sdRNAs Participate in the Maintenance and Onset of Prostate Cancer

By

Neil Y. Chaudhary

A thesis submitted in partial fulfillment of the requirements of the Honors College at the University of South Alabama and the Bachelor of Science in the Department of Biomedical Sciences.

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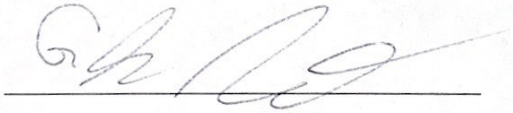
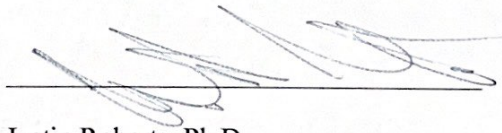
Approved by:

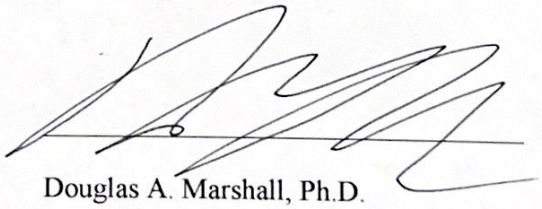


Glen M. Borchert, Ph.D.
Mentor



Justin Roberts, Ph.D.
Committee Member

Raymond Langley, Ph.D.
Committee Member



Douglas A. Marshall, Ph.D.
Dean, Honors College

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ABSTRACT

Studies have shown that small nucleolar RNAs (snoRNA) can be processed into smaller microRNA-like fragments known as sno-derived RNA (sdRNA). Utilizing The Cancer Genome Atlas's (TCGA) patient prostate tumor next-generation sequencing datasets, certain snoRNAs were noted to contribute to prostate cancer malignancy. The sdRNAs that arise from these particular snoRNAs are greatly overexpressed in prostate cancer cell types 8140 and 8550. Their mechanism of action involves the binding of Argonaute (Ago) proteins in order to influence the translation of messenger RNA (mRNA). Among 38 specifically excised, differentially expressed snoRNA fragments (sdRNAs) in TCGA prostate cancer (PCa) patient samples, snoRNA-derived fragments sdRNA-D19b and sdRNA-A24 emerged among the most differentially expressed as compared to normal prostate controls.

Phenotypic assays show that the overexpression of either sdRNA-D19b or sdRNA-A24 significantly increases cell proliferation of PC3 cells, a well-established model of castration-resistant prostate cancer (CRPC). Additionally, the overexpression of sdRNA-D19b in particular also markedly increases the rate of PC3 cell migration. This property of migration shows an increased potential for metastasis, a dangerous component of cancer that allows it to spread to other areas of the body. Alongside these effects, both sdRNAs provided drug-specific resistances with sdRNA-D19b levels correlating with paclitaxel resistance and sdRNA-24A conferring dasatinib resistance. Increased proliferation, heightened metastasis, and resistance to chemotherapy are core characteristics of CRPC that result in a disease ranked second in cancer-related deaths for men. Results indicate that sdRNA-D19b and sdRNA-A24 are active contributors to prostate cancer malignancy, and they have the potential to serve as novel biomarkers and therapeutic targets in clinical intervention.

LIST OF ABBREVIATIONS

Ago	Argonaute protein
h	hours
mRNA	messenger RNA
miRNA	microRNA
m	minutes
ncRNA	non-coding RNA
NGS	Next-Generation Sequencing
PCa	prostate cancer
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RPM	reads per million
sdRNA	small nucleolar derived RNA
snoRNA	small nucleolar RNA
snRNA	small nuclear RNA
sRNA	small RNA
s	seconds
TCGA	The Cancer Genome Atlas

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INTRODUCTION

Noncoding RNA (ncRNA) is a class of RNA that includes microRNA (miRNA) and small nucleolar RNA (snoRNA), among others (Kawaji, 2008). For a long time, no connection had been identified between these ncRNAs and cancer. In fact, many researchers began to characterize certain classes of ncRNAs as "transcriptional noise." These sentiments changed at the turn of the 21st century upon the discovery of a link between the deletion of certain miRNAs and B-cell chronic lymphocytic leukemias (Calin, 2002). This discovery prompted further research on ncRNAs as well as the fragments of noncoding-derived RNA (ndRNA) excised from them. A certain class of ndRNAs that are derived from snoRNAs have been denoted as Small Nucleolar Derived RNAs (sdRNAs). These sdRNAs have been shown to display regulatory functions on target genes, similar to the functions of miRNAs (see Fig. 1). The dysregulation of sdRNAs can contribute to oncogenesis and tumor progression, thus it is important to understand the biogenesis of sdRNAs and their roles in the regulation of human cancer.

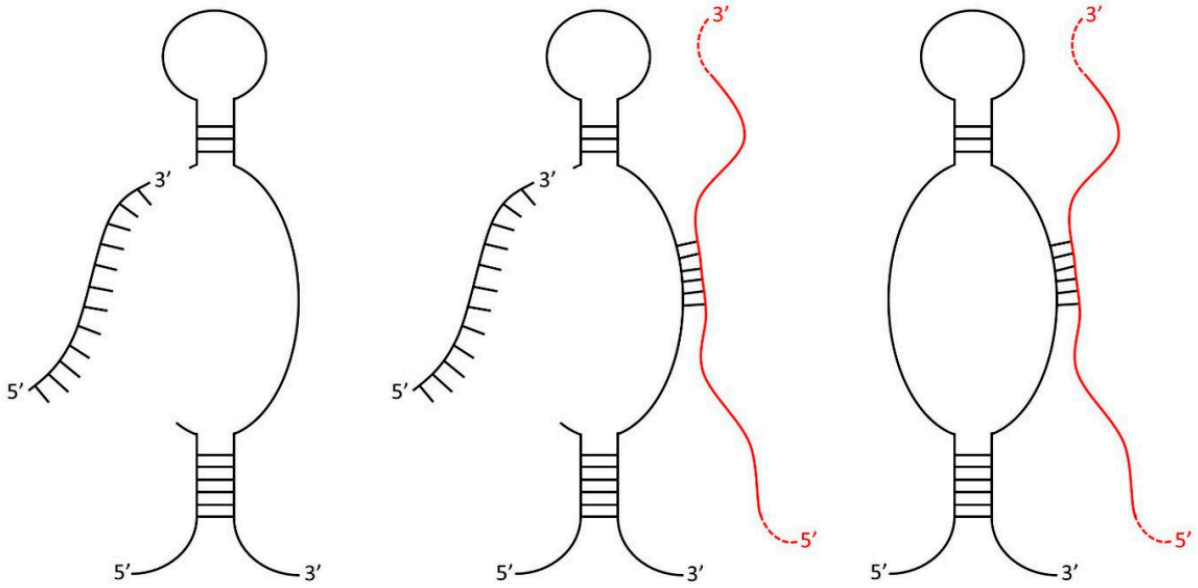

Figure 1. sdRNAs are excised fragments derived from snoRNAs. These snoRNAs can function exclusively as posttranscriptional RNA editors (right) or exclusively as functional sdRNA precursors (left). Some loci can both produce transcripts and engage in RNA regulation (center). sdRNAs are illustrated in black as excision products of a primary transcript. RNA editing targets are shown in red.

