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By

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

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Abstract

In recent studies small nucleolar RNAs (snoRNA) have been shown to be processed into smaller microRNA-like fragments known as sno-derived RNA (sdRNA). We recently identified 9 snoRNAs that contribute to prostate malignancy using The Cancer Genome Atlas (TCGA) patient prostate tumor next-generation sequencing datasets. These snoRNA were found to be processed into sdRNAs, heavily misexpressed in prostate cancer cell types 8140 and 8550, and shown to function by binding Ago proteins in order to impact mRNA translation. SdRNA-19b and sdRNA-24 in particular stood out as having significant differential expression in prostate cancer vs. control tissue.

Interestingly, the targets for sdRNA-19b and sdRNA-24 were then predicted to be well known tumor suppressors and oncogenes. SdRNA-19b aligned with STAT5b, CDK6 and CD44 while sdRNA-24 aligned with were predicted to be RHOH, Timp3 and AR, all of which were confirmed through the use of luciferase assays. Additionally, our phenotypic assays show that over-expressions of sdRNA-19b and sdRNA-24 lead to increased cell proliferation and over expressions of sdRNA-19b lead to increased cell migration rates, indicating a direct impact on sdRNA levels to the proliferative and migratory ability of prostate cancer cells. Current results also strongly indicate that when sdRNA-19b and sdRNA-24 are inhibited they are significantly less likely to survive the presence of chemotherapeutic agents, providing a new potential target for effective chemotherapy treatment. In summary, our results indicate that sdRNA-19b and sdRNA-24 actively contribute to the malignant phenotype of prostate cancer through miRNA-like regulation.

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List of Abbreviations

Ago	*Argonaute protein*
Bp	*base pairs*
lncRNA	*long non-coding RNA*
mRNA	*messenger RNA*
miRNA	*microRNA*
ncRNA	*non-coding RNA*
rRNA	*ribosomal RNA*
sRNA	*small RNA*
snRNA	*small nuclear RNA*
snoRNA	*small nucleolar RNA*
sdRNA	*sno-derived RNA*
RNA	*ribonucleic acid*
NGS	*Next-Generation Sequencing*
PC	*prostate cancer*
Nt	*nucleotide*
TCGA	*The Cancer Genome Atlas*

Introduction

Prostate cancer is the second highest cause of cancer-related deaths in men. The course for this disease is highly variable and despite extensive research it is still very difficult to distinguish between benign and aggressive disease. As of now, the best practice for diagnosing and determining the prognosis is digital rectal exams, PSA serum tests, and histological tissue biopsies [1]. In order to advance this process, identification of new biomarkers is needed. Interestingly, non-coding RNA (ncRNA) which are RNA products that are not translated to protein and make up over 80% of the genome, have been shown to display highly specific expression patterns restricted to certain tissues, organs, or developmental stages [2]. The expression of these ncRNAs is significantly altered in cancerous tissue, making ncRNAs prime candidates for functioning as biomarkers for the presence and progression of disease [1]. There has already been extensive research on one type of ncRNA known as microRNA. Mature microRNAs (miRNAs) consist of 18–25 nucleotides that associate with the RNA-induced silencing complex (RISC) and bind to specific mRNA targets in their 3′ untranslated regions (3′ UTRs), ultimately resulting in gene suppression through the translational repression or cleavage of their bound mRNAs.

Small nucleolar RNAs (snoRNAs) which have previously been perceived to be entirely unrelated to miRNA are localized within the nucleolus and have long been characterized as molecular guides for sequence-specific modifications to ribosomal RNAs (rRNAs) and small nuclear RNAs (snRNAs)[3,4]. A third of these snoRNA have no rRNA or snRNA complementarity, therefore, they either guide modification for another type of RNA or have a function that is still yet to be discovered. These snoRNAs are known as orphans [4]. SnoRNAs

can also be further processed into short stable miRNA-like fragments called small nucleolar RNA-derived RNAs (sdRNAs)[5]. These fragments can come from orphan snoRNAs or snoRNAs that already have another function [6,7] Some sdRNAs have now been shown to have miRNA like functions including dicer- dependent processing, Ago protein association, and mRNA silencing. These miRNA- like properties make sdRNAs a promising target for gene regulation and allow for miRNA mimics to be made and used for phenotypic testing [8,9].

