POST-TRANSCRIPTIONAL AND EPIGENETIC REGULATION OF MIEN1 IN PROSTATE CANCER

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ABBREVIATIONS

3'UTR	3' untranslated region
5-Aza-2'dC	5-Aza-2'deoxycytidine
Act-D	Actinomycin-D
ANOVA	Analysis of variance
Anti-DIG-AP	Anti-digoxigenin antibodies, conjugated to alkaline phosphatase
AUC	Area under the curve
BLAST	Basic Local Alignment Search Tool
BPH	Benign prostatic hyperplasia or hypertrophy
BSA	Bovine serum albumin
cDNA	complementary DNA
DNA	Deoxyribonuceic acid
DNMT	DNA methyltransferase
DRE	Digital rectal examination
EMT	Epitelial-to-mesenchymal transition
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GGTase-I	Geranylgeranyl transferase I
GS	Gleason Score
H&E	Hematoxylin and eosin

HER2/neu	human epidermal growth factor receptor 2
IgG	Immunoglobulin G
ITAM	Immureceptor tyrosine-based activation motif
LNA	Locked nucleic acid
MET	Mesencymal-to-epithelial transition
MIEN1	Migration and invasion enhancer 1
miR	miRNA or MicroRNA
miR-940	hsa-miR-940
MMP-9	Matrix metallopeptidase 9
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NBT-BCIP	Nitro-blue tetrazolium with 5-bromo-4-chloro-3'-indolyphosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PBS	Phosphate-buffered saline
PCN	Procainamide
PIN	Prostatic intra-epithelial neoplasia
PSA	Prostate specific antigen
qPCR	Real time polymerase chain reaction
qRT-PCR	Real-time reverse-transcription PCR
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
SINE	Short interspersed nuclear element
siRNA	Small interfering RNA

SSC	Saline-sodium citrate
TNM	Tumor-nodal-metastasis
tPSA	Total PSA
TURP	Transurethral resection of the prostate
U6snRNA, RNU6-2	Small nuclear RNA component of U6 small nuclear ribonucleoprotein
uPA	Urokinase-type plasminogen activator
USF	Upstream stimulatory factor
VEGF	Vascular endothelial growth factor

CONTRIBUTIONS OF THE AUTHORS

Chapter II

SR, SD and JKV conceptualized the study. SR designed, standardized and performed the assays and statistical analysis. AVP and RJH acquired patient specimens and scored the H&E, *in situ* hybridization and immunohistochemistry staining. SR and RKR performed cell line authentications. SR and JKV interpreted the data.

Chapter III

SR and JKV conceptualized the study and interpreted the results. SR designed, standardized and performed the assays and statistical analysis. SM extracted exosomes from cell culture supernatant. CO assisted with some qPCR. AH, AVH and YL collected patient serum and the associated clinicopathological variables and provided useful inputs from a clinical perspective.

Chapter IV

SR and JKV conceptualized the study and interpreted the results. SR designed, standardized, and performed the assays and statistical analysis. TVT helped with RNA isolations and luciferase assays.

CHAPTER I

INTRODUCTION

Cancer

In simple layperson terms, cancer is just an uncontrolled and abnormal growth of cells. Though this superficially seems to be a simple issue, one that our immune system should more than easily be able to tackle, in reality it is not. Cancer, or malignancy, could lead to interruption or alteration of the regular physiological processes. The tumor could also grow and destroy healthy tissues at the primary site or in regions further away. This process of the tumor spreading is called metastasis and most of the cancer related deaths are due to the spread rather than a benign tumor, which is usually confined within a well-defined area, like the primary site of origin.

Cancer is a leading cause of death, second only to cardiovascular diseases in the United States for about two decades [1]. The yearly statistics on cancer incidence, mortality and survival, collected and compiled by various organizations, including the American Cancer Society, National Cancer Institute, the Centers for Disease Control and Prevention, the North American Association of Central Cancer Registries and the National Center for Health Statistics, all project a total of 1,665,540 new cancer cases and 585,720 cancer deaths to occur in the United States in 2014. Though the cancer related deaths in the past two decades has dropped by about 20%, a reflection of advances made in cancer control and prevention with enhancing awareness as well as establishing better screening techniques for some cancers, the overall number of lives lost are still high; providing a need for research in the field.

Molecular Biology of Cancer Progression

There is increasing evidence that the process of tumor initiation is the ability of the tumor cell to accumulate genetic instabilities (due to various environmental and/or intrinsic factors) over time or rapidly, depending on the source/stimulus for the instability [2]. Apart from this, for tumor progression, the cells escape senescence and attain immortality, gain independence from growth factors for signaling and proliferation, undergo deregulated cell cycle control mechanisms, achieve anchorage independent growth abilities with altered cell adhesion mechanisms and facilitate neo-angiogenesis through a cascade of intra- and inter-cellular signaling events [3-8]. In short, cancer cells are known to acquire unique characteristics like diminished requirement of growth factors, loss of contact, and apoptosis evasion, apart from gaining the ability to invade and migrate to other regions. These are the results of various alterations occurring in the normal cells, which finally result in inappropriate gene expression, in turn leading to aberrant activation/inactivation of signaling pathways involved in normal cellular homeostasis maintenance; together facilitating cancer progression.

Molecular Biology of Cancer Metastasis (Figure 1)

Cancer progression eventually leads to the metastasis of cancer. Metastasis is a process of spreading of cancer from its primary site of origin to the other parts of the body through the circulatory and/or the lymphatic system. The favored sites of metastasis are usually dependent on the primary tumor site. Though many studies and current therapeutic approaches have succeeded in treating localized tumors, targeting and preventing the metastatic spread is still a challenging task; making metastasis the major cause of death in many cancers.

Metastasis is multi-step process involving various independent events and the accompanying signal transduction cascades, including evasion of cell death by immune cells and

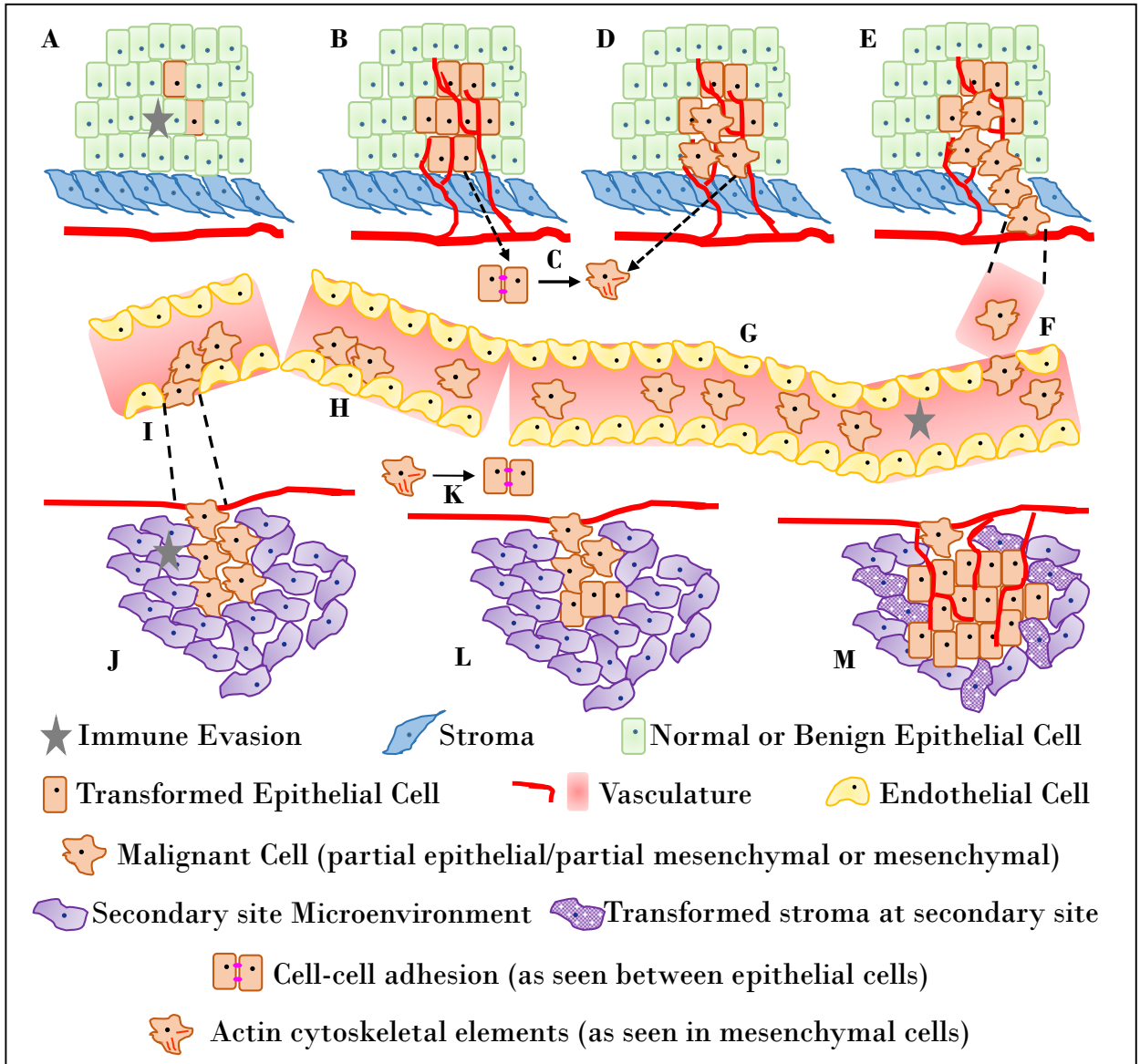

Figure 1: Cancer Progression and Metastasis

(A) Cellular transformation and immune evasion; (B) Angiogenesis and tumor cell proliferation; (C) EMT; (D) Beginning of cellular mobilization; (E) Invasion of matrix; (F) Intravasation (into circulation); (G) Immune evasion and migration; (H) Adherence to endothelial cells of vessel at secondary site; (I) Extravasation (out of circulatory system); (J) Homing in secondary site after immune evasion; (K) MET; (L) Micrometastatic nodule formation at secondary site; (M) Proliferation, angiogenesis and remodeling of the matrix in the secondary site

intracellular signaling at the primary site, during circulation and at the secondary site. The growth of the primary tumor, which precedes metastasis, is a result of both proliferation and angiogenesis. The cells re-structure themselves (morphological rearrangement; usually epithelial to mesenchymal) in order to detach from the colony and/or the extracellular matrix and invade the connective tissue. This process, called intravasation, is succeeded by the circulation of the tumor cells within the vasculature. Once the cells reach a potential secondary site, they bind to the endothelium and extravasate. Finally, the cells home (also altering their morphology again) in the secondary site, establishing the microenvironment to proliferate and form the secondary tumors, or metastatic nodules. Any cellular motility throughout the process of metastasis is referred to as migration. Thus, these steps form the hallmarks of cancer progression and metastasis [9-13].

The plasticity of tumor cells enable them to remodel their cytoskeletal structure to facilitate the process of metastasis [14]. The process of epithelial to mesenchymal transition (EMT) is the initial step during metastatic dissemination of cells. The tumor cells which are of epithelial morphology at the primary site, morph to gain characteristics that allow easy movement. The various steps include the loss of cell-cell contact followed by cell polarity, repression of epithelial genes, actin reorganization, secretion of proteins that aid extracellular matrix cleavage, and expression of mesenchymal genes. Once the mesenchymal phenotype is achieved, the cells are attributed to become more motile. Upon reaching the secondary site, the cells possibly undergo the reverse process, mesenchymal to epithelial transition (MET) in order to form the colonies [15]. MET, though a lesser studied process, is believed to be inverse of the EMT process, and equally vital for the metastatic progression.

Invasion and migration are key events in cancer metastasis [16]. Conventionally, the cells were postulated to invade and migrate individually, an amoeboid motion which would necessarily

involve mesenchymal morphology. But recent advances clearly show that these processes are more complex and dependent on the tumor type and microenvironment. While it is believed that the lower differentiated stages favor specific migratory patterns, it is also seen that some tumors exhibit both the patterns simultaneously [17]. It is essential to recognize that though many cells may be primed and possess the potential to undergo migration and invasion, only a certain set succeed in physically moving; this could be attributed to the signaling alteration in those cells. Additionally, interaction between the tumor and microenvironment (initiated by the tumor and/or the environment), to create a path for the motion of the tumor cells, includes the processes of neovascularization/angiogenesis and disintegration of the matrix/substratum. This occurs through the secretion of angiogenic factors and proteases respectively [18, 19].

In summary, metastasis is a complex cascade. Though many approaches have been targeted against specific events, like blocking proliferation, inhibiting blood vessel formation, preventing cell polarization, impeding morphological changes, or simply targeting specific proteins involved in the progression of cancer [20-27], the success has been minimal to negligible in the clinics. This implies that a deeper understanding of these processes is still required in order to identify more efficacious targets. Also, since the success has been better with targeting localized primary tumors [1], it is of utmost importance to increase awareness and enhance screening tools and techniques to diagnose cancers at early stages, thus improving the prognosis.

Prostate Cancer

Statistics, Risk Factors and Diversities, Signs and Symptoms

Prostate cancer is the second leading cause of cancer related deaths among American men, next only to lung and bronchial cancers, as reported by the American Cancer Society [1]. While it

is estimated that 1 in 7 men will be diagnosed with prostate cancer during their lifetime, this accounts for 27% of the new cases of incidence among men, making it the leading cancer in men. According to the American Cancer Society and National Cancer Institute estimations for 2014, the gross number of new cases of prostate cancer that will be diagnosed is 233,000 and 29,480 men will die of the disease [1]. The recent reports on the relative five-year survival for prostate cancer indicate that while almost all the local and regional cancer patients have excellent survival rates, those men that have the distant metastatic form of cancer have only a 28% survival chance [28].

The high incidence and prevalence of prostate cancer has been associated with various factors like age, family history, race and ethnicity and environmental exposures, apart from inherited genetic conditions [1, 28, 29] as reported by the prostate cancer foundation, apart from other organizations. Of these, age is the most well-established risk factor. The older the person, higher is the risk of prostate cancer; about 97% of men diagnosed with prostate cancer are over 50 years, with ~60% being over 65 years, according to prostate cancer foundation. Based on race [30], African American men not only have the highest incidence rates but are also about 2.5 times more likely to die of prostate cancer than Caucasian men. Though the reasons for this disparity is not yet clearly understood or well supported by scientific research, it is conceived that the reasons may include genetic factors and socioeconomic status (including access to care, lifestyle and nutritional habits) [28]. A third contributing risk factor that has evolved in the last decade is the relation of prostate cancer incidence to a family history of the disease [31-33]. Men who have had one or more relatives diagnosed with prostate cancer are at a higher risk for prostate cancer. This risk varies anywhere from 1.5 times to 5 times higher depending on the number of relatives diagnosed, age at diagnosis and closeness to the diagnosed relative. Finally, all cancers have some degree of genetic predisposition. In most cases, this is supported by additional events leading to the

development of cancer. In prostate cancer, though there is no evidence of any single gene mutation being a risk factor, various gene mutations, including but not limited to HPC1, HPC2, ELAC2, RNASEL, LEPR, Cytochrome 1, IL-4, ARVCF, AR, BRCA1 and BRCA2, seem to be involved [34, 35]. Though there is no strong evidence correlating the existence of these mutations to incidence of prostate cancer, there definitely is reason to believe that some of these genes are strong predictors of aggressiveness and mortality [36, 37].

Distinguishing aggressive prostate cancer from the indolent form has gained importance in the recent past and more studies are being conducted to obtain variances in risk factors between the two forms [38-41]. Apart from gene mutations, high body mass index, smoking, bad dietary habits, African American race and occupational exposures are the other factors associated with the aggressive prostate cancer. Determining these differences has become key to treating prostate cancer patients since more often than not, just active surveillance is sufficient for indolent tumors while more radical approaches may be needed when encountering aggressive prostate cancer. At an early stage, no specific symptoms are presented, making early detection highly challenging. But with the progression of the disease, the men experience discomfort and pain, trouble during urination and blood in the urine, and if there has been metastasis, severe pain in the various bones including the hips, chest and back.

Anatomy of the prostate and Prostatic conditions

The normal prostate is a small organ in the shape of a walnut, located in the pelvis, inferior to the bladder and surrounding the urethra. In order to understand the pathology of prostate cancer, it is essential to appreciate the anatomy and physiology of the normal prostate. An adult prostatic parenchyma can be divided in four major zones, namely, the peripheral, central, transitional and anterior fibromuscular regions [42]. The peripheral zone (encompassing posterior and lateral parts

of the prostate) consisting of 70% of the glandular tissue, is most susceptible to cancer and inflammation; while the central zone comprising of 25% of the glandular tissue, is most resistant. The transitional zone, surrounding the urethra with several tiny periurethral ducts, has 5% glandular tissue and is the region where benign prostatic hyperplasia (BPH) or nodular hyperplasia originates. During the later stages of BPH, large discrete nodules which further constrict the urethra, are pathologically observed. The anterior fibromuscular zone, containing a thick and non-glandular region, is the anterior of the prostate.

BPH is a disorder (benign condition, not carcinoma) [43-45] characterized by the enlargement of prostatic glands exclusively in the transitional zones. It is common in many older men and there is no concrete evidence associating BPH to a precancerous stage. It is accepted that BPH is a condition that occurs as a result of hormonal imbalance; excessive conversion of testosterone to dihydrotestosterone, resulting in cell proliferation. Depending on the extent of discomfort and pain, BPH is either treated (non-invasive: α blockers, 5α-reductase inhibitors; minimally invasive: interstitial laser coagulation, microwave hyperthermia, transurethral needle ablation; surgical: transurethral incision of the prostate, transurethral resection of the prostate, simple prostatectomy, vaporize the prostate tissue) or actively monitored [46-50]. Another important prostatic condition which has no correlation to increased risk of prostate cancer is known as prostatitis [44, 45]. Prostatitis is an infection of the prostate which usually results in inflammation and elevated prostate specific antigen (PSA), without necessarily enlarging the prostate. The treatments options are the use of antiobiotics and/or surgery [51, 52].

Pathology of prostate cancer

The precursor of prostatic adenocarcinoma is prostatic intraepithelial neoplasia (PIN) [53-55]. PIN, characterized by dysplasia in the epithelial lining of prostate with proliferating ducts,

ductules and acini, was first introduced as a stage between hyperplasia and carcinoma. PIN is further categorized into low grade (crowded and irregularly spaced epithelial cells with pleomorphic nuclei and small nucleoli) and high grade (more crowded and heaped up epithelial cells with prominent nucleoli). While low grade PIN is very unlikely to progress to form cancer, patients exhibiting high grade PIN have a much higher risk of developing cancer in the future. The major distinction between high grade PIN and invasive carcinoma is the disruption of the basal and association of this disruption with the PIN foci. Although PIN is a precancerous stage, the PSA levels are relatively normal; thus making their detection possible only histologically. Usually, PIN is treated with selective estrogen receptor modulators, anti-androgens, angiogenic inhibitors or chemotherapy, along with increased surveillance [56].

Over 90% of the clinically diagnosed prostate cancers are acinar adenocarcinomas arising from the peripheral zone of the prostate. Enlarged hyperchromatic nucleus with prominent nucleolus, and mitotic figures are the cytological features of prostatic adenocarcinoma. The adenocarcinoma is classified based on stages and grades of the disease [57-62]. Staging a prostatic adenocarcinoma is of extreme clinical importance since this assists the physician in determining the treatment as well as estimating the prognosis. A widely accepted and used staging system is the tumor, node and metastasis (TNM) staging. Based on TNM status, as well as Gleason scores and PSA, the tumor is grouped into four broad stages. The cancer starts as a primary localized tumor in the prostate in Stage I. It cannot be identified by digital rectal exam (DRE) and is only microscopic. In Stage II, the tumor grows within the prostate but does not extend beyond the gland and by Stage III, it starts to spread to the nearby tissues like the seminal vesicles. Stage IV is the final stage in the progression and the cancer metastasizes to the farther organs and tissues. The most common metastatic sites in prostate cancer are the bone followed by the brain, lungs and

lymph. The grading of prostate cancer based on the Gleason scores (2 to 10) of the histological sections relies on the most predominant pattern and the second most predominant pattern as observed by the pathologist. The two grades, 1 to 5 each, are then added to provide the Gleason score for the tumor. In general, grades 1 and 2 look very much like a normal prostate tissue and are often not classified as tumor; grade 3 exhibits single, separate, variable glands that infiltrate the benign glands, forming a cribriform pattern; grade 4 show larger nodules of cribriform and fused glands; and grade 5 appears to be sheets of tumor cells with extremely minimal differentiation or carcinomas with central necrosis. Overall, Gleason scores ≤ 6 are well differentiated (closer to normal), less likely to spread, slow growing or less aggressive tumors with little clinical significance; a score of 7 depicts moderately differentiated tumors; and 8 to 10 are poorly differentiated, aggressive tumors which may grow and spread rapidly. An additional consideration from a molecular perspective, for prostate cancer progression, is the switch from androgen (hormone) dependency to androgen independency status.

Current Diagnosis and Treatment Strategies

Developing awareness among the population and recommending the men at and above 50 years of age to undergo DREs is the epidemiological approach to detect cancer. In addition to DRE, total PSA (tPSA) blood test is also used as a diagnostic test [63-69]. If an irregular DRE (lump or hardening of prostate) and/or elevated tPSA are detected, with or without demonstration of other symptoms or risk factors, it is the physician's call on whether a transrectal ultrasound should be performed to obtain the size and density of prostate [63, 70-72]. Usually a biopsy is also performed simultaneously to examine the tissue microscopically. Presence of denser masses and Gleason scores ≥ 6 are often strong indicators of cancer. On the contrary, if the biopsy is negative, a repeat biopsy is suggested when PSA increases by about 25%.

In many cases, though just an active surveillance of the prostate cancer patients is rational (primarily depending on the age at diagnosis), in others, a combination of surgical procedures (radical prostatectomy**,** transurethral resection of the prostate (TURP), orchidectomy, percutaneous nephrostomy, robotic-assisted prostatectomy), radiotherapy (external beam, brachytherapy, or palliative), chemotherapy (Docetaxel, Cabazitaxel, Mitoxantrone, Estramustine, Doxorubicin, Etoposide, Vinblastine, Paclitaxel, Carboplatin, Vinorelbine), hormone therapies (luteinizing hormone-releasing hormone analogs and antagonists, anti-androgens, steroids) and biological therapies like cryosurgery and vaporization are used, to treat the localized tumor [73-82]. The treatment options for metastasized disease are more limited and include pain management along with some of the aforementioned therapies, personalized vaccine therapies (Provenge) and/or slowing osteoclastogenesis (Bisphosphonates, Denosumab) if metastasized to the bone [83-89].

Clinical Challenges in Diagnosis

Though PSA and DRE have served very well in the past as diagnostic tools for prostate cancer, a recent report by the U.S. Preventive Services Task Force stated "the potential benefits of the tPSA based screening does not necessarily outweigh the harms caused, especially the complications and the side effects of the repeated needle biopsies upon irregular DRE and tPSA levels" in asymptotic men [82, 90]. Additionally, many people have inherently high levels of tPSA and an enlarged prostate, resulting in extensive over-diagnosis and treatments causing not only psychological stress (fear of having cancer, a false positive) but also secondary infections related to biopsies. Also, the tPSA test lacks robustness because of its poor specificity and questionable overall survival benefit. However, though it is conceived that higher PSA values indicate higher risk of prostate cancer, the cancer can also be found at low levels of PSA and hence those cancers

that are aggressive could be missed in the screening process. The more recent PCA3 test [91-94], which measures PCA3 levels in the urine after a DRE, has led to an improved accuracy in diagnosing prostate cancer at the cost of a decrease in sensitivity and specificity compared to PSA. This test also fails to predict the requirement of repeat needle biopsies, the fate of the disease progression or the short-term or long-term outcomes (including distinction between indolent and aggressive disease). Thus, there is a critical need for identifying new biomarkers which will have an improved diagnostic and predictive value, in combination with the existing tests, for detection and monitoring the progression of prostate cancer.

Novel candidate biomarkers for prostate cancer

Advancements in the fields of genomics and proteomics through improved methodologies like deep sequencing, microarrays, immunostaining and high throughput assays, have paved the way for biomarker discovery [95, 96]. Many potential biomarkers that have been identified include, but are not limited to, a-Methylacyl Coenzyme A Racemase, Chromogranin A, Prostate-specific Membrane Antigen, B7-H3, Sarcosine, Caveolin-1, TMPRSS2-ERG gene fusion rearrangement, DAB2 interacting protein, and DNA, RNA, miRNA and metabolites secreted in urine after DRE [97]. Given that none of these aforementioned markers have yet reached the clinics, it seems that they are either still in validation stages or failed clinical validation. Even if these molecules do not make it to the clinics as biomarkers in future, the studies would unequivocally increase our knowledge of prostate cancer biology, and hence provide insights into development of better targeting strategies [98]. Taken together, there is still a need for identification and development of biomarkers for both early detection as well as differentiation of indolent from aggressive prostate cancer.

Gene Regulation

Gene regulation, an integral process in maintenance of disease-free state, is carried out at multiple interrelated and yet broadly distinct levels, in order to maintain homeostasis [99-101]. Altered gene regulatory patterns are involved in a broad spectrum of outcomes and could even potentially hold a diagnostic and/or therapeutic value, when properly understood. A classic example of this is the association of genetic risk factors with certain cancers. Understanding the mutations, amplifications, or loss or gain-of function of specific genes not only assist in cancer diagnosis and treatment but also serve as predictors of incidence, responses to therapy, and overall patient survival. In order to better understand the importance of gene regulation it is vital to appreciate the complexity of the processes that usually play a major role in one or many levels of regulation, thus keeping a check on the biological pathways and the ultimate fate of the cell, tissue and organ.

Mechanisms and their Overall Significance in Maintaining Cancer-free state

From a global perspective, the read outs of alterations in nucleic acids and/or proteins will determine the degree by which the cells/tissues are deviant from the normal state. The disrupted gene regulatory mechanisms include variations at the chromosomal level (DNA mutations, copy number variations, chromosomal translocations), transcriptional level (promoter accessibility, transcription factor binding), post-transcriptional level (RNA stability, alternate splicing), translational level (protein isoforms, protein-protein, protein-RNA and protein-DNA interactions), post-translational level (protein modifications, stability, localization), and epigenetic level (histone and DNA modifications) to name a few. Apart from these, the environmental factors (exosomal/endosomal shuttling from adjacent cells and tissues, autocrine and paracrine signaling, physical and mechanical stimulus) can also attribute to exhibition of gene regulation [102-107].

Together, it is sufficient to state that though these regulations may seem unrelated and independent (even inherited or just a circumstantial response), there is elaborate cross talk between them; more often, trying to bring the cell back to "normalcy". Hence, the cascade ensued, right from the initial point of variation, is critical in deciding the state of the cell - disease or normal.

Among the various events that are known to control gene expression, protein localization, stabilization/destabilization and interactions, have been widely used in the field of medicine, for both diagnosis and treatment. In the recent past, with the advent of whole genome analysis, global epidemiological studies and discovery of new classes of biological molecules, the possibilities of exploiting the post-transcriptional (microRNAs) and epigenetic (DNA methylation) regulations in the fields of prediction and/or detection of disease, along with provision of therapeutic interventions, have broadened.

Post-transcriptional Regulation: MicroRNA (Figure 2)

Studies conducted over the last two decades have established a new role for a class of non-coding RNAs called microRNAs (miRNAs) in the regulation of genes at the post-transcriptional level. Though the very first report of the involvement of a small RNA (22 and 61 nucleotides long) in regulating an mRNA by binding to its 3' untranslated region (3'UTR) was published in 1993, the term microRNA was only coined in 2001, when expression of multiple tiny RNAs were reported. The seminal studies conducted in the early 2000s demonstrated not only the presence but also the ability of the miRNAs to regulate the translation and/or stability of mRNAs [108-112]. Ever since, extensive genome-wide and targeted studies have been conducted to not only determine the conservation and mechanism of biogenesis of new miRNAs but also validate their roles in basic biology as well as medicine.

The endogenous miRNAs regulate gene expression by transcriptional repression or degradation of target mRNA based on the extent of complementarity with the untranslated regions (UTRs) and cellular context. The miRNAs are processed, after transcription, through the class II riboendonuclease III enzymes, Drosha and Dicer (with their binding partners, DGCR8 and TRBP, respectively) and the nuclear export protein, Exportin 5 to finally attain their mature form of 18 to 24 nucleotides [113-118]. Based on the proximity of one miRNA to another, many miRNAs could also be transcribed from the same pri-miRNA transcript [119]. Studies reveal that the only prerequisite for a miRNA-mRNA interaction is the absolute complementarity of the seed sequence of the miRNA (1 to 7 or 2 to 8 nucleotides from the 5' end of the miRNA sequence) to the mRNA. A recent study revealed that the miRNA targeting may be classified as non-canonical (lesser target downregulation) and canonical (higher target downregulation); which in turn is dependent on low or high GC content in the seed sequence, respectively [120]. Given that the miRNA sequence does not have to be a 100% complementary to the target mRNA, one miRNA could regulate multiple transcripts and many miRNAs could regulate one transcript. This has also led to the concept of competing endogenous mRNAs (ceRNAs) as miRNA sequesters rather than targets [121]. Although miRNAs were initially associated with the developmental genes, their importance in other aspects of cellular physiology and pathology have been identified over the years.

Studies demonstrating the involvement of miRNA in cancer were conducted not long after their initial discovery [122]. The role of miRNAs in oncogenesis and tumor suppression, either by down-regulating pro-apoptotic or anti-apoptotic genes, led to their classification as oncomiRs or tumor suppressor miRNAs [113, 123-126]. MicroRNA profiling has shown their extensive deregulations in various cancers and the possibility of using these deregulated miRNA signatures in detection of primary tumors in cancers of unknown primary [124, 125, 127-142]. Among all

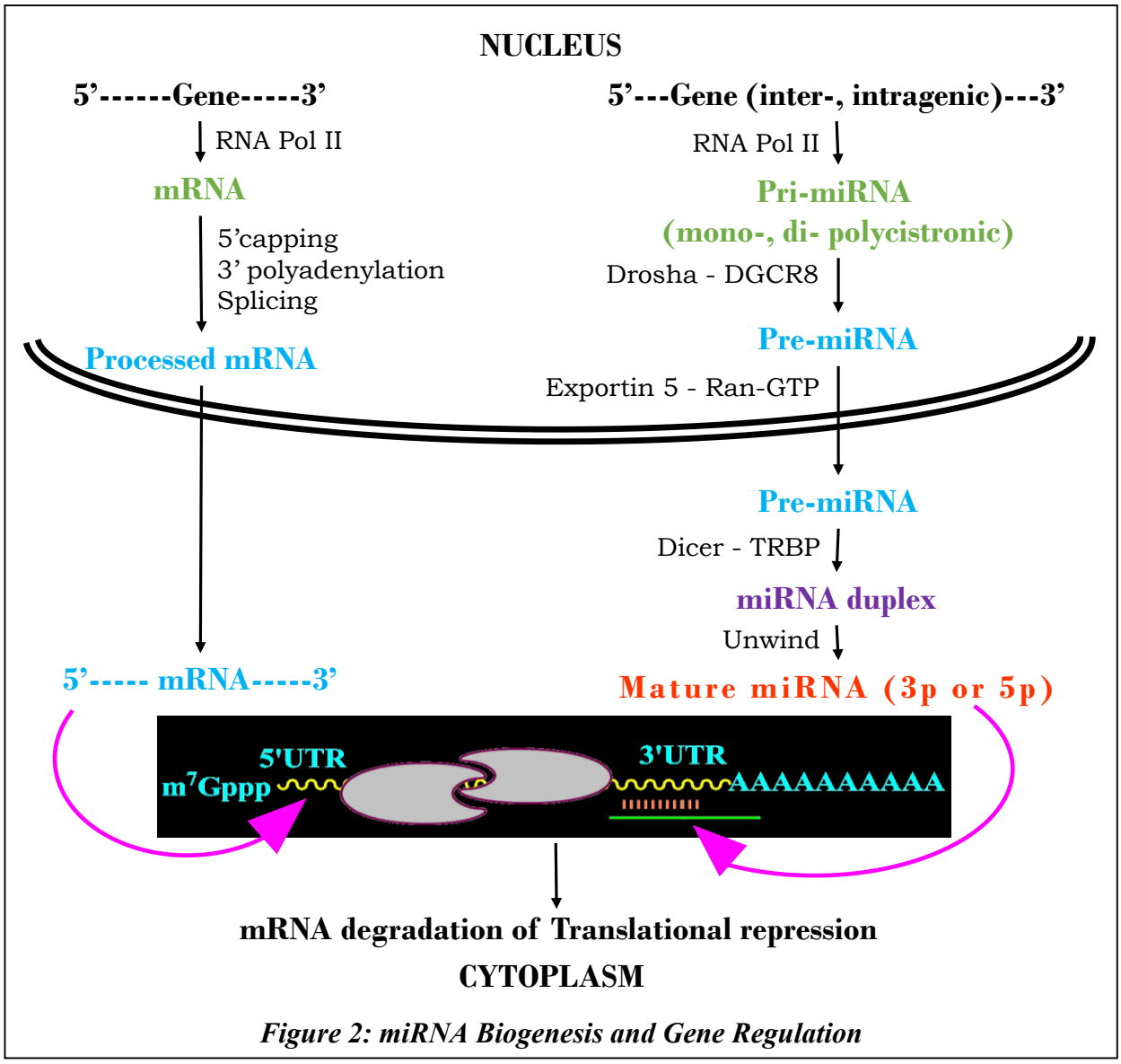

Figure 2: miRNA Biogenesis and Gene Regulation

cancers, prostate cancer has highly deregulated microRNA expression profiles. Literature review shows that microRNAs could be very useful targets or therapeutic agents depending on their endogenous role in tumor suppression or tumorigenesis. Hence, identifying the particular miRNA(s) regulating a gene differentially between normal and cancerous cells will provide a new dimension in treatment strategy development. Also, a clinical correlation between the miRNA(s) identified and the grade and stage of cancer may provide better patient prognosis. Additionally, recent studies have revealed that miRNAs could be secreted out of the cells under various conditions, in free form or in exosomes or bound to other lipids/proteins. These are also more stable than other nucleic acids in the serum. Alterations (either increase or decrease, depending on their status in normal individuals) in circulating miRNAs have a high potential to be used as serum based, non-invasive biomarkers for cancer. Once validated, such detection tests will have tremendous power in prostate cancer diagnosis, subsequently limiting the need for biopsies and hence the associated complications. Also, such tests may enable clinicians to detect and start treating certain potentially aggressive cancers they would have otherwise missed based on biopsies (because biopsies are only samples taken from specific areas of the prostate), at an earlier stage; thus preventing metastasis, the major cause of prostate cancer related deaths.