As discussed, sdRNAs originate as fragments of snoRNAs. SnoRNAs are a class of ncRNA localized to the nucleolus and can be transcribed either alone or alongside their host pre-mRNAs. They are subsequently processed and released from excised introns (Tycowski, 1996). Once full-length snoRNAs are produced, a microprocessor complex that is made of DGCR-8 and Drosha converts them into transcripts that are shorter in length. This process is similar to how the primary microRNAs are processed into precursor microRNAs (Coley, 2022). This transcript is then transported to the cytoplasm where it interacts with the enzyme DICER for further processing to produce the mature sdRNA (Taft, 2009). As described, this method of biogenesis distinguishes sdRNAs from miRNAs and warrants their separate classification. Understanding this unique method of development is crucial to determining the role of sdRNAs when it comes to influencing gene expression and cancer in humans. The mature sdRNA that is produced via DICER in the cytoplasm then associates with Argonaute proteins, which are their cellular binding partners, to compose the heart of gene-silencing effector complexes. This enables the formation of the RNA-induced silencing complex (RISC) (Ender, 2008). The mature sdRNAs act as signals to direct RISC's targeting of mRNAs for degradation to prevent protein translation. By silencing specific mRNA sequences, RISC prevents them from being translated into proteins in order to carry out their intended functions. This regulatory capability allows sdRNAs to influence cell growth and proliferation as well as other mechanisms relating to the development and progression of different types of cancer.

Breast cancer is the second leading cause of death from cancer in females around the world (Siegel, 2021). In 2017, data analysis was conducted by the Borchert lab to determine if any particular sdRNAs were overexpressed or underexpressed in this extremely prevalent type of cancer. Based on a study published in 2011, sdRNA-93 was of particular interest as it had displayed genomic silencing capabilities in the past (Brameier, 2011). Two well-known breast cancer cell lines were analyzed by the Borchert lab: primary MCF-7 and metastatic MDA-MB-231. It was discovered that sdRNA-93 was expressed to a much higher degree in MDA-MB-231 cells than MCF-7 cells. Furthermore, our lab discovered that downregulating sdRNA-93 leads to a reduction of breast cancer cellular invasion. On the contrary, upregulating sdRNA-93 enhances the invasive capability of these cells (Patterson, 2017). This is a significant finding because it demonstrates a tangible explanation for MDA-MB-231 cancer cells' characteristically greater invasive ability which has previously been described. The link between sdRNA-93 overexpression and cellular invasion was further quantified during MCF-7 cell analysis. As previously mentioned, these cells do not naturally express sdRNA-93 as highly as MDA-MB-231. Therefore, reducing the expression of sdRNA-93 did not have a significant impact on cellular invasion, but sdRNA-93 overexpression increased MCF-7 cellular invasion by about 80% (Patterson, 2017). This data supports the important regulatory effects that can be exerted by sdRNA-93 in multiple breast cancer cell types, even in those in which it is not as naturally abundant. The fact that sdRNA-93 increases cell invasion shows that it can promote metastasis, a dangerous component of cancer that allows it to spread to other areas of the body. Once metastasis occurs, cancer becomes much more difficult to control. The Borchert lab aims to continue our work to identify which specific mRNA sequences and proteins are silenced in the presence of sdRNA-93. This link between sdRNA expression and such a prevalent type of cancer

provided an important backing for further research into the roles of sdRNAs in other cancer types.

After previously identifying the contributing role of sdRNAs in breast cancer, our lab aimed to determine whether they play a role in prostate cancer which is the second-highest cause of cancer-related deaths in males in the United States (Siegel, 2021). We analyzed the small RNA transcriptome of 489 prostate cancer tissue samples and 52 healthy prostate tissue samples from The Cancer Genome Atlas (TCGA). Results showed that 9 snoRNAs were found to be processed into sdRNAs heavily overexpressed in prostate cancer. Of these snoRNA-derived fragments, sdRNA-D19b and sdRNA-A24 emerged among the most differentially expressed as compared to normal prostate controls. Target predictions showed relevant alignments between these particular sdRNAs and the 3' untranslated regions of known proto-oncogenes and tumor suppressor genes. Notably, sdRNA-D19b aligned with CD44, a surface marker for aggressive prostate cancer that regulates prostate cancer proliferation, migration, and invasion (Tai, 2011). Additionally, sdRNA-D19b aligned with CDK6 and STAT5B which are involved in cell cycle regulation and prostate cancer metastasis respectively (Badache, 2001). Similarly, sdRNA-A24 aligned with RHOH, TIMP3, and AR. RHOH is involved with prostate cancer invasion, TIMP3 relates to the progression of prostate cancer, and AR serves as a regulator of prostate cancer migration respectively (Adissu, 2015). In addition to exploring regulatory targets, this study also describes the phenotypic consequences of manipulating cellular levels of sdRNA-D19b and sdRNA-A24 in prostate cancer (PC3) cell lines. This includes the effects on cancer cell proliferation, migration, and invasion. This work shows that sdRNA-D19b and sdRNA-A24 are contributors to prostate cancer pathology. Furthermore, this study denotes the importance of

sdRNAs to malignancy as a newly studied ncRNA regulator while also pointing out much-needed potential biomarkers in clinical application.

AIMS

Overall Aim

To highlight the characteristic overexpression of sdRNA-D19b and sdRNA-A24 in prostate cancer to further explore their phenotypic effects.

Specific Aims

1. To display how the overexpression of these sdRNAs leads to increased cell proliferation.
2. To examine the impact of these sdRNAs on cell migration and the ability of cancer cells to metastasize.
3. To explore the roles of these sdRNAs on specific drug resistances.

