Identification and Characterization of a Human Prostate Cancer Specific Long Non-Coding RNA

Joseph Mazar and Ranjan J. Perera

***Abstract*—The human genome is transcribed in both (sense and anti-sense) directions and this dynamic process generates a large number of transcripts. Interestingly, the majority of these transcripts do not code for proteins, and among these non-protein-coding transcripts, exist a group of long non-coding RNAs (lncRNAs) known to play an important role in disease genesis and development in human. Here we report the identification and characterization of one such lncRNA specific to prostate cancer. Microarray and qRT-PCR analysis confirmed that lncRNA *AF086453* is consistently down-regulated in all prostate cancer cell lines surveyed in this study compared to normal prostate epithelial cells. Bioinformatics analysis revealed that this lncRNA is located immediately downstream of Forkhead Box Protein N1 (*FOXN1*). *FOXN1* is also down-regulated in prostate cancer; therefore, *AF086453* may likely to be regulated by the *FOXN1* regulatory units. Prostate cancer cell line PC3 was engineered to ectopically express *AF086453* and the transfected cells demonstrate defects in cell growth and invasion. These results suggest that *AF086453* may play an important role in prostate cancer development and may act as a useful diagnostic marker for prostate cancer detection in humans.**

***Index Terms*—Gene expression, invasion, long non-coding RNAs, prostate cancer.**

I. Introduction

The role of signaling molecules and transcription factors in cancer development is well appreciated. More recently, non-coding RNAs (microRNAs and long non-coding RNAs) have emerged as important regulatory molecules for the normal function of most eukaryotic cells. Some of these non-coding RNAs may influence cancer development because of their function as tumor suppressors or oncogenes. Long non-coding RNAs (lncRNAs) are transcribed and expressed in a developmentally and disease-regulated manner and their function in the genome is a source of great interest [1]–[7]. They are also known to control various aspects of transcriptional and post-transcriptional mRNA processing, and may further regulate mRNA function by binding and masking key elements within the coding sequence. Examples are elements that may be required for the binding of *trans*-acting factors in post-transcriptional gene expression, such as splicing, transport, translation and degradation of pre-mRNA [8]. Non-coding RNAs are also transcribed by mechanisms similar to those involved in the transcription of most protein-coding genes, and therefore, not surprisingly, abnormal regulation of non-coding RNA genes may induce prostate cancer development and progression in humans. Fundamental questions about the interaction of microRNAs, mRNAs and long non-coding RNAs with prostate cancer development remain unanswered. Systematic sequencing of protein-coding genes has led to the identification of novel mutated genes involved in several cancer types [9]–[13]. We have recently demonstrated that siRNA-mediated knockdown of the melanoma up-regulated lncRNA *SPRY4-IT1* causes defects in cell growth and differentiation, and increases the apoptotic rate of melanoma cells [14]. However, little is known about the molecular function of this long non-coding RNA.

The primary focus of this manuscript is the characterization of one specific lncRNA, (Genbank accession ID *AF086453*), which was recently identified in our laboratory as one of the most highly down-regulated lncRNAs in numerous prostate cancer cell lines compared to prostate epithelial cells. This lncRNA is located immediately downstream of the transcription factor *FOXN1* [15] and its regulation may be directly effected by *FOXN1*. We hypothesize that together; *FOXN1* and lncRNA *AF086453* may play a key role in prostate cancer development and progression in men.

Prostate Cancer is one of the leading causes of cancer deaths among American men. In 2010, an estimated 190,000 new cases of prostate cancer were diagnosed, and for 27,000 men the disease will likely be fatal. This observation suggests that there is an unmet need to develop better therapeutics and accurate biomarkers for prostate cancer. Our preliminary results show that ectopic expression of *AF086453* in prostate cancer cells causes significant defects in both cell growth and invasion. These preliminary data, together with the increasing recognition that lncRNAs play important roles in cell transformation has led us to examine *AF086453* to determine if it may be of use in future diagnostic & therapeutic applications.