Notably, in 2012, snoRNA-42 was found to function as an oncogene and potential therapeutic target for lung cancer [15]. Additionally, SNORD50 has been implicated in affecting prostate cancer colony growth and breast cancer development [16,17] Similarly, SNORD76 was found to act as a tumor suppressor in glioblastoma [18]. In 2017, however, the Borchert lab was the first to characterize a role specifically for a sdRNA (not a full length snoRNA) in malignancy by confirming sdRNA-93 directly contributes to tumor invasiveness in breast cancer. In this work, analysis of the snoRNA expression profiles of MDA-MB-231 as compared to MCF-7 resulted in the identification of 13 snoRNAs expressed at markedly higher levels (≥7.5x) in MDA-MB-231s. Importantly, 10 of these 13 were found in complex with the miRNA-associating protein Ago suggesting their active involvement in the RNAi pathway. SdRNA-93 in particular was found to be significantly more highly expressed (≥75x) in metastatic MDA-MB-231s as compared to primary MCF-7 cells. Notably, an examination of sdRNA-93 expressions in small RNA-seq data corresponding to 116 patient tumors and normal breast controls, found robust expression of sdRNA-93 in 92.8% of Luminal B Her2+ tumors versus negligible expression in matched controls[10]. Phenotypic examination demonstrated that silencing sdRNA 93 reduced MDA-MB-231 cellular invasion by >90% at 48 h post transfection as compared to cells transfected with scrambled control, and conversely that sdRNA-93 over expression could

reciprocally increase MDA-MB-231 cellular invasion by >100% in the same amount of time [10]. In summary, this work confirmed that snoRNAs can be processed into smaller sdRNA fragments with miRNA-like functions, and that these fragments can regulate phenotypic aspects of malignancy.

In a similar fashion, our lab recently analyzed the small RNA transcriptome of 499 prostate cancer tissue samples and 52 healthy prostate tissue samples from The Cancer Genome Atlas (TCGA). As a result, 9 snoRNAs were found to be processed into sdRNAs heavily mis-expressed in prostate cancer. SdRNA-19B and sdRNA-24 in particular were found to be highly misexpressed in prostate tumors relative to control tissue. Excitingly, target predictions identified significant alignments between these sdRNAs and the 3' UTRs of known proto-oncogenes and tumor suppressor genes. Notably, sdRNA-19b aligned with (and potentially targets) CD44, a surface marker for aggressive prostate cancer [11] that regulates prostate cancer proliferation, migration, and invasion. SdRNA-19b also aligned with CDK6 and STAT5B which are involved in cell cycle regulation and breast/prostate cancer metastasis respectively [12]. Similarly, sdRNA- 24 aligned with RHOH, TIMP3, and AR which are known to be involved with prostate cancer invasion, a surface indicator of prostate cancer progression, and a regulator of prostate cancer migration respectively[13,11,14]. In addition to exploring regulatory targets, this study also describes the phenotypic consequences of manipulating cellular levels of sdRNA-19b and sdRNA-24 in prostate cancer (PC3) cell lines (e.g. proliferation, migration, and invasion). In summary, this work successfully identifies sdRNA-19b and sdRNA-24 as contributors to prostate cancer pathology, highlighting the relevance of this relatively new form of noncoding RNA regulator to malignancy while also identifying much needed potential biomarkers for deciphering aggressive vs. indolent disease.

Preliminary Data

In order to determine the snoRNAs that are processed into sdRNAs and most likely to be functionally relevant to the progression of prostate cancer five data sets were evaluated. The first was the snoRNA atlas which was used to identify 18 full length snoRNAs that are mis-expressed in prostate cancer. The second involved the search for sequences that were highly expressed as sdRNA in prostate cancer tissue as opposed to normal tissue. The third was a patient analysis of the small RNA transcriptome of 499 prostate tumor samples and 57 controls looking for differential expression. Next, snoRNA expression was evaluated in prostate cancer cell types 8140 (adenocarcinoma, non-specific) and 8550 (acinar adenocarcinoma) which resulted in the identification of 24 snoRNAs that are differentially expressed in both cell types. Finally, Ago protein immunoprecipitation was used to identify 100 sdRNAs associating with Ago proteins in PC3 cells. The sdRNAs bind to the Ago proteins like miRNA in order to impact gene expression. The result of these queries was the identification of sdRNA-19b and sdRNA-24 as being highly misexpressed in prostate cancer cells as compared to controls and the most likely candidates for impacting the phenotypic characteristics of aggressive prostate cancer such as proliferation, migration, and chemoresistance.