Epigenetic Regulation: DNA Methylation (Figure 3)

Epigenetic regulations are heritable alterations that occur independent of changes in the genetic material (specifically DNA sequences). It is well known that every cell in the body contains the exact same genetic material, yet only a particular set of genes are "turned-on" at any given time and/or in any specific cell. DNA methylation is a common epigenetic regulatory mechanism that controls such gene expression patterns, thereby keeping the other genes in a "turned-off" or repressed state.

DNA methylation was first discovered as a regulatory tool in 1979. Over the following two decades, this concept was better understood with the use of methylation inhibitors 5-azacytidine, and its deoxy version, 5-aza-2′-deoxycytidine [143-145]. DNA methylation is a process where in there is transfer of a methyl group, by DNA methyltransferases (DNMTs), onto the C5 position of the cytosine, to form 5-methylcytosine. The DNMTs are classified as de novo or maintenance DNMTs based on their role in methylation. While the de novo DNMTs are responsible for the initial transfer of the methyl group to a cytosine residue, the maintenance DNMTs ensure proper copying of this modification in the new strand after DNA replication. Usually, the altered cytosine residue is followed by a guanine (CpG), thus exhibiting two diagonally methylated residues on the double stranded DNA. It has been demonstrated that the CpG residues are distributed in a non-random fashion across the mammalian genome and about 70% of these CpGs are methylated; however, it has also been seen that these residues cluster (termed as CpG islands) in the promoter regions of some genes making these regions CpG-rich and hence inaccessible for transcriptional activation [146-151]. There are two mechanisms by which DNA methylation represses transcription; inhibition of DNA-binding proteins (transcription factors) to their respective responsive elements (DNA sequences) by methylating residues within the binding site, and binding of methyl-CpG proteins to methylated DNA in a non-sequence specific manner, thus competing with transcription factors or restructuring the DNA to the tightly-coiled “closed” chromatin structure. Depending on the region, one or both the mechanisms may ensue.

Given the critical role of DNA methylation in gene regulation, it is not inconceivable that errors in this mechanism would result in a diseased state. Cancer is one of the most common and highly studied consequences of erroneous DNA methylation [152]. Two general phenomena by which DNA methylation could be altered have been postulated [144]. Though more emphasis has

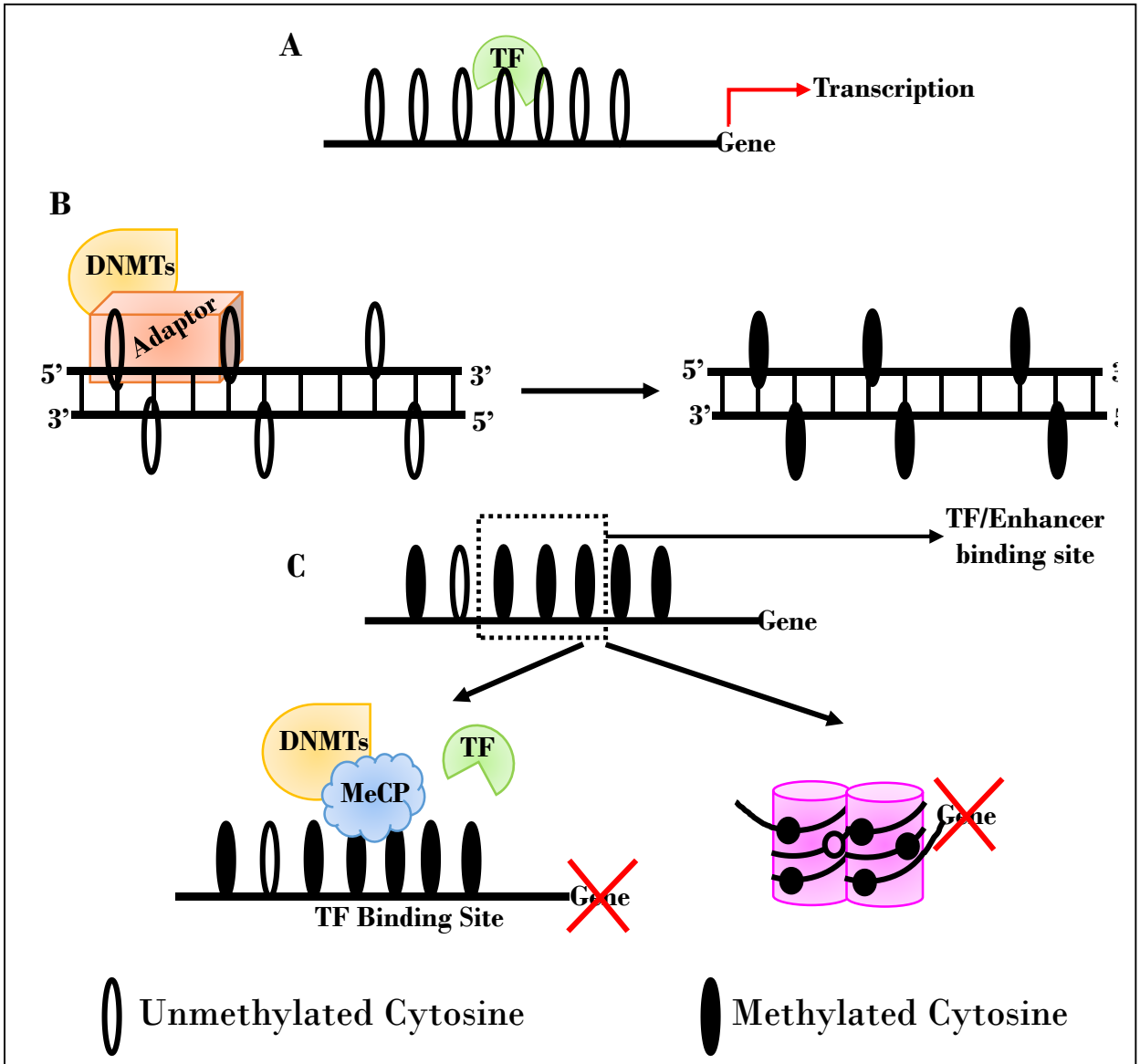

Figure 3: Gene regulation by DNA methylation

(A) Transcription of a gene with unmethylated promoter; (B) DNMTs methylate the cytosine residues with the help of certain scaffold proteins (DNMT3a and 3b during de novo methylation and DNMT1 during maintenance methylation); (C) Methylated promoter can result in transcriptional repression of the gene by one of the two methods: Inhibition of transcription factor binding (Left) or Facilitation of closed chromatin structure (Right)

been given to hypermethylation in cancer progression, recent genome wide studies show that hypomethylation holds as much importance in carcinogenesis [151, 153-158]. In general, global hypomethylation along with promoter specific hypermethylation has been observed in many cancers. The hypomethylation is associated with repetitive sequences and contributes to genomic instability and cellular transformation. On the other hand, the promoters that are hypermethylated usually correspond to tumor suppressor genes, thus leading to their repression in cancer. The other hypermethylated promoters studied are the ones involved in transcriptional activation of genes in the processes of DNA repair mechanisms, cell cycle progression, apoptosis, drug resistance and additional pathways which if active may prevent cancer progression and metastasis. Despite this general observation of methylation patterns in cancer, there is evidence supporting the hypomethylation of promoter regions of some genes, like proto-oncogenes and other genes that can promote tumor invasion and metastasis. Interestingly, in some cases, a more intricate repression of transcription is observed when DNA methylation promotes the neighboring histone deacetylation. In certain cancers like colorectal carcinomas, promoter hypermethylation could even serve as an early biomarker. In others, methylation abnormalities could act as drivers for progression [159-161]. Considering the growing importance of methylation patterns in cancer detection and treatment, extensive research has been and is being conducted to establish the methylome and epigenome for various cancers. Recent advances also implicate the role of DNA methylation in the regulation of noncoding RNAs such as microRNAs [162]. In light of identification of new genes and other regulatory elements in cancer progression, it is important to determine the alteration in methylation status of their promoters between the normal and cancer cells and tissues. Though global DNA methylation inhibitors may provide short-term anti-cancer

effects, their use and effectiveness in cancer therapy have to be considered with utmost caution, given the contradicting effects of methylation in cancer progression [153, 163, 164].

Gene of Interest: Migration and Invasion Enhancer 1 (MIEN1)

Migration and Invasion Enhancer gene 1 (MIEN1), alternately called C17orf37 or C35/MGC14832/ORB3/RDX12/XTP4, is a novel gene located next to Her-2/*neu* on the 17q12-21 region of the human chromosome in a tail-to-tail arrangement [165, 166]. Among other genes that are co-amplified with Her-2/*neu,* though MIEN1 shows the highest degree of positive correlation in amplification, MIEN1 is also independently expressed in metastatic prostate cancer in cases where Her-2 expression is lacking [166]. MIEN1 is expressed at higher levels in the various stages and grades of prostate cancer phenotypes, when compared to normal cells and tissues [165]. MIEN1 is also differentially expressed in breast cancer (undetectable in 38 different normal tissues except in the Leydig cells and is highly expressed when breast cancer metastasizes to the lungs and liver) and this predominant expression has led to MIEN1 being predicted as a novel breast cancer biomarker [166].

The 4 exons of the 776 nucleotide-long *MIEN1* transcribes into a 115 amino acid protein with a molecular weight of 12kDa. *MIEN1* is 505 nucleotides away from the 3' end of HER-2/*neu* oncogene in a tail-to-tail chromosomal rearrangement, on the negative strand. MIEN1 is the only known protein to possess both a prenylation and an immunoreceptor tyrosine-based activation motif (ITAM), in solid tumors [167, 168]. Covalent addition of a 15-carbon farnesyl or a 20-carbon geranylgeranyl isoprenoid to a conserved cysteine residue of a "CaaX" motif containing protein by the respective prenyltransferases is classified as prenylation of the protein. Apart from preferentially promoting membrane interactions, the modified proteins also play a role in

prenylation based protein-protein interactions [169]. The importance of prenylation is determined by the nature of prenyltransferase substrates; in cancer specifically, the proteins that are prenylated seem to play a role in growth, differentiation, actin cytoskeletal rearrangement, cell adhesion, vesicular trafficking and signal transduction, to name a few [170]. Though targeting prenylation using inhibitors was an attractive option to prevent cancer progression, the clinical trials on bisphosphonates and prenylation inhibitors have had limited success, in part, because of a lack of information on the complexity and the extent of molecular targets (proteins) that are impacted by it, and in part by poor patient selection, thus making this approach less prudent [171]. The ITAM domain, YxxL/Ix(6-8)YxxL/I, is a conserved sequence commonly seen in cytoplasmic tail of cell surface receptor proteins in the hematopoietic cells. Ligand binding immediately phosphorylates the receptors at the tyrosine residues in their ITAM domain, thus providing a binding site for Src homology 2 containing proteins [172]. The signal transduction cascade is dependent on the molecules associated with the phosphorylated ITAMs and together they regulate the migration and invasion of the cells. Additionally, ITAM containing receptors also facilitate osteoclastogenesis [173]. It is well known that prostate cancer mediates osteoclastogenesis via receptor activator of NF-κB ligand [174]. Since MIEN1 contains the ITAM domain, it is not far-fetched to believe that MIEN1 may be an adaptor molecule facilitating osteoclastogenesis in prostate cancer.

MIEN1 is localized to the inner leaflet of the plasma membrane after its prenylation, where it aids in filopodia formation, resulting in enhanced migration of the cells [167]. MIEN1 also promotes the migration and invasion of prostate cancer cells through up-regulation of Akt dependent NF-κB downstream target genes like uPA, MMP-9 and VEGF [165]. Using a xenograft mouse model, MIEN1 overexpression was shown to increase the metastatic potential of DU-145 cells [167]. Recent confirmation of the solution structure of MIEN1 predicts that Akt

phosphorylation via MIEN1 may be dependent on the active redox-like motif in the MIEN1 structure [175]. In breast cancer cells, MIEN1 is predicted to be an oncogene that promotes breast cancer progression by inducing EMT-mediated invasion and cellular transformation [166, 168]. The knockdown of MIEN1 induced apoptosis in breast cancer cells while its high expression is correlated to poor survival [176]. A more recent study in ovarian cancer implicated the role of MIEN1, along with ΔNp73, in ovarian cancer progression and cisplatin resistance [177].

Objectives of this study

Hypothesis and Specific Aims

Prostate Cancer is the second leading cause of cancer related death among men in the United States. Death is mostly the result of our current lacuna in diagnosing the disease at an earlier stage, thus leading to metastasis. The diagnostic tests using serum PSA and biopsies have their own disadvantages despite their current use in clinics. This has led to new biomarker discoveries, with limited success. MIEN1, a novel gene coded in the 17q12 region of the human chromosome, is known to be over-expressed in many cancers including prostate, with minimal to no expression in the normal cells and tissues, making it a plausible effective target. MIEN1 has been linked to migration and invasion, hence it is important in cancer progression (Figure 4). Despite having a clear understanding of the functional roles of MIEN1 in cancer, reasons for the aberrant expression between cancer and normal cells and tissues are still unknown. Understanding the mechanisms of the gene regulation and the regulators involved will be of importance in order to use them as potential therapeutic agent(s)/diagnostic markers for diagnosing and treating prostate cancer.

In this study, we have identified two molecular mechanisms that are deregulated with respect to MIEN1, leading to its aberrant overexpression in prostate cancer. Additionally, we have

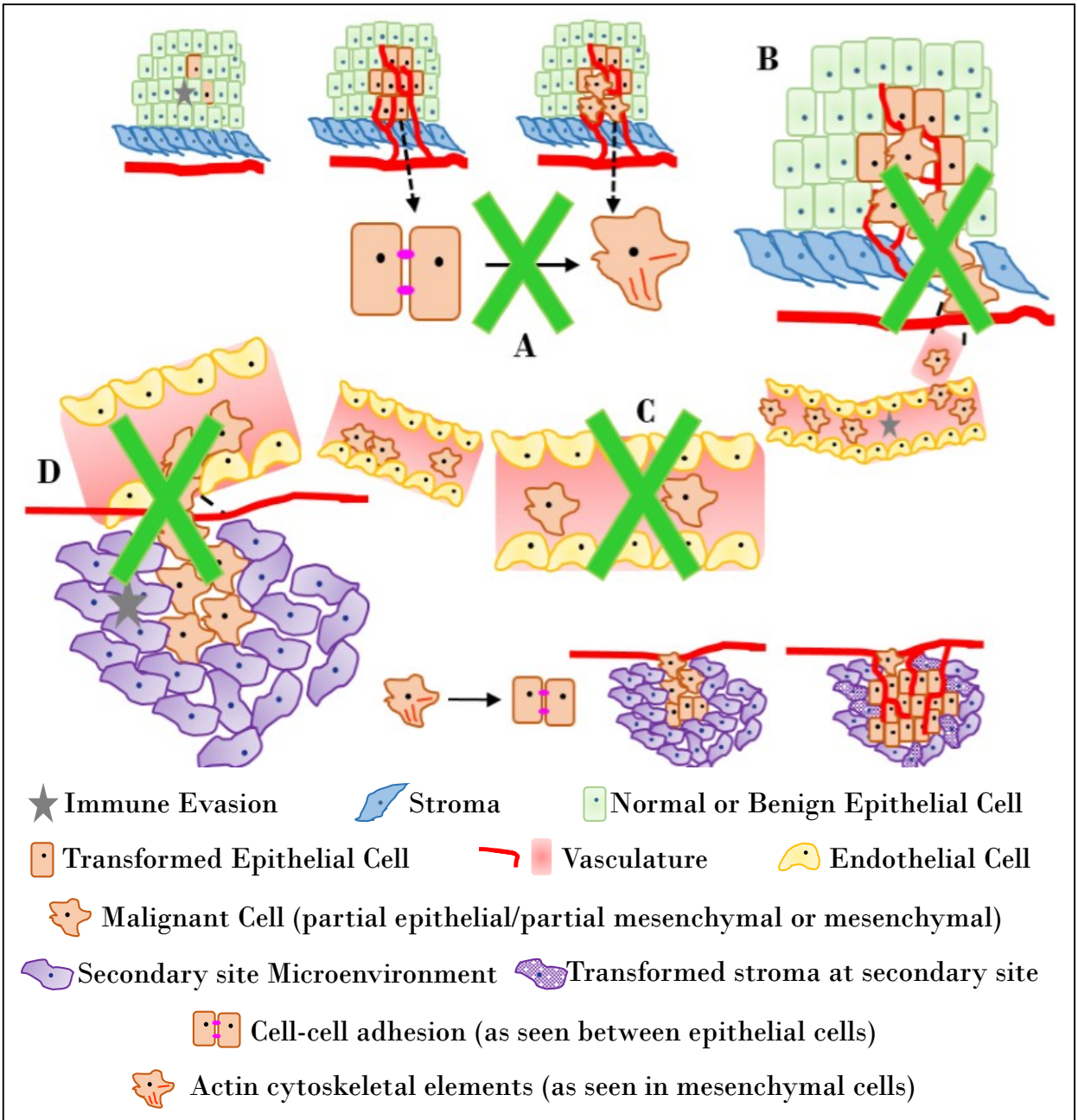

Figure 4: Effects of downregulating MIEN1 expression

(A) Inhibition of EMT; (B-D) Inhibition of Invasion and Migration; (C) Methylated promoter can result in transcriptional repression of the gene by one of the two methods: Inhibition of transcription factor binding (Left) or Facilitation of closed chromatin structure (Right)

attempted to validate one of the regulators as a non-invasive biomarker for prostate cancer. The **central hypothesis** is that ***the increased expression of MIEN1 in prostate cancer is the result of lower miR-940 levels and DNA hypomethylation in MIEN1 promoter in the cells and the secreted miR-940 in the serum by the cancer cells could be a diagnostic indicator of prostate cancer.*** Based on this, we have three specific aims (Figure 5):

Specific Aim 1: MicroRNA-940 suppresses prostate cancer migration and invasion by regulating MIEN1

Hypothesis: MIEN1 is under post-transcriptional regulation by miR-940.

Specific Aim 2: MicroRNA-940 in serum could be a potential biomarker for prostate cancer detection

Hypothesis: Secretion of miR-940 is higher by cancer cells than normal cells.

Specific Aim 3: DNA hypomethylation of MIEN1 promoter leads to transcriptional de-repression of MIEN1 in prostate cancer

Hypothesis: MIEN1 is transcriptionally repressed by DNA methylation.

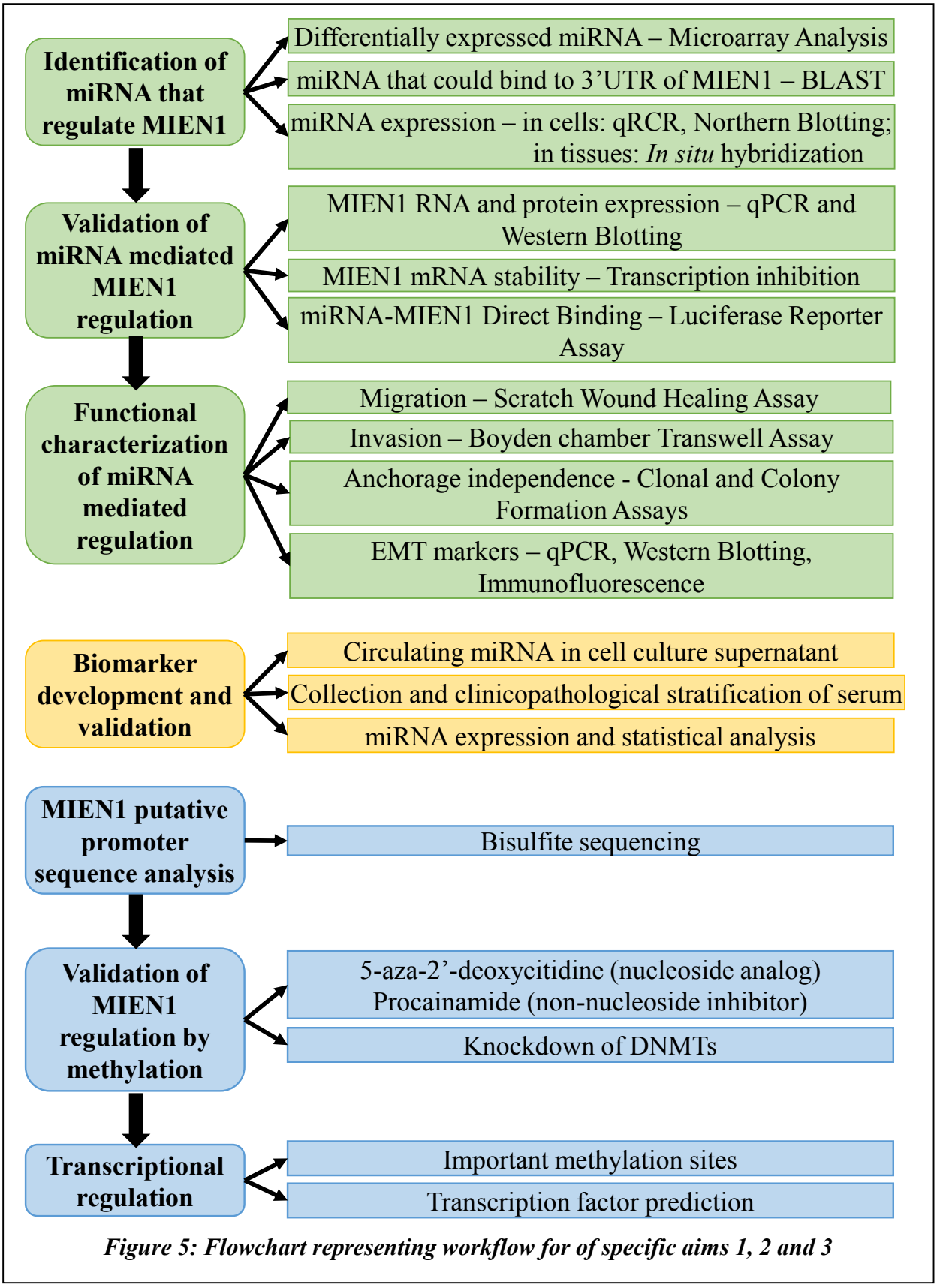

Figure 5: Flowchart representing workflow for of specific aims 1, 2 and 3

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CHAPTER II

MICRORNA-940 SUPPRESSES PROSTATE CANCER MIGRATION AND INVASION BY REGULATING MIEN1

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Abstract

Background

MicroRNAs (miRNAs) are crucial molecules that regulate gene expression and hence pathways that are key to prostate cancer progression. These non-coding RNAs are highly deregulated in prostate cancer thus facilitating progression of the disease. Among the many genes that have gained importance in this disease, Migration and invasion enhancer 1 (MIEN1), a novel gene located next to HER2/*neu* in the 17q12 amplicon of the human chromosome, has been shown to enhance prostate cancer cell migration and invasion, two key processes in cancer progression. MIEN1 is differentially expressed between normal and cancer cells and tissues. Understanding the regulation of MIEN1 by microRNA may enable development of better targeting strategies.

Methods

The miRNAs that could target MIEN1 were predicted by *in silico* algorithms and microarray analysis. The validation for miRNA expression was performed by qPCR and Northern blotting in cells and by *in situ* hybridization in tissues. MIEN1 and levels of other molecules upon miRNA regulation was determined by Western blotting, qPCR, and immunofluorescence. The functional effects of miRNA on cells were determined by wound healing cell migration, Boyden chamber cell invasion, clonal and colony formation assays. For knockdown or overexpression of the miRNA or overexpression of MIEN1 3'UTR, cells were transfected with the oligomiRs and plasmids, respectively.

Results

A novel miRNA, hsa-miR-940 (miR-940), identified and validated to be highly expressed in immortalized normal cells compared to cancer cells, is a regulator of MIEN1. Analysis of human prostate tumors and their matched normal tissues confirmed that miR-940 is highly expressed in the normal tissues compared to its low to negligible expression in the tumors. While MIEN1 is a direct target of miR-940, miR-940 alters MIEN1 RNA, in a quantity as well as cell dependent context, along with altering its downstream effectors. The miR-940 inhibited migratory and invasive potential of cells, attenuated their anchorage-independent growth ability, and increased E-cadherin expression, implicating its role in mesenchymal-to-epithelial transition (MET).

Conclusions

These results, for the first time, implicate miR-940, a regulator of MIEN1, as a potential novel therapeutic agent that could be used for prostate cancer treatment.

Introduction

Metastatic progression of prostate cancer is a major cause of death among men in the United States [1]. Though cancer metastasis is a highly complex multi-step process facilitated by several key events and molecular players, the most effective way known to prevent this progression is by identifying and targeting the various genes involved in the process(es) [2, 3]. Gene regulation is tightly controlled in the normal cells, thereby retaining the homeostatic expression of the appropriate genes for the functioning of the organism. Deregulation of these mechanisms in cancer causes the disrupted expression of the genes, which in turn furthers the cancer progression. MicroRNAs are a class of endogenous, small non-coding RNAs, 18 to 22 nucleotides long in their mature form, which can regulate a set of target genes and result in translational repression or mRNA degradation depending on the extent of complementarity and cellular context [4, 5]. Recent studies have shown extensive dysregulation of miRNAs in prostate cancer [6-8]. Many miRNAs have been implicated as tumor suppressors or oncomiRs depending on their target(s) and/or the global effects they have towards cancer progression [9-12]. Though studies have been performed with respect to certain miRNAs and their specific targets in prostate cancer [13, 14], not much is known about novel miRNAs targeting the players of cancer progression that can be used as diagnostic markers for early detection, or detection of a possible recurrence or resistance, or therapeutic agents to slow the progression. Identification of these novel miRNAs and their target gene(s), and the pathways they affect during cancer progression, will provide new insights into using them for diagnosis or determination of specific therapy regimens.

Migration and invasion enhancer 1 (*MIEN1*), alternately called C17orf37, C35, RDX12, XPT4, ORB3 or MGC14832, is located in the 17q12-21 region of the human chromosome next to HER2/*neu* in a tail-to-tail arrangement. MIEN1 is abundantly expressed in different stages and

grades of prostate cancer phenotypes when compared to normal cells and tissues [15]. MIEN1 has also been predicted as a novel breast cancer biomarker with increased expression in patients with metastatic progression to lung and liver, suggesting its importance in cancer metastasis [16]. MIEN1 plays a role in prostate cancer migration and invasion through enhancement of filopodia formation by facilitating actin cytoskeletal rearrangement and by up-regulating the Akt dependent NF-κB target genes [15, 17]. This was further confirmed by the recent determination of the solution structure of MIEN1 which predicts that Akt phosphorylation via MIEN1 may be dependent on the active redox-like motif in the MIEN1 structure [18]. MIEN1 is also post-translationally modified by prenylation, via GGTase-I, at its C-terminus CVIL motif. Deletion of the motif not only led to the disruption of MIEN1 membrane localization and reduced invasive and migratory potential but also decreased metastasis to the lungs [17]. Although abrogation of prenylation is a possible targeting strategy, it cannot be effectively used since it has been proven that many proteins involved in the regular functioning of the cell are prenylated, rendering this a very important modification. Hence, inhibition of prenylation could negatively impact multiple cellular processes [19]. On the contrary, since MIEN1 is differentially expressed between normal and cancer cells and tissues, deciphering the regulatory mechanism(s) that explain the aberrant expression of MIEN1 in cancer will enable targeting MIEN1 using mechanisms that are endogenously prevalent thus forming an intervention for prostate cancer progression.

In this study, we have identified a novel miRNA, hsa-miR-940 (miR-940), which targets and regulates MIEN1 expression. Our study indicates that miR-940 expression inversely correlates with tumor progression in clinical prostate cancer and the loss of miR-940 in cancer causes an increased expression of MIEN1 which in turn enables prostate cancer progression. Ectopic expression of miR-940 resulted in not only decreased MIEN1 and its downstream effector

molecules, but also reduced the migratory and invasive potential of the cells. Though the overall proliferation was unaltered, the ectopic expression of miR-940 reduced the anchorage-independent growth of cells, increased E-cadherin and decreased slug expression, suggesting facilitation of mesenchymal-to-epithelial transition (MET). Our results demonstrate that miR-940 may be a useful diagnostic marker as well as a therapeutic agent for prostate cancer.

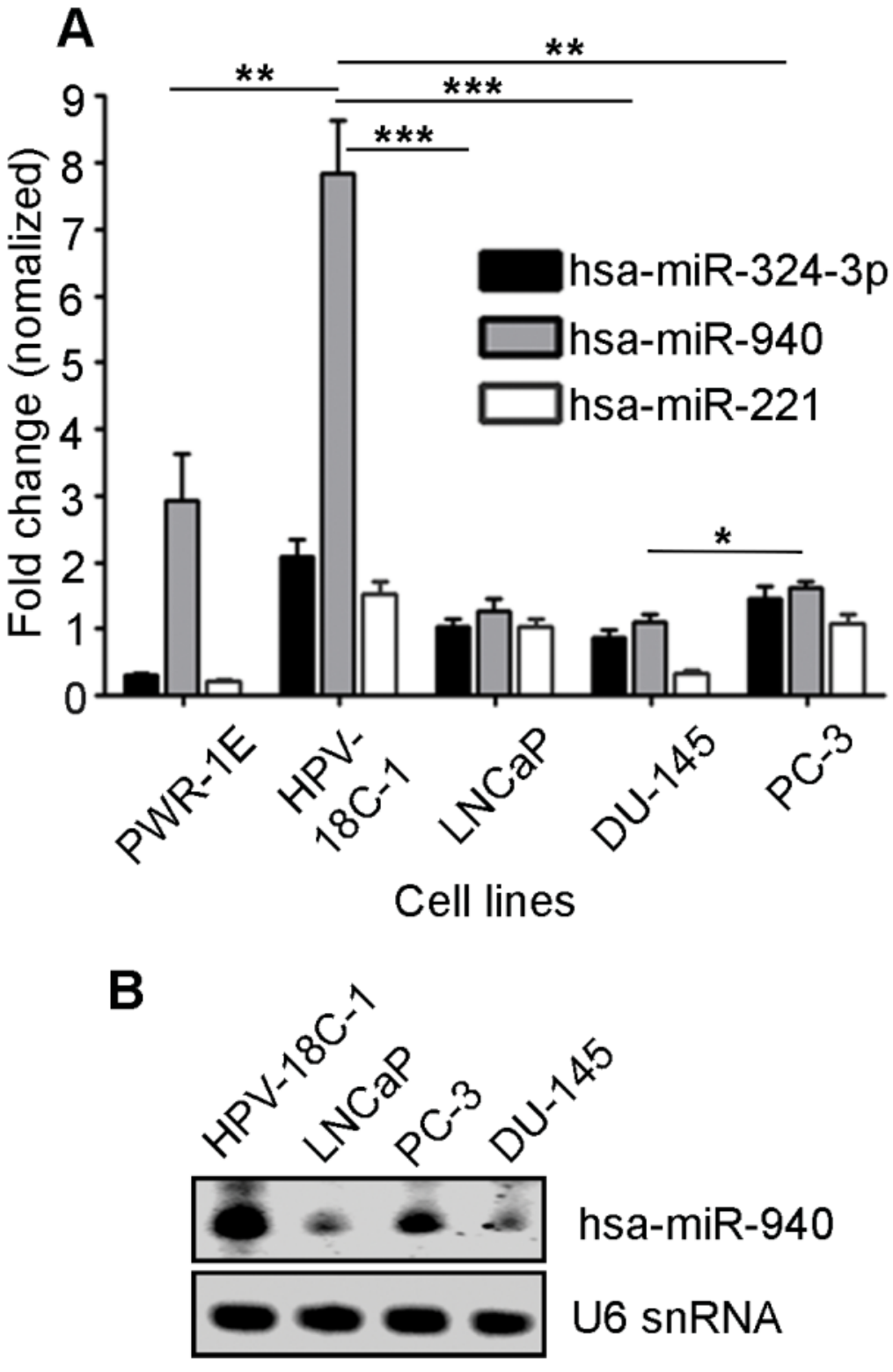
Results

MIEN1 is post-transcriptionally regulated by microRNAs.

