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14. ABSTRACT (200 words)

The overall goal of this project is to develop novel method to inhibit cancer metastasis. The major hypothesis to be tested is that aptamers (short oligonucleotides) can be developed that target the process of invasion, without prior knowledge of a target protein, and that these aptamers will inhibit the development of metastasis. We have identified two aptamers (Mod 12 and Mod 13) using a novel application of a process called "systemic evolution of ligands by exponential enrichment" (SELEX), that bind PCa cells that we selected for their high invasive ability. In the work done to date, we have optimized aptamer synthesis for quality and quantity; identified that the aptamers work best on PCa, as opposed to other cancer cell types; identified that Mod 13 is more effective at inhibiting in vitro invasion then Mod 12; determined that AIAs have no direct impact on overall cell growth in vitro; and established a novel bioluminescent method to measure invasion of cells in vitro. These results now have has poised to be able to assess the ability of Mod 13 to inhibit PCa metastasis in an in vivo model in ongoing studies.

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Metastasis, invasion, aptamer, anti-metastasis therapy

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INTRODUCTION

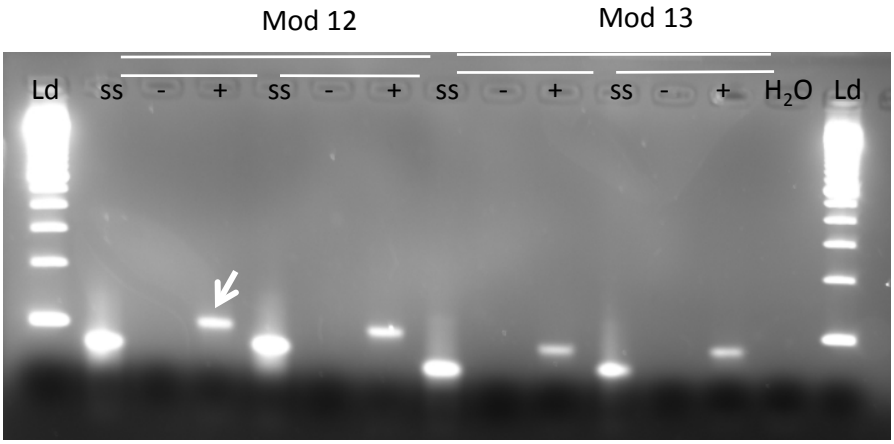
Metastasis and tumor progression at metastatic sites ultimately results in the demise of prostate cancer (PCa) patients. Currently there are no highly effective methods that can target these problems. Aptamers (reviewed in 1), which have proven clinical efficacy for non-neoplastic disease (2) and are generally more specific and stable than antibodies, may have clinical utility in PCa. However, defining aptamers that can prevent metastasis is challenging due to the fact that many proteins that play a role in the metastatic process are unknown. The overall goal of this project is to develop novel method to inhibit cancer metastasis. The major hypothesis to be tested is that aptamers (short oligonucleotides) can be developed that target the process of invasion, without prior knowledge of a target protein, and that these aptamers will inhibit the development of metastasis. We also hypothesize that the aptamers can be used to identify cell surface proteins that are important mediators of metastasis. This latter information is important as it may help identify further therapeutic targets. We have made some initial progress towards testing this hypothesis. Specifically, we have identified aptamers, using a novel application of a process called "systemic evolution of ligands by exponential enrichment" (SELEX) (reviewed in 3), that bind PCa cells that we selected for their high metastatic ability. We now propose to test these aptamers for their ability to inhibit metastasis and identify their target protein.

BODY

The technician that was working on this project originally unexpectedly left immediately prior to the initiation of the project. This necessitated hiring and training a new technician to work on this project, which resulted in a significant delay (i.e. 6 months) in the start of the specific tasks, although the new technician was working on project-relevant methods and essentially fine tuning invasion assays and producing high quality aptamer during this time period.