First, potential targets for sdRNA-19b and sdRNA-24 were found by using BLAST+ alignment tools from NCBI to align the sdRNA sequences with the 3' untranslated region (UTR) of known protooncogenes and tumor suppressor genes. The likely targets were found to be CD44, CDK6, and STAT5b for sdRNA-19b and RHOH, TIMP3, and AR for sdRNA-24. These targets were then confirmed through the use of luciferase assays. For the luciferase assays PC3 cells were cultured with DMEM in 12 well plates. At 85-90% confluency the cells were

transfected with sdRNA-19b and sdRNA-24 using the lipofectamine 2000 protocol. 24 hours after transfection, the cells were scraped and placed in Eppendorf tubes which were lysed by freezing and thawing 3 times. The tubes were centrifuged at 16,000 RCE for 1 min and 75uL of the supernatant were transferred to a 96 well plate. Finally, Dual-glo Luciferase Reporter System and a 96 well plate luminometer with an integration of 1000ms at 37 degrees was used to measure firefly and Renilla activities. The relative light units per well were determined by finding the quotient of Renilla/ firefly RLU. The target predictions were deemed successful because the sdRNAs bound to the fluorescent targets and inhibited expression. Knowing that sdRNA-19b and sdRNA-24 are over-expressed in malignant prostate tissue as compared to controls and having confirmed their targets as known protooncogenes and tumor suppressor genes, the next step was to explore the consequences of sdRNA expression on phenotypic attributes of PC3 cells.

Proliferation assays were performed by plating PC3 cells in 96 well plates at a concentration of 3x104 cells/mL in 100mL of RPMI media. The cells were transfected 24 hours after plating and were collected at 24 hr and 72hr time points. At these points the media was removed, the cells were washed twice with PBS, trypsinized, and mixed with trypan blue at a 1:1 ratio and counted using a Countess Automatic Cell Counter. Results from the assay conclude that when sdRNA-19b and sdRNA-24 are overexpressed in PC3 cells, cell proliferation increases significantly. The inverse was also observed when sdRNA-19b and sdRNA-24 were inhibited; proliferative ability decreased.

Migration assays were performed by culturing PC3 cells in a 24 well plate to 90-95% confluency before aspirating the media and transfecting with sdRNA-19b and sdRNA-24 mimics and inhibitors as well as controls using lipofectamine 2000. A 1mm-wide scratch was made

down the center of each well. Pictures were taken every 3 hours and ImageJ was used to assess the rate of migration by measuring the area of the scratch at each timepoint. The area was then divided by the height of the image to find average width. The width from one time point was subtracted from the width of the time point before and divided by 3 to produce the average rate of migration. Our results showed that over-expression of sdRNA 19b leads to a distinct increase in migratory ability. This preliminary information identifies sdRNA-19b and sdRNA-24 as having phenotypic consequences for PC3 cells. Therefore, the next step was to determine the impact of sdRNA expression on chemoresistance.

Aims

Overall Aims:

The overall objective is to examine the functionally relevant role of sdRNA-19b and sdRNA-24 expression in prostate cancer metastasis.

Specific Aims:

1. To analyze the impact of the differentially expressed target sdRNAs on the chemoresistance of PC3 cells
2. To describe the phenotypic patterns of PC3 cells when sdRNA-19b and sdRNA-24 are altered
3. To describe the targets of sdRNA-19b and sdRNA-24

Methods

Acquisition and Culture of PC3 Cells

The human prostate cancer cell lines (PC3) were acquired from the Mitchell Cancer Institute. The cells were grown and maintained in DMEM (Dulbecco's Modification of Eagle's Medium) with 4.5 g/L glucose and L-glutamine without sodium pyruvate and supplemented with 10% fetal bovine serum and 1% pen strep. The cells were kept at 37° C with 5% CO_2.

Acquisition of sdRNA Mimics and Inhibitors

Antisense oligonucleotides were designed to target sdRNA-19 (5′-AUCAGAGUUGGAUCUU GUAA-3′) and sdRNA-24 (5'-GUCAUCACCAUCUCUCAGAUA-3'), and ordered as custom IDT® miRNA Inhibitors from IDT (Integrated DNA Technologies, Coralville, IA). A scrambled nonspecific oligonucleotide was also ordered as a negative control (5′-GTGAGCTGTTTCAGTGGTTTGAGT-3′). Similarly, sdRNA-19b mimic, sdRNA-24 mimic and the scrambled control sdRNA-CUI (5'-GAUUCAAUUUGAUUUGCCCGUGGA-3') were ordered as custom miRIDIAN mimics from Dharmacon (GE Healthcare Dharmacon, Inc, Chicago, IL).