In various androgen dependent and independent prostate cancer cells, both MIEN1 mRNA and protein are highly expressed compared to the immortalized normal cells of the prostate [15]. Interestingly, in PC-3 cells, which are androgen independent, though MIEN1 mRNA was expressed, the protein was absent. Hence, we predicted an active role of post-transcriptional regulation of MIEN1. Downregulation of microRNA processing restriction endonucleases, Drosha and/or Dicer [5] using RNAi resulted in a significant transcriptional up-regulation of MIEN1 in HEK-293T cells, which do not express MIEN1 mRNA or protein (Supplementary Figure 1A). In PC-3 cells, the knockdown of the miRNA maturation enzymes resulted in an increase in MIEN1 protein expression by ~4-fold (Supplementary Figure 1B). We next performed a microarray analysis to determine the miRNAs that were differentially expressed in immortalized normal cells (PWR-1E) and cancer cells (DU-145). Subsequently, using BLAST and *in silico* algorithm-based predictions, we identified three microRNAs, hsa-miR-324-3p, hsa-miR-221, and hsa-miR-940, that were differentially expressed between DU-145 and PWR-1E cells and could potentially target MIEN1 3'UTR (Supplementary Figure 2A and 2B) [20, 21]. Using qPCR, the expression levels of these microRNAs were quantitated as fold change normalized to U6 snRNA in the different cell

lines. A significantly higher expression of miR-940 was observed in the non-malignant cells, PWR-1E (~3-fold) and HPV-18C-1 (~7.5-fold), compared to DU-145 and LNCaP, while, PC-3 showed ~1.5-fold higher expression of miR-940 (Figure 1A). The expression of miR-221 and miR-324-3p were neither consistently higher in the immortalized cells compared to the cancer cells, nor were they significantly different, together indicating that miR-940 may be the most relevant regulator of MIEN1 among the three miRNAs. Next, we performed northern blotting to confirm the expression levels of the 21nt miR-940 with a biotin-labeled probe. Consistent with the pattern observed by PCR, the expression of miR-940 in HPV-18C-1 was significantly higher (Figure 1B). To validate the regulation of MIEN1 by miRNA(s), we ectopically overexpressed the miRNA mimics or inhibitors in the various cell lines. We observed a decrease in the expression of MIEN1 protein by ~3- and ~2-fold in DU-145 (Figure 2A, left) and LNCaP (Figure 2A, right) cells, respectively, when transfected with miR-940 mimic. Conversely, inhibiting the endogenous miR-940 in PWR-1E (Figure 2B, left) and PC-3 (Figure 2B, right) using anti-miR-940 increased the MIEN1 protein by ~2- and ~4-fold, respectively. Since miR-221, was significantly higher in PC-3 compared to DU-145, we also ectopically expressed miR-221 mimic in DU-145, together with miR-940, or by itself, and observed a decrease in the MIEN1 protein (Figure 2A, left). But, when we inhibited miR-221 alone in PC-3, we did not see any increase in MIEN1 (Figure 2B, right), implying miR-940 to be a more potent regulator of MIEN1. Together, these results demonstrate that miR-940 is differentially expressed between normal and cancer cells and that it targets and regulates MIEN1 expression. Hence, from here on, we only examined the relevance and role of miR-940 in key processes of prostate cancer progression.

Figure 1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Fold change (nomalized)
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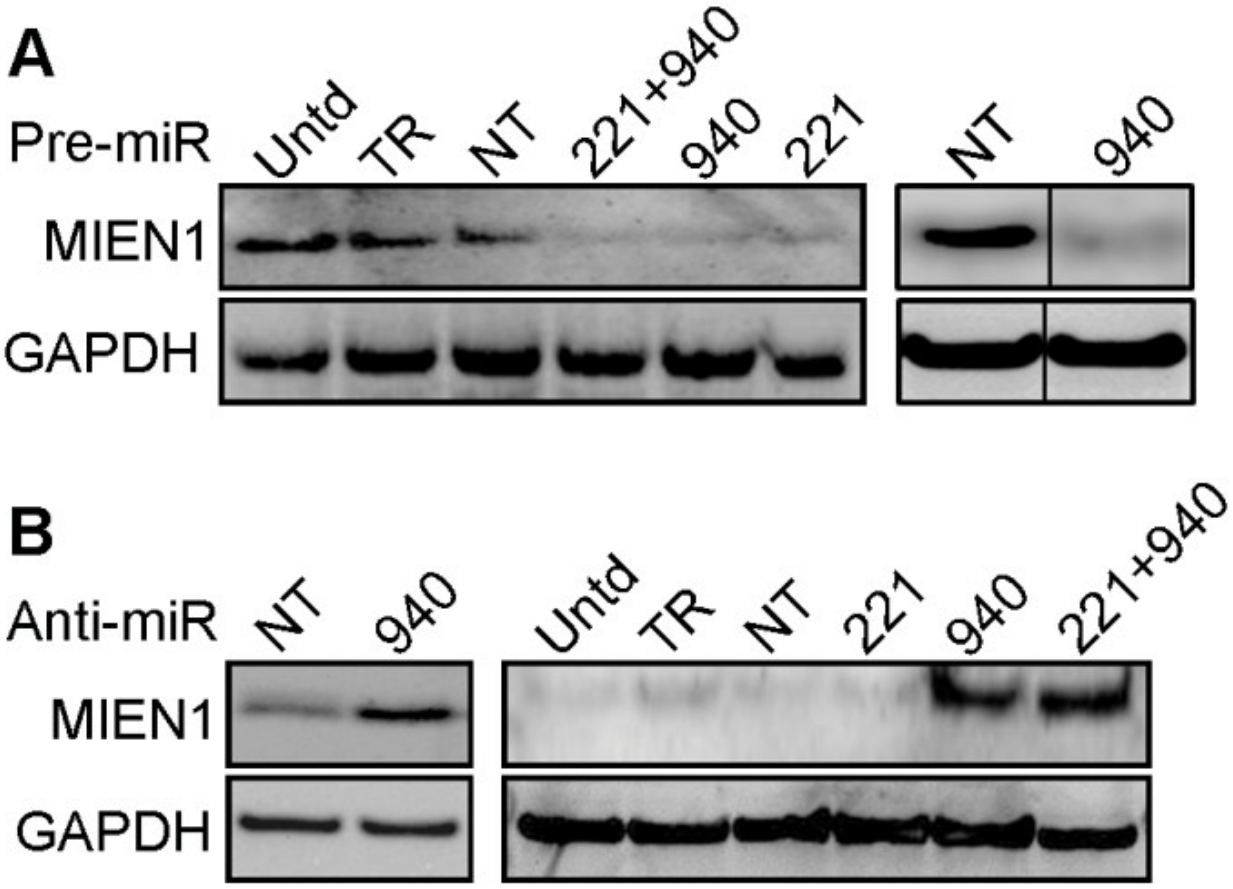
8
9
hsa-miR-324-3p
hsa-miR-940
hsa-miR-221
*
PWR-1E
HPV-18C-1
LNCaP
DU-145
PC-3
Cell lines
B
HPV-18C-1
LNCaP
PC-3
DU-145
hsa-miR-940
U6 snRNA

Figure 1

miR-940 expression. (**A**) qPCR shows expression of the three identified microRNAs: hsa-miR-324-3p, hsa-miR-940 and hsa-miR-221 normalized to U6 snRNA in immortalized prostate derived cell lines, PWR-1E and HPV-18C-1 and different prostate cancer cells, LNCaP, DU-145 and PC-3. (**B**) Northern Blot depicting the expression of hsa-miR-940 in immortalized prostate cell line HPV-18C-1 and prostate cancer cells LNCaP, PC-3 and DU-145. ***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$.

Figure 2


A
Pre-miR
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221
NT
940
MIEN1
GAPDH
B
Anti-miR
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Untd
TR
NT
221
940
221+940
MIEN1
GAPDH

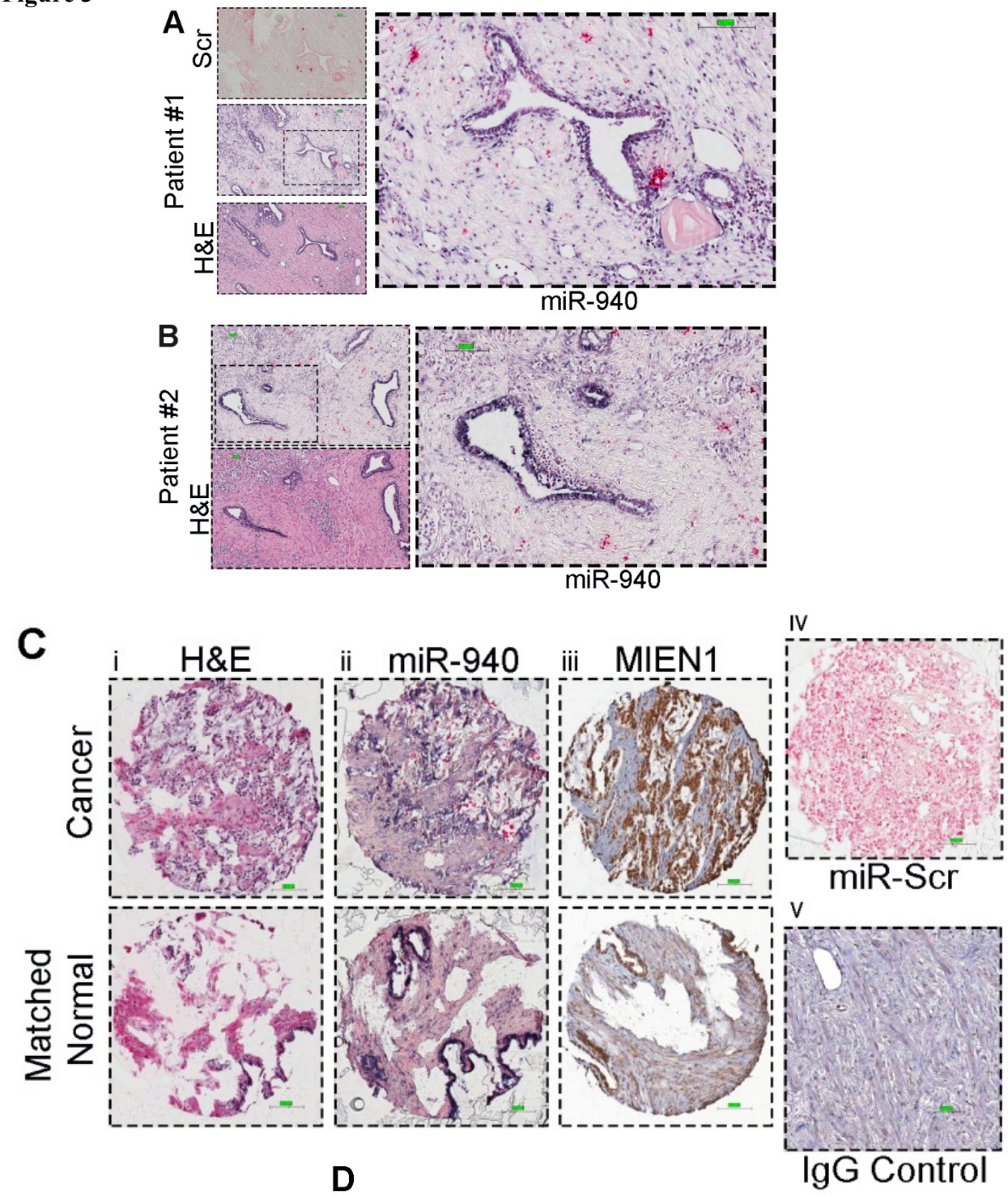
Figure 2

MIEN1 expression upon transfection of miRNA mimic(s) or inhibitor(s) in different cell lines. (**A-B**) Western blot reveals levels of MIEN1 in the cell lines transfected with miR-221 (221), miR-940 (940) or the combination (221+940) while untransfected (Untd), scrambled miR (NT) or transfection reagent (TR) transfected cells are controls in (**A**) DU-145 (left), LNCaP (right) and (**B**) PWR-1E (left), PC-3 (right). GAPDH was used for normalization.

Loss of microRNA, hsa-miR-940, is an indicator of prostate cancer.

The expression levels of miR-940 were next examined in a clinical sample cohort of prostate cancer patient tissues by *in situ* hybridization with miR-940 or scrambled miRNA probe as described in the methods. The tissues were independently scored and graded (based on the corresponding H&E performed) by two pathologists; only those that matched were used to draw any conclusions. Using a tissue microarray (69 cores) with predominant pattern scoring categorized as Gleason score 3 or 4 in a prostate progression cohort, we observed that the miR-940 levels were not significantly different between the groups; though the staining intensities were consistently low (≤ 3 staining intensity) in over ~80% of the tissues (Supplementary Figure 3). In two specific patients who had undergone surgical resection via radical prostatectomy, we observed that miR-940 expression was high in normal glands and benign prostatic hyperplasia with the expression being lower in infiltrating prostate cancer cells (Figure 3A and 3B). In the small pilot cohort of 15 samples, we observed that the miR-940 expression was higher in the matched normal sections in contrast to the low expression in the tumor cells (represented in Figure 3C). Correspondingly, the expression of MIEN1 was relatively higher in the cancer sections compared to the normal (represented in Figure 3C). We observed that the miR-940 staining intensity was high (4 and 5) in 12 of the normal tissues, while only 2 out of 15 tumor sections showed staining intensity of 4. Complementarily, 3 of the 15 normal tissues showed a staining intensity of 3 as opposed to tumors exhibiting lower intensities (1, 2, and 3) in 13 of the cases (Figure 3D). Together, our results indicate that even in a clinical setting (supported by the *in vitro* data), miR-940 expression is consistently higher in the normal tissues as opposed to the tumor cells.

Figure 3



D

% Staining Intensity (N = 15)	≤ 3	> 3
Tumor	87	13
Matched Normal	20	80

Figure 3

miR-940 expression in human prostate cancer and normal tissues. (**A-B**) In situ hybridization of the scrambled miR (miR-Scr) or miR-940 and H&E staining in normal/benign glands compared to the infiltrating tumor. (**C**) H&E staining (i), miR-940 (ii), and MIEN1 (iii) in cancer (top) and matched normal (bottom) tissues with miR-Scr (iv) and IgG control (v) representing the negative controls. (**D**) ~87% (n = 13) tumor shows low (intensity ≤3) expression of miR-940 compared to ~20% (n = 3) of the matched normal tissue with low (intensity ≤3) miR-940 expression; conversely, ~13% (n = 2) of the tumor expressed more (>3) miR-940 compared to higher expression (>3) in ~80% (n = 12) of the matched normal (n = 15).

MIEN1 is a direct target of miR-940.

Next, we wanted to determine if the MIEN1 mRNA stability was altered directly by miR-940. DU-145 cells were transfected with either miR-940 or the control miRNA and treated with Actinomycin-D (Act-D). The half-life of MIEN1 mRNA was observed to be ~6 hours with almost no detectable mRNA in 12 hours after treatment with Act-D in miR-940 transfected cells compared to the control transfected cells (Figure 4A), thus indicating that miR-940 decreases MIEN1 mRNA levels significantly.

We then examined if miR-940 directly binds to the MIEN1 3'UTR using a luciferase plasmid cloned with MIEN1 3'UTR. Binding of the miRNA directly to the 3'UTR of MIEN1 is expected to inhibit the luciferase luminescence compared to the luminescence when the miRNA is unable to bind to the empty luciferase vector control. When MIEN1 3'UTR containing luciferase plasmid (MIEN1) was co-transfected with miR-940 in DU-145 (Figure 4B), there was ~2-fold reduction in luminescence as opposed to luminescence from empty luciferase plasmid (Vec) and miR-940 co-transfections. Additionally, co-transfection of the miR-NT with either the Vec or the MIEN1 plasmid did not show any significant changes in the luminescence in DU-145 (Figure 4C). Similar results were observed in LNCaP (Figure 4D and E). Further, in PC-3 cells which express some endogenous miR-940, ectopic over-expression of miR-940 or the miR-NT with the MIEN1 plasmid showed significantly lesser luminescence (~1.5-fold) compared to Vec co-transfections (Figure 4F and G), thus providing direct evidence of miR-940 – MIEN1 mRNA interaction.

Figure 4

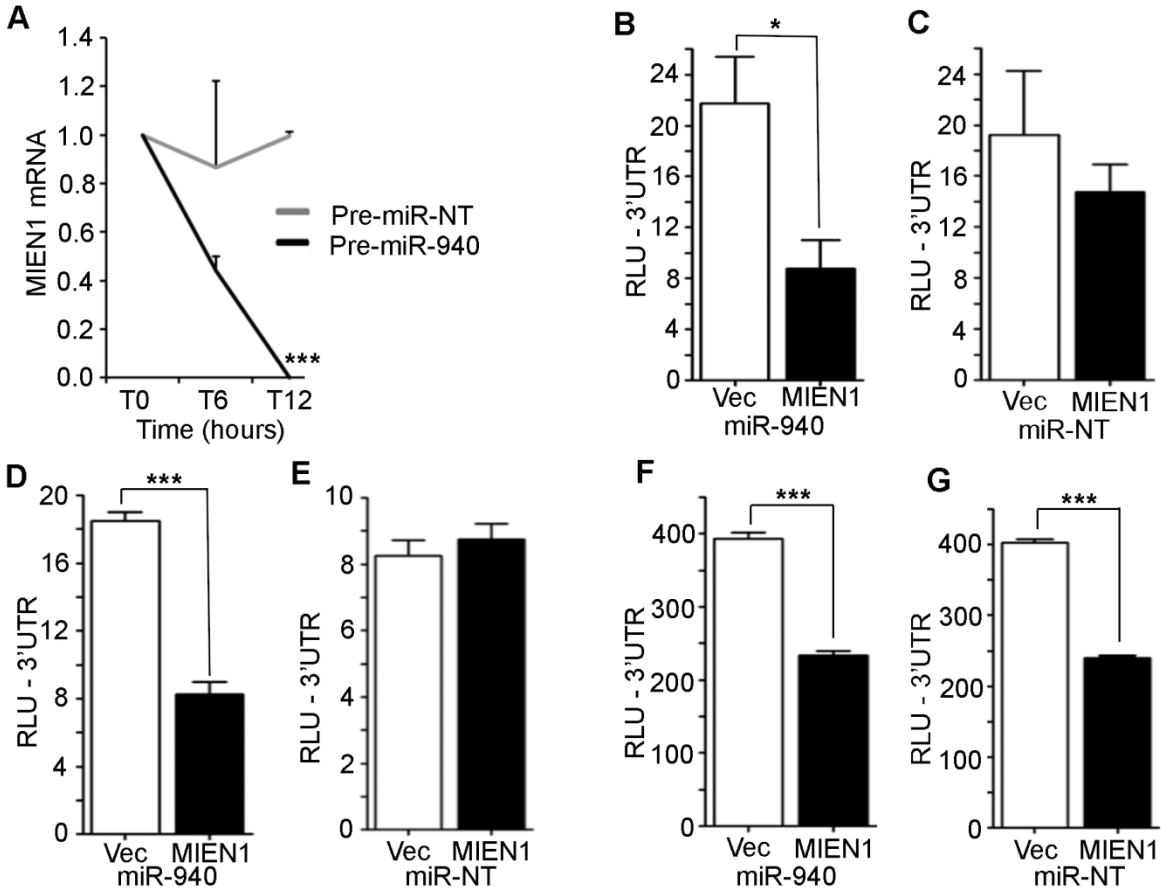

A
MIEN1 mRNA
Pre-miR-NT
Pre-miR-940
***
T0
T6
T12
Time (hours)
B
RLU - 3'UTR
*
Vec
MIEN1
miR-940
C
RLU - 3'UTR
Vec
MIEN1
miR-NT
D
RLU - 3'UTR
***
Vec
MIEN1
miR-940
E
RLU - 3'UTR
Vec
MIEN1
miR-NT
F
RLU - 3'UTR
***
Vec
MIEN1
miR-940
G
RLU - 3'UTR
***
Vec
MIEN1
miR-NT

Figure 4

miR-940 directly binds to MIEN1. (**A**) MIEN1 mRNA level expressed as fraction of the initial value (T0) plotted over time, upon 10 μg/ml Actinomycin-D treatment following transfection with the Pre-miR-NT or Pre-miR-940 for 48 hours in DU-145 (N=3). (**B-G**) Luciferase Reporter Assay showing relative luminescence upon co-transfection of miR-940 (**B, D, F**) or Pre-miR-NT (**C, E, G**) with either empty luciferase vector (Vec) or MIEN1 3'UTR luciferase vector (MIEN1) in DU-145 (**B, C**), LNCaP (**D, E**) or PC-3 (**F, G**) respectively. ***$P \leq 0.001$; *$P \leq 0.05$.

miR-940 affects target genes in a cellular context dependent manner.

Since it is known that silencing MIEN1 decreases NF-κB mediated downstream effectors MMP-9, uPA and VEGF [15], we examined if ectopic over-expression of miR-940 had the same effect on downstream effectors of MIEN1. Upon over-expression of miR-940 in DU-145, a decrease was observed in MMP-9, uPA and VEGF, along with pNF-κB S536 (an indicator of nuclear NF-κB that is responsible for the transcription of target genes) at the protein level (Figure 5A) compared to the control. Reductions from ~2- to 3-fold in MMP-9, uPA and VEGF transcripts in DU-145 (Figure 5B) further confirmed the inhibition of the NF-κB mediated transcriptional activity. Conversely, the knock-down of endogenous miR-940 in PWR-1E increased transcript levels of MMP-9, uPA and VEGF by ~1.5- to 2-fold (Figure 5C).

Interestingly, the effects observed with PC-3 were quite unique. It is well known that the miRNA regulation can result in either mRNA degradation or translational repression of the target [22, 23]. Though PC-3 has an increased level of MIEN1 protein upon treatment with anti-miR-940 (Figure 2B, right), there was no increase in the mRNA levels of MIEN1 (Figure 5D). Further, ectopic overexpression of miR-940 in PC-3 resulted in a decrease in MIEN1 and its downstream targets, MMP-9, uPA and VEGF compared to the control at the mRNA level (Figure 5E), indicating that the mechanism by which miR-940 affects MIEN1 mRNA is dependent on the amount of miRNA present, which in turn is dependent on the cell type being considered and thus the general cellular context.

Figure 5

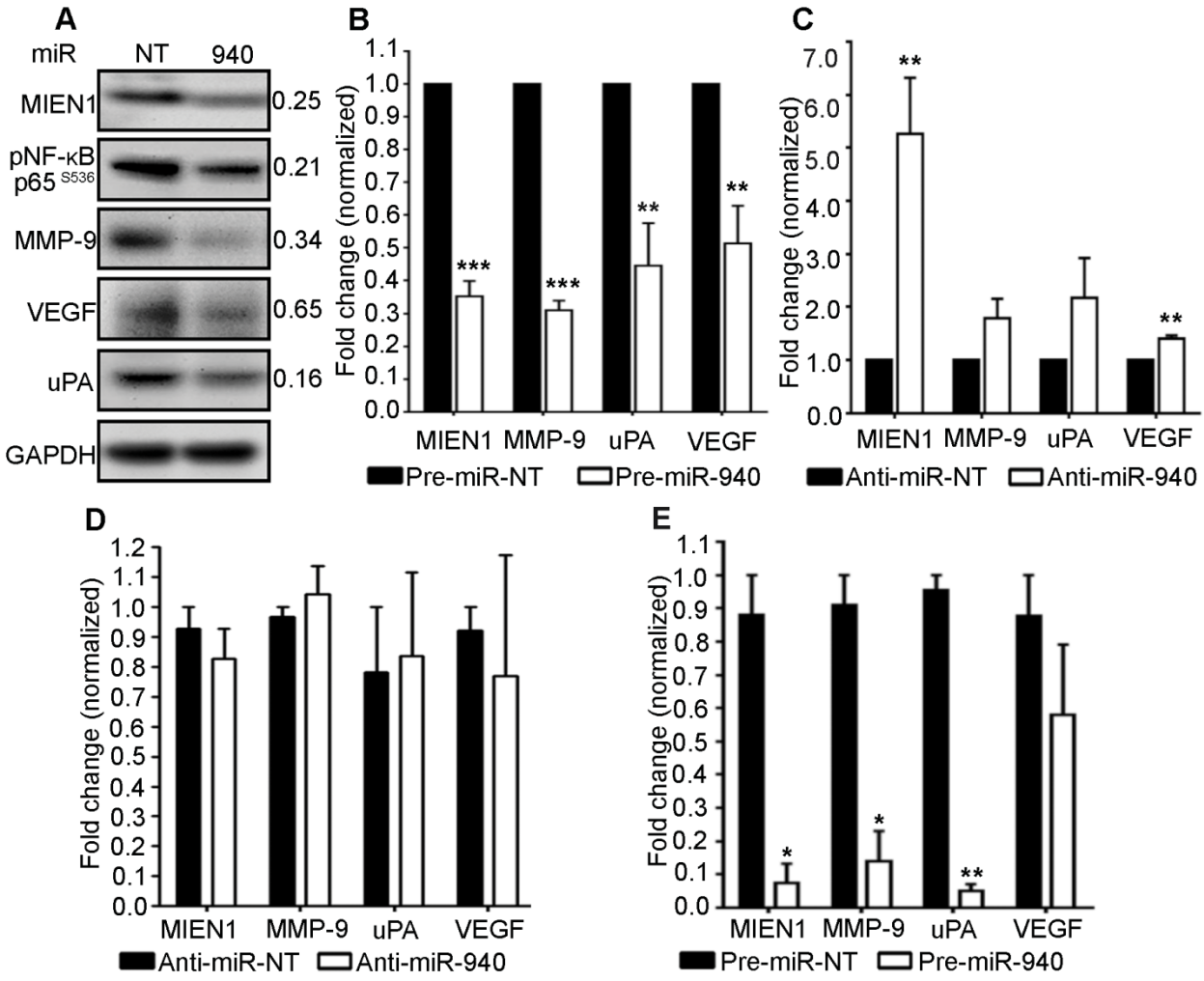

A
miR
NT
940
MIEN1
0.25
pNF-κB p65 S536
0.21
MMP-9
0.34
VEGF
0.65
uPA
0.16
GAPDH
B
Fold change (normalized)
MIEN1
MMP-9
uPA
VEGF
Pre-miR-NT
Pre-miR-940
C
Fold change (normalized)
MIEN1
MMP-9
uPA
VEGF
Anti-miR-NT
Anti-miR-940
D
Fold change (normalized)
MIEN1
MMP-9
uPA
VEGF
Anti-miR-NT
Anti-miR-940
E
Fold change (normalized)
MIEN1
MMP-9
uPA
VEGF
Pre-miR-NT
Pre-miR-940

Figure 5

miR-940 targets MIEN1 and affects MMP-9, uPA and VEGF expression in a cellular context-dependent manner. (**A-B**) Expression of the downstream targets of MIEN1 upon transfection of Pre-miR-NT or Pre-miR-940 in DU-145 at both the translational and transcriptional levels as shown by western blotting (**A**) and qPCR (**B**) respectively. (**C-E**) qPCR showing the expression of MIEN1 and the effectors upon transfection with Anti-miR-940 in PWR-1E (**C**), with Anti-miR-940 in PC-3 (**D**) and with Pre-miR-940 in PC-3 (**E**). ***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$.

miR-940 attenuates the migration and invasion of prostate cancer cells along with inhibiting their anchorage-independent growth potential.

MicroRNAs have multiple targets; hence, the overall effects of a particular microRNA on global cellular functions may vary depending on the regulation of the various targets and their combined implications [24, 25]. Migration and invasion are key processes that facilitate cancer progression and MIEN1 is known to increase these processes [2, 15]. Since, MIEN1 is one of the direct targets of miR-940, we sought to determine if the ectopic over-expression of miR-940 could attenuate these processes, independent of the effect that miR-940 may have on other transcripts. A scratch wound healing migration assay showed that lesser migration (~0.6-fold) was observed in DU-145 cells treated with miR-940 compared to the non-targeting control 24 hours after the initial scratch (Figure 6A and B). Knockdown of the miR-940 using anti-miR-940 in PC-3 resulted in ~1.8-fold increase in its migratory potential (Figure 6C). To investigate whether miR-940 affects the cell viability, MTT assays were performed with ectopic expression of miR-940 mimic in DU-145 and anti-miR-940 in PC-3 cells. Though transfection with anti-miR-940 in PC-3 cells showed a statistically significant decrease in cell viability after 48 hours (Supplementary Figure 4A) this was only a 15% change and the effect was abrogated after 72 hours. No significant differences were observed in the cell viability after 48 or 72 hours of transfection in DU-145 (Supplementary Figure 4B), proving that miR-940 has no dramatic effect on cell viability. Furthermore, cell cycle analysis revealed no significant differences between DU-145 cells transfected with miR-NT or miR-940 mimics (Supplementary Figure 4C). The invasive potential of the cells was determined using miR-940 mimic or inhibitor transfected DU-145 or PC-3 cells through the transwell matrigel invasion assay system. The ectopic expression of miR-940 decreased the invasiveness of DU-145 ~8-fold (Figure 6D), while the invasiveness of PC-3 was ~3.5-fold higher upon inhibition of endogenous

Figure 6

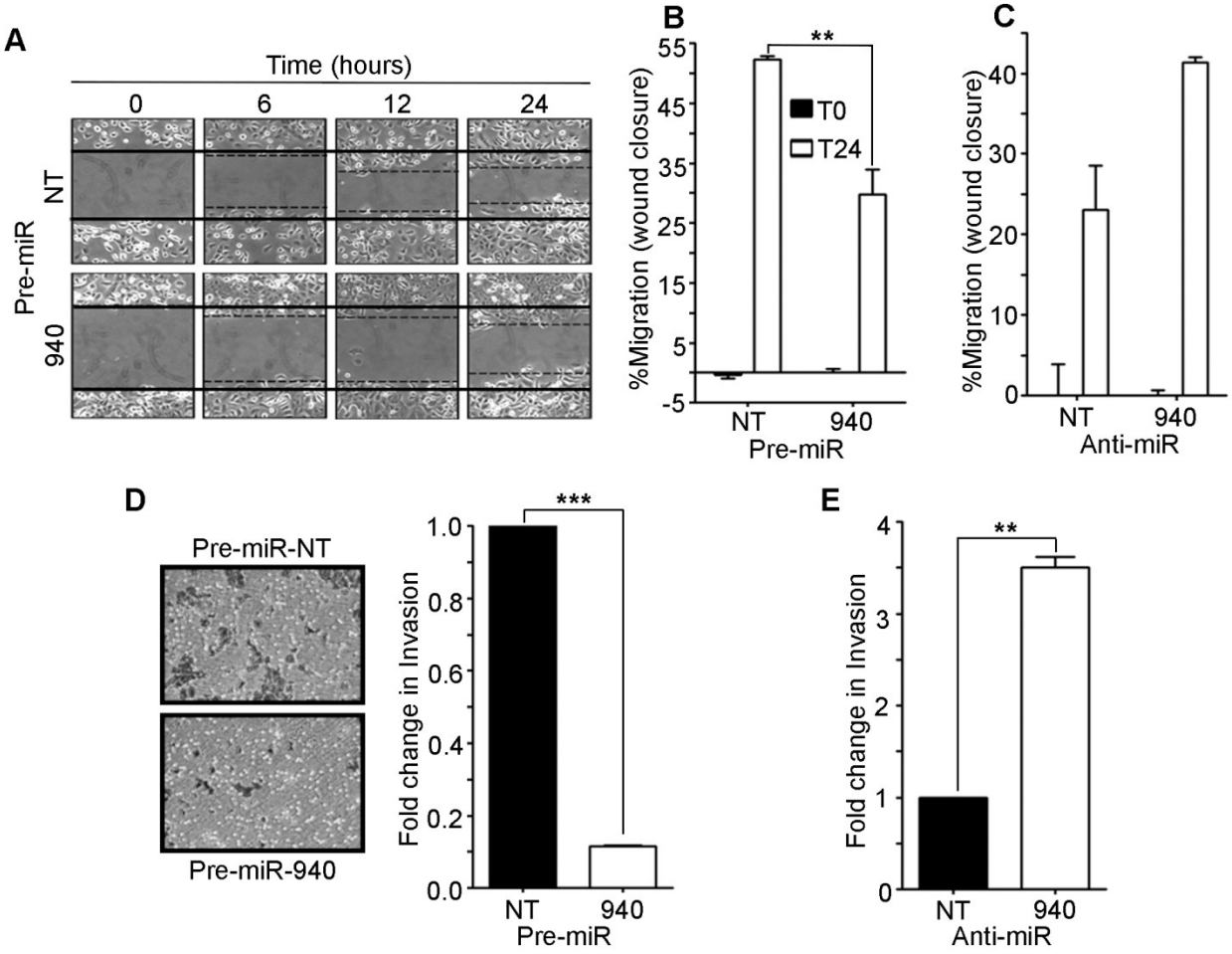

A
Time (hours)
0
6
12
24
Pre-miR
NT
940
B
**
T0
T24
%Migration (wound closure)
NT
940
Pre-miR
C
%Migration (wound closure)
NT
940
Anti-miR
D
Pre-miR-NT
Pre-miR-940
***
Fold change in Invasion
NT
940
Pre-miR
E
**
Fold change in Invasion
NT
940
Anti-miR

Figure 6

miR-940 affects the cellular migratory and invasive potential. (**A-B**) Migration of DU-145 upon transfection with either Pre-miR-NT or Pre-miR-940 depicted by a wound healing assay (**A**) and quantified as a percentage of the wound area closed (**B**) after 24 hours. (**C**) Quantification of the migratory potential of PC-3 cells upon transfection with Anti-miR-NT or Anti-miR-940, 24 hours after making a wound. (**D-E**) Representation and quantification of the invasive potential of DU-145 (**D**) and PC-3 (**E**) cells as determined by the Boyden chamber matrigel invasion assay when transfected by miRNA mimic and inhibitor respectively. ***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$.

miR-940 by anti-miR-940 (Figure 6E). Taken together, these results show that miR-940 inhibits both the migratory and invasive potential of the cells without affecting cell viability.