In order to perform this project, we needed to have high quality aptamer that can be administered in vivo. Our original plan was to purchase this from a company, but then they could not guarantee a feasible production amount (i.e. they did not guarantee a high yield). Thus we took on the challenge of synthesizing our own aptamer to ensure production quality and sufficient yield. We initially attempted to PCR amplify aptamer sequences from plasmid clones; however, even at 35 cycles we often found contaminant bands and very feint aptamer-specific bands, indicating low yield with possible contaminants. To overcome the problem of amplifying from a plasmid, we ordered full DNA sequences of the aptamer sequence alone to use as PCR template to create aptamer RNA synthesis template. We initially had to test a variety of PCR conditions to optimize the PCR to minimize contaminating non-specific PCR products, while maximizing specific target yield. This allowed us to perform just 5 cycles of PCR to create sufficient DNA template for RNA aptamer synthesis. We then needed to optimize the amount of DNA template to use, as our initial attempts indicated we were not using sufficient amounts; however, once we precipitated and concentrated the DNA templates, we were able to perform RNA transcription that yielded good amounts of uncontaminated RNA aptamer (Fig. 1).

Unfortunately, the process to get to this final success point, including the unsuccessful attempts took 6 months. But now we have this aspect optimized and can produce aptamer as needed.

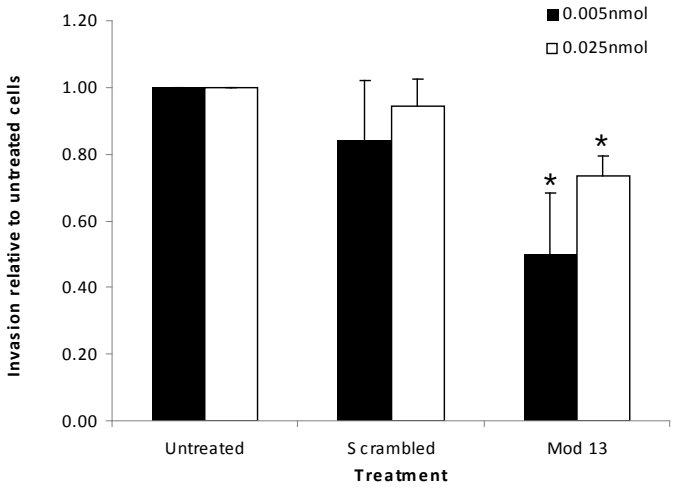


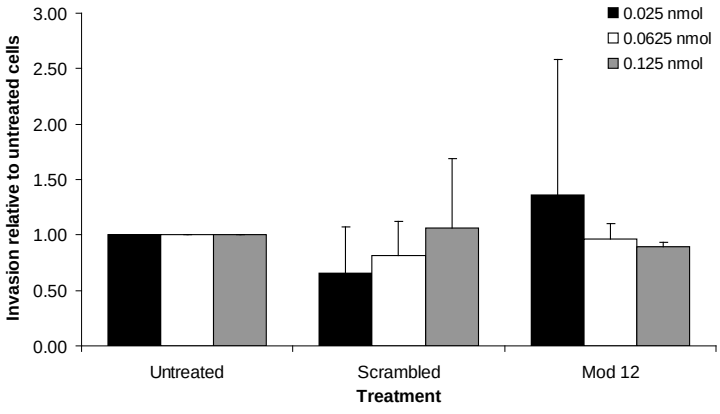
indicated times.**Figure 1. Production of RNA aptamers.** SS DNA template was used to create Mod 12 and Mod 13 aptamers (also called AIA 1 and AIA 2). RNA aptamers with T7 RNA polymerase. The RNA aptamers are indicated by the single strong intensity bands in the "+" lanes (arrow gives example). SS=single stranded DNA; "-" =No RNA polymerase; "+"= RNA polymerase' Ld = ladder.

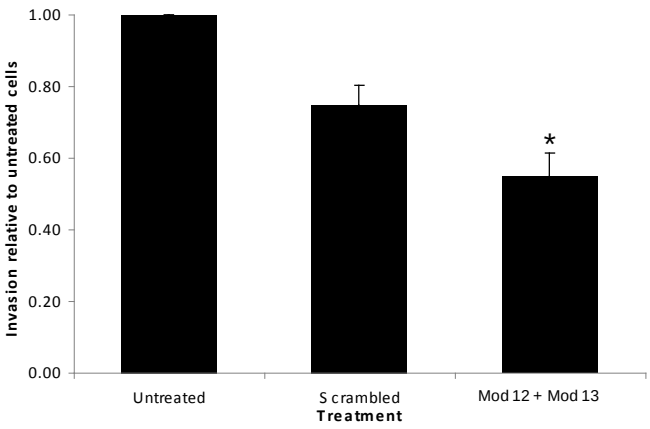
Task 1.1 Compare the anti-invasive aptamers (AIAs) efficacy and synergy on in vitro invasion.