PC3 Transfection

Cells were cultured in 24- well plates and transfected after 24 hours with mimics or inhibitors using Lipofectamine 2000 (Invitrogen). For the transfection a 1:1 ratio of sdRNA and media (30µL) was combined with a 1:1 ratio of lipofectamine 2000 and media (30µL). A total of 4µL of transfection mixture was added to each well along with media raising the volume to 600µL.

PC3 Chemoresistance Assay

On the same day of transfection, the transfected cells are allowed to incubate at 37° C with 5% CO_2 for 20 minutes. At that point 4µL of the respective chemo agent are added to the cells. The concentration used were .025g/50mL of Cisplatin, .584uL/L of Dasatinib and 2.04µL/600mL for Paclitaxel. Methylene blue dye was added at 50% total volume to identify dead cells. After a 5 min incubation period hr 0 pictures were taken of each well. Pictures were then taken every 6 hrs. for 24 hrs. The number of dead and live cells were counted manually using ImageJ.

Analysis of Chemoresistance Assays

ImageJ was used to count the number of live and dead cells for each well for every time period. The ratio of dead to live cells were calculated for each well. All of the wells from the same time window and mimic or inhibitor for averaged to calculate average death rate.

Results/Discussion

The inhibition of sdRNA-19b and sdRNA-24 impacts PC3 cell chemoresistance.

Cells transfected with sdRNA-19b and sdRNA-24 mimics survived the introduction of chemo agents at a higher rate than PC3 cells transfected with the respective inhibitors. The ratio of dead to live cells was consistently higher for cells transfected with sdRNA-19b and sdRNA-24 inhibitors, suggesting that the respective sdRNAs play a role in maintaining the survivability of PC3 cells when exposed to chemo agents. SdRNA-19b mimics and overexpressions had significantly variable survivability when exposed to Cisplatin **(Figure 6b)** while sdRNA-24 displayed a significant contrast when treated with Paclitaxel **(Figure 6a)** over the course of 24 hours. The effect of sdRNA expression on resistance to Dasatinib requires further study as the results were less consistent.

Analysis of chemoresistance in response to the expression of other sdRNAs requires further study

Although results for respective mimics vs. inhibitors were consistent, further trials including controls of other various sdRNAs are needed to draw significant conclusions. Although this data does show a promising pattern mimics and inhibitors of unrelated sdRNAs are needed to confirm that sdRNA-19b and sdRNA-24 have a unique effect on survivability.

SdRNA-19b and sdRNA-24 are misexpressed in prostate cancer and impact phenotypic characteristics

The computational analysis, target analysis, proliferation study, and migration assays point to the impact of sdRNA-19b and sdRNA-24 overexpressions on increased malignancy. Chemoresistance results are in further agreement with these patterns as overexpression generally lead to greater chemoresistance and better survival while inhibition leads to greater cell death. All of these conclusions point to the potential role of sdRNA-19b and sdRNA-24 as vital to understanding prostate cancer progression.

Conclusions

Prostate cancer, the second most common type of cancer related death in men, has a highly variable course that is difficult to distinguish. New biomarkers are needed for early detection, intervention, and outcome predictions that are all necessary for successful treatment. Recent studies suggest that the answer to this quest are sno-derived RNAs that function in a manner similar to miRNAs. Our lab recently published a paper characterizing sdRNA-93 as an active contributor to aggressive phenotypes in breast cancer. Inhibiting sdRNA-93 led to decreased invasion while the inverse was true for over-expressions. This study that suggests the direct role of sdRNAs in breast cancer pathology and led to the inquiry of other highly misexpressed sdRNAs in other forms of malignancy such as prostate cancer.

