described by Hanson et al. Further, Zubakov et al found that miRs could be detected using qPCR from just picograms of total RNA, far below the known detection limit of mRNAs. Recent reports have shown that miRs are detectable and coextracted in silica-column based DNA extracts at a similar level to RNA extracts. Because of the preliminary results reported from these authors, it is clear that miRs are detectable from forensic samples, and that it is an area that should be evaluated thoroughly. The purpose of this work was to evaluate the stability of microRNAs in blood, semen, urine, and saliva under the harsh treatment conditions that evidence samples could be subjected to at the crime scene.

Methods & Materials

Sample Collection & Treatment

Blood, urine, semen, and saliva were collected from volunteers under an approved human subjects research protocol. One body fluid collection from a single individual was used for all treatments, in order to eliminate variation seen in person-to-person and collection-to-collection miR levels. Urine, semen, and saliva were deposited into sterile collection cups and 50 µL (semen, saliva) or 100 µL (urine) was applied to
cotton swabs or cloth. Blood was collected into a Vacutainer® containing EDTA (Beckton, Dickinson & Company, Franklin Lakes, NJ), inverted for 15 seconds, and 50 µL immediately applied to cotton swabs or cloth. All samples were allowed to dry for 24 hours, and swabs or stains cut and placed into 1.5 mL microcentrifuge tubes, and stored at -20ºC before and after subjection to treatment.

Samples undergoing irradiation were placed on a Bio-Rad 464BR ultraviolet transilluminator (Bio-Rad Laboratories, Hercules, CA) for 4 hours. Samples undergoing heat treatment were incubated at 55ºC or 95ºC for 0.5, 1, 2, 4, or 24 hours. For samples exposed to chemical conditions, 100 µL of 87 mM or 870 mM sodium hypochlorite (1:10 dilution and full-strength household bleach, respectively), glacial acetic acid, or household dishwashing detergent were applied to the samples. The microcentrifuge tubes containing the treated samples were left open to air dry for 72 hours. The samples were then stored at -20ºC until isolation.

RNA Isolation and Analysis

RNA isolation was conducted using the Qiagen miRNeasy mini kit (Qiagen N.V., Venlo, The Netherlands). Briefly, the entire swab or fabric cutting was placed in 700 µL of QIAzol lysis reagent, and incubated for 5 minutes at room temperature, vortexing every minute. The swab or cutting was then placed in a DNA IQ™ Spin Basket (Promega, Madison, WI, USA), and centrifuged at 13000xg for 3 minutes. The resultant lysate was then processed according to protocol and total RNA eluted in 30 µL of RNase-free water. RNA was quantified using the NanoDrop ND-2000 UV Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA).
Quantitative reverse transcription was carried out via the qScript™ microRNA Quantification System (Quanta Biosciences Inc., Gaithersburg, MD). Reverse transcription was carried out according to the manufacturer's protocol using 7 µL of RNA extract. qPCR reactions were prepared in triplicate for each sample using a modified protocol: 6.25 µL of PerfeCTa SYBR Green SuperMix (2X), 0.25 µL (2.5 µM) PerfeCTa microRNA Assay Primer (miR-16, 21, or 24), 2 µL of cDNA reaction, and 4 µL of nuclease-free water. Thermal cycling was conducted on the Life Technologies Prism 7300 instrument (Life Technologies, Foster City, CA) using the following parameters: 95ºC for 2 minutes, followed by 40 cycles of 95ºC for 5 seconds, 60ºC for 15 seconds, and 70ºC for 30 seconds (data collection). qPCR analysis was conducted using SDS software, v1.3.1 (Life Technologies).

All treatments were performed on a minimum of two treated samples, with 3 technical (qPCR) replicates for each treated sample, with the exception of the full-strength bleach, detergent, and acetic acid treatments, which had one biological sample with a minimum of 3 technical replicates.

Data Analysis

In order to determine relative changes in sample integrity, a dC_T was calculated in which the C_T of the treated sample was subtracted from the C_T of the untreated control of the same body fluid. Given that the literature regarding normalization miRs for body fluids is variable, and effect on integrity is as yet unknown, this is the most discriminating measure. Using identical RNA extraction, reverse transcription, and qPCR parameters provide a consistent comparison of miR quality from treated sample
to control. An unpaired two-tailed t–test was applied to determine significance between treated and untreated samples.

**Results & Discussion**

*RNA Isolations*

Total RNA yield was not affected by any treatment method (data not shown). This is consistent with a previous report by Setzer et al\textsuperscript{178}, in which mRNA stability in environmentally challenged samples was evaluated.