MATERIALS AND METHODS

1. Sequence Alignment and Data Analysis

The samples used in this study are publicly available and were obtained from The Cancer Genome Atlas (TCGA) Research network PRAD dataset. The Short Uncharacterized RNA Fragment Recognition (SURFR) tool that performs in-depth analyses of ncRNA-derived RNAs from input RNA-seq data. Rstudio was used to classify each sdRNA by cancer prevalence (% of TCGA samples that expressed the sdRNA) and differential expression. Significant results were constricted to those sdRNAs that displayed a change of at least 2× in prostate cancer and were expressed in at least 30 reads per million (RPM) in at least half of the TCGA PRAD small RNA-seq files. Small RNA-seq files were obtained for the TCGA PRAD dataset in order to confirm findings from SURFR. Alignments between snoRNAs and reads were obtained via BLAST+. The frequency of alignments to sdRNAs included reads that contained at least 20 nucleotides and perfect matches (100% identity). PC3 cell Ago pulldown data were obtained from the NCBI SRA. Alignments between sdRNAs and Ago pulldown reads were obtained via BLAST+.

2. Validation of sdRNA Expression via Quantitative RT-PCR

The mirVana miRNA Isolation Kit was used to isolate small RNA. The All-in-One miRNA qRT-PCR Kit was used to confirm the presence of sdRNA and its degree of prevalence via real-time, quantitative PCR. Three trials were performed for each of the reactions in 96-well plates using 0.2 μM of each custom forward and universal reverse primers and 1.5 μg of total RNA in nuclease-free water. qRT-PCR was conducted on the iQ-5 Real-Time PCR Detection System with the following settings:

initial polymerase activation and DNA denaturation at 95°C for 10 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s, 72 °C for 15 s.

3. Obtaining and Growing PC3 Cells

The human prostate cancer cell lines (PC3) used in this experiment were obtained from the Mitchell Cancer Institute (MCI). The cells were grown and maintained in DMEM (Dulbecco's Modification of Eagle's Medium) with 4.5 g/L glucose and L-glutamine. The medium excluded any trace of sodium pyruvate and was bolstered with 10% fetal bovine serum and 1% pen strep. The cells were incubated at 37°C with 5% CO_2.

4. Manipulating sdRNA-D19b and sdRNA-A24 levels

Antisense oligonucleotides were designed to target sdRNA-D19b (5′-AUCAGAGUUGGAUCUU GUAA-3′) and sdRNA-A24 (5'-GUCAUCACCAUCUCUCAGAUA-3'). These oligonucleotides were ordered from Integrated DNA Technologies located in Coralville, IA. A scrambled nonspecific oligonucleotide was also ordered as a negative control (5′-GTGAGCTGTTTCAGTGGTTTGAGT-3′). Similarly, sdRNA mimics and the scrambled control sdRNA-CUI (5'-GAUUCAAUUUGAUUUGCCCGUGGA-3') were ordered as custom miRIDIAN mimics from Dharmacon located in Chicago, IL. Cell migration, proliferation, and invasion assays were then performed to observe the effects of manipulating sdRNA-D19b and sdRNA-A24 levels. For transient transfections, the cells were cultured in 12-well plates and grown to 60% confluency before transfection with mimics or inhibitors using Lipofectamine RNAiMAX.

5. Phenotypic Assays

Proliferation assays. PC3 cells were first transfected with either 100 nmol/L of RNA mimic, antisense RNA (inhibitor), or negative control using Lipofectamine RNAiMAX (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. The cell number was determined by trypan blue staining. The cells were counted manually at intervals of 24, 36, and 48 h post-transfection. Proliferation was determined as the relative cell number compared with the vehicle-treated (0.1% DMSO) controls ($n \geq 8$).

Cell migration assays. In order to determine the cells' ability to migrate, a thin scratch was introduced along the center of the dish. PC3 cells were transfected with inhibitors or mimics in standard Petri dishes (Corning), as described for examining the cell proliferation, and then grown to 100% confluence. A 1 cm-wide zone was scratched across the center of each dish utilizing a pipette tip and images were taken every 3 h using an EVOS XL Core inverted microscope imaging system to assess the rate of migration.

Examining chemoresistance. Following transfection, the cells were incubated for 20 min in 5% CO_2 at 37 °C, after which they were treated with paclitaxel (5 nM), dasatinib (50 nM), cisplatin (50 μM), or DMSO control. Cells were stained with methylene blue and their degree of survival was determined by manual counting at intervals of 0, 6, 12, 18, and 24 h after transfection. Viability was determined as the relative live cell number compared with vehicle-treated (0.1% DMSO) controls ($n \geq 3$).

Cell invasion assays. PC3 transfected cells were used for assessment of invasion using a matrigel invasion chamber kit (BD Bioscience, Sparks, MD, USA). The matrigel-coated plates were rehydrated in a warm DMEM serum-free medium for 2 h at

37 °C. After removing the medium, cells were suspended in 500 μL blank medium, and then the 750 μL chemoattractant (medium with 10% fetal bovine serum) was added to the well chamber. Cells were then incubated for 36 h in 5% CO_2 at 37 °C. For the measurement of invading cells, non-invading cells were removed from the upper surface of the membrane by scraping using cotton swabs, and invading cells through the matrigel to the bottom of the insert were fixed with paraformaldehyde and then stained with crystal violet for counting ($n \geq 3$). Cells were observed and photographed using an EVOS XL Core inverted microscope imaging system. Ten random fields of view for each well were quantified by counting the cells in each field and averaging the results.

6. Vector Construction

Unless otherwise indicated, PCR amplifications were performed in 40 μL reactions at standard concentrations (1.5 mM MgCl2, 0.2 mM dNTP, 1x Biolase PCR buffer, 0.5 U Taq (Bioline USA, Inc., Randolph, MA, USA), 0.5 μM each primer) and using standard cycling parameters (94 °C—3 min, (94 °C—30 s 55 °C—30 s, 72 °C—60 s) × 30 cycles, 72 °C—3 min), then, they were cloned into Topo PCR 2.1 (Invitrogen) and sequenced. Antisense reporters were constructed by the standard PCR with primers containing 50 Xho-I and 30 Not-I restriction enzyme sites. Following digestion, amplicons were ligated into the Renilla luciferase 3 0UTR of psiCheck2 (Promega, Madison, WI, USA) vector linearized with Xho-I and Not-I. Reporter assays were performed as previously described, where the presence of an independently transcribed firefly luciferase in these reporters allowed normalization for transfection efficiency

7. Luciferase Assays

Human embryonic kidney (HEK293) cell line was obtained from GenLantis (San Diego, CA, USA) and cultured in MEM (Mediatech, Herndon, VA, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 25 mg/mL streptomycin, and 25 I.U. penicillin (Mediatech). Cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C. For luciferase assays, HEK293 cells were cultured in MEM (10% FBS and 1% PS) in 12-well plates. At 90% confluency, cells were transfected following the Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) protocol. At 36 h post-transfection, cells were scraped from the bottom of the wells and moved to 1.5 mL Eppendorf tubes. Eppendorfs were centrifuged at 2000 RCF for 3 min, followed by supernatant aspiration and cell resuspension in 300 µL of PBS. Cells were lysed by freeze thaws and debris was removed by centrifuging at 3000 RCF for 3 min. A total of 50 µL of supernatant was transferred to a 96-well MicroLite plate (MTX Lab Systems, Vienna, VA, USA), then, firefly and Renilla luciferase activities were measured using the Dual-glo Luciferase® Reporter System (Promega) and a 96-well plate luminometer (Dynex, Worthing, West Sussex, UK). RLUs were calculated as the quotient of Renilla/firefly RLU and normalized to mock.