Thus, a comprehensive study of snoRNA derived fragments in prostate cancer was conducted and concluded that sdRNA-19b and sdRNA-24 were dramatically differentially expressed between cancer and control groups (Figure 2). The predicted molecular targets for these sdRNAs were also found to be directly involved with tumor progression. Among predicted targets for sdRNA-19b are the 3'UTR regions of CDK6, Stat5b, and CD44, all of which have a known role in regulating prostate cancer proliferation (Figure 3). SdRNA-24 also had intriguing targets including RHOH, Timp3, and AR. These targets have been described as facilitating prostate cancer invasion and migration (Figure 3). Following the determination of heavily misexpressed sdRNA in prostate cancer tissue samples and their respective targets, the phenotypic effect of manipulating sdRNA levels on PC3 proliferation, migration, and chemoresistance needed to be evaluated.

Proliferation assay results indicate that sdRNA-19b and sdRNA-24 levels have a positive relationship with increased cell proliferation. Overexpressions of both sdRNAs led to increased proliferation and the reverse was also true for inhibitors (Figure 4). Migration data suggests that cells transfected with sdRNA-19b mimic experienced faster migration rates whereas, when inhibited, the PC3 cells showed a decrease in migration rates (Figure 5). Preliminary results from the chemoresistance assay also indicate a direct role between sdRNA expression level and cell survivability across three types of chemo agents: Dasatinib, Cisplatin, and Paclitaxel.

When sdRNA-19b and sdRNA-24 were inhibited, both showed significantly reduced survivability compared to their respective mimics meaning PC3 cells with sdRNA-19b and sdRNA-24 inhibited could not withstand the chemo agents and died at a significantly higher rate than their controls. Inhibition of sdRNA-19b had the most profound effect when introduced to Cisplatin (Figure 6a) while sdRNA-24 inhibition had the most significant impact when tested with Paclitaxel (Figure 6b). SdRNAs that are shown to impact the cell's ability to resist chemotherapy treatment could be useful targets when trying to increase the effectiveness of a chemo agent. They could also serve as indicators when deciding on a plan for intervention. This experiment, however, must be confirmed and the inclusion of further sdRNA controls as well as placebo transfection controls are necessary in order to standardize results. Additionally, more experiments beyond the scope of this study will be necessary in order to fully describe the phenotypic impact of sdRNA-19b and sdRNA-24 on prostate cancer progression.

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Appendix of Figures

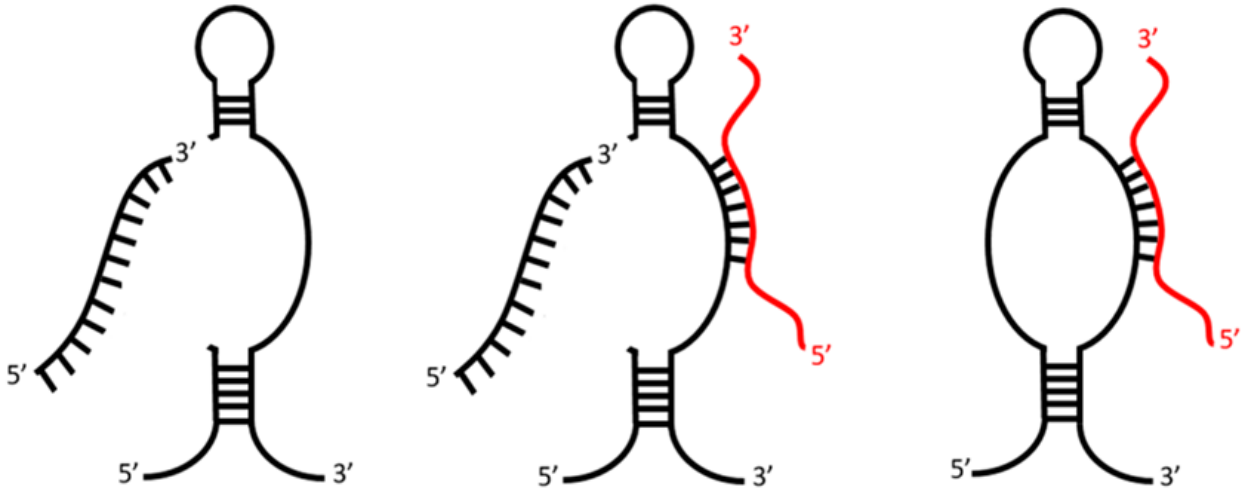


Figure 1. SnoRNAs can function as RNA editors, miRNA precursors, or participate in both forms of non-coding RNA regulation.