II. Materials and Methods

A. Cell Lines

Cell lines were purchased from the American Type Culture Collection (Manassas, VA). Experimental studies illustrated in this manuscript used the human prostate cancer cell lines PPC-1 (stage D2), 22RW1, Du145 (stage 4), LNCaP (stage 4), and PC3 (stage 4). Prostate cancer cells were cultured at

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The authors are with Sanford-Burnham Medical Research Institute, Orlando, FL 32827 USA (e-mail: jmazar@sanfordburnham.org, rperera@sanfordburnham.org).

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37℃ [5% (v/v CO_2)] in vitro using RPMI 1640 media supplemented with 10% (v/v) fetal bovine serum.

B. NCode lncRNA Array

One microgram of total RNA was labeled and hybridized to NCode human microarrays (Life Technologies™, Carlsbad, CA, USA) according to the manufacturer's protocols (www.Lifetechologies.com). An Agilent 2 μm scanner was used to scan the slides and the data was normalized and analyzed using GeneSpring software (www.agilent.com). The NCode human array contains over 10,000 putative lncRNAs (>200 nt) including most of the known lncRNAs in human. Lack of coding potential was estimated by a previously described algorithm [14] that scores various characteristics of protein-coding genes, including open reading frame length, synonymous/non-synonymous base substitution rates and similarity to known protein. These arrays are the first generation of tools designed to investigate the dynamic expression of a large subset of lncRNAs in human to identify candidate genes for more detailed functional analysis. In addition to the lncRNA content, probes targeting mRNA content from RefSeq are also included, allowing discovery of coordinated expression with associated protein-coding genes.

C. Quantitative Real-Time PCR

Total RNA was acquired using the Trizol method (Invitrogen/LifeTechnologies) with quantitation and integrity of the sample performed using an Agilent 2100 Bioanalyzer (AgilentTechnologies, Santa Clara, CA, USA). Human prostate epithelial cell total RNA was acquired from ScienCell (ScienCell Research Laboratories). From these samples, total RNA (100 ng) was then reverse transcribed with a High Capacity cDNA kit (Applied Biosystems/Life Technologies) and qRT-PCR was carried out using SYBR Green mRNA Assays on a 7500 Real-Time PCR System (Applied Biosytems/Life Technologies) according to the manufacturer's protocols. SYBR Green primers included AF086453 qPCR For – ggaagcacaagggataccttggc & AF086453 qPCR Rev – ggccaacctgtggaagttcttgtcac and FOXN1 qPCR For – gccattgttcccacagccgg & FOXN1 qPCR Rev – ggccaacctgtggaagttcttgtcac. SDS1.2.3 software (Applied Biosystems/Life Technologies) was utilized for comparative Ct analysis with GAPDH or b-actin acting as the endogenous controls.

D. Construction of a Plasmid Expressing lncRNA AF086453

Oligonucleotides complimentary to the lncRNA AF086453 genomic sequences were constructed (AF0806453 clone For – taagcttcacatagtttaggtcctggcttaactctac & AF0806453 clone Rev – actcgaggagaggggccagaaatgaaagcttg), containing HindIII and XhoI sites on their respective 5' and 3' ends, then amplified from human prostate epithelial cell genomic DNA (ScieCell Research Laboratories) using Amplitaq Gold (Applied Biosystems/Life Technologies). The product (620 bp) was then gel purified from a 1% Agarose gel (QIAquick gel extraction kit, Qiagen Corp.) and TOPO cloned into pCR4-TOPO (Invitrogen/Life Technologies). The vector construct was sequenced and the lncRNA AF086453 fragment was sub-cloned into pcDNA6/V5-HisA (Invitrogen/Life Technologies) using the HindIII and XhoI sites to create pcDNA6/AF086453.