*Heat Treatment*

Blood, treated for any length of time at both 55 and 95°C, was highly resistant to degradation, with no failed reactions (Figures 7-1a & b). miR-16 levels are high in blood, which resulted in low C\textsubscript{T} values regardless of treatment. Saliva too was remarkably stable under heat treatment at 55°C (not tested at 95°C) and was always detectable. However, seminal fluid and urine showed themselves to be more susceptible to the degradative effects of heat treatment. Semen had a 92.8% failure rate (4 biological replicates, 14 technical replicates) at 30', 50% failure rate at 1 hour, and 0% failure rates at 2 and 24 hours. This pattern is seen in several instances of treatment, and is cause for deliberation, as it too, was seen in multiple biological and technical replicates. Urine, as a high-volume, low cell-content fluid, understandably has low levels of miRs to begin with (C\textsubscript{T} range of positive control miR-16 – 32.4 ± 1.4).

Thus, any degradation can quickly result in loss of signal. Thus, sample failure rates at 1 and 2 hours of 55°C were not unexpected. What was unexpected, however, were excellent amplification of miR-16 in urine at 24 hours at 55°C, and 100% sample
success at 1, 2, or 24 hours at 95°C. As these results are somewhat more variable than expected, several biological and technical replicates of the failed treatments were repeated; however, results were consistent and since native miR-16 levels are low in untreated urine, not statistically significant.
Figure 7-1: microRNA stability under heat conditions over time. Relative miR-16 levels as determined by the difference in C\textsubscript{T} from treated sample to control of the same body fluid.  **A**: 55°C Heat Treatment. Blood and saliva miR levels did not change significantly at 30 and 60 minutes, but differences were significantly higher at 2 and 24 hours (p<0.05). Urine and semen had significantly higher dC\textsubscript{T} levels and failed reactions. N≥2 biological and 6 technical replicates for each treatment.  **B**: 95°C Heat Treatment of blood and urine (semen and saliva not tested). Differences in urine were not statistically significant; however, all differences in blood were statistically significant (p<.005). No failed reactions. N=1 biological and minimum 3 technical replicates for each treatment.
Stability at 55°C over time

Stability at 95°C over time
Ultraviolet Treatment

All body fluids tested were highly resistant to ultraviolet treatment, yielding detectable miR levels. Blood and semen C_Ts were statistically higher than the control (p<.01), but still detectable with no reaction failures. (Figure 7-2A). As seen in heat treatment, saliva samples actually had a modest increased in miR levels based on C_T values, but the results were not statistically significant. While there is very little research in the area of UV damage to RNAs, and none for small RNAs, the primary mode of action for ultraviolet damage is the fusion of pyrimidine doublets, and ultraviolet exposure has been shown to impact RNA in pure extracted form^{179}. However, this is not relevant to the matrix of a dried body fluid sample; ultraviolet light has been shown to affect mRNA levels only after 90 and 180 days of exposure from mock forensic samples^{178}.

Chemical Treatment

Bleach treatment resulted in two widely variant results. While blood and urine were consistently resistant to both diluted and household strength bleach application, semen and saliva proved vulnerable, resulting in a majority of sample failures at 10% bleach treatment, and 100% failures at full-strength bleach application (Figure 7-2B). This is enigmatic, as application with glacial acetic acid (pH ~2.4) yielded minimal difference from the control for all body fluids (Figure 7-2C). Given the fact that RNA is more stable under slightly acidic conditions than DNA, it would be interesting to evaluate samples that had been exposed to similar conditions for DNA quantity and
Figure 7-2: microRNA stability under various conditions. Relative miR-16 levels as determined by the difference in C\textsubscript{T} from treated sample to control of the same body fluid. A: Ultraviolet exposure. miR levels were minimally affected by ultraviolet exposure. Only blood and semen showed statistically significant differences vs. control (p <0.01) N=2 biological and ≥5 technical replicates for each treatment. No failed reactions were observed. B: Bleach treatment. 10% bleach: Urine miR levels did not change significantly (p=0.263), and blood was significantly affected (p<0.001) but still readily detectable. Saliva and semen had mainly failed reactions N≥2 biological and 6 technical replicates for each body fluid treatment. Full-strength bleach: Urine was not significantly affected by whole bleach treatment (p=0.483), but blood, semen, and saliva were significantly affected (p<0.001). While blood miR-16 levels were affected by treatment, but still detectable, saliva and semen were degraded to the point that miR-16 was undetected. N=1 biological and 3 technical replicates for each body fluid treatment. C: Acid and Detergent treatment. Glacial Acetic Acid: All body fluids were detectable, with no significant deviation from positive control detection threshold. N=1 biological and 3 technical replicates for each body fluid treatment. Dishwashing detergent: Saliva and urine were readily detectable, with no significant deviation from positive control detection threshold. Blood too was easily detectable, but significantly affected (p=0.03). Semen had a 50% failure rate with dishwashing detergent application. N≥1 biological and 3 technical replicates for each body fluid treatment.
A) Ultraviolet Exposure

B) Bleach Treatment

C) Acid & Detergent Treatment
STR profile analysis. Again, saliva seemed to gain miR levels during treatment as compared to the control (difference not statistically significant).