The ability of the cancer cells to adhere and grow by anchorage-dependent and -independent mechanisms is very important to determine their clonogenic ability and hence their potency to evade cell death and finally metastasize [26]. We observed that the DU-145 cells transfected with miR-940 formed smaller, smooth edged colonies compared to bigger, disseminated colonies formed by the control miR transfected cells, after 12 days (Figure 7A, left and Supplementary Figure 5A) under anchorage-dependent conditions. The total number of individual colonies remained unchanged between the treatments (Figure 7A, right). Conversely, the soft agar colony formation assay demonstrated that the anchorage-independent growth potential of the miR-940 transfected cells was highly hindered (~8-fold) compared to the control transfected cells after 12 days (Figure 7B). Dissemination of the cells is an indicator of the cells undergoing epithelial-to-mesenchymal transition (EMT), a phenomenon crucial to the initial detachment of the cells from the tumor site, leading to the invasion and migration of cells, and finally resulting in progression of cancer [27]. The considerable morphological difference observed in terms of compactness of the colonies between cells transfected with miR-940 and miR-NT suggested loss of the ability of the miR-940 transfected cells to undergo EMT. To further confirm the possibility of the involvement of miR-940 in hindering EMT, we performed immunostaining for E-cadherin, a cell adhesion marker that is down-regulated if the cells undergo the process of EMT, and Vimentin, a mesenchymal marker. Our results show an increase in E-cadherin in miR-940 transfected cells compared to control cells, while total Vimentin levels remained relatively unaltered with just the disruption of membrane localization (Figure 7C and D). Additionally, the mRNA expression of

Figure 7

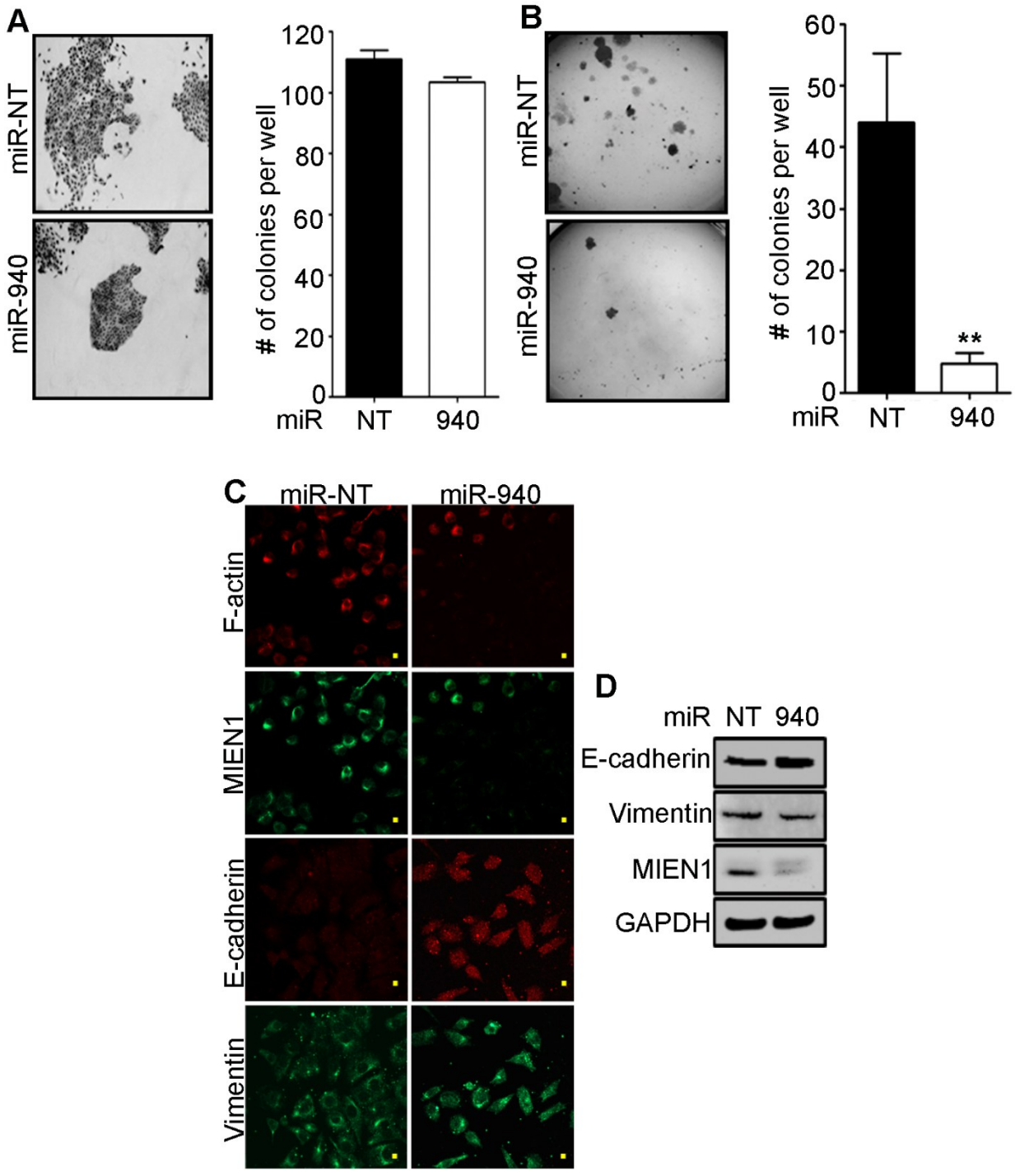

A
miR-NT
miR-940
# of colonies per well
120
100
80
60
40
20
0
miR NT 940
B
miR-NT
miR-940
# of colonies per well
60
50
40
30
20
10
0
**
miR NT 940
C
miR-NT
miR-940
F-actin
MIEN1
E-cadherin
Vimentin
D
miR NT 940
E-cadherin
Vimentin
MIEN1
GAPDH

Figure 7

miR-940 alters the anchorage-dependent and -independent growth of DU-145 cells. (**A**) Morphology (left) and number (right) of colonies formed by Pre-miR-NT or Pre-miR-940 transfected DU-145 cells after 12 days on tissue culture treated adherent plates. (**B**) Soft agar colony formation assay shows the colonies in the agar (left) and their quantification (right) after 12 days. (**C-D**) Immunofluorescence (representative image from two independent experiments, and 3 fields in each experiment) (**C**) and western blotting (**D**) show the expression and localization of various proteins upon over-expression of Pre-miR-940 compared to Pre-miR-NT. $^{**}P \leq 0.01$.

Slug, a transcriptional regulator of E-cadherin, decreased in miR-940 transfected cells compared to the control (Supplementary Figure 5B). In PC-3 cells, transfection of the miR inhibitor caused a decrease in E-cadherin transcript levels (Supplementary Figure 5C). Together, this suggests the possible involvement of miR-940 in MET, the reverse process of EMT.

Discussion

MIEN1, a novel gene in the 17q12 region of the human chromosome, is differentially expressed between cancer and normal cells and tissues [16]. Previous studies indicate that MIEN1 plays an important role in prostate cancer progression [15, 17]. In this study, we show that MIEN1 undergoes post–transcriptional regulation by miR-940. Our data show that miR-940 decreases the migratory and invasive potential of prostate cancer cells along with facilitation of MET. Also, decreased expression of miR-940 in prostate cancer specimens proves the clinical relevance of this miRNA, leading to our belief that miR-940 is a potential diagnostic marker and therapeutic agent.

The use of miRNAs for therapy and/or diagnosis of cancer is currently under consideration due to the accumulating evidence demonstrating their extensive deregulation in many cancers, including prostate cancer [7, 28-30]. MiRNA mediated regulation of a target gene depends on multiple parameters including: 1) properties of miRNA responsive elements, such as the degree of complementarity and accessibility; 2) number of miRNAs that could target a single transcript; 3) expression of competitive endogenous mRNAs for a miRNA in a specific cellular context; 4) stimulus for the miRNA transcription/splicing and hence expression and stability; and, 5) other factors influencing the target mRNA stability and expression [22-25]. Careful examination of the degree to which a gene is regulated by a miRNA and the overall effects of the miRNA mimic or inhibitor, are essential to determine the global role of the miRNA in any cellular context.

The distinct difference in the expression of MIEN1 and lack of protein despite mRNA expression in PC-3 cells directly implied involvement of post-transcriptional regulation. Our experiments confirmed that MIEN1 is indeed regulated by miRNA and led to the identification and validation of miRNA miR-940. Alterations in mRNA half-life in the presence of the miRNA elucidated the effect of miR-940 on MIEN1 mRNA stability. Furthermore, using luciferase assays, we ascertained that miR-940 binds directly to the 3'UTR of MIEN1 to cause its suppression.

MIEN1 is minimally expressed in several normal tissues compared to its overexpression in cancer [16]. Its proximity to the HER2/*neu* locus on chromosome 17 explains its frequent amplification (in 79% of breast cancers) with HER2 amplicon [31]. A recent study using a set of eight genes, including MIEN1, revealed a moderate response to adjuvant Trastuzumab therapy even in HER2 negative breast cancer, confirming the importance of this gene in responses to neo-adjuvant therapies [32]. Katz *et al.* have shown that the overall survival of breast cancer patients is low in cases where MIEN1 is highly expressed while lower expression indicates better prognosis [33]. It is well known that prostate cancer-related deaths are due to metastasis rather than the presence of a primary tumor alone. Metastasis is a complex process involving multiple intermediate steps, from detachment of the cells at the primary site to formation of secondary tumor. The tumor cells evade the resistances faced at every step by different mechanisms [2, 3]. Our previous study has shown that cells overexpressing MIEN1 have a higher metastatic potential, though it does not mean quicker onset or initiation of the tumor [17]. We have also previously shown that MIEN1 increases phosphorylation of Akt causing the translocation of NF-κB to the nucleus and then transcriptionally activating downstream effectors like MMP-9, uPA and VEGF [15]. These proteases and angiogenic factors are known to cleave extracellular matrix, hence facilitating migratory and invasive potential of the cells. Taken together, these studies confirm that

MIEN1 plays an important role in the progression of cancer rather than in the initiation of the tumor. Here, we show that upon ectopic reintroduction of miR-940, the mRNA as well as protein levels of the effector molecules decrease in DU-145. Conversely, down-regulation of the miRNA using inhibitors increases the effector molecules in PWR-1E. Hence, miR-940, indirectly through MIEN1, is capable of decreasing expression levels of specific proteins that facilitate migration and invasion.

It is known that miRNAs can affect expression by causing mRNA degradation through complex formation with RNA-induced silencing complex or by repressing the translation of the mRNA, thus inhibiting formation of the protein [22]. The degree of complementarity and the competitive endogenous mRNAs determine the fate of the miRNA-mRNA complex [24]. Here, we see that miR-940 expression is highest in the immortalized PWR-1E cells, followed by PC-3 cells and lowest in DU-145. In addition to this expression pattern, our data prove that inhibition of miR-940 has different effects on MIEN1 mRNA and protein levels in the various cell lines. This implies that the inhibition of MIEN1 using miR-940 affects MIEN1 in a manner dependent on not only the cellular context (other competitive endogenous mRNA in the specific cells) but also on the endogenous miRNA levels. While in PWR-1E, the endogenous miR-940 potentially degraded MIEN1 mRNA; the over-expression of anti-miR-940 resulted in attenuation of MIEN1 mRNA degradation, thus causing an increase in both MIEN1 mRNA and protein levels. Conversely, the loss of the endogenous miR-940 in DU-145 possibly led to the over-expression of both MIEN1 mRNA and protein; and hence ectopic over-expression of the miR-940, as we have observed, caused MIEN1 mRNA degradation resulting in significant depletion of both transcript and protein levels. However, in PC-3, where MIEN1 mRNA is expressed but protein is low, inhibition of miR-940 with anti-miR-940 resulted in no further increase of MIEN1 mRNA but only increased MIEN1

protein. Additionally, the inhibition of endogenous MIEN1 mRNA with ectopic miR-940 in PC-3 decreased MIEN1 transcript. Together, this indicates that the endogenous miR-940 was causing translational repression of the MIEN1 mRNA rather than degradation in PC-3. Thus, these results show that while miR-940 causes MIEN1 mRNA degradation in DU-145 (ectopic and conceivably endogenous expression) and PWR-1E (endogenous), it causes translational repression of MIEN1 in PC-3 cells (endogenous). The unique ability of this miRNA to perform both mRNA degradation as well as translational repression of the same target depending on the levels of the miRNA and the cellular context seems like a novel finding.

MicroRNAs could target multiple transcripts, thus eliciting a response which is dependent on the combined effects on its targets [24]. A recent study implied that miR-940 could be one of the regulators of alpha-1 antitrypsin [34]. Loss of this serine proteinase inhibitor results in increased risk of lung and liver cancers while its elevated serum levels are associated with prostate cancer [35], supporting our hypothesis that the miR-940 is lost in cancer cells and tissues. In our study, we performed experiments to determine the effects miR-940 would have on migration and invasion, thus delineating the mechanism by which miR-940 could affect cancer progression, based on its regulation of MIEN1, a validated player in the regulation of prostate cancer migration and invasion. We observed a decrease in both the migratory and invasive potential of the cells upon ectopic expression of the miRNA and the converse was seen when the miRNA was inhibited. Hence, we are the first to report that miR-940 inhibits prostate cancer migration and invasion via MIEN1 and other probable targets. The ability of cells to form disseminated colonies without attaching to the substratum is very important to determine the tumorigenicity of the cells [26, 27, 36]. Previous reports indicate that MIEN1 enhances EMT in breast cancer [33]. Our study demonstrates that miR-940 completely inhibits this ability of prostate cancer cells along with

promoting MET by increasing the E-cadherin expression. Additionally, the decrease in slug, an indicator of cells losing their mesenchymal trait [37], was also observed in corroboration with the increased E-cadherin. Since EMT is a very complex yet important process in prostate cancer progression [38-40], further investigation to identify the exact mechanism by which miR-940 facilitates this transition is required. It is also important to determine the different global pathways and the proteins that may be altered by miR-940 that culminates in miR-940 mediated inhibition of prostate cancer progression. Since miR-940 is a very novel miRNA whose function has never been validated or reported in any pathway before, in our study we used a set of common genes predicted to be targets of miR-940 by multiple algorithms. The extensive gene list was then categorized into known and validated pathways using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway mapping and annotation table available in the Database for Annotation, Visualization and Integrated Discovery 6.7 (DAVID 6.7) [41, 42]. The preliminary examination of the results (obtained from DAVID) was then represented as a function of the number of genes involved within the pathway that could be down-regulated by miR-940 (Supplementary Table 1A), and further classified based on the significance of the overall pathway alteration (Supplementary Table 1B). Interestingly, the pathway with most number of genes affected was a global pathway in cancer. Also, many other pathways predicted within the threshold set indicated the regulation of other pathways considered important in cancer. Thus, miR-940 may be eliciting the responses we have observed via other targets in addition to MIEN1 and this needs further validation.

This study is the first to identify miR-940 as a novel regulator of MIEN1, a molecule involved in prostate cancer progression. With our *in vitro* studies, we established the role of miR-940 in several key processes of metastasis; including migration, invasion, anchorage-independent

growth and EMT. Additionally, with the clinical investigations in a small sample cohort, we demonstrated that miR-940 expression is low in tumor, contrary to MIEN1 expression pattern. Together, this could be an important regulator-target combination to study and use as prognostic indicators for prostate cancer.

Materials and Methods

Cell lines, cell culture, siRNA, miRNA and transfection - Human prostate carcinoma cells DU-145 (ATCC HTB-81), PC-3 (ATCC CRL-1435), and LNCaP (ATCC CRL-1740) were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum (Life Technologies). Immortalized non-tumorigenic prostate epithelial cell line HPV-18C-1 (a kind gift from Dr. Jhong S. Rhim, Frederick Cancer Research and Development Center, National Cancer Institute, Frederick, MD) and PWR-1E (ATCC CRL-11611) were maintained in Keratinocyte-SFM (Life Technologies) supplemented with bovine pituitary extract (25 μg/ml) and recombinant epidermal growth factor (0.15 ng/ml). Cells were cultured at 37°C with 5% CO_2. The cell lines were authenticated according to "Authentication of Human Cell Lines: Standardization of STR Profiling" using *GenePrint*® 10 System (Promega); all cell lines and their passages exhibited >80% match to the initial cell line STR profile provided by ATCC [45]. The smart pool siRNAs were obtained from Dharmacon (Thermo Fisher Scientific), while the precursor and inhibitor miRNA oligos (Pre- and Anti-miR) were purchased from Ambion (Life Technologies). The final concentration of the miRNA oligos used for transfection was determined by preliminary concentration-dependent studies and remained constant for all the experiments. Plasmid transfections were performed using Lipofectamine 2000 while Lipofectamine RNAiMAX was used for RNAi transfections, performed according to the manufacturer's protocols (Life Technologies).

Antibodies and reagents - The following antibodies and reagents were used: Mouse monoclonal and mouse polyclonal MIEN1 (Abnova; antibody specificity tested and proven in previous studies[15, 17]), rabbit polyclonal MIEN1 (Life Technologies; antibody specificity tested in previous studies[15]), mouse monoclonal GAPDH (Santa Cruz Biotechnology), rabbit monoclonal pNF-κB p65 S536 and rabbit polyclonal MMP-9 (Cell Signaling Technology), mouse monoclonal VEGF and uPA (R&D Systems), mouse monoclonal Phalloidin (Life Technologies), mouse monoclonal E-cadherin (BD Biosciences), Vimentin (supernatant developed in mouse and tested against human antigen, Developmental Studies Hybridoma Bank), anti-mouse and anti-rabbit IgG (Promega), sheep anti-DIG-AP antibody and NBT-BCIP ready-to-use tablets (Roche), sheep serum (Jackson ImmunoResearch), rabbit IgG, BSA, levamisole hydrochloride, Tris-HCl (pH 7.4), nuclease free water, SSC buffer, Xylene, Tween-20, Nuclear Fast Red, Hematoxylin and Eosin (Sigma-Aldrich) and Permount and PBS (Thermo Fisher Scientific).

Bioinformatics and microarray analysis - *In silico* analyses were performed to determine the putative miRNAs that could target MIEN1. The software programs used included miRANDA [20], PicTar [46], miRBase [47] and TargetScan [21], all of which used the 3'UTR as the target region to determine miRNA recognition elements and provided scores to determine predictive values.

For microarray based hybridization, DU-145, LNCaP and PWR-1E cells were trypsinized, spun down, washed with sterile PBS and frozen immediately at -80°C. The samples were de-identified and shipped to LC Sciences (Houston, TX) for microarray hybridization. In brief, total RNA was isolated from the cells and enriched for small RNA (<300nt). Subsequently, the small RNAs were 3' extended with polyA tail and an oligonucleotide tag was ligated to it for fluorescent dye staining (Cy3). The samples were then hybridized to the probe set on the plate (probes consisted of sequences complementary to miRNA from miRBase as well as the specially requested custom

probes). After hybridization, the miRNA expression was detected by fluorescence labeling using tag-specific dye. Images collected were analyzed using Array-Pro image analysis software. Data analysis involved subtraction of the background along with normalization. ANOVA and paired t-test results were provided for further interpretation and study.

qPCR - Total RNA was isolated from the cell lines using TRIzol (Life Technologies) and quantified. Equal amount of RNA was used for the one-step or two-step qPCR performed using the Superscript III SYBR Green qRT-PCR kits, according to manufacturer's instructions (Life Technologies). For miRNA, PCR was performed using NCode VILO miRNA cDNA Synthesis and EXPRESS SYBR GreenER miRNA qRT-PCR Kits (Life Technologies), according to the manufacturer's protocol. The primers (sequences provided in the Supplementary materials and methods; Additional file 7) were designed using Primer 3 [48] and synthesized by Integrated DNA Technologies (Coralville, IA). PCR was performed using Mastercycler ep gradient S thermal cycler (Eppendorf).

Western blotting - Western blotting was performed according to standard protocols. Briefly, total protein was isolated using NP-40 lysis buffer and estimated using the standard Micro BCA Protein Assay Kit (Pierce Biotechnology). NuPAGE® Novex® 4-12% Bis-Tris Gels were used and the samples were transferred onto nitrocellulose membranes using an iBlot (Life Technologies). Membranes were blocked in 5% non-fat dry milk or 1% BSA prior to antibody subjection. The chemiluminescent reaction was captured by the AlphaImager (ProteinSimple) and bands were analyzed using ImageJ software [49].

Northern blotting - Northern blotting was performed using miRNA Northern Blot Assay Kit and custom ordered biotin-labeled miR-940 and U6 control probes (Signosis) with one microgram of

total RNA from each cell line, according to manufacturer's instructions. This experiment was performed only once.

RNA stability assay - Cells were transfected with the precursor oligomiRs and 48 hours after transfection, treated with 10 µg/ml Act-D (Sigma-Aldrich). RNA was isolated at several time points and quantified. Equal amounts of RNA were used to run qPCR to determine MIEN1 levels.

Luciferase reporter assay - Cells were transfected with 3'UTR constructs (Origene) - 3'UTR-Vector (Vec) or 3'UTR-MIEN1 (MIEN1) and miR-940 or miR-NT in duplicate. Luciferase assay was performed using the Luciferase Assay System (Promega) according to manufacturer's instructions and luminescence read using Synergy2 Alpha Microplate Reader (BioTek). Five independent experiments were performed and averaged to obtain the relative luminescence.

Migration assay - For migration assay, a scratch was made to a monolayer of transfected cells using a pipet tip, 48 hours after transfection. Fresh media was added immediately to remove the floating cells and image the scratch and surrounding cells at T0 (immediately after scratching). Images were captured at specific time points from at least ten independent fields to determine the wound closure. Migration was calculated as a percentage of the area covered by the cells compared to the original wound area.

Invasion assay - Invasion assay was performed with transwell invasion assay inserts and 24-well plates (BD Biosciences) according to manufacturer's protocol. In brief, cells were transfected with the miRNA oligomiRs and the inserts were coated with Matrigel (BD Biosciences). Cells were trypsinized 48 hours after transfection and 500 µl of the cell suspension (concentration of 5 X 10^4 cells/ml) was plated in duplicate in Matrigel-coated and non-coated transwell inserts with 750 µl of fetal bovine serum as a chemoattractant in the bottom well. The lower side of the transwell membranes were fixed and stained with 0.05% crystal violet 24 hours after plating. Fold change

in invasion was calculated as a ratio of cells invading the Matrigel matrix-coated insert membrane to the cells migrating through the uncoated membrane. The non-targeting miRNA transfected set-up was considered as 1 and the fold change was calculated accordingly.

Anchorage-dependent and -independent growth assays - For the anchorage-dependent clonal assay, cells were treated with precursor miR-NT/940 for 48 hours and seeded (2500 cells per well) on polystyrene coated 6-well plates. After 12 days, the colonies were fixed and stained with 0.05% crystal violet or subjected to immunofluorescence. Only individual colonies (>50 cells per colony) were considered to obtain the average number of colonies for each treatment.

For anchorage-independent colony formation assay, cells were treated with precursor miR-NT/940 for 48 hours before re-plating (5000 cells per ml per well) on soft agar (cells in 2X media:agar = 1:1). Colonies were stained with 0.05% crystal violet and counted after 12 days of incubation in soft agar.

Immunofluorescence - Cells were plated as described in anchorage-dependent assay on coverslips, fixed with 4% paraformaldehyde, permeabilized with 100% methanol, blocked with 1% BSA and stained for the specific proteins.

In situ hybridization and immunohistochemistry - Archived paraffin-embedded prostate tumor with matched normal and tumor infiltrating normal gland tissue sections from multiple patients were used. The anatomic pathologists independently read the slides and graded the Hematoxylin & Eosin (H&E) stained sections to provide Gleason scores (1-5; based on predominant primary pattern) and hybridized sections to determine miR-940 intensity scores (1-5; 1 being basal to very low to 5 being high intensity; a chromogenic assay based on DIG labeled probes detected by alkaline phosphatase conjugated anti-DIG and NBT-BCIP substrate). The Exiqon (Denmark) miRCURY LNA™ microRNA ISH Optimization Kit (FFPE) was used to standardize and perform

in situ hybridization, using scrambled miRNA and the 5'- and 3'-DIG double labeled miR-940 probes. The extent of Proteinase-K treatment, the hybridization time and temperature, and incubation with the substrate were all standardized for the probes. Correspondingly, MIEN1 and isotype-specific rabbit IgG antibodies were used for immunohistochemistry that was performed on the serial sections according to standard protocols. The images were captured as described previously [50].

Statistical analyses - The results were represented as mean ± S.E.M of three independent experiments, unless mentioned otherwise. The p-value was calculated according to Student's t-test using GraphPad P-value calculator and considered significant if *p-value* was at least ≤ 0.05.

Supplementary Materials and Methods

Primer list

MIEN1 FP-5'*CAGTGCTGTGGAGCAGT*3',

MIEN1 RP-5'*GACGGCTGTTGGTGATCTTT*3';

GAPDH FP-5'*GAGCGAGATCCCTCCAA*3',

GAPDH RP-5'*ACTGTGGTCATGAGTCCTTC*3';

MMP-9 FP-5'*TTGACAGCGACAAGAAGTGG*3',

MMP-9 RP-5'*GCCATTCACGTCGTCCTTAT*3';

uPA FP-5'*TGCGTCCTGGTCGTGAGCGA*3',

uPA RP-5'*CTACAGCGCTGACACGCTTG*3';

VEGF FP-5'*CCTGGTGGACATCTTCCAGGAGTA*3',

VEGF RP-5'*CTCACCGCCTCGGCTTGTCACA*3';

E-cadherin FP-5'*CGGGAATGCAGTTGAGGATC*3',

E-cadherin RP-5'*AGGATGGTGTAAGCGATGGC*3';

Slug FP-5'*AATATGTGAGCCTGGGCG*3',

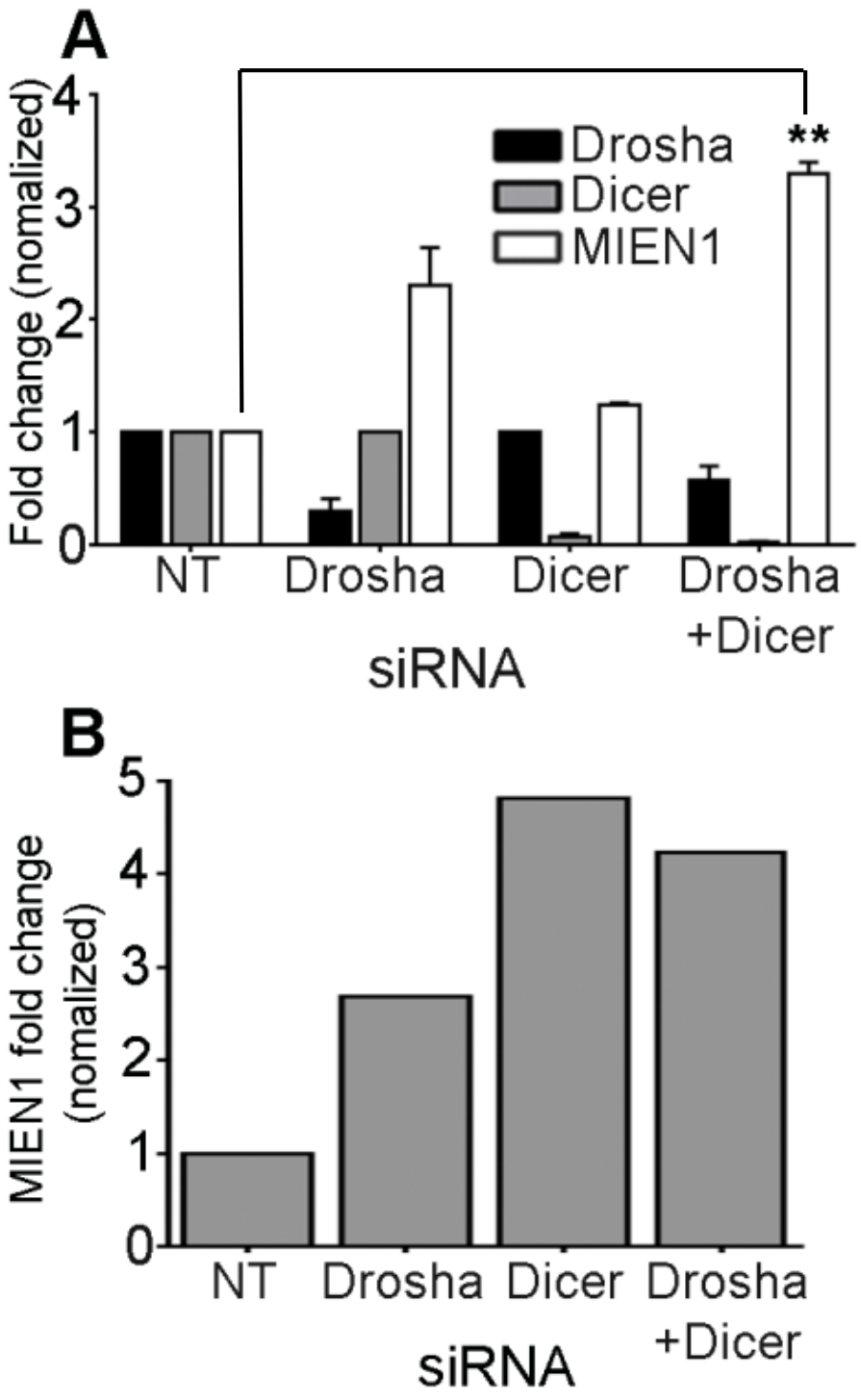
Slug RP-5'*CTCTGTTGCAGTGAGGGCAAG*3'

MTT assay - DU-145 and PC-3 cells were transfected with precursor and inhibitor oligomiRs respectively. The cells were trysinized, counted and plated in complete media on a 96-well plate, 24 hours after transfection. Percent viability was measured 48 and 72 hours after reseeding by 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) assay. The absorbance was measured by Synergy2 plate reader (BioTek) at 570 nm wavelength.

Flow cytometry - DU-145 cells were transfected with miRNA mimic and subjected to cell cycle analysis using Propidium Iodide in a Beckman Coulter Cytomics FC 500 Flow Cytometer. In brief, transfected cells were trypsinized, washed with PBS, counted and resuspended to a concentration of 1.5X106cells/ml. Cells were fixed in cold ethanol at 4°C, overnight. After washing with PBS and centrifuging the suspension, the pellet was resuspended in PI with RNaseA and incubated at 4°C for about 3hours in the dark before analysis.

Bioinformatic Analysis - For identification of pathways that could be affected by miR-940, a common list of genes that were predicted by four independent algorithms, miRanda [20], TargetScan [21], DIANAmT [51] and miRWalk [52], was generated. This list was then analyzed using Database for Annotation, Visualization and Integrated Discovery 6.7 (DAVID 6.7) to classify genes according to the pathways they were involved in based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway mapping function [41, 42]. Pathways containing 20 or more genes from the common list were first tabulated. This list was then rearranged to obtain a hierarchy of pathways with significant Fisher Exact P-values (< 0.05).