E. Growth Rate Assays for Transient Expression of AF086453 in PC3 Cells

PC3 cells were grown to log phase, trypsinized, counted using an automated cell counter (Countess, Invitrogen/Life Technologies), and then seeded into wells of a 6-well plate at 10^5 cells per well (in triplicate per time point per sample). Cells were then transfected with 5 ug of either pcDNA6/V5-HisA (Vector Control) or pcDNA6/AF086453 using Fugene 6 (Roche), or no plasmid at all (cells only). Media was changed after 4 hours, and cells were harvested at 24, 48, & 72 hours to be counted (samples included: untransfected cells, "vector control" & and expression vector transfected cells).

F. Invasion Assays

BD BioCoat growth factor reduced insert plates (Matrigel Invasion Chamber 24 well plates) were prepared by hydrating the matrix coating of the inserts with 0.5 mLs of RPMI 1640 media (no serum) for 2 hrs at 37°C/5% CO_2 [16]. The hydration solution was carefully removed from the inserts and 0.75 mLs RPMI 1640 media containing chemoattractant (10% FBS) was added to the wells of the plate with 0.5 mLs of cell suspension containing 30000 cells (either transfected with pcDNA6/AF086453 or pcDNA6/V5-HisA, or no plasmid) in serum-free medium added to each insert well. Invasion assay plates were incubated for 48 hrs at 37°C/5% CO_2. Following incubation, the non-invading cells were removed by scrubbing out the upper surface of the insert. The cells on the lower surface were stained with crystal violet, and then the trans-well membranes were mounted onto a microscope slide for visualization and analysis. All slides were scanned utilizing the Scanscope digital slide scanner, with the number of cells migrating counted using Aperio software. All data were expressed as percent invasion through the membrane relative to migration through the control membranes:

% invasion = Mean number of cells invading through the Matrigel membrane/Mean number of PC3 (wild type) cells migrating through membrane.

III. Results and Discussion

A. Identification of a Long Non-Coding RNA Differentially Regulated in Prostate Cancer Cells

Total RNA was isolated from prostate epithelial cells as well as the prostate cancer cell line PC3 and expression of lncRNAs were analyzed by using a non-coding microarray (NCode) to identify differentially regulated lncRNAs. NCode human microarrays contain probes to target 12,784 lncRNAs and 25,409 mRNAs. In total, we identified 22 lncRNAs that were significantly differentially expressed ($P < 0.015$) in PC3 prostate cancer cells relative to prostate epithelial cells. Individual lncRNAs were further screened by qRT-PCR to confirm microarray results. Next, these differentially regulated ncRNAs were tested in a panel of five commonly used prostate cancer cell lines (PPC-1, DU145, 22RW1, LNCap and PC-3) to identify consistent trends of mis-regulation. We found that one lncRNA (Genbank accession ID *AF086453*) was highly down-regulated in all five prostate cancer cell lines compared to epithelial cells

(with four of the five cell lines yielding less than 10% of the expression seen in epithelial cells). Fig.1 depicts the relative gene expression levels of *AF086453* in all five prostate cancer cells lines compared to prostate epithelial cells.

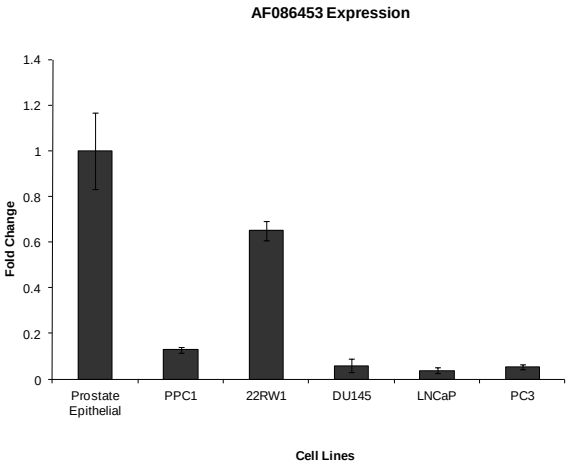

Fig. 1. *AF086453* expression profiles of the prostate cancer cells lines PPC-1, 22RW1, DU145, LNCaP, and PC3 normalized to prostate epithelial cells.