8. Statistical Analyses

Cell proliferation and migration assays. Treatment effects were assessed using a two-tailed Student's t-test at each time point measurement. To assess the longitudinal effects of treatment, a mixed model was utilized to examine the difference across all groups and between each pair of groups for the whole study period. Data were presented as mean ± SD from no less than three independent experiments, and a p-value < 0.05 was

considered significant. For imaging, five microscopic fields randomly chosen from each assay were counted individually, then, the results were averaged.

Luciferase assays. Data are presented as the average intensity ± standard deviation in four independent experiments. Quantitative RT-PCR. Gene expression was calculated via the Delta–Delta cycle threshold method and qRT-PCR data were analyzed by Fisher's exact test.

RESULTS & DISCUSSION

1. sdRNA-D19b and sdRNA-A24 are overexpressed in prostate cancer samples

The Borchert lab developed the web resource SURFR to locate ncRNA fragments present in small RNA sequence datasets. This program aligns next generation sequencing (NGS) datasets to a frequently updated database of all human ncRNAs and performs a computational analysis to find the location and expression of ncRNA-derived fragments (ndRNAs). SURFR then conducts an expression analysis to identify significantly differentially expressed ndRNAs. For this project, we employed SURFR to determine sdRNA expressions in 489 PCa and 52 normal prostate TCGA patient RNA-seq datasets. This allowed us to rank differentially expressed sdRNAs in prostate cancer. As seen in Table 1 below, control sdRNAs such as sdRNA-D30 and sdRNA-D61 are not significantly differentially expressed between prostate cancer and normal tissue controls. We chose to focus on sdRNA-A24 and sdRNA-D19b for in vitro characterization because sdRNA-D19b is expressed (avg. 384 RPM) in 91.6% of 489 TCGA PCa samples versus only 42.3% of normal tissue controls (avg. 162 RPM), and sdRNA-A24 is expressed (avg. 711 RPM) in 97.5% of 489 TCGA PCa samples versus only 30.8% of normal tissue controls (avg. 150 RPM) (see Table 1).

	Prevalence (%) in 489 PCa Samples	Average Expression (RPM) in PCa Samples	Prevalence (%) in 52 Tissue Controls	Average Expression (RPM) in Tissue Controls	Differential Expression Fold Change (Cancer/Control)
sdRNA-A24	97.5	711	30.8	150	4.74x
sdRNA-D19b	91.6	384	41.3	162	2.4x
sdRNA-D30	99.6	31067	100.0	19719	1.6x
sdRNA-D61	53.2	215	17.3	119	1.9x

Table 1. Significant overexpression of sdRNA-D19b and sdRNA-A24. These two sdRNAs are significantly overexpressed in TCGA prostate cancer patient datasets when compared to other sdRNAs. The SURFR algorithm was used to identify sdRNAs abundantly expressed in prostate cancer patient tumors versus normal prostate tissues.

2. sdRNA-D19b and sdRNA-A24 levels significantly impact the proliferation of PC3 Cells

We decided to utilize the PC3 cell line to determine whether sdRNA-D19b and sdRNA-A24 are contributors to the phenotype of castration-resistant prostate cancer (CRPC). This cell line was chosen in particular since PC3 cells are a common model of aggressive CRPC. This is because they omit expression of the androgen receptor and thus they grow without the influence of androgen signaling (Tai, 2011). In order to introduce variation in sdRNA expression, we used a custom mimic/inhibitor system to manipulate specific sdRNA levels. RNA sequences identical to sdRNA-D19b and sdRNA-A24 were commercially synthesized. Then, PC3 cells were transfected with these specific sdRNA mimics to simulate the overexpression of sdRNA. Similarly, RNAs complementary to sdRNA-D19b or sdRNA-A24 were synthesized and introduced as sdRNA inhibitors (Anti-sd) via transfection of PC3 cells. The first parameter we measured with the

manipulation of sdRNA-D19b and sdRNA-A24 was the impact on PC3 proliferation. We determined that the misexpression of either sdRNA-D19b or sdRNA-A24 profoundly impacted PC3 proliferation in comparison to two control sdRNAs-A61 and -93 which are not significantly expressed in prostate cancer samples as determined via The Cancer Genome Atlas database. The overexpression of sdRNA-D19b increased PC3 cell proliferation by 24% at 24 h and by 32% at 72 h. On the other hand, inhibiting the expression of sdRNA-D19b reduced PC3 cell proliferation by 22% at 24 h and by 32% at 72 h. The case was similar when it came to analyzing sdRNA-A24. Overexpression of sdRNA-A24 increased PC3 proliferation by approximately 25% at both 24 h and 72 h. Inhibiting sdRNA-A24 decreased proliferation by 14% at 24 h and by 40% at 72 h (see Fig. 2). All of the preceding percent changes were calculated in relation to cells transfected with controls. Notably, PC3 proliferation was not impacted by varying the expression of two control sdRNAs that are expressed in PC3 cells but are not differentially expressed in prostate cancer malignacy. These results indicate functional roles for both sdRNA-D19b and sdRNA-A24 in PC3 proliferation.