Supplementary Figure 1



Supplementary Figure 1

Post-transcriptional regulation of MIEN1. (**A**) Drosha, Dicer and MIEN1 expression levels upon knockdown of miRNA maturation enzymes, Drosha and/or Dicer compared to control siRNA (NT) in HEK293T as shown by qPCR. (**B**) Fold change in MIEN1 protein levels upon knockdown of miRNA maturation enzymes, Drosha and/or Dicer compared to control siRNA (NT) in PC-3. $^{**}P \leq 0.01$

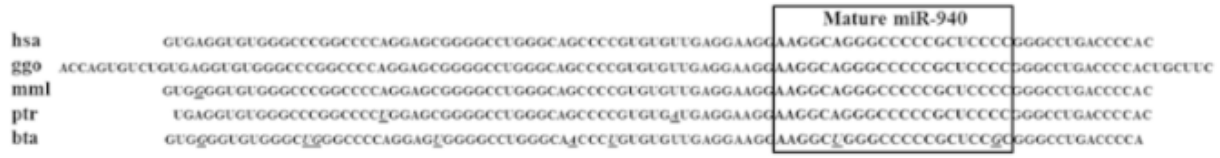
Supplementary Figure 2

A

miRNA	p-Value	Log_2 (PWR-1E DU-145)	5' ---- MIEN1 3'UTR ---- 3' 3' miRNA 5'	Prediction Tool
hsa-miR-324-3p	4.65 e-01	0.60	GAUUCCGUGGCCUUGGGGGCAGG GGUCGUCGUGGACCCCGUCA TCGAGTCGCGCCTCGGGGGCACAG GGUCGUCGUGGACCCCGUCA	miRanda, TargetScan, Micro-Inspector
hsa-miR-221	9.64 e-03	0.64	AACCCAG--GGCAATGTCAGCT UUGGGUCGUCUGUUACA-UCGA	miRanda
hsa-miR-940	3.63 e-02	0.90	GCUGGGAGCUCCC--CCUGCCUC CCCCUCGCCCCCGGGACGGAA	TargetScan miRanda

B

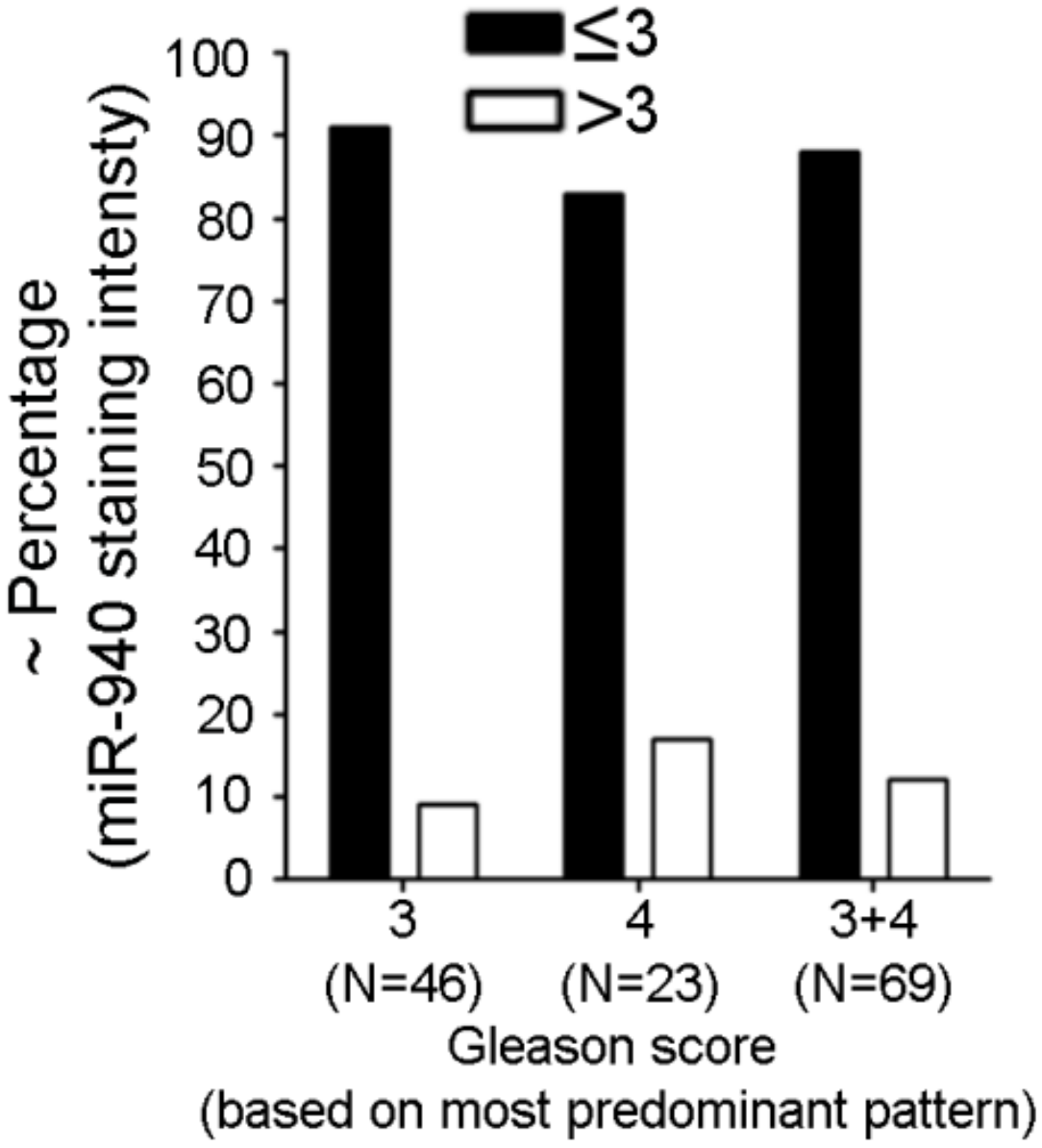
C



Supplementary Figure 2

Potential miRNA regulators of MIEN1. (**A**) miRNA identified by miRNA microarray*, in silico* algorithms and BLAST showing putative binding sites in the 3'UTR of MIEN1. (**B**) hsa-miR-940 precursor miRNA – stem-loop-stem structure. (**C**) Conservation of miR-940 between different species.

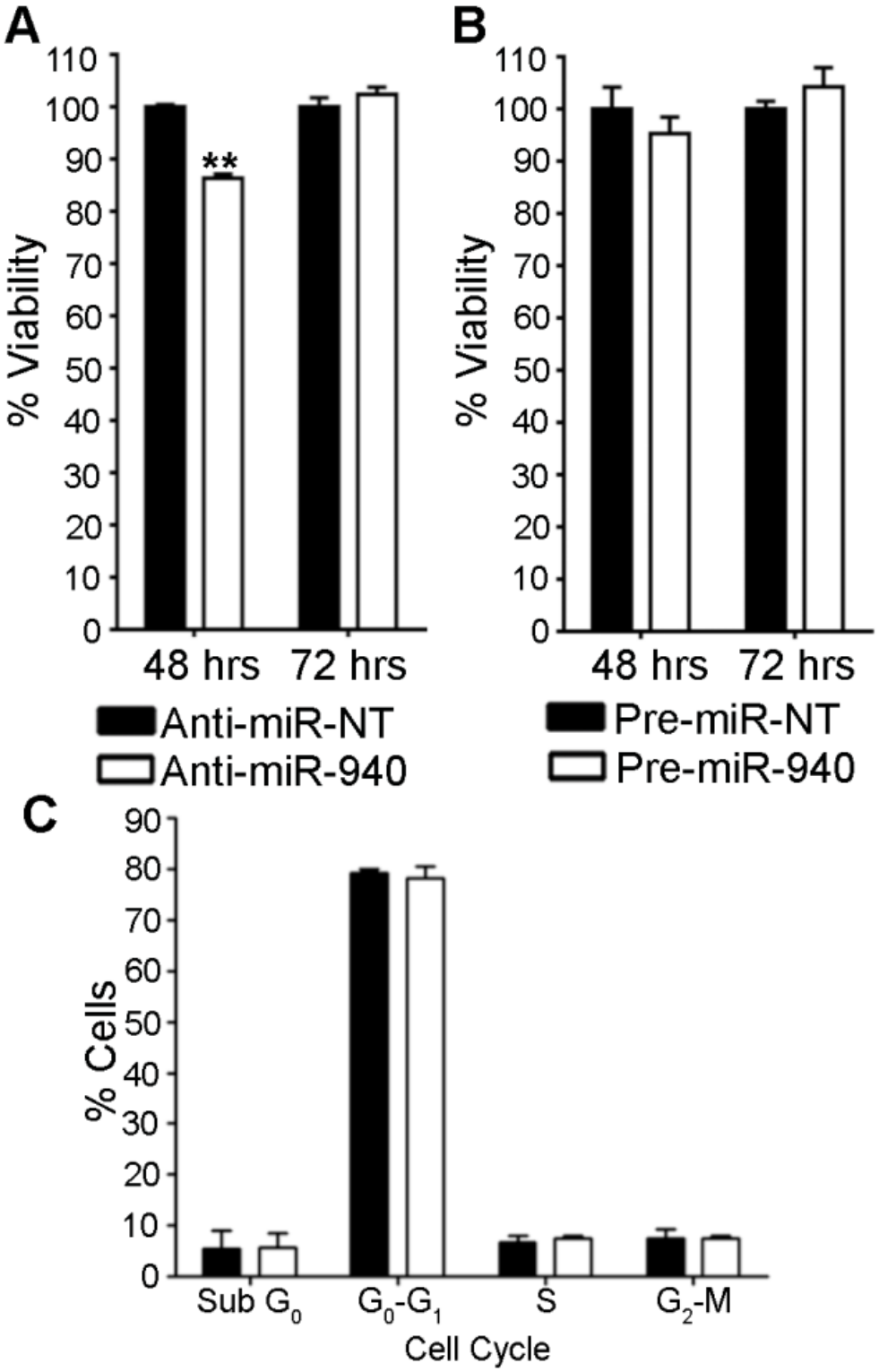
Supplementary Figure 3



Supplementary Figure 3

miR-940 expression pattern in prostate tumors. Graphical representation of the miR-940 expression (staining intensities) based on scoring of *in situ* hybridization of a tissue microarray with prostate tumors of predominant pattern Gleason scores 3 or 4.

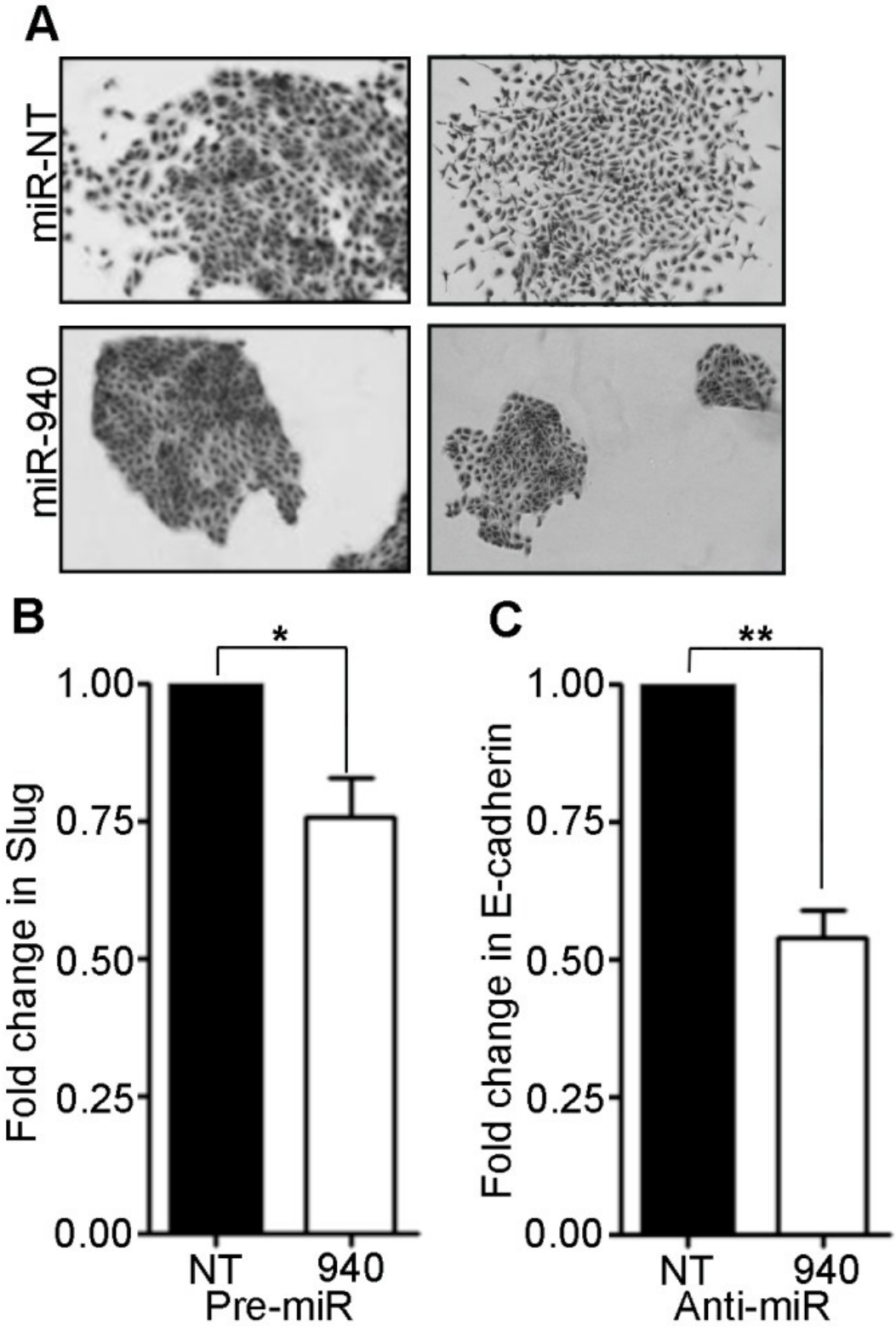
Supplementary Figure 4



Supplementary Figure 4

miR-940 does not alter the cell viability. (**A-B**) MTT assay to determine % Viability in (**A**) PC-3 upon transfection of Anti-miR-940 and (**B**) DU-145 upon transfection of Pre-miR-940. (**C**) Cell cycle analysis showing the percentage of the cell population in the Pre-miR-NT and Pre-miR-940 transfected DU-145 cells. $^{**}P \leq 0.01$.

Supplementary Figure 5



Supplementary Figure 5

miR-940 attenuates EMT and promotes MET. (**A**) Morphology of colonies formed by Pre-miR-NT or Pre-miR-940 transfected DU-145 cells on adherent plates. (**B**) Slug mRNA expression in DU-145 cells transfected with Pre-miR-940 or Pre-miR-NT. (**C**) E-cadherin transcript levels when PC-3 cells were transfected with Anti-miR-940 or Anti-miR-NT. $^{**}P \leq 0.01$; $^{*}P \leq 0.05$.

Supplementary Table 1

A

Term	EASE P-value	Count	%	Fisher Exact P-value
Pathways in cancer	0.0180	71	2.6	0.0130
MAPK signaling pathway	0.0097	61	2.2	0.0065
Endocytosis	0.0001	52	1.9	0.0001
Cytokine-cytokine receptor interaction	0.2400	50	1.8	0.1900
Focal adhesion	0.0160	47	1.7	0.0100
Regulation of actin cytoskeleton	0.1300	44	1.6	0.0960
Neuroactive ligand-receptor interaction	0.6100	43	1.6	0.5500
Insulin signaling pathway	0.0007	39	1.4	0.0003
Chemokine signaling pathway	0.1200	39	1.4	0.0900
Wnt signaling pathway	0.0270	36	1.3	0.0170
Jak-STAT signaling pathway	0.0390	36	1.3	0.0250
Calcium signaling pathway	0.1700	36	1.3	0.1200
Axon guidance	0.0041	35	1.3	0.0022
Purine metabolism	0.0510	35	1.3	0.0330
Huntington's disease	0.2600	35	1.3	0.2100
Lysosome	0.0180	30	1.1	0.0100
Apoptosis	0.0003	29	1.1	0.0001
Neurotrophin signaling pathway	0.0930	28	1	0.0610
Alzheimer's disease	0.5800	28	1	0.5000
Chronic myeloid leukemia	0.0001	27	1	0.0000
Leukocyte transendothelial migration	0.0870	27	1	0.0570
Tight junction	0.2500	27	1	0.1900
Cell adhesion molecules (CAMs)	0.3000	26	0.9	0.2300
GnRH signaling pathway	0.0340	25	0.9	0.0190
Vascular smooth muscle contraction	0.1200	25	0.9	0.0830
Melanogenesis	0.0640	24	0.9	0.0380
T cell receptor signaling pathway	0.1400	24	0.9	0.0910
Colorectal cancer	0.0200	23	0.8	0.0100
Natural killer cell mediated cytotoxicity	0.5800	23	0.8	0.4900
Adherens junction	0.0150	22	0.8	0.0072
ErbB signaling pathway	0.0520	22	0.8	0.0300
Prostate cancer	0.0640	22	0.8	0.0380
Dilated cardiomyopathy	0.0860	22	0.8	0.0530
Small cell lung cancer	0.0650	21	0.8	0.0370
TGF-beta signaling pathway	0.0870	21	0.8	0.0530
Fc gamma R-mediated phagocytosis	0.1700	21	0.8	0.1100
Ubiquitin mediated proteolysis	0.8000	21	0.8	0.7300
Pancreatic cancer	0.0270	20	0.7	0.0140
Hypertrophic cardiomyopathy (HCM)	0.1200	20	0.7	0.0720
Pyrimidine metabolism	0.2500	20	0.7	0.1700
Toll-like receptor signaling pathway	0.3400	20	0.7	0.2500

B

Term	EASE P-value	Count	%	Fisher Exact P-value
Endocytosis	0.0001	52	1.9	0.0001
Chronic myeloid leukemia	0.0001	27	1	0.0000
Apoptosis	0.0003	29	1.1	0.0001
Insulin signaling pathway	0.0007	39	1.4	0.0003
Axon guidance	0.0041	35	1.3	0.0022
MAPK signaling pathway	0.0097	61	2.2	0.0065
Adherens junction	0.0150	22	0.8	0.0072
Focal adhesion	0.0160	47	1.7	0.0100
Pathways in cancer	0.0180	71	2.6	0.0130
Lysosome	0.0180	30	1.1	0.0100
Colorectal cancer	0.0200	23	0.8	0.0100
Wnt signaling pathway	0.0270	36	1.3	0.0170
Pancreatic cancer	0.0270	20	0.7	0.0140
GnRH signaling pathway	0.0340	25	0.9	0.0190
Jak-STAT signaling pathway	0.0390	36	1.3	0.0250
Purine metabolism	0.0510	35	1.3	0.0330
ErbB signaling pathway	0.0520	22	0.8	0.0300
Melanogenesis	0.0640	24	0.9	0.0380
Prostate cancer	0.0640	22	0.8	0.0380
Small cell lung cancer	0.0650	21	0.8	0.0370

Supplementary Table 1

Predicted pathways altered by miR-940. (**A-B**) Pathways potentially affected by common genes predicted by multiple algorithms as identified by KEGG pathways generated in DAVID based on the number of genes present in the pathway (**A**) and the P-value of the pathways with highest number of genes (**B**).

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CHAPTER III

NOVEL MIRNA-940 IS A POTENTIAL SERUM BIOMARKER

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Abstract

Background

Prostate cancer is one of the leading causes of deaths despite an astounding 98% survival rate for localized tumors. This is primarily due to the current methods being insufficient in differentiating indolent from aggressive tumors and the lack of specificity and sensitivity in the detection techniques used in clinics. Though PSA test has been used for a while and is reasonably accurate, there are major caveats. Also, biopsies, the confirmatory diagnostic test for prostate cancer have some associated shortcomings. Together, there is a need for new diagnostic tool development. MicroRNAs are stable circulatory molecules that can be detected in serum.

Methods

Here, we investigated the expression of circulatory miR-940, a novel miRNA known to play a role in prostate cancer progression, by qPCR, from cell culture supernatants (PWR-1E and DU-145 exosomes) and in serum from 25 normal, 32 cancer (untreated, Gleason Score (GS) $\geq$ 7 in 26 and GS = 6 in 6 patients), and 31 cancer (treated, surgical and/or radiation and/or hormonal therapy) patients.

Results

We found that miR-940 was significantly higher in cancer cell supernatant as well as serum from cancer patients. Though the odds ratio of using this miRNA as a biomarker was better than what was exhibited by PSA, the area under the curve (AUC) from the receiver operating characteristic (ROC curve) did not show a significantly higher diagnostic accuracy of this test compared to PSA. The serum miR-940 was better able to distinguish clinically significant tumors (GS $\geq$ 7) from the low grade tumors (GS = 6). Additionally, miR-940 expression was observed to be very significantly higher in patients who were treated, in the past or during the period when the serum was collected, compared to both the normal as well as untreated cancer patients, leading to the question of miR-940 expression regulation by the treatments.

Conclusion

The use of miR-940 as a biomarker for prostate cancer has potential due to the ability of miR-940 expression to distinguish between tumors over GS 6 compared to the GS 6 tumors. Since the area under the curve from the receiver operating characteristic curve is not significantly higher for miR-940 compared to PSA, this test does not have a better diagnostic accuracy than PSA. Larger number of samples need to be evaluated to determine the true diagnostic accuracy of miR-940 in prostate cancer detection.

Introduction

Prostate cancer is one of the leading causes of cancer related death among men in the US [1]. Though many prostate cancers are slow growing, some forms are very aggressive and metastatic, resulting in death. Men over the age of 50 are recommended to undergo digital rectal exam (DRE) and serum total prostate specific antigen (tPSA) screening. While active surveillance

is recommended in some cases, repeat biopsies, surgery and other treatments are currently followed in others who demonstrate irregularities in the DRE or tPSA levels. Unfortunately, some people inherently have high levels of tPSA and enlarged prostate, resulting in extensive over-diagnosis and treatments [2]. Additionally, a recent report by U.S. Preventive Services Task Force recommended against tPSA based screening. Though PCA3 urine test is under consideration as a better prognostic marker, there is insufficient evidence of extensive improvements in sensitivity and specificity compared to tPSA [3-5]. Also, though the test currently marginally improves the diagnostic accuracy of prostate cancer, it does not predict short-term or long-term outcomes or the need for biopsies [3, 6-12]. Further, it does not necessarily help distinguish aggressive from indolent disease [13].

MicroRNAs (miRNAs) are a class of small RNA that regulate gene expression post-transcriptionally [14-18]. Various studies report miRNAs to be highly deregulated in prostate cancer [19-23]. Additionally, recent studies have revealed that miRNA are highly stable in the serum, providing scope for using these circulating miRNA as detection agents in diagnosis of cancer [24-31]. The miRNA signatures are also implicated in determination of the primary site in tumors of unknown origin. Use of the miRNAs as blood based markers has tremendous potential when used in combination with the existing PSA screening techniques for the status of the disease – cancer or not [20, 32-36].

We have recently identified miR-940, expressed on chromosome 16 in humans, as a novel miRNA that impedes prostate cancer progression in an *in vitro* system. Here, we have tried to determine if circulatory miR-940 could allow early detection of prostate cancer and distinguish between the high grade (GS $\geq$ 7) and low grade (GS = 6) tumors. Our results demonstrate miR-940 as a biomarker that can distinguish between normal and cancer states. To a degree, the miR-

940 even distinguishes between low grade and high grade tumors, but owing to the small sample cohorts in the various groups, the statistical significance we observed is not appreciably better than the existing test (PSA) characteristics [37]. Finally, the expression of miR-940 was significantly higher in the serum of patients who had, in the past or during the time of sample collection, undergone treatment implicating other possible roles of miR-940 after treatment.

Results

miR-940 is highly secreted by the cancer cells in vitro.

Based on our previous study, miR-940, a novel miRNA differentially expressed in prostate cancer was chosen to be tested as a biomarker for diagnosis of prostate cancer. To determine if miR-940 was a secretory miRNA, we evaluated the expression of both the mature and the precursor miR-940 levels in the cell culture supernatant. The exosomes from cells that had been serum starved for 24 to 48 hours was collected as described in the methods. The qPCR analysis for miR-940 from exosomes revealed that miR-940 was highly expressed in the exosomes derived from the cancer cells, DU-145, compared to the immortalized normal cells, PWR-1E (Figure 1A). We then quantified the ratio of the miR-940 with the levels of miR-940 within the same cells. We observed that the miR-940 secreted into the serum was significantly higher when the ratio was calculated (Figure 1B). This inverse expression pattern of miR-940 within and outside the cells suggested that miR-940 could be secreted out of the cells during cancer progression, in order to prevent the function of miR-940 mediated suppression of cancer progression.

Circulatory miR-940 is high in cancer patients' serum.

The expression of miR-940 was next assessed in a cohort of clinical serum samples. MiR-940 levels were significantly (P-value = 0.0012) higher in serum obtained from cancer patients who

Figure 1

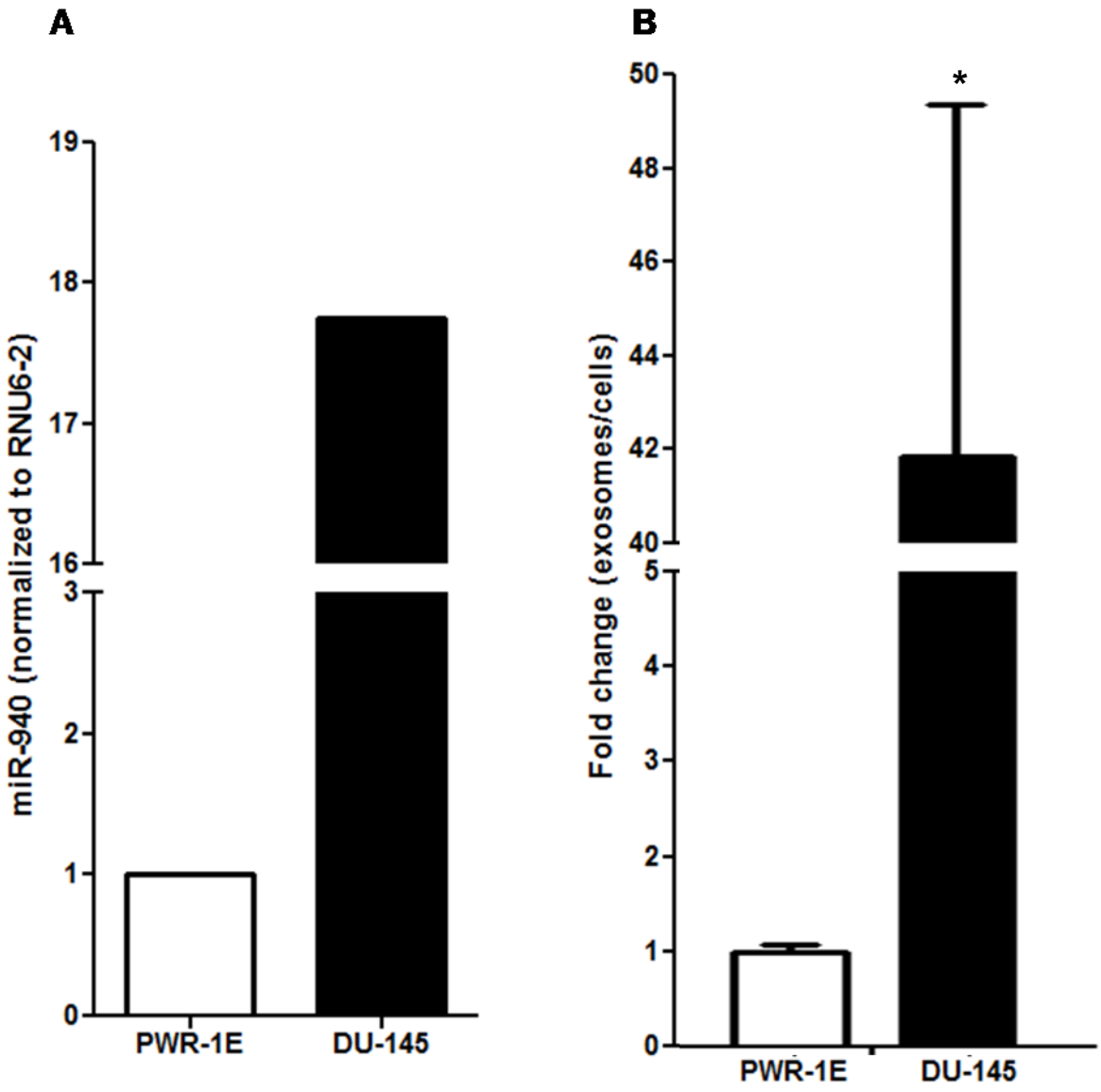
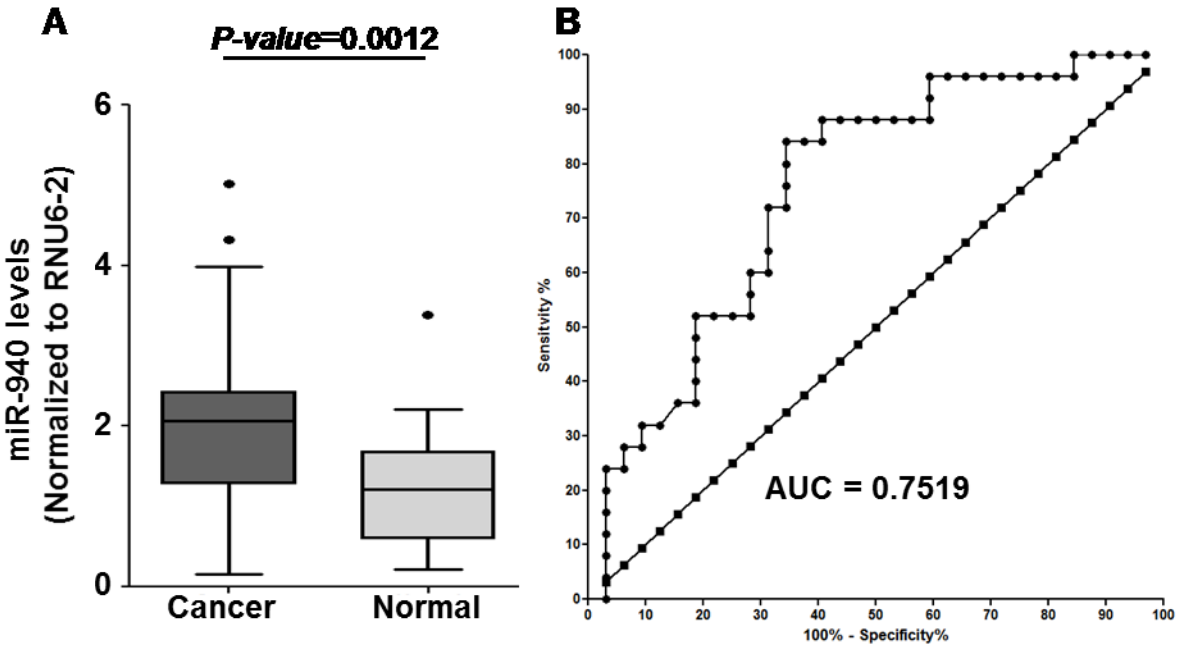

A
miR-940 (normalized to RNU6-2)
19
18
17
16
3
2
1
0
PWR-1E
DU-145
B
Fold change (exosomes/cells)
50
48
46
44
42
40
5
4
3
2
1
0
*
PWR-1E
DU-145

Figure 1

miR-940 expression in cell culture supernatant. (**A**) Total RNA collected from the exosomes isolated from serum starved DU-145 and PWR-1E cell culture supernatants (as described in the materials and methods) was used to determine miR-940 levels compared to RNU6-2 control by qPCR. (**B**) Exosomes were first isolated from the serum starved cell culture supernatants and RNA was extracted from the exosomes as described in the methods. RNA was simultaneously isolated from the cells and by qPCR the levels of miR-940 within and outside the cells was obtained. The ratio of miR-940 (secreted out through exosomes) to the miR-940 within the cells is represented. The experiment was repeated two independent times. $*P \leq 0.05$.

Figure 2



	Cancer	Normal
Sample, N	32	25
Mean ± SEM	2.094 ± 0.1889	1.240 ± 0.1476

C

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B)	
								Lower	Upper
Step 1[a]	PSA	.147	.065	5.117	1	.024	1.159	1.020	1.317
	Foldchange	.975	.428	5.179	1	.023	2.651	1.145	6.137
	Constant	-2.378	.826	8.290	1	.004	.093		

a. Variable(s) entered on step 1: PSA, Foldchange.

Figure 2

Serum miR-940 is significantly higher in cancer compared to normal serum. (**A**) Serum miR-940 levels were detected by qPCR after their isolation using Ambion miRVana PARIS kit from untreated cancer serum and healthy normal control serum as described in the methods. The box and whisker plot shows the median (central line) and the outliers (dots beyond the whiskers. The table provides the mean (±s.e.m.) for each group. (**B**) ROC curve depicts the sensitivity (true positive: cancer in reality and according to test variable; miR-940 fold change) and specificity (100% - false positive: 100% - normal in reality with fold change in miR-940 predicting cancer) of the use of secreted miR-940 as an independent test to determine cancer versus normal states. (**C**) Binary logistic regression analysis comparing secreted miR-940 measured by qPCR and PSA reported by the clinic depicts the significance of each of the covariates (fold change in miR-940 and PSA) along with the odds ratio and their 95% confidence intervals in determining the disease state.

had not undergone any treatment compared to the normal serum (Figure 2A). While the mean(±s.e.m.) for the two groups were 2.094(±0.1889) and 1.24(±0.1476), the ROC curve determining the sensitivity and specificity of the test showed the area under the curve to be 0.7519 (Figure 2B). To determine how well this test performed in comparison to PSA, the current standard for prostate cancer detection, we performed binary logistic regression analysis with PSA and miR-940 fold change as covariates (Figure 2C). Our results indicate that this test is slightly better than PSA, exhibiting a significance of 0.023 and odds ratio of 2.651 with a 95% confidence interval of 1.145 to 6.137.