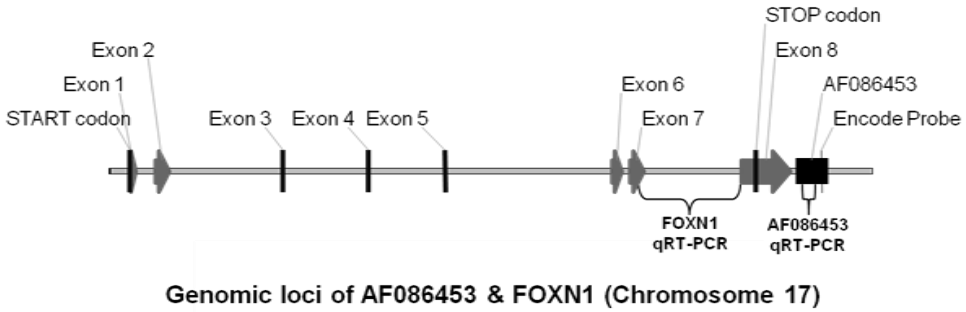

Fig. 2. Genomic loci of *FOXN1* and lncRNA *AF086453*. Annotations include the locations selected for qRT-PCR in reference to FOXN1 exons and the EnCode probe initially used to measure AF086453 expression.

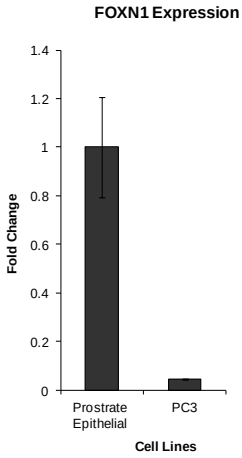

Fig. 3. qRT-PCR of FOXN1 expression in PC-3 prostate cancer cells compared to prostate epithelial cells. Experiment was performed in triplicate.

In order to determine a possible regulatory association between *FOXN1* and *AF086453*, we performed qRT-PCR for FOXN1 in PC-3 prostate cancer cells and compared the results to prostate epithelial cells (Fig. 3). The results demonstrate that FOXN1 is highly down-regulated (~20-fold) in prostate cancer cells. These results lead us to believe that *AF086453* may be under *FOXN1* regulatory control, but we are gathering additional supportive evidence in our laboratory.

B. FOXN1 May Regulate the Gene Expression of AF086453

An examination of the genomic loci of *AF086453* revealed that it is located less than 300 bp downstream of the final exon of the *FOXN1* gene (Fig. 2). *FOXN1* is a winged-helix transcription factor that has been shown to induce terminal differentiation in keratinocytes and defects have led to T-cell immunodeficiency in the thymus [17], [18]. A study by *Karanam et al.* [15] indicated that *FOXN1* binding sites were found to be significantly enriched in genes that were over-expressed in prostate cancer.

Previous studies have suggested coordinated regulation/function when a lncRNA appears within the loci or in close proximity to an Open Reading Frame [19], [20]. Our own studies involving the melanoma up-regulated lncRNA *SPRY4-IT1* have supported such an observation for a possible co-regulation with the SPROUTY 4 (*SPRY4*) gene [14].

C. Biological Function and the Physiological Relevance of AF086453 Down-Regulation in Prostate Cancer Cells.

Although the down-regulation of *AF086453* is clear, the physiological relevance of this lncRNA in prostate cancer cells has not been determined. In order to address this, the genomic sequence of *AF086453* was cloned into a eukaryotic gene expression plasmid and the plasmid was transiently transfected *into* prostate cancer cells (PC-3). A growth curve was performed to measure the effect of the ectopic expression of AF086453 and compared to an "empty vector" (Fig. 4A). The results indicated that after 72 hours, transfection of PC-3 cells with the *AF086453*-expressing plasmid (pcDNA6/AF086453) grew ~45% slower than either wild type or "empty vector" (pcDNA6/V5-HisA). Next, an invasion assay was performed to measure the effect of the ectopic expression of *AF086453* on cell mobility. PC-3 cells were again transfected with the same construct and compared to "empty vector" or untransfected cells. All cell samples were introduced into modified Boyden invasion chambers and membranes were harvested after 48 hours. The results indicated that cells transfected with the *AF086453*-expressing plasmid migrated nearly 30% slower than vector-only cells. Together, these results indicate that *AF086453* expression has an adverse effect on both the growth and invasive properties of prostate cancer cells.