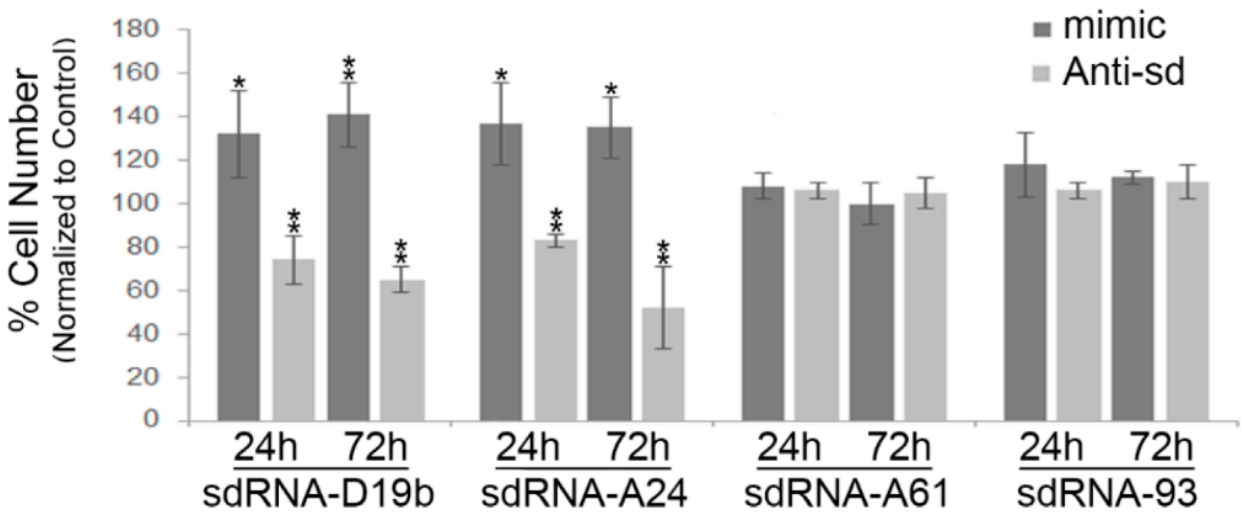

Figure 2. sdRNA-D19b and sdRNA-A24 levels significantly impact PC3 cell proliferation. PC3 cells were transfected with indicated sdRNA mimic or antagomiR (Anti-sd). Cell counts were performed at 24 and 72 h then normalized to scrambled control transfections (n = 8). * indicates $p \leq 0.05$; ** indicates $p \leq 0.01$; p-values obtained via unpaired two-tailed t-test.

3. Overexpression of sdRNA-D19b markedly increases the migration of PC3 cells

As identified, the increased cell proliferation caused by sdRNAs is a significant contributor to oncogenesis and is a quantifiable indicator of cancer (Hanahan, 2011). The ability of cancer cells to gain migratory capabilities allows primary tumors to move beyond their original location. When these cancer cells spread, they give rise to metastases in other areas of the body that were previously unaffected. Metastases are primary contributors to patient mortality. In fact, of all the attributes of cancer, metastases are responsible for the greatest number of cancer-related deaths (Fares, 2020). Prostate cancer is characteristically metastatic, and this leads to its high associated morbidity. Due to the significant impacts of metastasis on patient survival, we elected to examine whether sdRNA-D19b and sdRNA-A24 have the ability to impact PC3 cell migration in addition to their effects on PC3 cell proliferation. We performed this analysis via the

wound-healing assay. In this method, culture dishes were transfected with sdRNA mimics, inhibitors, or scrambled controls. Then, confluent cells were bisected with a scratch through the middle of the dish (see Fig. 3 and Fig. 4).

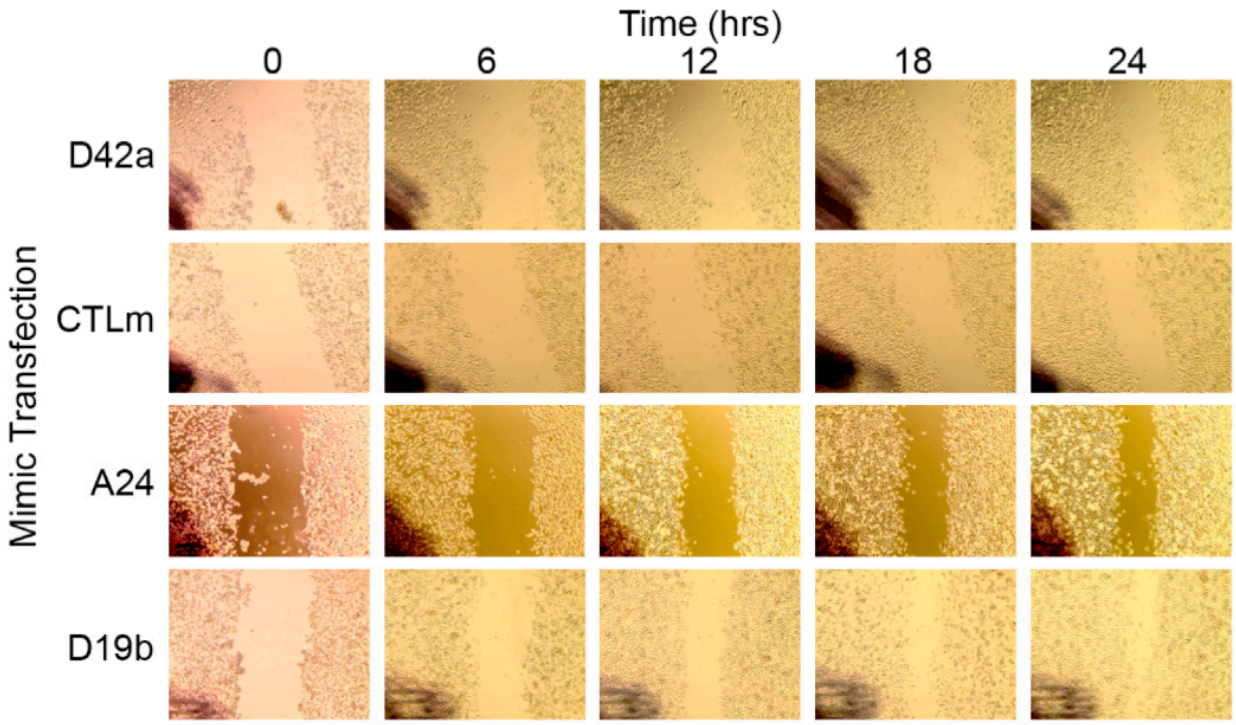

Figure 3. Effects of sdRNA mimic transfection on PC3 cell migration. Representative migration (wound-healing) assays for PC3 cells transfected with the indicated sdRNA mimic. D42a = sdRNA-D42a mimic; CTLm = scrambled mimic; A24 = sdRNA-A24 mimic; D19b = sdRNA-D19b mimic.