Application of dish detergent was performed for multiple reasons. First, it is a canonical treatment for forensic samples, given the fact that crime scene cleanup by perpetrators are typically attempted using common and convenient household chemicals. Secondly, it stands to reason that because some secreted miRs are encapsulated within a microvesicle, which contains large amounts of cell membrane lipid components, or co-transported with HDL particles, stability could be affected by disrupting such particles and exposing the microRNAs. Interestingly, blood, urine, and saliva were minimally affected by detergent application, with saliva again yielding better amplification results than the control (Figure 7-2C). However, semen yielded only a 50% success rate (2 biological, 6 technical replicates).

*Overall miR stability (by body fluid, different miRs)*

Three candidate microRNAs were evaluated for stability purposes; miR-16, 21, and 24. These miRs are commonly detected in tissues and body fluids, were evaluated in all body fluids and found to be expressed at high levels. miR-16 was used for this study, but miR-21 and miR-24 were also evaluated in a number of treatment types, and were consistent with the results seen in miR-16 (data not shown). This indicates that degradation seen in this study is likely to be representative of the integrity levels of the miR population in the body fluids assessed. In an effort for consistency, miR-16 was used across all body fluids; however, highly expressed miRs in each body fluid could be
Figure 7-3: Overall Stability Parameters. Compiled average Ct data for all treatments, all replicates. As miR-16 was highly abundant in blood, even severe treatment resulted in detectable miR levels. As the abundance of the miR decreased (as determined by Ct), treatment resulted in more amplification failures in a 40-cycle amplification.
found and used as a better indicator, as they would likely result in less failed reactions in low-abundance body fluids such as urine.

**Conclusions**

This study sought to characterize the stability of microRNAs for forensic samples. Given the explosion in recent interest in microRNAs, it is important to understand the limitations, if any, to the forensic analysis of a new species of nucleic acid. Overall, the microRNAs assayed, as an indicator of the species as a whole, were remarkably stable, with detection in the majority of treatments (Figure 7-3). The microRNAs present in blood and saliva were both more abundant and robust, and were detectable throughout all treatments. Urine and semen were more susceptible to treatment, but were detectable in the majority of treatment scenarios. Larger volumes of each body fluid could have been used, but it was felt that keeping the volume small was more indicative of the sample sizes often seen in forensic evidence. Given that the miRs tested were very readily and consistently detectable in such small samples, it is highly possible that stability is even better than seen in these results, once more abundant and indicative miRs are identified for each body fluid. The results from this study underscore the need to comprehensively evaluate the miRnome in each forensically relevant body fluid. Other researchers have begun to do this through the use of microarray qRT-PCR, but there are still inconsistencies between research laboratories in regards to which miRs are diagnostic for the primary body fluids. The secondary, accessory body fluids have not been assayed (urine, feces, sweat) in most cases. Additionally, there is disagreement and confusion, not only in the forensic community, but also within the analytical and research community, regarding appropriate endogenous markers for
miRs in body fluids. Certainly once there is more standardization and better means of normalization, a better evaluation and identification of unique markers for each body fluid can be undertaken.
REFERENCES


25. Zhang X, Ladd A, Dragoescu E, Budd WT, Ware JL, Zehner ZE. MicroRNA-17-3p is a prostate tumor suppressor in vitro and in vivo, and is decreased in high grade prostate


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APPENDIX 1: Expression Analysis of modulated microRNAs

Table 1: Average \( C_T \) values of miR RT-qPCR selected for further analysis in prostate cell lines. Further processing included normalization of all CT values using RNU48, which reached the cycle threshold consistently around 19-20 in the RNA of all cell lines tested. Each value is the average of a minimum of one biological replicate with technical triplicates. Undet = undetected to 40 amplification cycles. *For the miR-299-5p M12 evaluation, RNU48 had an atypical amplification plot (\( C_T \) of ~24).

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Table 3: Relative miR expression of laser-captured microdissected tissues from 5 patients. miR-levels were normalized to RNU48, and expression level calculated relative to the benign tissue of the same patient. Undet = both benign and the tissue were undetected to 40 amplification cycles. Red shading indicates that the miR in question was undetected in the benign sample to 40 cycles, and green indicates that miR in question was undetected in the tissue type being tested. miR-133b was not examined due to inconsistent readings in the cell line analysis.

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VITA

Sarah Joy Seashols was born on February 24, 1978, in Pensacola, Florida, and is an American citizen. She graduated from Denbigh Baptist High School in Newport News, Virginia in 1996. She received her Bachelor of Science in Biology from The College of William & Mary in Williamsburg, Virginia in 2000. She received her Masters of Science in Criminal Justice, with a concentration in Forensic Science, from Virginia Commonwealth University in 2003, and subsequently worked as a forensic scientist for 4 years, followed by a faculty appointment in the Department of Forensic Science at Virginia Commonwealth University since 2007.

Publications

7. Peromyscus leucopus p450c17alpha (cyp17) gene, complete cds, & protein (NCBI cDNA & Protein submissions -- Accession Nos. AY054747 & AAL12229)