We compared the effect of Mod 12 and Mod 13 and their combination on PC-3 luciferase cells. Mod 12 had minimal anti-invasive ability compared to scrambled control; whereas, Mod13 had marked anti-invasive ability (~50% reduction of invasive cells) compared to scrambled control (Fig. 2). The combination of both anti-invasive aptamers was less anti-invasive than Mod 13 alone.

Figure 2. MOD 13 has greater anti-invasive properties than MOD 12 in PC-3 cells. PC-3-lux cells were plated in modified Boyden invasion chambers with scrambled aptamers and either Mod 12, Mod 13 or the combination of both at various concentrations. After 24 hours, the invasive cells were counted. Result are shown as relative to untreated cells. *p<0.05 versus untreated cells.



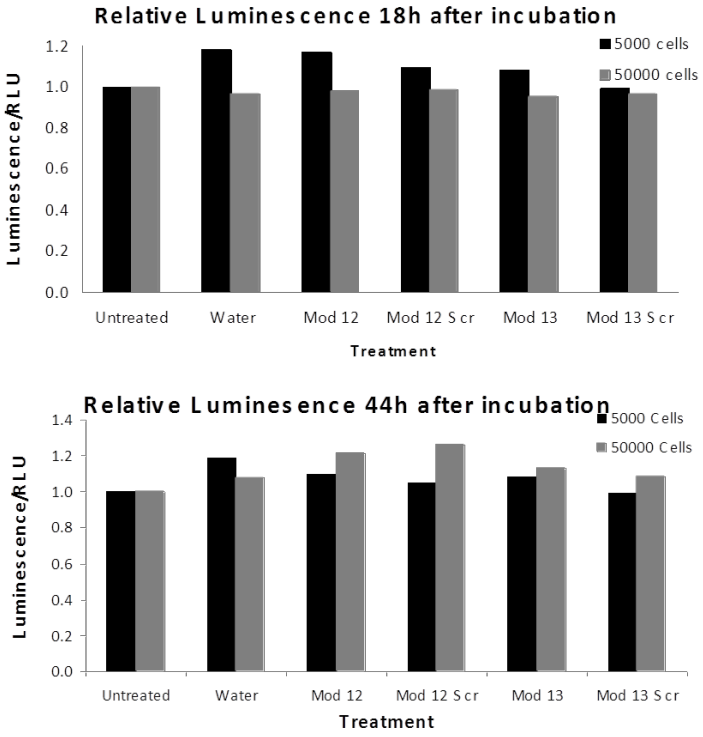




Task 1.2 Determine AIAs effect on cell's growth.

In order to determine if the AIAs had a direct impact on cell growth, we incubated PC-3-luciferase cells with Mod 12 or Mod 13 and measured total cell number (as indicated by overall luminescence) at 18 hours and 44 hours after beginning incubation with AIAs. We also evaluated the impact with a low and high tumor cell density. Neither AIA had an impact on overall cell growth at either high or low cell density or at either time point (Fig. 3). These results indicate that the AIA's impact on invasion or metastasis is not through a direct impact on cancer cell growth. These results also indicate that there is no impact on cell proliferation or cell apoptosis. However, we cannot rule out that there are opposite effects on these parameters, which would result in no impact on overall growth. Accordingly, we will perform specific tests on cell cycle and apoptosis in the next fiscal year.

Figure 3. The AIAs have no impact on cell growth in vitro. PC-3-lux cells were plated at the indicated densities in 96-well plates and the indicated treatment was given. Aptamers were given at 5 μg/ml final concentration. Luminescence was quantified at the



Task 1.3 Determine if the AIA's anti-invasive effect is PCa specific or impacts other cancers.