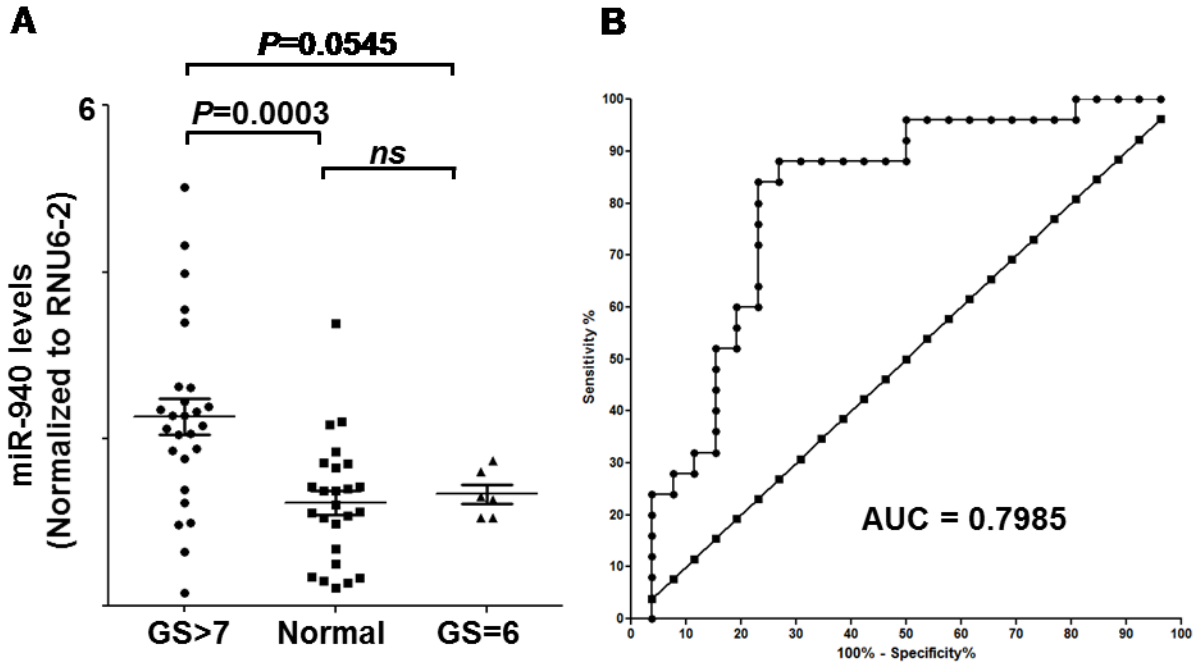
Serum miR-940 has a better diagnostic accuracy when determining moderately and poorly differentiated tumors compared to normal.

We classified the tumor serum further into moderately and poorly differentiated (GS ≥ 7) and well differentiated (GS = 6) tumors, based on the pathological Gleason scoring rendered to the tumors after surgery. The miR-940 levels in serum from moderately and poorly differentiated tumors was very significantly different from their levels in normal serum (Figure 3A). Though there was an overall increase in miR-940 in GS ≥ 7 tumor samples compared to GS 6 tumors, this was statistically insignificant. The area under the curve for the ROC curve increased to 0.7985, from the previously observed 0.7516, when we eliminated the clinically insignificant tumor cohort and tested the diagnostic accuracy of this miRNA (Figure 3B). Correspondingly, the significance and odds ratio also improved (Figure 3C).

miR-940 levels are elevated dramatically after treatment for prostate cancer.

The last patient cohort that we examined in this pilot study included serum from cancer patients who had previously undergone some form of treatment, including one or more of the following: prostatectomy, radiation, brachytherapy, and hormone therapy; or were at present under treatment.

Figure 3



	GS>7	Normal	GS=6
Sample, N	26	25	6
Mean ± SEM	2.267 ± 0.2177	1.240 ± 0.1476	1.343 ± 0.1156

C

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B)	
								Lower	Upper
Step 1[a]	PSA	.160	.068	5.494	1	.019	1.173	1.027	1.341
	Foldchange	1.187	.472	6.328	1	.012	3.276	1.300	8.257
	Constant	-3.177	.996	10.176	1	.001	.042		

a. Variable(s) entered on step 1: PSA, Foldchange.

Figure 3

Secreted miR-940 levels from serum is significantly higher in patients with clinically significant tumors. (**A**) Serum miR-940 were detected by qPCR after their isolation using Ambion miRVana PARIS kit from patients presenting with tumors of various pathological scores. The secreted miR-940 levels from patients who had primary tumors of GS 7, 8 , 9 and 10 were grouped together as one category, separating the clinically significant tumor patients' serum (GS $\geq$ 7, sample size = 25) from the insignificant tumor patients' serum (GS = 6, sample size = 6). A comparison was then drawn between these two groups using Student's t-test. These two groups were also separately compared with the miR-940 levels from the normal healthy controls (Student's t-test). The horizontal line for each group in the graph indicates the mean expression level (normalized to RNU6-2) within each group. The table provides the mean (±s.e.m.) for each group. (**B**) ROC curve depicts the sensitivity (true positive: cancer in reality and according to test variable; miR-940 fold change) and specificity (100% - false positive: 100% - normal in reality with fold change in miR-940 predicting cancer) of the use of secreted miR-940 as an independent test to determine clinically significant cancers (GS $\geq$ 7) versus normal states (healthy controls). (**C**) Binary logistic regression analysis comparing secreted miR-940 measured by qPCR and PSA reported by the clinic depicts the significance of each of the covariates (fold change in miR-940 and PSA) along with the odds ratio and their 95% confidence intervals in determining the disease state (clinically significant tumors, GS $\geq$ 7 versus healthy normal controls).

Figure 4

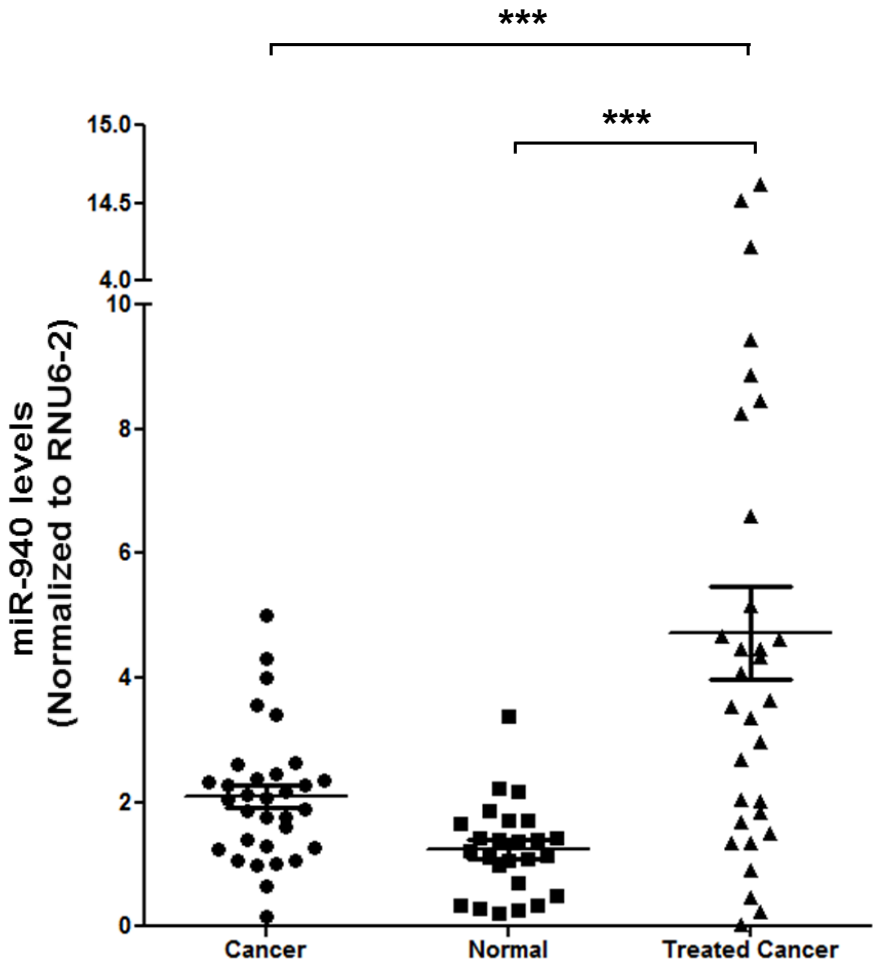

***
***
15.0
14.5
4.0
10
8
6
4
2
0
miR-940 levels
(Normalized to RNU6-2)
Cancer
Normal
Treated Cancer

Figure 4

miR-940 levels in serum obtained from patients previously treated or undergoing treatment is significantly elevated. The expression of miR-940 was computed by qPCR and normalized to RNU6-2. The horizontal line for each group in the graph indicates the mean expression level (normalized to RNU6-2) within each group. The one-way ANOVA showed significant differences between the groups (*P-value < 0.0001*), since our goal was to draw pairwise comparisons between the three groups, we performed post-hoc pairwise comparisons. These comparisons were made between the untreated cancer samples (N=32) and treated cancer samples (N=31) and normal healthy controls (N=25) and the treated samples (N=31) as the depicted in the plot. ***$P \leq 0.001$.

This group was used to determine the effects of the treatments on miR-940 circulation levels. As seen in figure 4, almost any kind of treatment elevated the miR-940 in serum.

Discussion

This study presents the possible use of a novel miRNA, miR-940, as a diagnostic biomarker for prostate cancer. We have shown in a small cohort of samples that miR-940 levels are high in serum from cancer patients compared to healthy controls; making this a potential biomarker. Also, the diagnostic accuracy of miR-940 in predicting a moderately or poorly differentiated cancer is marginally better than PSA (0.799 for miR-940 versus 0.782 for PSA) [37].

Our previous study has shown miR-940 to be highly expressed in normal cells and tissues compared to cancer counterparts. The expression of miR-940 in the serum is contradictory to this intracellular pattern as observed in a study conducted in breast cancer where 28 miRNAs were opposing in terms of their expression patterns between the tissue and the serum [38]. This may be attributed to the many ways miRNAs are trafficked between cells or exported out of the cells, if their intracellular targets potentiate cancer progression or to facilitate cell-cell communication [39-43]. We believe that miR-940 is exported out of the cancer cells to prevent its regulation of MIEN1, and other proteins predicted as targets inside the cell, thus exhibiting an increased level in the serum from cancer patients.

An interesting finding, however, is that the miR-940 levels in circulation radically increased in the serum from patients who have been treated or are currently undergoing treatment. This could be due to a variety of reasons: the miRNA expression may be increased as a compensatory mechanism for its loss in cancer cells and the excess may be passed into circulation; or the miRNA could be under the regulation of factors that are part of the treatment regimen, for

example, androgen; or the miRNA may be synthesized in the adjacent normal environment and be shuttled. Alternatively, the excess of miR-940 in the serum after treatment may be used as a read out for treatment response; higher miR-940 levels being indicative of lesser chance of recurrence or metastasis [39, 43]. Since these are speculations made based on a very small cohort of samples, it warrants further investigation, including longitudinal short-term and long-term follow-up studies.

The current standard for prostate cancer detection includes DRE with PSA, followed by biopsies, for confirmation of the clinical diagnosis. With the relatively low diagnostic accuracy of PSA, and secondary infections associated with biopsy, there has been a clinical deadlock in prostate cancer diagnosis. To overcome this predicament, extensive research is underway to find reliable non-invasive markers which will not only increase the precision of diagnosis but also reduce patient discomfort. Considering that our study strongly alludes to miR-940 being one such molecule, larger scale validations are warranted.

Materials and Methods

Cell lines and cell culture - Human prostate carcinoma cells DU-145 (ATCC HTB-81), and LNCaP (ATCC CRL-1740) were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum (Life Technologies). Immortalized non-tumorigenic prostate epithelial cell line PWR-1E (ATCC CRL-11611) was maintained in Keratinocyte-SFM (Life Technologies) supplemented with bovine pituitary extract (25 μg/ml) and recombinant epidermal growth factor (0.15 ng/ml). Cells were cultured at 37°C with 5% CO_2.

Exosome isolation from cell culture supernatant - Exosomes from cell culture supernatant were isolated as described previously [45, 46]. Briefly, the cells were grown in serum free medium for

at least 24-48 hours. The conditioned medium was transferred to conical tubes and centrifuged at 300 x g for 10 minutes at 4°C to pellet the cells. Next, the supernatant was transferred to ultracentrifuge tubes and centrifuged at 16500 x g for 20 minutes at 4°C to further remove cells and cell debris. The supernatant was passed through a 0.2μm filter to remove particles larger than 200nm. Next, the filtered supernatant was transferred to new ultracentrifuge tubes and centrifuged at 120000 x g for 70 minutes at 4°C to pellet the exosomes. The supernatant was discarded and resuspended in TRIzol and processed for RNA isolation.

Patients and serum samples - Serum samples (N=32) from prostate cancer patients who had not undergone any treatment at the time of sample collection was obtained by Dr. Yair Lotan (UT Southwestern, Dallas, TX) between 2001 and 2010. Of this pool, the pathological Gleason Score was ≥ 7 in 26 patients and 6 in 6 patients. Normal serum samples (N=25) was collected by Dr. Jamboor Vishwanatha's group, through a collaboration with Harris Methodist Hospital at Fort Worth, TX, during prostate cancer screening. Serum samples (N=31) from prostate cancer patients who had or are still undergoing some treatments (surgery and/or radiation and/or hormonal therapy) was collected by Dr. Allen Van Horn (Southewest Urology Associates, Duncanville, TX) between 2011 and 2012. The cohorts selected for primary analysis were the untreated prostate cancer patient samples (N=32; further divided into GS ≥7 and GS =6) and the normal serum (N=25). The third group of samples collected after treatment were used to determine if treatment altered the trend of miR-940 expression. The collection process was the standard blood draw procedure, performed by a well-trained phlebotomist at the respective sites. Subjects were consented to the study and no personal health was revealed. In short, venous blood was collected from each patient and centrifuged. The supernatants recovered and aliquoted and stored at –80 °C

until further use. The study was approved by the Institutional Review Boards at the respective sites.

RNA isolation and qPCR - Total RNA was isolated from the exosomes collected using TRIzol (Life Technologies) according to the standard protocol. Total RNA from the serum was isolated according to the manufacturer's instructions using the miRVANA PARIS kit (Life Technologies). Equal amount of quantified RNA was used for the first step of cDNA synthesis using NCode VILO miRNA cDNA Synthesis Kit (Life Technologies). For the expression of the miRNA, first the miRNA specific forward primers (mature and precursor) and normalization control specific forward primers were designed using Primer 3 [47] and NCBI BLAST softwares [48] and synthesized by Integrated DNA Technologies (Coralville, IA). Next, using EXPRESS SYBR GreenER miRNA qRT-PCR Kits (Life Technologies), the qPCR for the miRNA and controls were carried out, according to the manufacturer's protocol on a Mastercycler ep gradient S realplex^2 thermal cycler (Eppendorf). Normalization of samples was carried out with respect to RNU6-2 expression. The average of the ΔCt values of all the normal samples was used to obtain the ΔΔCt values for each individual sample (cancer, with or without treatment, or normal). This ΔΔCt was then used to determine the fold change (the normalized expression values).

Statistical analyses – ROC curve was generated based on the sensitivity and the specificity of the different data points by the GraphPad Prism software in order to obtain the maximized area under the curve. The box and whisker plots and scatter plots were generated and *p-value* was calculated according to Student's t-test or one-way ANOVA followed by Tukey's Post-hoc test between the groups of interest using GraphPad Prism 5.0. The outliers have been denoted as points outside the whiskers. To perform binary logistic regression analysis, IBM SPSS version 21 was used. The

PSA and fold change in miR-940 were used as covariates to generate the odds ratio, 95% confidence interval for odds ratio and the significance.

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CHAPTER IV

MIEN1 IS TIGHTLY REGULATED BY SINE ALU METHYLATION IN ITS PROMOTER

Smrithi Rajendiran, Timothy Van Treuren and Jamboor K. Vishwanatha

Abstract

Background

Migration and invasion enhancer 1 (MIEN1), a gene located next to HER2/*neu* in the 17q12 amplicon of the human chromosome, is a relatively novel gene that has gained importance in prostate cancer progression by enhancing prostate cancer cell migration and invasion. MIEN1 is differentially expressed between normal and cancer cells and tissues, making it an effective target for therapy. DNA methylation, an important epigenetic regulation, is one of the most widely altered mechanisms in prostate cancer. Evidence for the use of methylation inhibitors in cancer treatment is not substantially justified due to the contradictory effects these agents may have on independent genes which may turn into oncogenic drivers, instead of just increasing tumor suppressive genes. Hence, a better understanding of the epigenetic regulation of the molecules involved in prostate cancer progression is essential; framing the basis to study the DNA methylation patterns in the promoter region of MIEN1.

Methods

Bisulfite pyrosequencing was used to ascertain the role of DNA methylation in MIEN1 transcriptional activation. Validation of methylation inhibition on MIEN1 transcript and protein levels were performed using both nucleoside analogs and non-nucleoside inhibitors, 5-Aza-2'-deoxycitidine and Procainamide, respectively. Further, individual DNA methyltransferases were knocked down using RNA interference technologies to determine effects on MIEN1 transcript and protein. Finally, dual luciferase reporter assays and *in silico* analysis were performed to identify the region for transcriptional factor binding.

Results

MIEN1 promoter contains a short interspersed nuclear Alu element (SINE Alu) repeat sequence. Inhibition of methylation resulted in an increase in both MIEN1 RNA and protein in the normal cells. A sequence immediately upstream of the transcription start site has a site for binding of putative transcription factors, USF. Thus, hypomethylation of the SINE Alu, along with potential binding of USF, facilitates the transcriptional activation of MIEN1 in cancer.

Conclusions

MIEN1 promoter has a SINE Alu region that is hypermethylated in normal cells leading to repression of the gene. In cancer, the hypomethylation of a part of this repeat results in MIEN1 expression.

Introduction

Epigenetic regulation of genes involve non-genetic modifications of DNA and/or histones. Such regulation leads to the transcriptional activation or repression, thereby maintaining an accurate spatial-temporal expression pattern, culminating in cellular homeostasis [1].

Subsequently, the deregulation of the epigenetic mechanisms result in aberrant gene expression. DNA methylation, an important epigenetic modification, is often deregulated in various cancers [2-4]. Usually, a global hypomethylation of repeat sequences including interspersed non-coding region, accompanied by gene-specific DNA hypermethylation in the promoters of the tumor suppressor genes are detected in many cancers [4-7]. With the developments in the field of genomics, including next generation sequencing, many genes that are altered in various cancers have been identified. A vast majority of the genes that have been down regulated among this list correlate to genes exhibiting hypermethylated promoters, thus corroborating the previous observations. These studies, along with some others in the past, allude to the potential use of the methylation pattern signatures as biomarkers for early detection of cancer [8-12].

Prostate cancer, next only to lung cancer in terms of cancer related deaths, is estimated to account for 29,480 deaths in 2014 [13]. Mortality in prostate cancer is the result of metastasis of the cancer, a complex process involving several players. Many genes that are involved in apoptosis, cell cycle regulation, and hormone regulation, apart from genes that act as tumor suppressors by inhibiting cell proliferation, invasion and metastasis, have been shown to be hypermethylated in prostate cancer [3, 7, 10, 11, 14, 15]. Though most of the focus has been in terms of DNA hypermethylation, it is important to also consider the hypomethylation patterns – which directly or through repeat elements drives the expression of oncogenes resulting in genomic instability, during tumor progression [4, 6, 12, 16-19]. Thus, generalized demethylation may not be the most effective approach to consider when trying to treat cancer. Hence, a clearer understanding of the different genes involved in the tumor progression will open new avenues in developing more effective methylation based targeting strategies.

Studies previously conducted by us and a few other groups have identified Migration and invasion enhancer 1 (MIEN1) as an important gene involved in cancer progression [20, 21]. MIEN1 is located in the 17q12 region of the human chromosome, a loci of extreme importance in various cancers, including breast and ovarian, due to the presence of HER2/*neu* [22, 23]. This region has also been shown to be important with respect to castration resistant prostate cancers, the more aggressive form to prostate cancer with poor prognosis. While increased MIEN1 facilitates tumor progression, its expression is low to negligible in various normal cells and tissues, making MIEN1 and attractive biomarker and target.

In the present study, we determined the DNA methylation mediated epigenetic regulation of MIEN1. Our study shows that the MIEN1 promoter has a SINE Alu region that is hypomethylated in cancer, resulting in an increased expression of MIEN1 in cancer [5, 24]. Inhibition of methylation in the immortalized normal epithelial cells by various methods including knocking down of the DNA methyltransferases led to an increase in MIEN1 transcript and protein. Additionally, scanning the promoter revealed a binding site for a transcription factor that could potentially bind and regulate the MIEN1 expression in conjunction with the methylated SINE Alu. Thus, we believe that MIEN1 promoter methylation is very important in repressing the gene, an observation to be considered when using general inhibitors of methylation for prostate cancer therapy.

Results

MIEN1 putative promoter has DNA methylation responsive elements.

Based on UCSC and NCBI, the sequence of MIEN1 putative promoter region was obtained. Upon scanning, we observed that this region contained numerous CpG dyads, islands and a short

interspersed nuclear (SINE) Alu element repeat. A portion of the SINE Alu region, a pre-transcription start site region and a translation start site region (Figure 1A) were then examined in detail by bisulfite sequencing (Supplementary Figure 1A) in immortalized normal prostate epithelial cells (PWR-1E), and androgen dependent (LNCaP) and independent (DU-145 and PC-3) cancer cells. Since SINE Alu repeats constitute 11% of the human genomic sequence, first, a bisulfite primer set (BSP, Supplementary Table 1) was used to extract the entire region of interest to us. Subsequently, the pyrosequencing of the three-100bp regions were performed using the sequence specific primers (SEQ, Supplementary Table 1) to determine the % methylation at each of the CpG sites within the region. The sequencing data showed that the % methylation was less that 30% at any given CpG site in both the pre-transcription start site as well as the translation start site regions across all the cell lines (Supplementary Figure 1B and 1C). On the contrary, the methylation pattern between the normal and cancer cells was variant in about half of the CpG sites located within the sequenced SINE Alu region (Figure 1B). Within this differential region, PWR-1E exhibited 100% methylation in all the 5 sites, while 2 sites in LNCaP were 100% and 80% methylated. In contrast, DU-145 and PC-3 demonstrated methylation in only 2 sites totally. Together, these results demonstrate that the methylation of SINE Alu region in the MIEN1 putative promoter is definitely lost in cancer compared to normal cells, supporting the known phenomena of global hypomethylation in cancer.

MIEN1 expression is altered upon pharmacological inhibition of DNA methylation.

Since MIEN1 promoter has a SINE Alu repeat which was hypermethylated in normal cells but hypomethylated in cancer cells, a pattern corresponding to the high expression of MIEN1 in cancer compared to normal cells, we next determined the effects of DNA demethylation on MIEN1 expression. The most effective and commonly used global inhibitor of DNA methylation is 5-Aza-

Figure 1

A

-1057

ccagagctgcaaaatgggaataatgaCGccagccccccagtgagaggctggatgagaagggatgCGcagaaactgttgggggcacagagcctgAAGA
GCATCTACTGTTTTCCAGTGCAGGCAAGAAGCAGCTGCAAAAGACTGGAAAAAGCAGTTTAGAGC
TTTGGGATCAGAAACACTTGTGTATGTAACTACAGGGTGGTTCCAAATGCCATCAGCAcCGggCGtgg
tggctcaCGcctgtaatcccagcactttgggaggccaaggcaggcagatcatttgaggtcaggagttCGagaccagtctggCGaacatggtgaaaccctgtc
tctactaaaacaaacaaacaaacaaacaaacaaAAACTGggccaggCGCCGgtggctcatgtctgtaattccagcactttaggaggcCGaggctggtggatc
acctgaggtcaagagttCGagaccagcctgaccaacatgatgaaaccctgtctctactaagaatacaaaattaacCGggtgCGgtggcaCGCCGcctgta
gtcccagctactCGggaagctgaggcagaagaatCGcttgaacccGGgaggcagaggttgcagtgaaccGagatCGCCGccattgcactccagcctg
ggcaacaagagCGaaactcCGtctcaaaaaaaaaaaaaaaaaaaaTGCCATCGGCATGTTAGCGAGAAAATGTCAACTAT
TTCATGCATCAGCCACACCCACCCCCATCCCCAGGATGCTTGCCCCACCACTTTCTCTTTCTTTTGTA
AACTGAAAAGCGCTTTGCAGTCTAAGATAGTCCATTAGAGTAAATGAAGCCATGAAGTCCAGCGG
ACACCGGGAGTGGGGAGTGGGGAAGCCCGGCACTCCGGGAGACCGGGCCAGGGAAGGAGGGTCT
GGACCGGACCCAGCCCCTGCCCGGGGAGCGAGCTCCGGAGCTGCCCTACGAGGTCAAAACGTAG
CAGTGGCGGAGACCCGCAGGGGGCGCCCGAACGCCACCCTCGGCCCCTCCCCGCTCCAGAGGCC
CCGCCCCGTCACGTGCCCGCGGTTCGCGTCACACCCGGAAGCAGGGGCCCGAGCGGAGCCGGC
CGCCGATGAGCGGGGAGCCGGGGCAGACGTCCGTAGCGCCCCCTCCCGAGGAGGTCGAGCCGGG
CAGTGGGGTCCGCATCGTGGTGGAGTACTGGTGAGCGGCCCCGGCTGGAGGACCCGCACCCTGGT
CCCGCGGGCCGGACGGAGGTGGGTCCACGGAGGCCCCACCCCCGAATCCCCAGCCCAGCCCCA
TCTCTTGACTCCCCAGTGAACCCTGCGGCTTCGAGGCGACCTACCTGGAGCTGGCCAGTGCTGTGA
AGGAG **+299**

Transcription Start Site **Translation Start Site**

SINE Alu Region (SEQ 1)

Pre-Transcriptional Start Site Region (SEQ 2)

Translation Start Site Region (SEQ 3)

B

SINE Alu Region (SEQ 1)	**CpG methylation (100% - black; ≥80% - grey; <80% - white)**								
Relative to TSS	**-552**	**-546**	**-538**	**-536**	**-516**	**-494**	**-484**	**-460**	**-454**
PWR-1E									
LNCaP									
DU-145									
PC-3									

Figure 1

MIEN1 promoter. (**A**) MIEN1 promoter sequence from UCSC: CpG dyads and islands are underlined and in red; the SINE Alu repeat is represented in lower case. (**B**) Bisulfite sequencing based % methylation at the CpG sites within the SINE Alu region with respect to the technical control of DNA treated with SssI methyltransferase (100% methylation).

2’-deoxycitidine (5-Aza-2’dC). PWR-1E and DU-145 (which have doubling times of ~ 35 and 26 hours respectively) cells were exposed to varying concentrations of 5-Aza-2’-dC for 72 hours. The RNA and protein were extracted and subjected to qPCR and western blotting to determine the expression of MIEN1 transcript and protein. Our results clearly show an increase in MIEN1 RNA (Figure 2A) and protein (Figure 2B) in PWR-1E cells treated with 5-Aza-2’dC compared to the vehicle treatment. Interestingly, the RNA (Figure 2A) and protein (Figure 2C) levels remained unaltered in DU-145 cells upon the treatment. When we shortened the 5-Aza-2’-dC treatment to 48 hours, such that the cells would only undergo one replication cycle before collecting the RNA and protein, we observed the same patterns; an increase in MIEN1 expression in PWR-1E (Supplementary Figure 2A and 2B) and no change in DU-145 (Supplementary Figure 2A and 2C) cells. The expression of MIEN1 remained constant in PC-3 cells also (Supplementary Figure 2D). Next, to determine if the effects observed were a result of hindering maintenance DNA methyltransferase, DNMT1, we treated PWR-1E and DU-145 cells with the pharmacological inhibitor of DNMT1, procainamide (PCN), at a range of concentrations. MIEN1 expression was significantly induced at the transcriptional and protein levels after 96 hours of procainamide treatment in PWR-1E cells, which inherently express low to negligible MIEN1 (Figure 3A and 3B). As seen earlier with the 5-Aza-2’dC treatment, no induction of MIEN1 was observed in DU-145 cells upon treatment with any concentration of procainamide (Figure 3C and 3D). Additionally, procainamide treatment of PC-3 cells also showed no changes in MIEN1 expression levels (Supplementary Figure 2E and 2F). A closer look at the fold changes revealed that the MIEN1 mRNA was about 3- to 5-fold higher than the control upon 5-Aza-2’dC treatment (depending on the concentrations used), but with procainamide treatment, the maximum increase was ~2-fold, thus indicating the role of both maintenance as well as *de novo* methyltransferases in

Figure 2

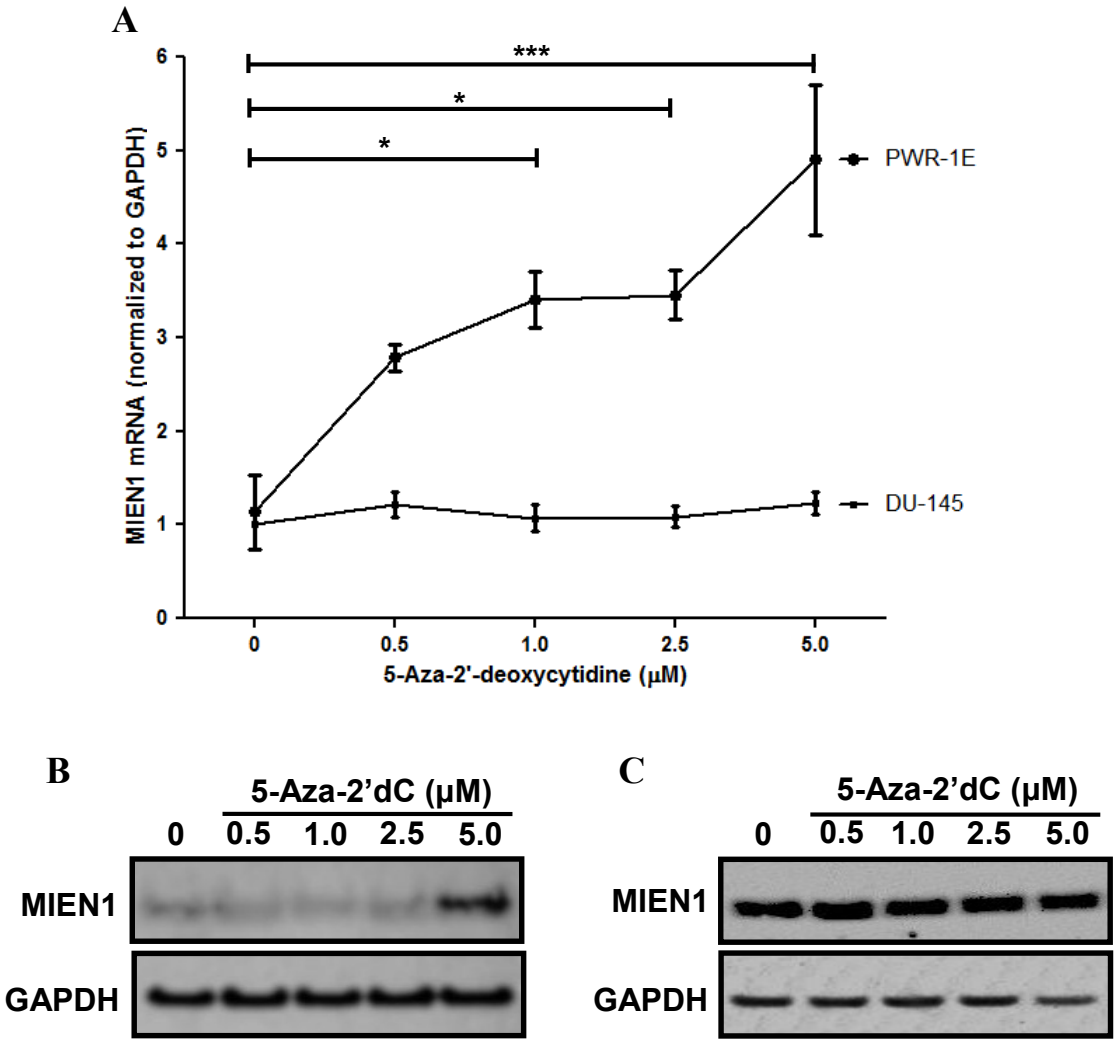

A
***
*
*
MIEN1 mRNA (normalized to GAPDH)
PWR-1E
DU-145
0
0.5
1.0
2.5
5.0
5-Aza-2'-deoxycytidine (μM)
B
5-Aza-2'dC (μM)
0 0.5 1.0 2.5 5.0
MIEN1
GAPDH
C
5-Aza-2'dC (μM)
0 0.5 1.0 2.5 5.0
MIEN1
GAPDH

Figure 2

MIEN1 expression upon treatment with the nucleoside analog, 5-Aza-2'-deoxycitidine. (**A**) qPCR showing the MIEN1 expression normalized to GAPDH (internal control) upon different concentrations of 5-Aza-2'-dC treatment in PWR-1E and DU-145 cells. (**B-C**) Western Blotting showing the MIEN1 expression upon 5-Aza-2'-dC treatment in (**B**) PWR-1E and (**C**) DU-145 cells; GAPDH was used for normalization.

The *P-values* were computed using Student's t-test between the control and the indicated concentrations of 5-Aza-2'dC treatments. $***P \leq 0.001$, $*P \leq 0.05$.