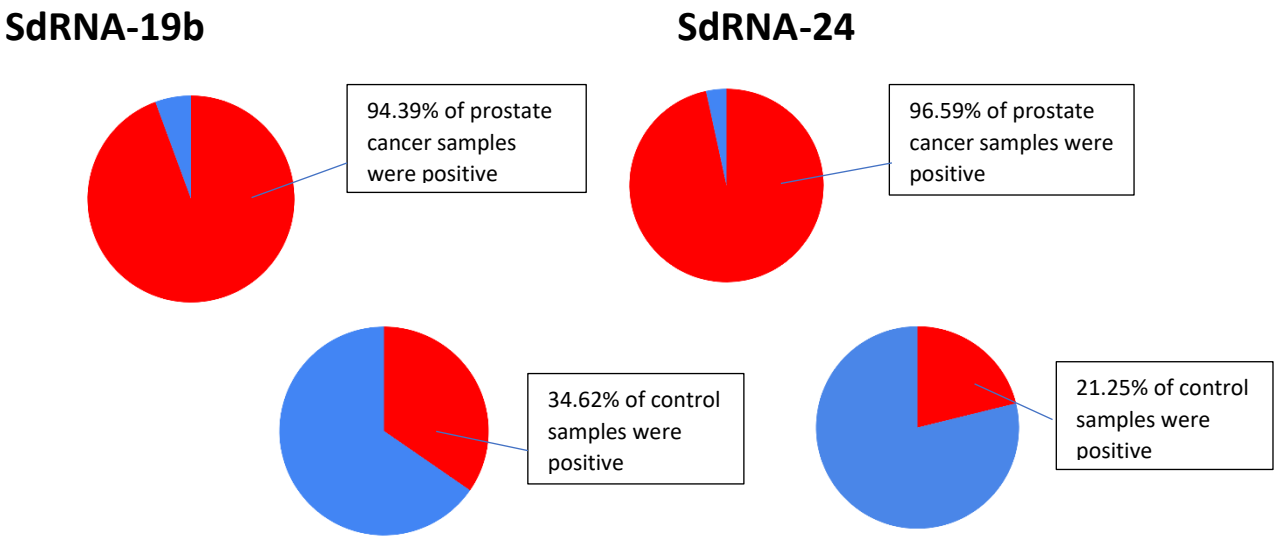


Figure 2. SdRNA-19b and sdRNA-24 are differentially expressed in prostate cancer vs. control tissue. Out of 499 prostate cancer tissue sample sequences and 52 controls, sdRNA-19b and sdRNA-24 show significant differential expression