We evaluated the effect of Mod13 on in vitro invasion of several other cancer types. It decreased the invasion of UC6, a bladder cancer cell line, but not of MDA-231 (breast cancer) (Fig. 4). It did decrease invasion of 82L osteosarcoma cells, which is a highly invasive variant, of the OS-187 osteosarcoma cells, which it did not decrease. This latter observation indicates that it successfully targets invasive; however, a caveat is that the scrambled aptamer also inhibited 82L and UC6 invasion. Thus, this may be a non-specific effect in these cell lines. We will re-evaluate the in vitro invasion with our more precise method of quantification (see following paragraph on optimizing invasion assays). Also, we need to still evaluate Mod 12 for its effect. At this point, it appears a specific anti-invasive effect is seen only in prostate cancer, as the aptamers can inhibit prostate cancer invasion above and beyond that of the scrambled controls.

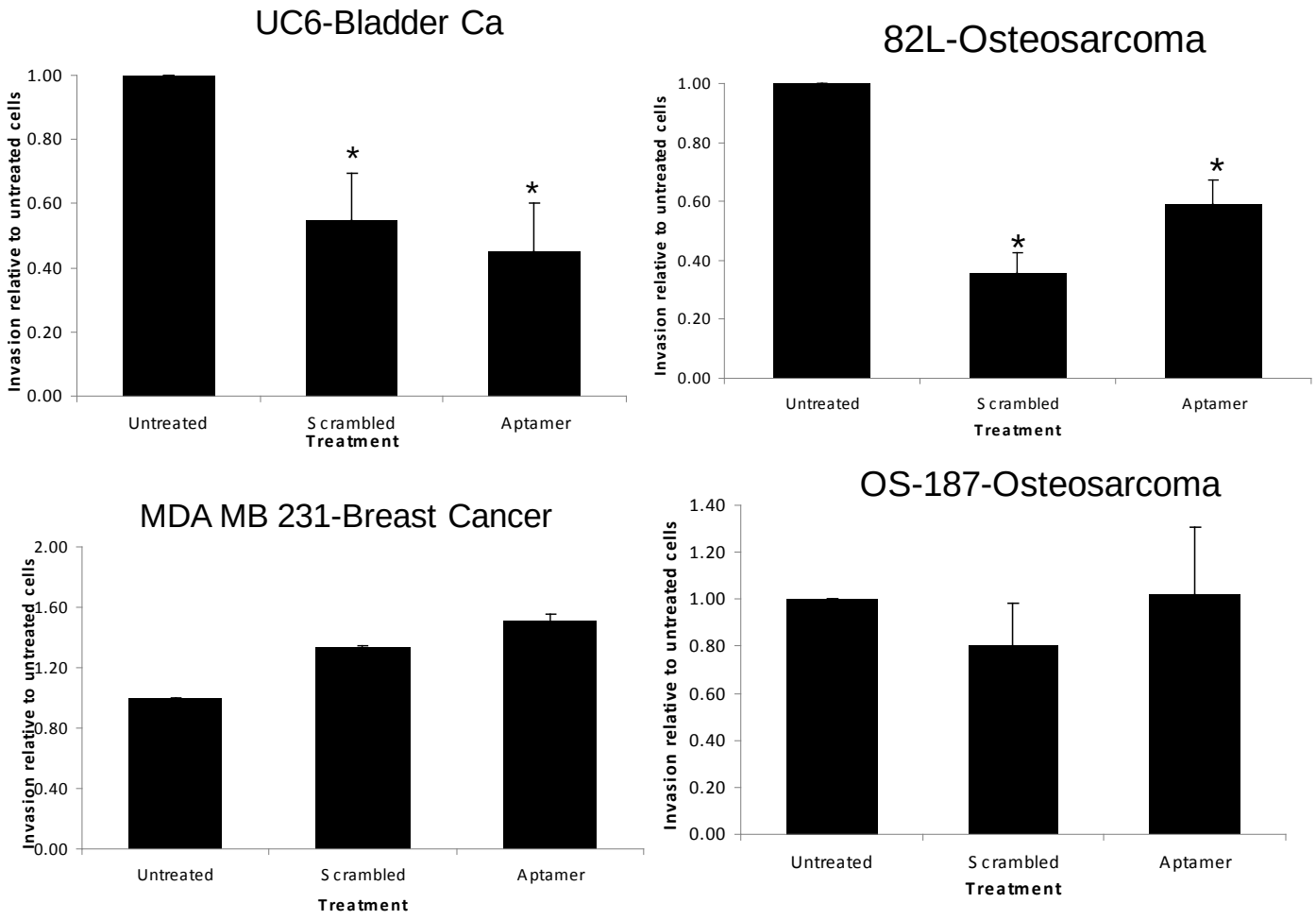
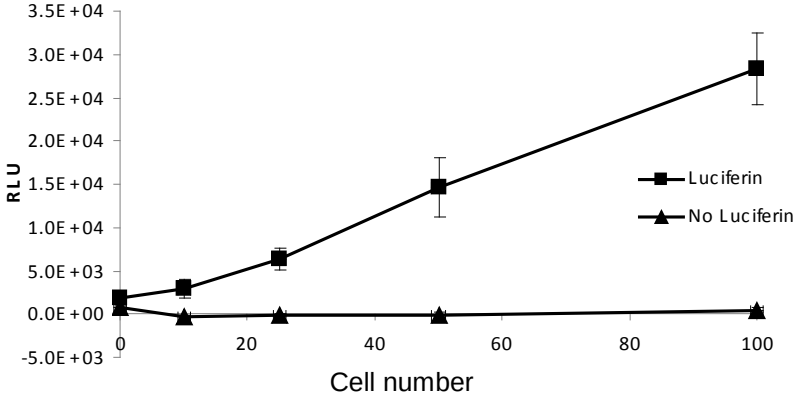