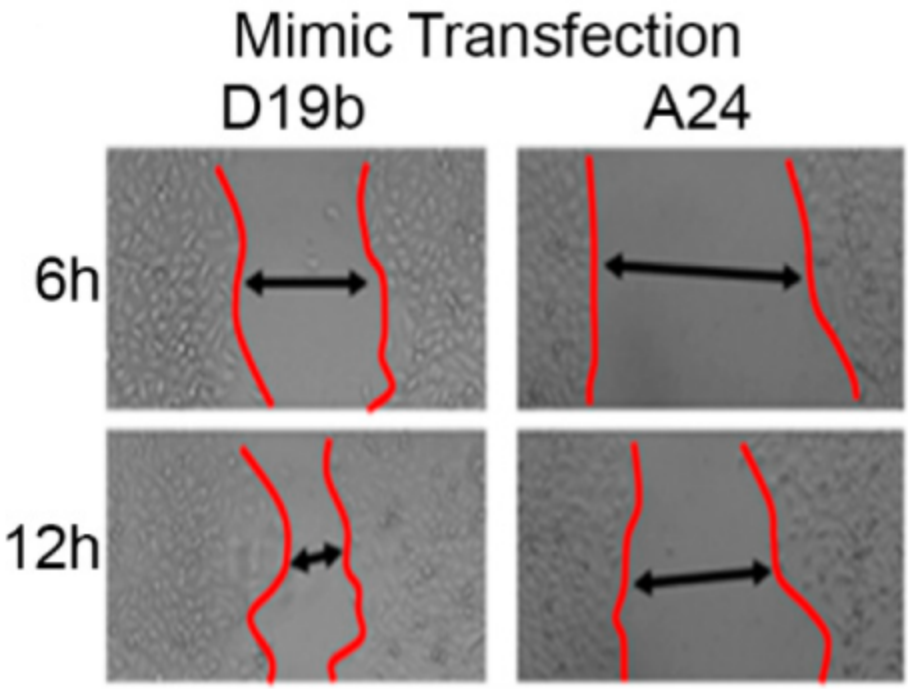

Figure 4. sdRNA-D19b overexpression markedly increases cell migration. Wound border closure is indicated by the black arrows. A24 = sdRNA-A24 mimic; D19b = sdRNA-D19b mimic.

Results showed that inhibiting the expression of sdRNA-D19b and sdRNA-A24 did not have a significant impact on PC3 migration compared to the controls. The same was true for overexpression of sdRNA-A24. Our findings showed that the inhibition or overexpression of sdRNA-D42A, which is significantly overexpressed in TCGA PCa samples but not expressed in PC3 cells, also did not significantly alter PC3 migration (see Fig. 3). In striking contrast, however, we found sdRNA-D19b overexpression markedly increased migration (avg 86.8%) between 6 h and 24 h (see Fig. 4 and Fig. 5).

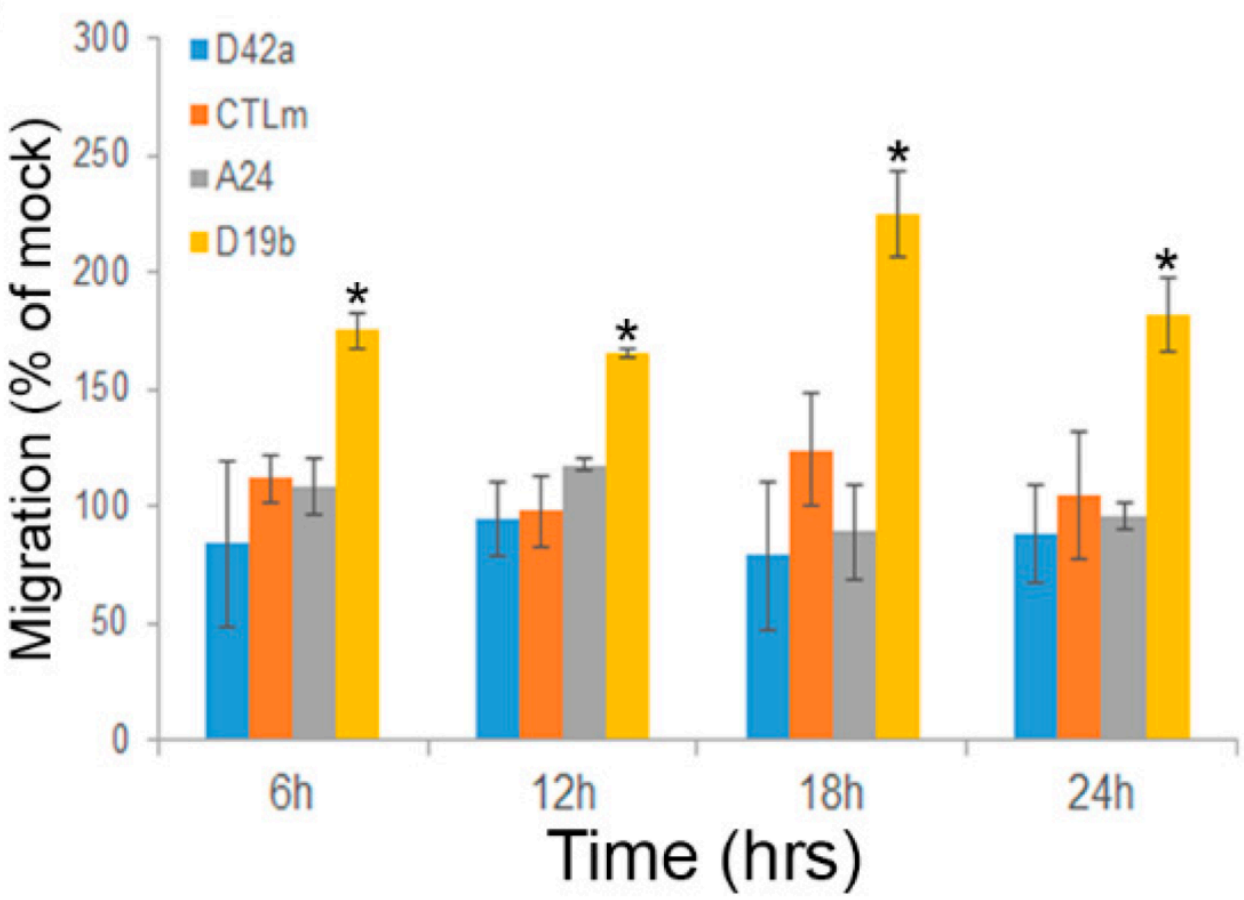

Figure 5. Quantification of PC3 migration assays. Images were captured at the indicated times (X-axis) and wound healing was quantified by utilizing ImageJ as % migration normalized to scrambled control ($n \geq 3$). * indicates $p \leq 0.05$; p-values by unpaired two-tailed t-test. D42a = sdRNA-D42a mimic; CTLm = scrambled mimic; A24 = sdRNA-A24 mimic; D19b = sdRNA-D19b mimic.