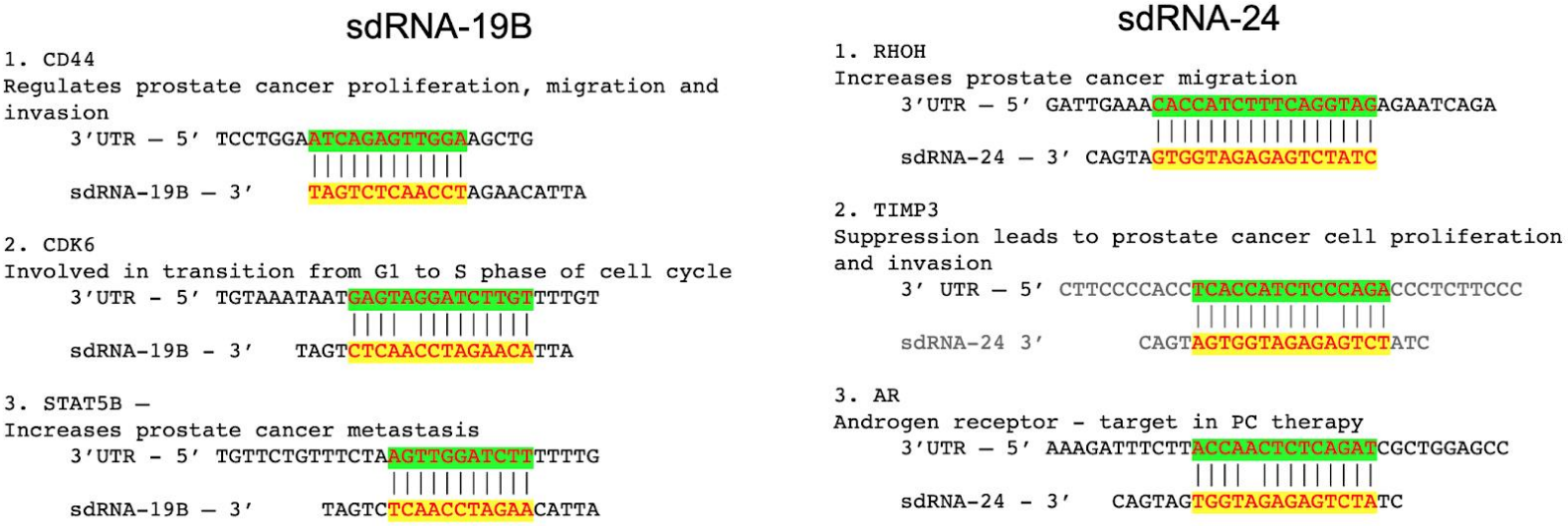


Figure 3. SdRNA-19b and sdRNA-24 align to the 3'UTR of known oncogenes and tumor suppressors. SdRNA-19b aligns with CD44, CDK6, and STAT5b, all related to prostate cancer progression. SdRNA-24 aligns with RHOH, TIMP3, and AR.

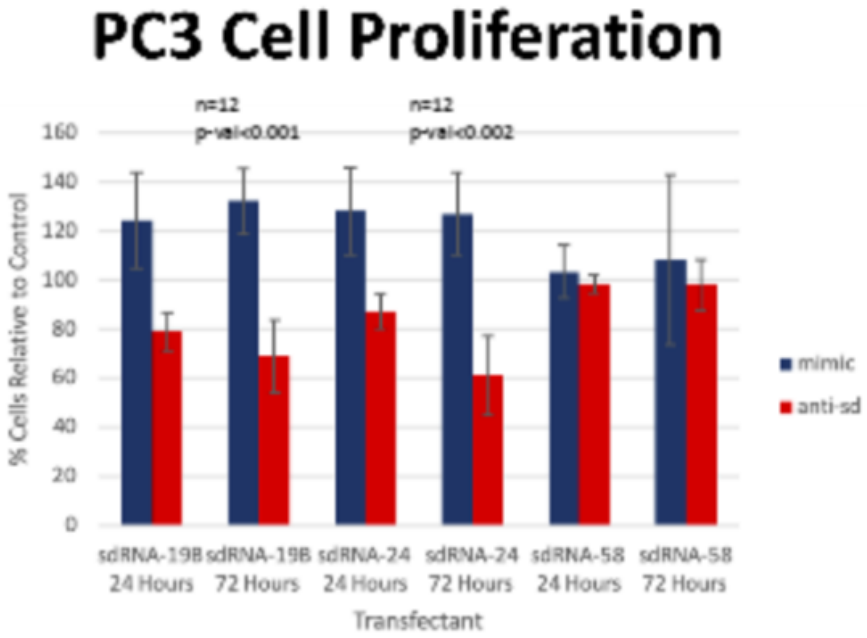


Figure 4. Impact of sdRNA-19b and sdRNA-24 on PC3 cell proliferation. Over-expressions of sdRNA-19b and sdRNA-24 resulted in increased cell growth shown at 24 and 72 hr.