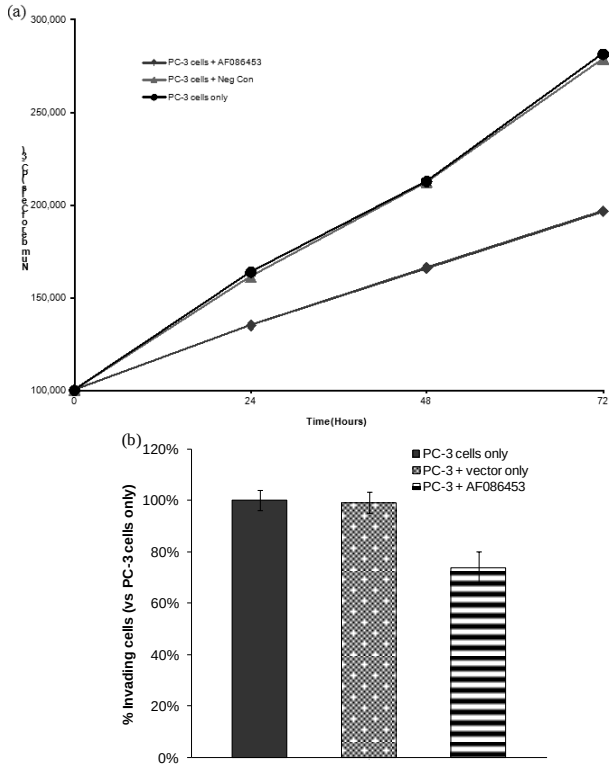

Fig. 4. The effect of ectopic expression of the lncRNA AF086453 on growth and invasion in PC-3 prostate cancer cells. A) Growth curve of PC-3 cells transfected with a plasmid expressing AF086453 or an "empty vector" plasmid versus untransfected cells. The growth curve was performed over 72 hours in triplicate. B) Invasion assay of PC-3 cells transfected with a plasmid expressing AF086453 or an "empty vector" plasmid versus untransfected cells. The invasion assay was performed over 24 hours in triplicate.

IV. Conclusion

Prostate cancer represents one of the leading causes of cancer death in American men, yet the success rate of diagnosis suggests an urgent need for novel and more comprehensive approaches for future diagnostic and prognostic evaluation.

The study of long non-coding RNAs (lncRNAs) represents a new avenue of research for prostate cancer biology and may lead to the discovery of novel biomarkers and therapeutics. Our previous evaluation of non-coding RNAs in normal skin and the skin cancer melanoma offered new appraisals on the mechanisms of cellular regulation and mis-regulation associated with cancer and it is our belief that this model can be applied to the study of prostate cancer.

The results of non-coding RNA (NCode) microarrays revealed that numerous lncRNAs were differentially expressed in prostate cancer cells. One particular lncRNA, Genbank accession ID *AF086453*, was highly down-regulated in a panel of prostate cancer cell lines compared to prostate epithelial cells. Located immediately upstream to *AF086453* is the transcription factor *FOXN1*, known to regulate numerous cancer specific genes. Gene expression analysis confirmed that *FOXN1* is also down-regulated in prostate cancer which suggests a coordinated gene expression between these two genes. It is our hypothesis that *AF086453* may influence *FOXN1* regulation and together *FOXN1* and *AF086453* may play key roles in prostate cancer development in men.

Preliminary cell biology studies to examine the physiological ramifications of the ectopic expression of *AF086453* in prostate cancer cells revealed defects in both cell growth and invasion. This suggests a possible role of *AF086453* as a tumor suppressor either alone or in conjunction with *FOXN1*. Together, these results strongly justify the need for future detailed examinations of *AF086453* and *FOXN1* molecular functions in prostate cancer development in humans and continues to be an ongoing study in our laboratory.

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