4. Altering the levels of sdRNA-D19b and sdRNA-A24 changes drug sensitivities in vitro

Paclitaxel and dasatinib are both common drug therapies employed in the treatment of prostate cancer. Therefore, we selected these drugs to determine if either sdRNA-D19b or sdRNA-A24 play a role in modulating PCa drug resistance. PC3 cells were treated with one of the chemotherapeutic drugs alongside a sdRNA mimic,

inhibitor, or scrambled control. We counted the cells every 6 h to denote the effect of sdRNAs and their expression on chemoresistance. Manipulating levels of sdRNA-D19b did not significantly alter the effect of paclitaxel on PC3 cells. On the other hand, sdRNA-A24 overexpression improved PC3 resistance to paclitaxel, increasing cell viability between 28.9% and 70.3% at all of the measured time points in comparison to controls. Although not statistically significant, inhibiting sdRNA-A24 appeared to sensitize PC3 cells to paclitaxel by 43.2% and 23.9% at 18 and 24 h, respectively (see Fig. 6). In contrast, sdRNA-D19b overexpression markedly desensitized PC3 cells to dasatinib treatment, increasing cell viability by over three times at 24 h as compared to controls. However, neither sdRNA-D19b inhibition nor sdRNA-A24 overexpression nor inhibition produced any identifiable effect (see Fig. 7). These results depict a complex, significant role for sdRNAs in relation to PC3 drug resistance. They imply that sdRNA-D19b and sdRNA-A24 occupy different mechanistic roles in greater drug resistance.

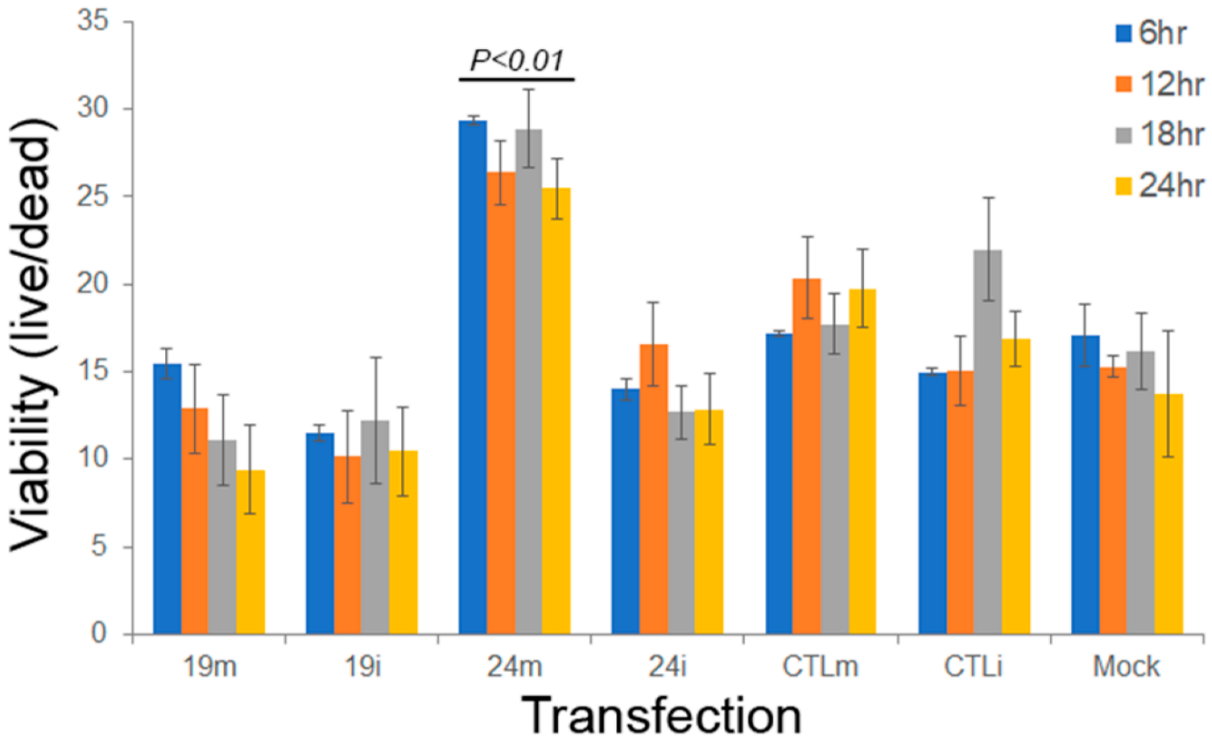

Figure 6. sdRNA-A24 overexpression protects PC3 cells from paclitaxel. Cells were cultured in 24-well plates and transfected at 70% confluency with mimics or inhibitors. Next, these cells were treated with 5 nM of paclitaxel. Cell death was observed at 6 h intervals for 24 h total using ImageJ and methylene blue dead cell staining. 19 m = sdRNA-D19b mimic; 19i = sdRNA-D19b inhibitor; 24 m = sdRNA-A24 mimic; 24i = sdRNA-A24 inhibitor; CTLm = scrambled mimic; CTLi = scrambled inhibitor; Mock = vehicle-treated control. ($n \geq 3$). * indicates $p < 0.001$; p-values by unpaired two-tailed t-test as compared to Mock.