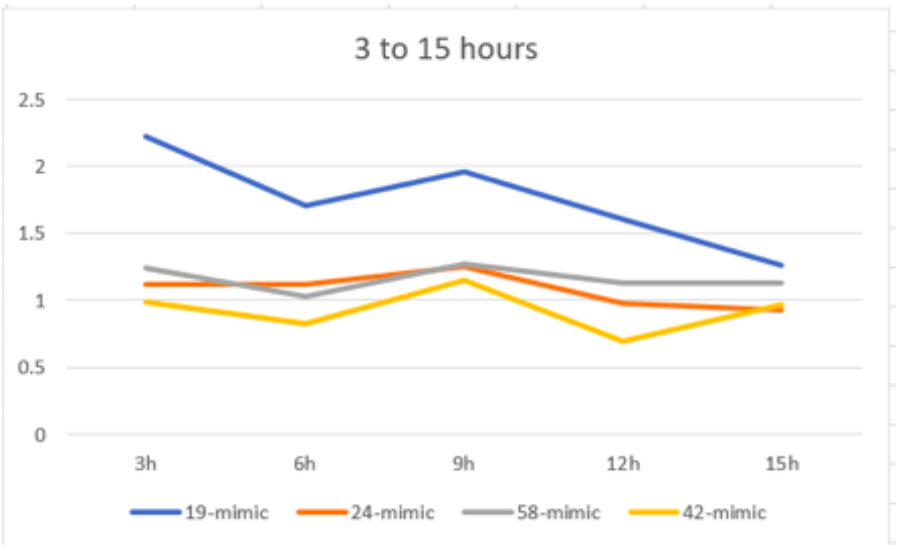
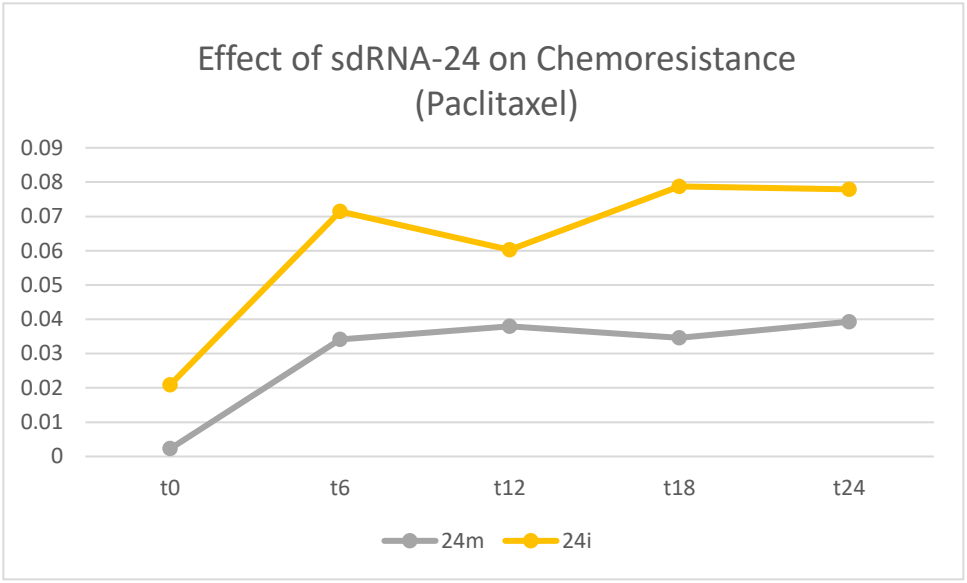


Figure 5. Effect of sdRNA-19b over-expression on migration. Transfection with sdRNA-19b mimic leads to faster migration over 24 hours at a 97.4% confidence level.

A.



B.

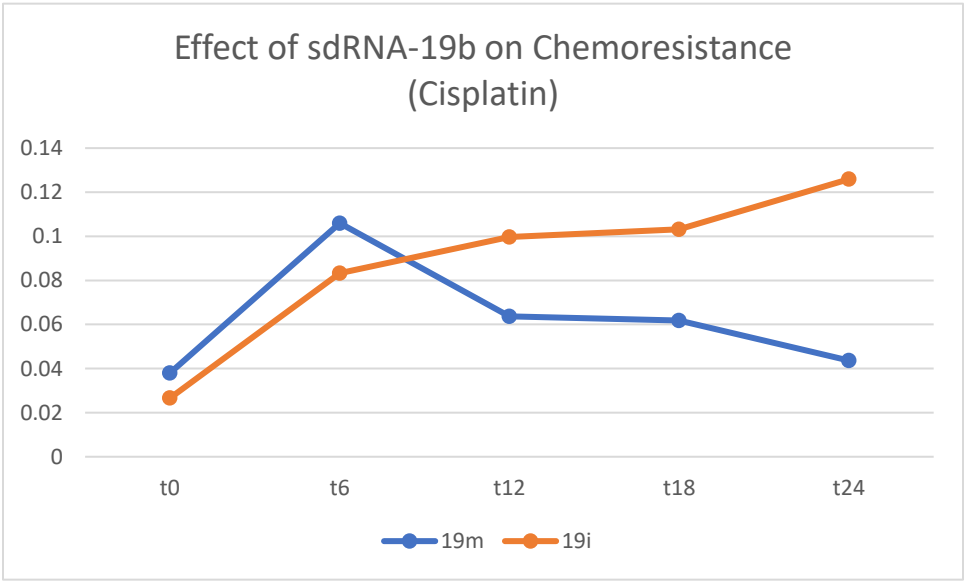


Figure 6. Effect of sdRNA-19b and sdRNA-24 on chemoresistance. When PC3 cells were transfected with inhibitors of sdRNA-19b and sdRNA-24 they were more susceptible to chemotherapeutic agents and experienced death at a higher rate.