Figure 3

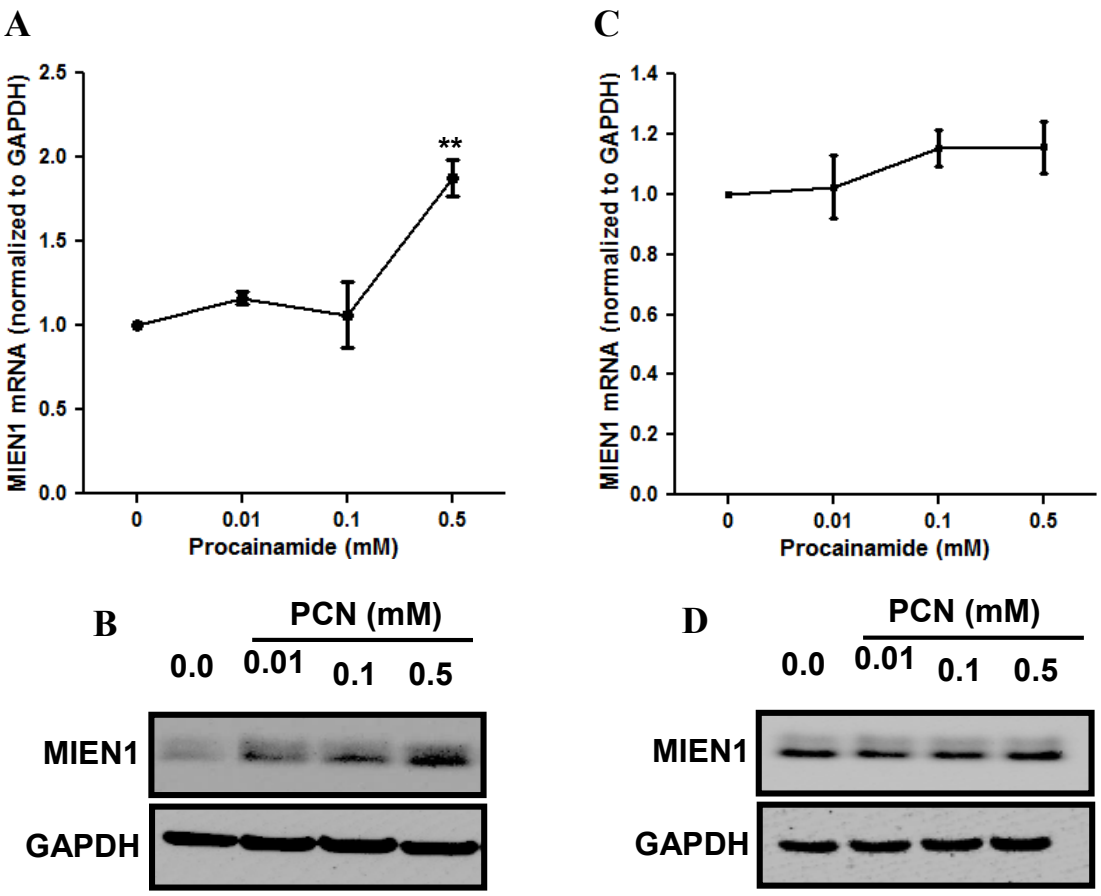

A
MIEN1 mRNA (normalized to GAPDH)
2.5
2.0
1.5
1.0
0.5
0.0
**
0
0.01
0.1
0.5
Procainamide (mM)
C
MIEN1 mRNA (normalized to GAPDH)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
0.01
0.1
0.5
Procainamide (mM)
B
PCN (mM)
0.0
0.01
0.1
0.5
MIEN1
GAPDH
D
PCN (mM)
0.0
0.01
0.1
0.5
MIEN1
GAPDH

Figure 3

MIEN1 expression upon treatment with the non-nucleoside inhibitor, Procainamide. MIEN1 expression normalized to GAPDH (internal control) upon different concentrations of Procainamide treatment in (**A-B**) PWR-1E and (**C-D**) DU-145 cells, as depicted by (**A, C**) qPCR and (**B, D**) Western blotting.

The *P-values* were computed using Student's t-test between the control and the various concentrations of procainamide. $^{**}P \leq 0.01$.

the methylation of the MIEN1 promoter region.

A combinatorial inhibition of DNMTs is necessary for the complete demethylation of MIEN1 promoter, leading to MIEN1 expression.

The use of pharmacological inhibitors is often accompanied by side effects and extreme cellular toxicity, not to mention the chances of mutation. Hence, we next used RNA interference technology to determine the effects of each individual DNMT on MIEN1 expression. The PWR-1E cells were transfected with siRNA against DNMT1, DNMT3a, DNMT3b or a combination (DNMT1, DNMT3a and DNMT3b). GFP targeting siRNA was used a non-targeting control. The qPCR analysis showed MIEN1 expression to be slightly elevated upon DNMT1 and DNMT3a knockdown (~1.5-fold), though this was not significant (Figure 4A). On the other hand, silencing all the three DNMTs significantly increased MIEN1 mRNA (Figure 4A). The efficiency of the knockdown of DNMTs was determined by qPCR of the DNMTs at the same time that MIEN1 expression was tested (Supplementary Figure 3). To validate if the increase in mRNA did indeed result in an increase in the MIEN1 protein, total protein was isolated after PWR-1E cells were transfected with siRNA against the DNMTs. DNMT3b was not knocked down since no increase in MIEN1 mRNA was observed upon DNMT3b knockdown. Our results showed an increase in MIEN1 upon knocking down the DNMTs (Figure 4B), though the combined knockdown did not have any additive effect, unlike what was seen at the mRNA level. In DU-145, the knockdown of the DNMTs led to no alteration in MIEN1 mRNA, as anticipated (Figure 4C). Taken together, these results imply that MIEN1 is indeed under the influence of methylation (DNMT1, DNMT3a and DNMT3b). In normal cells, the SINE Alu region in the MIEN1 promoter is methylated thus keeping the transcription of this gene under check; but in cancer, the hypomethylation results in transcription of the gene.

Figure 4

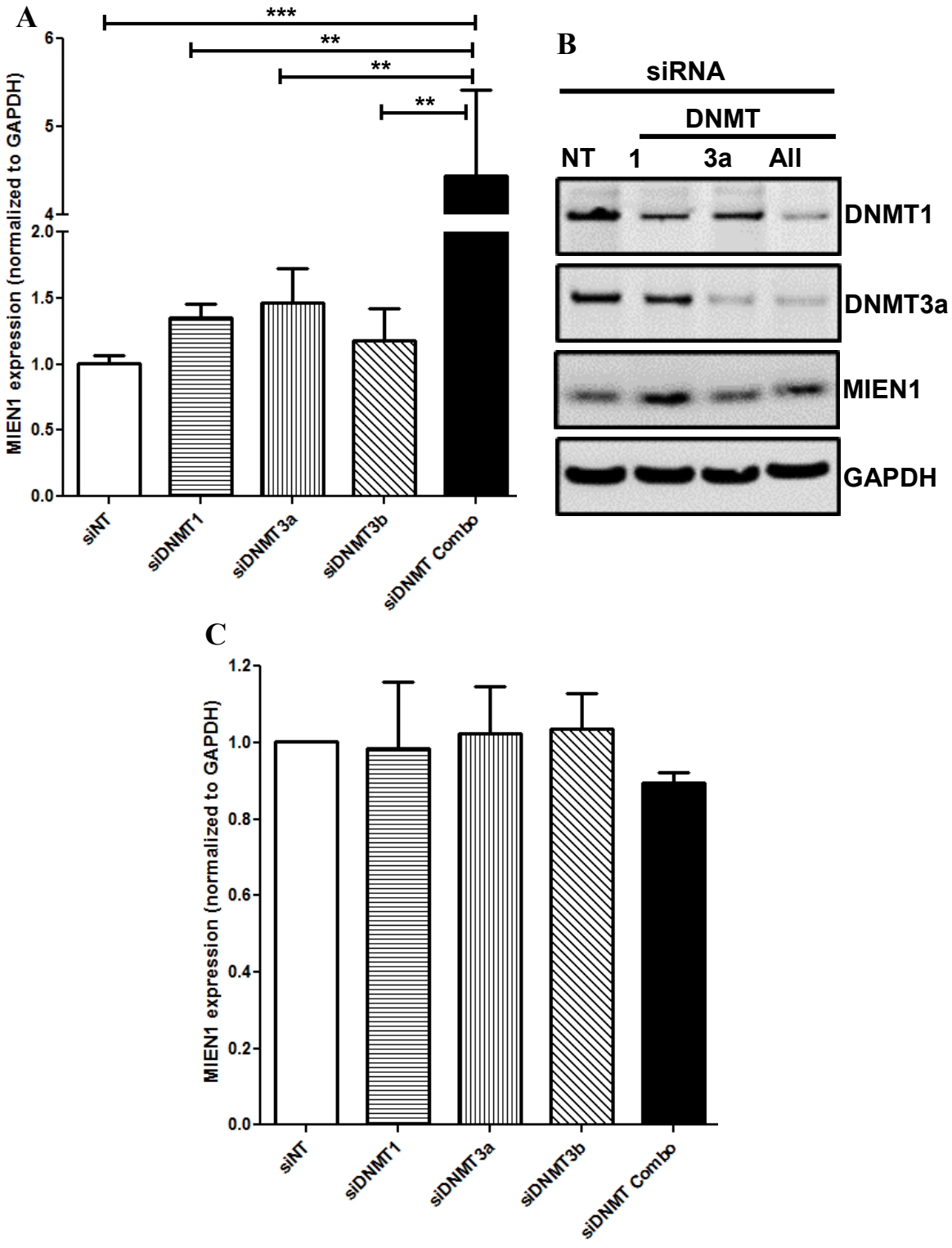

A
***
**
**
**
MIEN1 expression (normalized to GAPDH)
6
5
4
2.0
1.5
1.0
0.5
0.0
siNT
siDNMT1
siDNMT3a
siDNMT3b
siDNMT Combo
B
siRNA
DNMT
NT
1
3a
All
DNMT1
DNMT3a
MIEN1
GAPDH
C
MIEN1 expression (normalized to GAPDH)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
siNT
siDNMT1
siDNMT3a
siDNMT3b
siDNMT Combo

Figure 4

MIEN1 expression upon knockdown of DNA methyltransferases in PWR-1E. (**A-B**) MIEN1 expression upon different DNMT knockdown in PWR-1E cells as shown by (**A**) qPCR and (**B**) Western Blotting. (**C**) qPCR showing the MIEN1 expression in DU-145 cells after silencing DNMTs.

The *P-values* were computed using One-way ANOVA to compare all the groups and then followed by Tukey's Post Hoc comparison to obtain the pairwise significances between the treatments. ***$P \leq 0.001$; **$P \leq 0.01$.

Activity of MIEN1 promoter is influenced by SINE Alu.

To determine the MIEN1 promoter activity, we cloned the different fragments upstream of pGL3-Luciferase vector as described in the methods. Since our results show that methylation reduces the transcription of MIEN1, we constructed fragments of the putative promoter with and without the SINE Alu repeat (Figure 5A and 5B). The reporter assay confirmed our previous findings; the plasmids containing the SINE Alu region (-674/+99, -581/+99) exhibited significantly lower promoter activity compared to plasmids lacking the SINE Alu (-314/+99) in DU-145 (Figure 5C) and PWR-1E (Supplementary Figure 4) cells.

Next, we wanted to determine if the -460 and -454 sites in the SINE Alu region played a role in suppressing the promoter activity, since these sites were 100% methylated in DU-145 cells, according to the bisulfite sequencing (Figure 1B). We transfected DU-145 cells with the various plasmids (Figure 6A) and evaluated the luciferase activity. Our results showed that though the loss of -460 and -454 sites significantly increased the luminescence compared to the cells transfected with the plasmid containing the SINE Alu region, this increase was not significantly different from the loss of the complete SINE Alu (Figure 6B).

It is known that an interplay between various cellular mechanisms and gene repression by methylation exists. We sought to identify elements in the proximity of the transcription start site that could assist the epigenetic regulation by the SINE Alu (Figure 6A). TFSEARCH analysis of the sequence, with stringent parameters, predicted binding sites for Myeloid Zinc Finger 1 (MZF1) and upstream stimulatory factor (USF) (Figure 6C). The luciferase reporter assay showed no significant difference in the activity when the site for MZF1 was deleted; but then loss of the region containing the USF binding site (between -127 and -3) very significantly abrogated the transcriptional activity (Figure 6B).

Figure 5

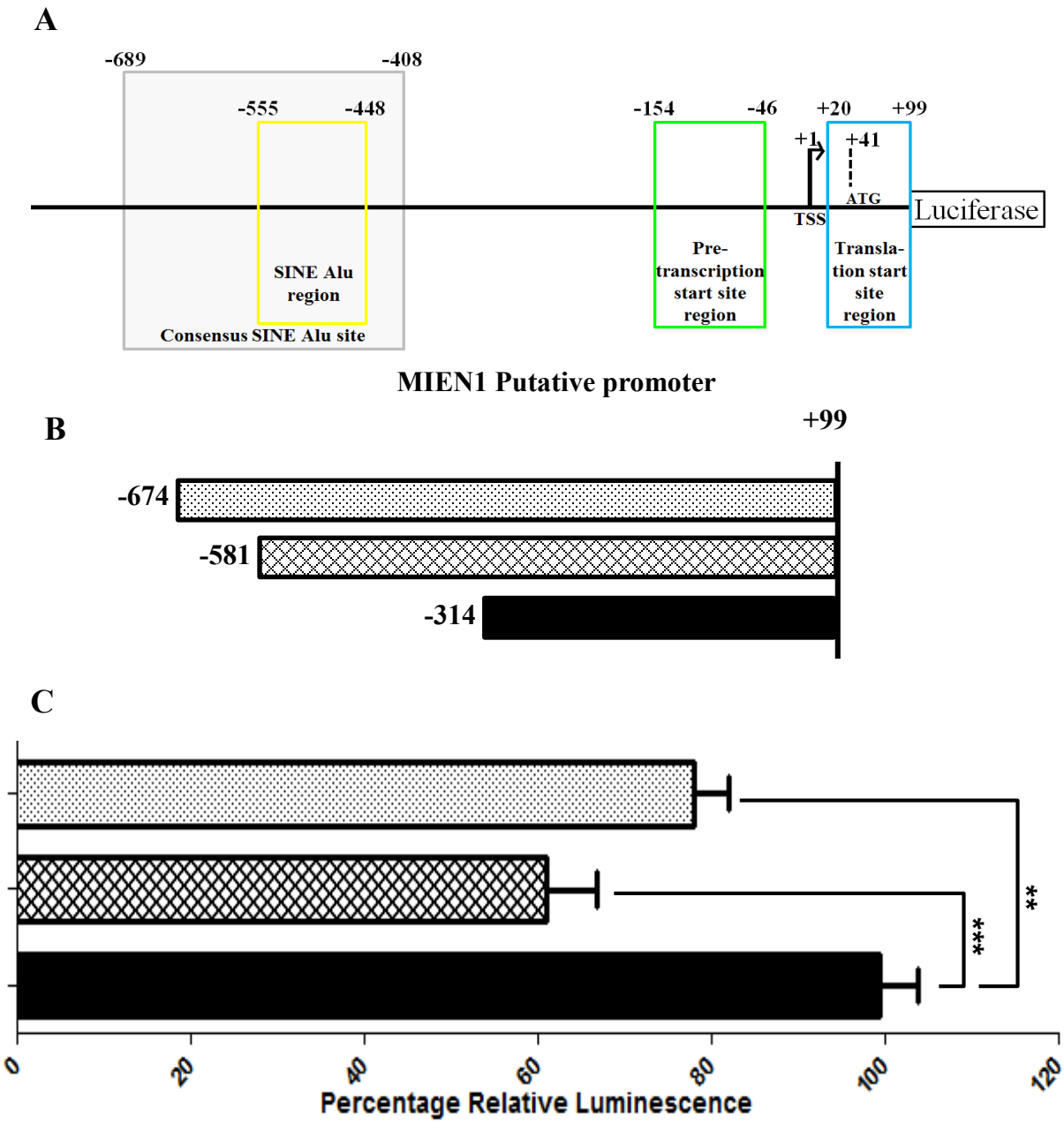
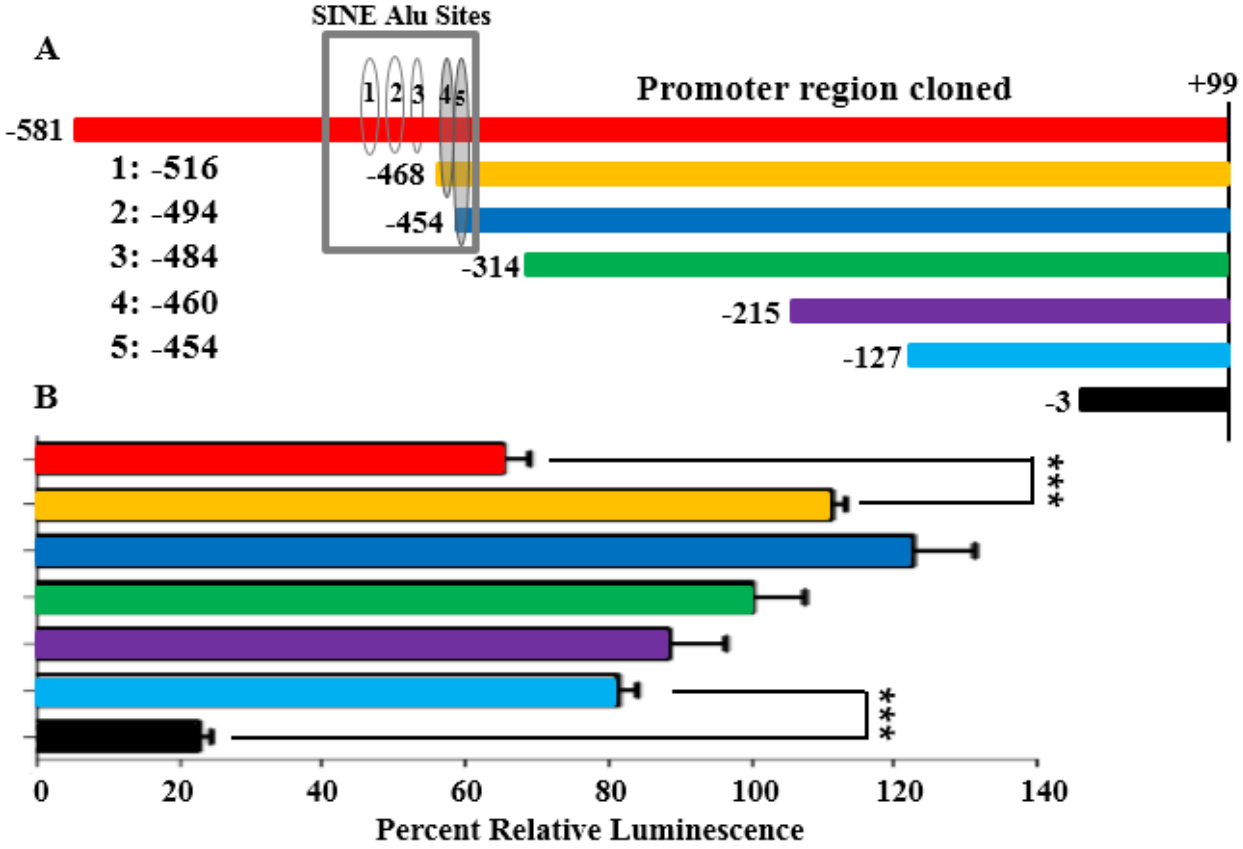

A
-689
-408
-555
-448
-154
-46
+20
+99
+1
+41
ATG
TSS
Luciferase
SINE Alu region
Consensus SINE Alu site
Pre-transcription start site region
Transla-tion start site region
MIEN1 Putative promoter
B
+99
-674
-581
-314
C
**
***
0
20
40
60
80
100
120
Percentage Relative Luminescence

Figure 5

Activity of MIEN1 promoter is influenced by SINE Alu. (**A**) Schematic representation of the regions tested for methylation status. (**B**) Cloning of MIEN1 putative promoter regions in pGL3-basic vector. (**C**) Promoter activity of different MIEN1 promoter constructs, after transfection, read out as relative (Firefly/*Renilla* ratio) luminescence signals.

The *P-values* were computed using Student's t-test between the constructs containing the SINE Alu repeat (-674/+99, -581/+99) and the construct without the SINE Alu (-314/+99). ***$P \leq 0.001$; **$P \leq 0.01$.

Figure 6



C

```
-600 CTGACCAACA TGATGAAACC CTGTCTCTAC TAAGAATACA AAATTAACCG
-550 GGTGCGGTGG CACGCGCCTG TAGTCCCAGC TACTCGGGAA GCTGAGGCAG
-500 AAGAATCGCT TGAACCCGGG AGGCAGAGGT TGCAGTGAAC CGAGATCGCG
-450 CCATTGCACT CCAGCCTGGG CAACAAGAGC GAAACTCCGT CTCAAAAAAA
-400 AAAAAAAAAA AAATGCCATC GGCATGTTAG CGAGAAAATG TCAACTATTT
-350 CATGCATCAG CCACACCCAC CCCCATCCCC AGGATGCTTG CCCCACCACT
-300 TTCTCTTTCT TTTGTAAACT GAAAAGCGCT TTGCAGTCTA AGATAGTCCA
                                                    MZF1
-250 TTAGAGTAAA TGAAGCCATG AAGTCCAGCG GACACCGGGA GTGGGGAGTG
-200 GGGAAGCCCG GCACTCCGGG AGACCGGGCC AGGGAAGGAG GGTCTGGACC
-150 GGACCCAGCC CCTGCCCGGG GAGCGAGCTC CGGAGCTGCC CTACGAGGTC
-100 AAAACGTAGC AGTGGCGGAG ACCCGCAGGG GGCGCCCGAA CGCCACCCTC
                                                  USF
 -50 GGCCCCTCCC CGCTCCAGAG GCCCCGCCCC GTCACGTGCC CGCGGTTCGC
  +1 GTCACACCCG GAAGCAGGGG CCCGAGCGGA GCCGGCCGCG ATGAGCGGGG
 +51 AGCCGGGGCA GACGTCCGTA GCGCCCCCTC CCGAGGAGGT CGAGCCGGG
```

Figure 6

Regulatory elements in the MIEN1 promoter. (**A**) Cloning of various regions upstream of MIEN1 transcription start site into pGL3-basic vector. (**B**) Relative luminescence (Firefly/*Renilla* ratio) obtained upon transfecting cells with the different pGL3 constructs. (**C**) The -600 to +99 region that contained the SINE Alu region and the proximal putative promoter of MIEN1 based on the known transcription start site (NCBI, UCSC) was used as the template, with a threshold set higher than default, in TFSEARCH software to obtain the putative transcription factor binding motifs in the region.

The *P-values* were computed using One-way ANOVA followed by Tukey's Post Hoc test for pairwise comparisons. For promoter activity, the difference between every two consecutive constructs was considered relevant. $***P \leq 0.001$.

Discussion

Cancer progression is a very complex process involving deregulation at various levels [25]. Prostate cancer is one of the few cancers where patients have a high survival chance, if the cancer is diagnosed at an early stage. Despite this advancement, the mortality associated with this cancer is very high. Prostate cancer related death is the result of metastasis [13]. The complexity of the metastatic cascade including the extensive cross-talk [26] that occurs has made treating this aggressive cancer a challenging task. Since cancer is the culmination of alterations at the chromosomal, DNA, RNA and protein levels, logically, the attempts to treat cancer should first involve gaining a better understanding of the altered gene regulatory mechanisms and the molecules that play a role in the process(es) of cancer progression.

MIEN1, a novel gene in the 17q12 region of the human chromosome, plays an important role in prostate cancer progression by enhancing the migration and invasion of cells [21]. This is achieved by activation of the Akt/NF-κB pathway, thereby mediating an increase in certain proteases (MMP-9 and uPA) and angiogenic factors (VEGF), and by altering the actin cytoskeleton structures, namely filopodia, to propel cellular movement [21, 27, 28]. Studies have revealed that MIEN1 expression has poor prognosis for breast cancer [20, 29], and that its interaction with ΔNp73 promotes cisplatin resistance in ovarian cancer [30]. MIEN1 was also implicated to be a "driver" gene in breast and ovarian cancers where HER2/*neu* amplification is evident [22]. In prostate cancer, MIEN1 overexpression led to increased metastatic colonization of the cancer cells [27]. Given that MIEN1 is highly expressed in cancers (breast, prostate, ovarian, oral) compared to normal cells and tissues [20, 21, 30], and that it plays an important role in cancer progression, it was essential to find the mechanism of its regulation in order to use it as a target. MicroRNA mediated regulation of MIEN1 by hsa-miR-940 (submitted), explained how the

MIEN1 mRNA in cells could be prevented from translating into the functional protein. This did not provide any concrete evidence of whether the transcriptional activation machinery for MIEN1 was inherently higher in cancer compared to normal cells.

Epigenetic modifications are key in regulating transcription of genes [2, 9]. Epigenetic modifications include alteration to the histone and/or DNA. DNA methylation is highly deregulated in many cancers [7, 14, 19]. Overall, the belief is that DNA hypermethylation in promoters of specific tumor-suppression related genes causes their repression in cancer. This is accompanied by global hypomethylation of repeat elements causing genomic instability [4, 10]. Even though most of the focus in the past has been with respect to gene-centric hypermethylation, there are increasing reports supporting the importance of hypomethylation in cancer [3, 11, 12, 12, 16-19, 31-34]. Some studies even show that the differences between normal and cancer are more pronounced in terms of hypomethylation status as the cancer progresses and this distinction is at its peak in castration resistant prostate cancers [12, 35]. With the current technology to perform genome wide studies, attempts to identify new biomarkers, for early diagnosis, based on the methylation pattern variance between cancer and normal cells are underway [3, 8, 10, 11, 34, 36]. As a treatment modality, inhibiting global methylation rather than a gene-centric demethylation, has been tried under the premise that such treatments will reactivate the tumor suppressors [2, 7, 18, 37, 38]. Nevertheless, due to the non-specific de-repression mechanism, along with tumor suppressor and cell cycle regulation associated genes, these agents may, in the long run, enhance global hypomethylation which may involve activating tumor promoting genes, resulting in amplification of genomic instability [4, 17]. A perfect example to this scenario is the increase observed in urokinase plasminogen activator because of its demethylation, leading to a more invasive prostate cancer phenotype [31, 33]. Hence, the success of methylation based therapies

reside in identification of the methylation status of target genes that are not only indicative but also integral to cancer progression, and then perform gene specific methylation or demethylation.

In this study, the sequence analysis of the putative MIEN1 promoter region in immortalized normal epithelial cells and prostate cancer cells revealed a constant pattern of hypomethylation of the SINE Alu segment in cancer cells. This is in agreement with the existing literature that SINE Alu regions are very commonly hypomethylated in cancers. The SINE Alu elements belong to class of gene regulatory elements called retrotransposons [24, 39] which have been shown to become less mobile upon genetic regulation [6, 40]. A genome wide study conducted showed that the SINEs enriched in cancers tend to be close to the transcriptional start sites of genes that are hardly methylated in cancer [5, 6], implying the presence of this region less than 550 bases from the transcription start site of MIEN1 to be strong indicator of lower methylation of MIEN1. Also, of the three predicted regions, only the SINE Alu region seemed to show any distinction in terms of methylation between the cancer and normal cells, suggesting the importance of such repeat elements in gene regulation in cancer.

The use of 5-Aza-2'dC as an epigenetic modulator in solid tumors has many associated complications including induction of mutations, lack of stability and cytotoxicity to normal cells, including the neutrophils [2]. Yet, from a molecular biology stand-point, this is the most potent demethylating agent [41], affecting methylation by all the DNA methyltransferases, justifying its use to determine the methylation based regulation of a gene. The immortalized normal epithelial cells exhibited an increase in MIEN1 upon treatment with 5-Aza-2'dC, but no concomitant increase in cancer cells was observed, implying the existence of complete demethylation in cancer instead of a hemimethylated state. Though the toxic effects of the nucleoside analogs was overcome with the use of non-nucleoside inhibitors of methylation, these molecules were in

general much less effective than the former in inhibiting global methylation [42-44]. Procainamide, a specific DNMT1 inhibitor, has been widely studied under this category of drugs [44] primarily due to its existing approval to treat cardiovascular diseases [45]. In this study, though procainamide treatment increased MIEN1 transcript, a more pronounced effect, comparable to 5-Aza-2'dC mediated increase, was achieved only upon silencing all the DNMTs. In contrast, the MIEN1 protein levels were not remarkably different between inhibition of only DNMT1 (by procainamide or siDNMT1) or all the DNMTs, implying the interplay of other factors in RNA and/or protein stabilization when individual DNMTs are targeted.

The presence of SINE repeats in a region near a protein coding gene (usually, gene promoter) has been shown to alter the gene transcription by providing binding access to promoter and enhancer elements for the gene activation, thus leading to oncogenic signaling, during cancer progression [5, 6]. TFSEARCH showed a binding site for USF in the MIEN1 putative promoter, from site -18 to -13 relative to the transcription start site [46]. USF is known to bind to E-box consensus sequence CACGTG and result in overall increase in transcription [47, 48]. This is accomplished by USF first recruiting histone modifying enzymes, leading to H3K4 methylation and histone acetylation at multiple sites, which then acts as a barrier for H3K9 methylation, thereby preventing chromatin condensation and heterochromatin spreading [49]. Reports show that genes bound by USF have a higher chance of possessing a SINE repeat [5]. In terms of Alu elements of the SINE family specifically though, there is an equal likelihood of USF binding to a CpG rich or a non-CpG containing promoter. Also, the fraction of bound to unbound sites is almost equal in either case [5]. While methylation of the E-box element reduces USF binding, USF binding in gene promoters insulates SINE repeat based gene inactivation better than unbound E-box site [5, 47]. Hence, further studies are not only needed to confirm the USF binding in the MIEN1 promoter

(which could lead to aberrant gene activation in cancer), but also to determine the differences in methylation status of the CpG in the E-box and binding of USF between normal and cancer cells. Also, the clinical correlation of this methylation pattern is warranted in order to consider epigenetic targeting of MIEN1.

In conclusion, this study is the first to identify methylation as an important modulator of MIEN1 in prostate cancer progression. With our *in vitro* studies, we established that the SINE Alu in the MIEN1 putative promoter region is hypermethylated in normal cells. The methylation of the MIEN1 putative promoter is dependent on both *de novo* as well as maintenance methyltransferases. Loss of this methylation could potentially open the chromatin structure and make the MIEN1 promoter accessible for transcriptional activation, thereby increasing processes of metastasis, including migration, invasion and EMT. Together, this is an important finding contributing to the knowledge of methylation based regulation of tumor promoting genes in prostate cancer.

Materials and Methods

Cell lines and cell culture - Human prostate carcinoma cells DU-145 (ATCC HTB-81), and PC-3 (ATCC CRL-1435) were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum (Life Technologies). Immortalized non-tumorigenic prostate epithelial cell line PWR-1E (ATCC CRL-11611) was maintained in Keratinocyte-SFM (Life Technologies) supplemented with bovine pituitary extract (25 µg/ml) and recombinant epidermal growth factor (0.15 ng/ml). Cells were cultured at 37°C with 5% CO_2.

Genomic DNA isolation, bisulfite modification and sequencing - Genomic DNA from PWR-1E, LNCaP, DU-145 and PC-3 cells was isolated using the Genomic DNA isolation kit (Sigma)

according to manufacturer's protocol. Bisulfite treatment and sequencing were carried out at University of Nebraska Medical Center. Bisulfite treatment was carried out using 1000 ng of the genomic DNA and the EZ DNA Methylation-Direct kit (Zymo Research, Orange, CA), to deaminate the unmethylated cytosine residues to uracil and leave methylated cytosine residues unchanged. To perform PCR reactions, 32 ng of bisulfite-modified DNA was used as template. The PCR reactions were performed in a total volume of 25 µl for 35 cycles using Roche Diagnostic Corporation (Indianapolis, IN) FastStart Taq DNA Polymerase (1.0U), MgCl2 solution (3.5 mM), dNTP's (0.2 mM), sense primer (0.24 µM), antisense primer (0.18 µM) (Supplementary Table 1), with denaturation at 95 ºC for 30 seconds, annealing temperature for 45 seconds at annealing temperature indicated in Supplementary Table 1, and extension at 72 ºC for 1 minute. A bisulfite sequencing (BSP) primer set was used to PCR and capture the sequence that contained the region to be analyzed. This was necessary to identify this specific region that contained a repetitive SINE Alu element. This PCR product was used as the template for the internal pyrosequencing primer sets SEQ1, SEQ2 and SEQ3 (Supplementary Table 1). All PCR products were electrophoresed on 0.8% agarose gel, stained with ethidium bromide, and visualized for appropriate and pure product before proceeding with all analyses using a Bio-Rad Laboratories (Hercules, CA) Gel-Doc UV illuminator. Methylation percentage of each CpG were determined using a Qiagen (Valencia, CA) Pyromark Q24 pyrosequencer and sequencing primer indicated in Supplementary Table 1, according to manufacturer's recommendations. This experiment was performed once.

Chemicals and treatments - 5-Aza-2'dC and Procainamide were purchased from Sigma-Aldrich and dissolved in DMSO and water respectively. The 1mM 5-Aza-2'dC and freshly prepared 1M Procainamide stock solutions were further diluted in the media (Keratinocyte-SFM or RPMI 1640) to obtain the appropriate final concentrations, as indicated for each treatment. The media with the

chemical was carefully added to cells which were seeded one day before and placed in the incubator for the duration of the experiment. Whenever the duration of the treatment was over 48 hours, fresh media with the same concentrations of the chemicals was added to the cells without removing the existing media.