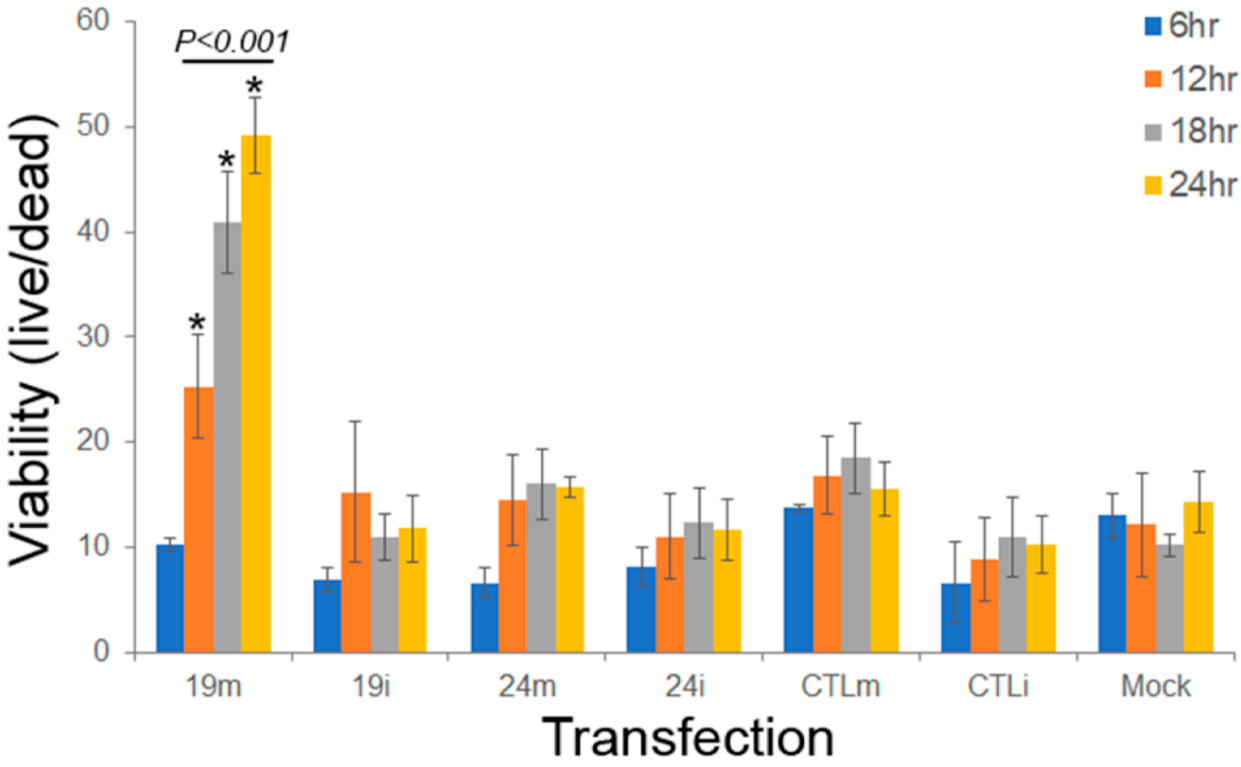

Figure 7. sdRNA-D19b overexpression protects PC3 cells from dasatinib. Cells were cultured in 24-well plates and transfected at 70% confluency with mimics or inhibitors. Next, these cells were treated with 50 nM of dasatinib. Cell death was observed at 6 h intervals for 24 h total using ImageJ and methylene blue dead cell staining. 19 m = sdRNA-D19b mimic; 19i = sdRNA-D19b inhibitor; 24 m = sdRNA-A24 mimic; 24i = sdRNA-A24 inhibitor; CTLm = scrambled mimic; CTLi = scrambled inhibitor; Mock = vehicle-treated control. ($n \geq 3$). * indicates $p < 0.001$; p-values by unpaired two-tailed t-test as compared to Mock.

CONCLUSION

In 2021, prostate cancer was the most prevalent malignant cancer type in American men (Fujita, 2019). Although there are therapeutic and surgical interventions available, these remissions can often result in a return of the more aggressive CRPC (Feng, 2019). There are not very many ways to treat CRPC effectively which leads to prostate cancer's second place standing as a leading cause of cancer death in American men. The only other type of cancer that ranks higher in this category is lung cancer (Fujita, 2019).

The previously cited research alongside novel data presented in this study clearly highlights the fact that small RNAs are functionally relevant sequences that arise from full-length snoRNAs. These sdRNAs have a similar form and function when compared to miRNAs, differing mainly in their methods of transcriptional origin. These sdRNAs become further differed from miRNAs by their usage of processing pathways aside from DICER/DROSHA. Like miRNAs, sdRNAs have the ability to post-transcriptionally regulate gene expression to cause varying effects on oncogenesis and malignant pathology. It is important to distinguish sdRNAs from miRNAs to avoid the current practice wherein they are routinely discarded from miRNA databases and omitted from any miRNA-focused studies that follow. Instead of discarding sdRNAs, they deserve inclusion in miRNA databases. By exploring the relevant roles of these sdRNAs in such a prevalent disease as prostate cancer, we aim to further raise awareness and stress their importance.

In 2017, our lab found that sdRNA-93 could limit the migration of breast cancer cells (Patterson, 2017). We decided to further investigate how sdRNAs function similarly in other cancer types, which led to this study's identification and characterization of direct roles for sdRNAs-D19b and -A24 in modulating CRPC. This work focuses on identifying and

characterizing sdRNA misexpressions directly involved with CRPC pathogenesis. We used PC3 cells to assess the impact of sdRNA misexpression, as PC3 cells are widely used as a model of aggressive CRPC. These cells provide an ideal environment to test our hypothesis that sdRNAs contribute to the CRPC phenotype and their recalcitrance towards therapies (Tai, 2011). A core characteristic of CRPC is enhanced metastasis, a factor largely responsible for the marked morbidity and high death rate among men in the US (Siegel, 2021). As such, the striking phenotypic consequences associated with manipulating sdRNA-D19b and sdRNA-A24 expressions described in this work strongly indicate an important role occupied by sdRNAs in promoting CRPC malignant traits.

In this study, we confirmed that manipulating the presence of sdRNA-D19b and sdRNA-A24 influences prostate cancer malignancy. When prostate cancer patient samples were compared to normal prostate controls, these two sdRNAs were clearly the most differentially expressed. Conducting tests on PC3 showed that overexpressing either sdRNA-D19b or sdRNA-A24 caused cell proliferation to increase. Another notable discovery was that increasing the expression of sdRNA-D19b also increased the rate of PC3 cell migration. This was untrue of sdRNA-A24 which did not have a significant impact on migration. Increased cell proliferation is a notable characteristic of cancer. Unlike normal healthy cells, cancer cells will grow at uncontrollable rates. Similar to the breast cancer study which explained the increased invasive capability of cells expressing sdRNA-93, the increased cell migration in PC3 cells was attributed to sdRNA-D19b levels. This further shows how sdRNAs contribute to the spread of cancer in not just one type, but across multiple types. A unique finding in this study correlated sdRNA expression to specific drug resistances. We have shown how sdRNA-D19b levels correlate with paclitaxel resistance and sdRNA-A24 levels are linked to dasatinib resistance. Therefore, not

only can sdRNAs promote cancer malignancy, but they can also prevent common forms of drug therapy from having a beneficial effect. When expressed, this makes certain sdRNAs more dangerous than previously hypothesized since they simultaneously promote the negative effects of cancer metastasis and inhibit the positive effects of common cancer therapies.

Our results show that sdRNA-D19b overexpression decreases PC3 sensitivity to dasatinib, a receptor tyrosine kinase (RTK) inhibitor, and that sdRNA-A24 overexpression significantly desensitizes PC3 cells to treatment with the microtubule-stabilizing agent paclitaxel (Rivera-Torres, 2019). In addition to implicating sdRNA-D19b and/or sdRNA-A24 as putative drug targets to sensitize PCa to treatment, these results suggest that sdRNAs may be involved with the regulation of core drug resistance components as paclitaxel and dasatinib largely represent mechanistically distinct chemotherapies.

In summary, research into the effects of sdRNAs on human cancer is ongoing and highly prevalent. Discoveries are bringing light to the importance of these noncoding-derived RNAs that were previously disregarded as irrelevant to the field of cancer research. As more studies are performed, we hope that sdRNAs may one day serve as therapeutic targets or identifiers for specific cancer types. By having denoted sdRNA-D19b and sdRNA-A24 as potential contributors to CRPC, the goal is that these noncoding RNA fragments can one day be used to assess the risk of certain cancers during early stages to employ preventative measures.

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