RNA isolation and qPCR - Total RNA was isolated using TRIzol (Life Technologies) and quantified. Equal amount of RNA was used for the one-step qPCR performed using the Superscript III SYBR Green qRT-PCR kits, according to manufacturer's instructions (Life Technologies) on a Mastercycler ep gradient S realplex^2 thermal cycler (Eppendorf). The primers were designed using Primer 3 [50]and synthesized by Integrated DNA Technologies (Coralville, IA). The sequences of the primers used are: MIEN1 FP-5'*cagtgctgtggagcagt*3', MIEN1 RP-5'*gacggctgttggtgatcttt*3'; GAPDH FP-5'*gagcgagatccctccaa*3', GAPDH RP-5'*actgtggtcatgagtccttc*3'; DNMT1 FP-5'*tacctggacgaccctgacctc*3', DNMT1 RP-5'*cgttggcatcaaagatggaca*3'; DNMT3a FP-5'*tattgatgagcgcacaagagagc*3', DNMT3a RP-5'*gggtgttccagggtaacattgag*3'; DNMT3b FP-5'*ggcaagttctccgaggtctctg*3', and DNMT3b RP-5'*tggtacatggcttttcgatagga*3'.

Antibodies and Western Blotting - The following primary and secondary antibodies were used: Mouse monoclonal MIEN1 (Abnova), mouse monoclonal GAPDH (Santa Cruz Biotechnology), rabbit DNMT1 and DNMT3a (Cell Signaling Technology), and anti-mouse-HRP conjugated and anti-rabbit-HRP conjugated IgG (Promega). Western blotting was performed according to standard protocols. Briefly, once the total protein was isolated using NP-40 lysis buffer, the concentration was estimated using the standard Micro BCA Protein Assay Kit (Pierce Biotechnology). Equal quantity of protein for each sample was run in NuPAGE® Novex® 4-12% Bis-Tris Gels prior to transferring onto nitrocellulose membranes using an iBlot (Life

Technologies). The membranes were blocked in 5% non-fat dry milk and subjected to primary and secondary antibodies before the chemiluminescent reaction was captured by the AlphaImager (ProteinSimple).

Transfections - The smart pool siRNAs against GFP, DNMT1, DNMT3a and DNMT3b were obtained from Dharmacon (Thermo Fisher Scientific) and used at the final concentration of 100nM. The siRNA transfections for RNA interference were performed using Lipofectamine RNAiMAX according to the manufacturer's protocols (Life Technologies). The plasmids (described below) were transfected using Lipofectamine LTX and Plus reagent according to the manufacturer's instructions (Life Technologies).

Plasmids and Luciferase Assay - The Firefly-luciferase plasmid, pGL3-basic, a kind gift from Dr. Myoung Kim (UNT Health Science Center, Fort Worth, TX) was used to construct the different reporter plasmids to measure MIEN1 promoter activity. A series of DNA fragments comprising of the nucleotides -674 (KpnI-5'-ctgggccaggcgcggtacctcatgtctgtaattc-3'), -581 (KpnI-5'-gcctgaccaacatgatggtaccctgtctctactaaga-3'), -468 (KpnI-5'-cgggaggcagaggtaccagtgaaccgagat-3'), -454 (MluI-5'-gtgaaccgagaacgcgtcattgcactccag-3'), -314 (NheI-5'-catccccaggatgctagccccaccacttt-3'), -215 (KpnI-5'-gaagtccagcgggtaccgggagtgG-3'), -127(NheI-5'-cggggagctagctccggagct-3') or -3(MluI-5'-tgcccgcggtacgcgtcacac-3') to +99 (XhoI-5'-cccggctcgagctcctcgggag-3'), relative to the know transcription start site of MIEN1, were PCR amplified and cloned at the KpnI/XhoI or NheI/XhoI or MluI/XhoI sites, upstream of the luciferase gene in pGL3-basic vector. After the sequences were verified (Seqwright), luciferase assay was performed with the plasmids using the dual luciferase assay kit (Promega), according to the manufacturer's instructions and luminescence was measured by a Synergy2 Alpha Microplate Reader (BioTek). In short, the cells were transiently transfected with the firefly pGL3-constructs and *Renilla* pRL-CMV (a kind gift from

Dr. Porunellor Mathew at UNT Health Science Center, Fort Worth, TX) plasmids. Approximately 72 hours after transfection, cells were lysed and both the firefly and *Renilla* luciferase activities from the extracts were detected. The relative luminescence was calculated as a ratio of firefly to *Renilla* luminescence (normalization of firefly with respect to *Renilla* luminescence to account for inter-sample variability). The relative luminescence obtained for -314 to +99 pGL3 construct was designated as 100% and the % relative luminescence was correspondingly calculated for the other plasmids. Each construct was transfected at least five independent times and the relative luminescence for each sample was the average of at least three readings.

Statistical analyses - The results were represented as mean ± S.E.M of three independent experiments, unless indicated otherwise. The *p-value* was calculated according to Student's t-test, or one-way ANOVA with Tukey's Post Hoc test, based on the comparisons made, using GraphPad P-value calculator. *P-value* ≤ 0.05 was considered significant.

Supplementary Figure 1

A

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ttagagttgtaaaatgggaataatgaCGttagttttttagtgagaggttggatgagaagggatgCGtagaaattgttgggggtatagagtttgAAGAGTATT
TATTGTTTTTTTAGTGTAGGTAAGAAGTAGTTGTAAAAGATTGGAAAAAGTAGTTTAGAGTTTTGGG
ATTAGAAATATTTGTGTATGTAATTATAGGGTGGTTTTAAATGTTATTAGTAtCGggCGtggtggtttaCGttt
gtaattttagtattttgggaggttaaggtaggtagattatttgaggttaggagttCGagattagtttggCGaatatggtgaaattttgtttttattaaaataaataaataaat
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tttgaattCGggaggtagaggttgtagtgaatCGagatCGCGttattgtattttagtttgggtaataagagCGaaatttCGttttaaaaaaaaaaaaaaaaaaa
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AGGATGTTTGTTTTATTATTTTTTTTTTTTTTTGTAAATTGAAAAGCGTTTTGTAGTTTAAGATAGTT
TATTAGAGTAAATGAAGTTATGAAGTTTAGCGGATATCGGGAGTGGGGAGTGGGGAAGTTCGGTA
TTTCGGGAGATCGGGTTAGGGAAGGAGGGTTTGGATCGGATTTAGTTTTGTTCGGGGAGCGAGT
TTCGGAGTTGTTTTACGAGGTTAAAACGTAGTAGTGGCGGAGATTCGTAGGGGGCGTTCGAACGT
TATTTTCGGTTTTTTTTCGTTTTAGAGGTTTCGTTTCGTTACGTGTTCGCGGTTCGCGTTATATTCG
GAAGTAGGGGTTCGAGCGGAGTCGGTCGCGATGAGCGGGGAGTCGGGGTAGACGTTCGTAGCG
TTTTTTTTCGAGGAGGTCGAGTCGGGTAGTGGGGTTCGTATCGTGGTGGAGTATTGGTGAGCGGTT
TCGGTTGGAGGATTCGTATTTTGGTTTCGCGGGTCGGACGGAGGTGGGTTTACGGGAGGTTTTAT
TTTCGAATTTTTAGTTTAGTTTTATTTTTTGATTTTTTAGTGAATTTTGCGGTTTCGAGGCGATTTAT
TTGGAGTTGGTTAGTGTTGTGAAGGAG +299

B

Pre-transcription Start Site Region (SEQ 2)	CpG methylation (> 15 – black; ≥ 10% - grey; < 10% - white)									
Relative to TSS	-151	-134	-127	-120	-107	-96	-85	-77	-68	-64	-60
PWR-1E											
LNCaP											
DU-145											
PC-3											

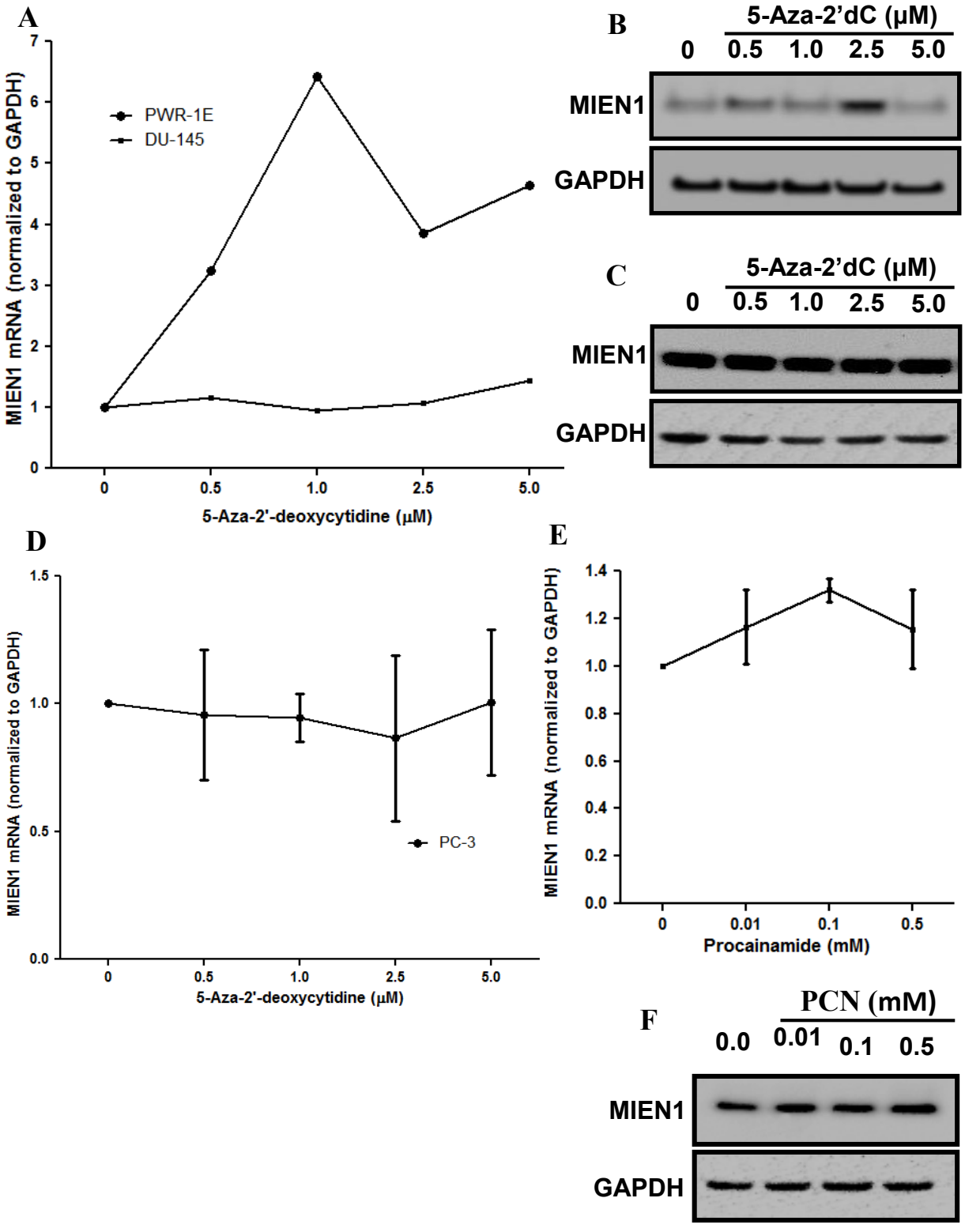
C

Translation Start Site Region (SEQ 3)	CpG methylation (> 15 – black; ≥ 10% - grey; < 10% - white)													
Relative to TSS	23	27	33	37	39	46	54	63	67	72	82	91	96	110	115
PWR-1E															
LNCaP															
DU-145															
PC-3															

Supplementary Figure 1

MIEN1 promoter. (**A**) Sequence that will be obtained upon bisulfite treatment if all the CpGs in the regions of interest are methylated. The three regions of interest here are represented in yellow (SINE Alu), green (Pre-transcription start site) and blue (Translation start site). The primers used are indicated in other colors as in Supplementary Table 1. (**B**) Bisulfite sequencing based % methylation at the CpG sites within the pre-transcription start site region. (**C**) Bisulfite sequencing based % methylation at the CpG sites within the translation start site region.

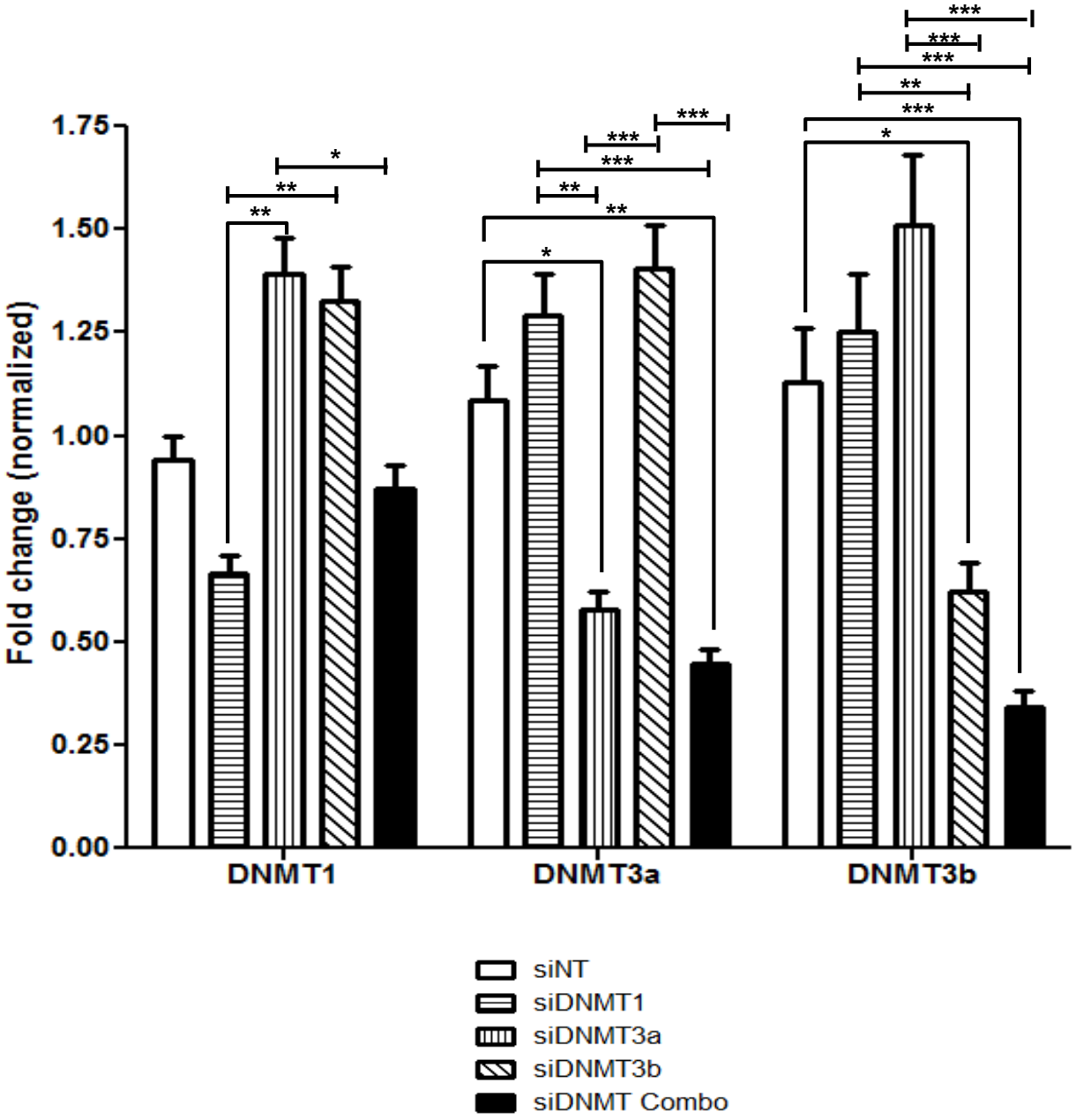
Supplementary Figure 2



Supplementary Figure 2

MIEN1 expression upon treatment with pharmacological inhibitors. (**A-D**) Nucleoside analog, 5-Aza-2'-deoxycitidine treatment: (**A**) qPCR showing the MIEN1 expression in PWR-1E and DU-145 cells, (**B-C**) Western Blotting showing the MIEN1 expression in (**B**) PWR-1E and (**C**) DU-145 cells, (**D**) qPCR showing the MIEN1 expression in PC-3 cells. (**E-F**) Treatment with the non-nucleoside inhibitor, Procainamide showing MIEN1 expression in PC-3 cells, as depicted by (**E**) qPCR and (**F**) Western blotting. GAPDH was used for normalization.

Supplementary Figure 3

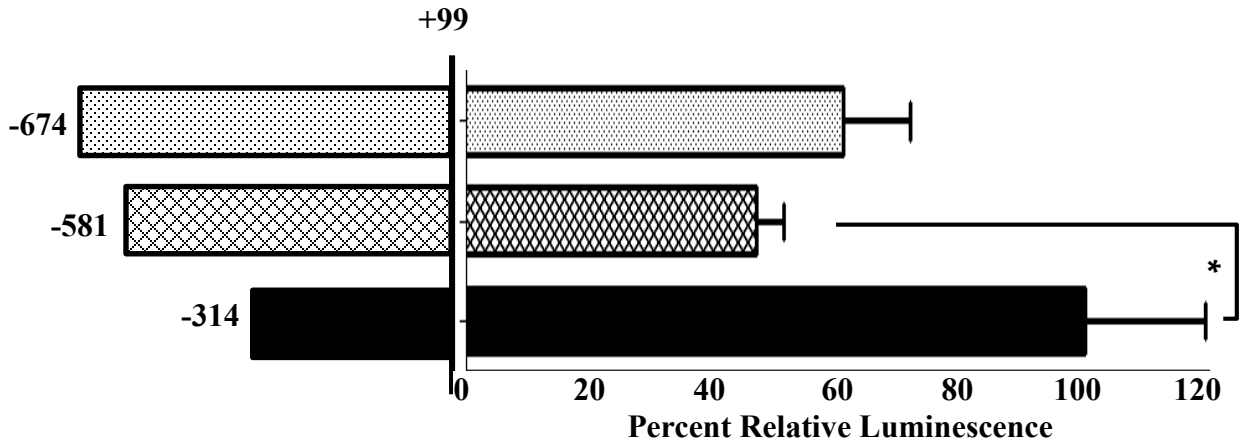


Supplementary Figure 3

qPCR for DNMTs. DNMT1, DNMT3a and DNMT3b expression in PWR-1E upon knockdown of DNA methyltransferases, DNMT1, DNMT3a, DNMT3b or the combination of DNMT1, 3a and 3b compared with the respective control non-targeting knockdown.

The *P-values* were computed using one-way ANOVA between the groups for each mRNA measured (mRNA: DNMT1, DNMT3a or DNMT3b; groups: siNT, siDNMT1, siDNMT3a, siDNMT3b and siDNMT Combo), followed by the Post Hoc test to obtain the pairwise significances between the groups. $***P \leq 0.001$; $**P \leq 0.01$; $*P \leq 0.05$.

Supplementary Figure 4



Supplementary Figure 4

Activity of MIEN1 promoter is influenced by SINE Alu. (**Left**) pGL3 plasmids cloned with the different MIEN1 putative promoter regions. (**Right**) Promoter activity of different MIEN1 promoter constructs, after transfection in PWR-1E, read out as relative (Firefly/*Renilla* ratio) luminescence signals.

The *P-values* were computed using Student's t-test between two consecutive constructs. $*P \leq 0.05$.

Supplementary Table 1

Primers for PCR amplification of the promoter regions and for bisulfite sequencing.

	Sequence	PCR (bp)	Anneal (°C)
BSP Sense primer (5' to 3')	GGTTGGTGGATTATTTGAGGTTAAGAG	935	63
BSP Anti-sense primer (5' to 3')	CTTCACAACACTAACCAACTCCAAATAA		
SEQ 1 Sense primer (5' to 3')	GAAATTTTGTTTTTATTAAGAATATAA	164	58
SEQ 1 Anti-sense primer (5' to 3')	TCTTATTACCCAAACTAAAATACAATA		
SEQ 1 Sequencing Primer (5' to 3')	TTTTTATTAAGAATATAAAATT		
SEQ 2 Sense primer (5' to 3')	GGAGATYGGGTTAGGGAAGGAGGGTTT	161	62
SEQ 2 Anti-sense primer (5' to 3')	AAACRAAACCTCTAAAACRAAAAAAAAC		
SEQ 2 Sequencing Primer (5' to 3')	GGTTAGGGAAGGAGGGTTTG		
SEQ 2 Sense primer (5' to 3')	GTTYGYGTTATATTYGGAAGTAGGG	152	62
SEQ 2 Anti-sense primer (5' to 3')	CAACCRAAACCRCTCACCAATACTCCAC		
SEQ 2 Sequencing Primer (5' to 3')	TTATATTYGGAAGTAGGGG		

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CHAPTER V

SUMMARY AND FUTURE DIRECTIONS

Discussion and Implications in Cancer

Over a century ago, Dr. Theodor Boveri, a German biologist, made some predictions about cancer initiation and progression. The understanding we have gained through experiments confirm that his predictions are alarmingly accurate; right from the presence of cell cycle checkpoints, oncogenes and tumor suppressors, tumor progression and genetic predisposition, to the sensitivity of cancer cells to radiation [1]. Apart from the proof of existence of these phenomena, various scientists have also tried to provide explanations on how these aberrations are caused. Gene regulation is the simple, yet all-encompassing explanation, which includes control at the chromosomal, transcriptional, post-transcriptional, translational, post-translational and epigenetic levels, to maintain homeostasis. Deregulation of a variety of these mechanisms results in genomic instability, as observed in cancer. Of the many cancers, prostate cancer is the second leading cause of death, a consequence of metastasis. Metastasis, a multi-step process including escape of cell death, proliferation, angiogenesis, invasion, migration and homing and growth of tumor at secondary site, is primarily facilitated by various signaling molecules that not only promote the tumor growth but also alter the microenvironment [2, 3]. These signaling molecules are the direct result of disrupted gene regulatory events.

This study focuses on the post-transcriptional and epigenetic regulation of a novel protein, MIEN1, in prostate cancer. MIEN1, expressed in the 17q12 region of the human chromosome, 505 nucleotides away from the HER2/*neu* gene in a tail-to-tail arrangement, plays an important role in cancer progression by promoting the Akt/NF-κB pathway and facilitating filopodia formation [4-6]. MIEN1 also has an important selenium binding domain and an ITAM motif which are currently under study in our laboratory. Interestingly, immunohistochemical analysis by Evans *et al.*, showed that while many normal tissues including adrenal gland, blood and bone marrow lacked MIEN1 expression, the Leydig cells and some normal breast ductal epithelium exhibited some expression of the protein [4]. In contrast, MIEN1 expression was remarkably high in many cancers including breast and prostate [4, 5]. Other tumor antigens which demonstrate such tissue distribution have been classified as cancer/testis antigens and are potentially implicated as diagnostic and therapeutic targets [7]. In order to validate the potential use of MIEN1 as a target for prostate cancer, identification of the molecular mechanisms that regulate this gene in normal cells was needed. This study is an effort to achieve this goal, and determine if these regulatory elements are disturbed in cancer. This understanding will advance our knowledge with respect to MIEN1 and its regulators in prostate cancer and ultimately may even help in development of diagnostic and/or therapeutic agents. To study the regulation of MIEN1, we used the *in vitro* cell culture system; PWR-1E and HPV-18C-1, immortalized normal epithelial cells, LNCaP, an androgen dependent prostate cancer cell and DU-145 and PC-3, androgen independent prostate cancer cells, in the various experiments.

Our rational to study post-transcriptional regulation of MIEN1 by miRNA was based on the lack of protein expression in PC-3 cells, though there was mRNA, apart from the distinctive differential expression of MIEN1 mRNA and protein between normal and cancer cells. The

bioinformatics and microarray based analysis helped in identifying potential miRNA that could regulate MIEN1. Further experiments demonstrated that MIEN1 could be regulated by a novel microRNA, hsa-miR-940. Upon examination, we found that the miR-940 was highly expressed in normal cells while its expression was lost in cancer, an inverse correlation to MIEN1 expression pattern. The higher expression of miR-940 in the normal and benign glands compared to the matched cancer tissues, from 15 patients, proved the clinical relevance of miR-940. To validate the regulation, we ectopically overexpressed or inhibited miR-940 in the different cells. Our results confirmed the ability of miR-940 to directly alter MIEN1 expression. MiRNAs are known to be able to target many mRNAs based on the availability of the target mRNA pool and the seed sequence complementarity of the miRNA to the mRNA [8-10]. Hence, the overall function of any miRNA cannot be assumed based on its regulation of a single target. To define the function of miR-940 in cancer progression, the effect of miR-940 on some of the hallmarks of cancer was tested. We observed that miR-940 decreased migratory and invasive capacities of the cells, functions facilitated by MIEN1. Anoikis resistance, another critical step in cancer progression and metastatic dissemination, includes anchorage-independent growth and epithelial-to-mesenchymal transition capabilities of the cells [11]. Ectopic expression of miR-940 in cancer cells not only abrogated EMT but also their anchorage-independent growth, thus implicating the role of miR-940 in induction of anoikis sensitivity. Analysis using bioinformatics algorithms predicted many targets of miR-940 to be involved in cancer progression.

The expression variances observed in miR-940 between cancer and normal cells led us to the next important question: could miR-940 be a serum biomarker, along with PSA. To address this, our preliminary experiments were to determine miR-940 expression in the conditioned media (cell culture supernatant). Interestingly, our results showed elevated expression of the miRNA in

the supernatant. This was contradictory to the expression pattern observed in cells. But, miRNA are known to be secreted out of the cells in various forms and reports predict the use of such circulating miRNA as biomarkers for disease prediction and prognosis [12-17]. We predicted that miR-940 was packaged into exosomes and secreted out of the cancer cells, thereby inhibiting its role in prevention of cancer progression. As hypothesized, isolated exosomes from cancer cell supernatant showed an increase in both the mature as well as the precursor forms of the miRNA compared to the normal counterpart. Based on these observations, our next task was to evaluate the expression of the miR-940 in human serum from healthy donors as well as cancer patients. Though there was a change, this did not provide any significant differences compared to the PSA test, owing to the small sample size. Thus, a more detailed study, with larger number of samples, is required to determine the use of miR-940 as a better biomarker for (a) prostate cancer diagnosis, and (b) differentiation between indolent and aggressive disease.

Even though miR-940 was identified as a regulator of MIEN1, there was still no clear understanding of the deregulation that led to its increased transcriptional activity in cancer. The proximity of MIEN1 with HER2/*neu* was sufficient to explain co-amplification of the gene (possibly copy number variations) [18]. But presence of MIEN1 on the negative strand (opposite to HER2), suggested that the transcriptional regulation leading to increased mRNA expression was not an amplicon effect. Scanning the putative promoter region of MIEN1 showed CpG rich islands. Almost all of the cancer/testis antigens have been demonstrated to be regulated by DNA methylation [7]. Bisulfite sequencing showed MIEN1 to be hypermethylated in its SINE Alu region compared to hypomethylation of the same region in cancer cells. Demethylation reversed the repression on MIEN1 transcription in the normal cells. Closer look at the region adjacent to the transcription start site revealed an E-box element. This implies that MIEN1 transcription may

be activated by binding of USF to an unmethylated E-box in cancer cells, which are known to exhibit global hypomethylation [19]. Conversely, since SINE repeats that are insulated by USF binding in the gene promoters are better tolerated, we believe that USF binding is lost in normal cells and the SINE Alu is methylated, resulting in MIEN1 transcriptional repression [20, 21].

In conclusion, this study successfully identified a post-transcriptional and an epigenetic regulation of MIEN1 (Figure 1). The miR-940 not only down regulates MIEN1 but also negatively influences cancer progression, globally. Though use of miR-940 as a biomarker in tissues and serum shows potential, it is still at its infancy and needs extensive validation. Finally, the loss of methylation leading to increased expression of MIEN1 in cancer questions the existing approach of using global hypomethylating agents as cures for cancer.

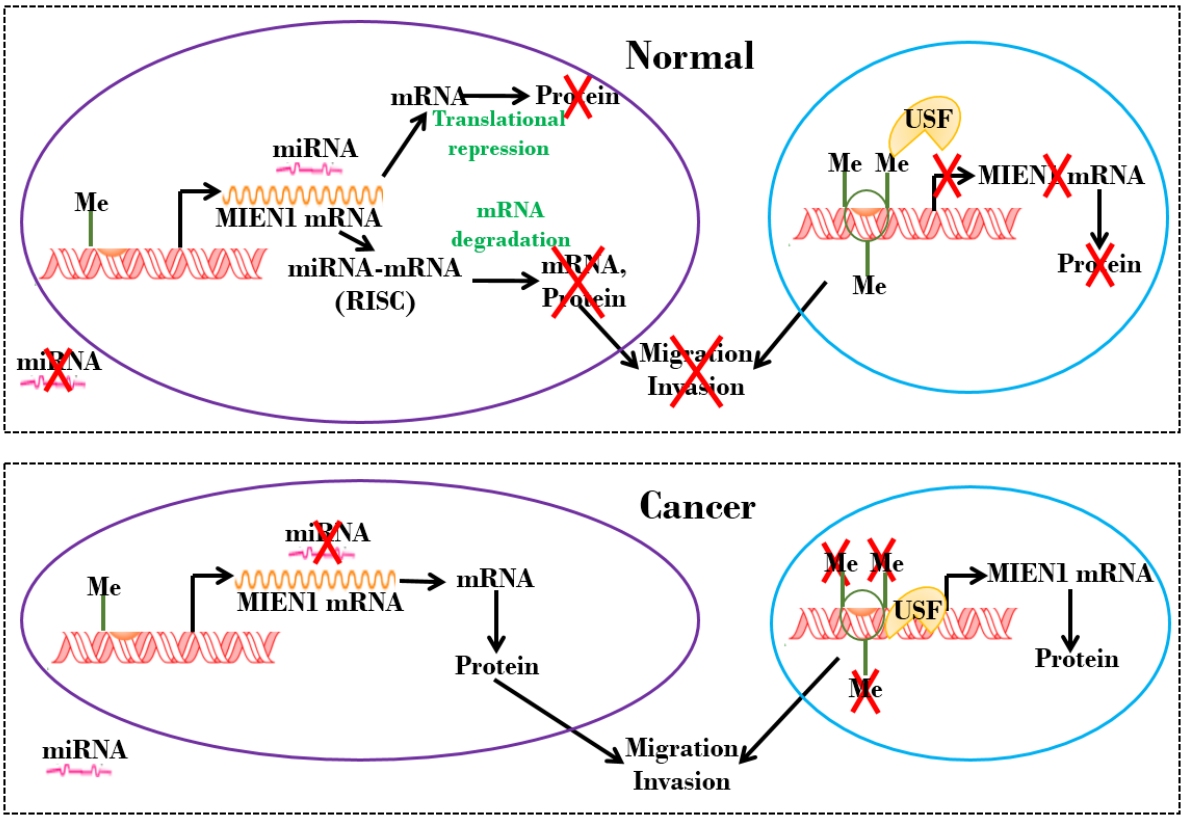

Figure 1: MODEL

(Top) In normal cells, MIEN1 promoter is methylated, preventing transcription; additionally, if any transcript is made, it is targeted by miR-940; together, these mechanisms inhibit MIEN1 expression. (Bottom) In cancer, MIEN1 promoter is hypomethylated, USF potentially binds, activating MIEN1 transcription; additionally, the miRNA transcribed is shuttled out of the cells and into circulation thereby preventing its action on MIEN1 transcript; together, these mechanisms enhance MIEN1 expression.

Future Directions

The global role of miR-940 in cancer progression was examined on an *in vitro* platform. The efficacy of using miR-940 as a therapeutic agent is dependent on its ability to affect tumor progression *in vivo* [22]; experiments towards achieving this goal are in progress. In order to use miR-940 as a marker/therapy, its expression in other cells and tissues and the other target mRNAs have to be carefully examined [23]. Additionally, a large scale validation study with appropriate controls have to be used to determine the use of miR-940 as a serum biomarker.

The role of histones in epigenetic regulation is, at times, closely linked with the DNA methylation [24]. In our study, since we only confirmed DNA methylation pattern, histone modifications are yet to be explored. Though we predict USF binding and activation of MIEN1, this needs to be experimentally demonstrated, along with confirmation of E-box methylation in normal cells. Assessing methylation status of MIEN1 in clinical tissues may add to the list of epigenetic markers for prostate cancer diagnosis [23]. The effects of demethylating agents, that are currently in clinical trials, on MIEN1 expression and hence, tumor progression, have to studied [25].

There is an increasing amount of evidence suggesting extensive cross-talk between the regulatory mechanisms [24, 26, 27]. In our study, we have identified miRNA and DNA methylation mediated regulation of MIEN1 as independent mechanisms; but they could be interrelated and this needs further investigation. Additionally, though we believe both the mechanisms are equally importance, the precedence of these regulations, specifically with respect to MIEN1 expression pattern and globally with respect to prostate cancer progression have to be evaluated.

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