Figure 4. Effect of Mod 13 on in vitro invasion of various cancer cell lines. Cells were plated in chambers of modified Boyden chambers and either untreated or treated with scrambled control or Mod12 aptamer (5 μg/ml). 24 hours later the numbers of invasive cells were quantifed. Data are presented relative to non-treated cells. *$p<0.05$ versus untreated cells.

Optimizing invasion assays. As we progressed through many of these in vitro invasion assays, it became very apparent that these assays are prone to several deficiencies. Current methods to quantify invasion assays include the need to manually count the number of cells that invade through the bottom of the membrane filter. This is very time consuming and prone to operator error resulting in large variance that can preclude the ability to identify statistical significance. We decided to pursue a method to optimize this assay. Specifically, we felt that if the total number of cells on the bottom of the filter could be quantified through a non-subjective and rapid approach, this would result in enhancing this assay. To that end, we determined if we could measure the total luminescence of the cells that invade through the filter as a measure of cell number. To that end, cells were plated on top of invasion chamber membranes in increasing numbers and then after 24 hours, the cells that invaded were gently washed off the bottom membrane filter, the subjected to luciferase measurement, then manually counted. The data were then plotted as the relation between manual cell count and luminescence. The correlation

between the two was outstanding (Fig. 5) indicating that this assay will be useful as we move forward in our experiments.

Figure 5. Invasive cell luminescence correlates with invasive cell number. PC-3-luciferase cells were plated at increasing numbers in in vitro assays and 24 hours later cells that were on the bottom of the membrane were collected and subjected to luminometer assay after addition of luciferin (on none as negative control) and then manual cell count. RLU = relative light units.



Task 2.1. Effect of aptamer on spontaneous PCa metastasis.

This task has not been initiated as we are still optimizing which aptamer is best and due to the delays due to aptamer synthesis optimization.

KEY RESEARCH ACCOMPLISHMENTS

- Optimized aptamer synthesis for quality and quantity
- Identified that AIAs work best on prostate cancer, as opposed to other cancer cell types
- Identified that MOD-13 is more effective at inhibiting in vitro invasion then MOD-12
- Determined that AIAs have no direct impact on overall cell growth in vitro
- Established a novel bioluminescent method to measure invasion of cells in vitro

REPORTABLE OUTCOMES

None

CONCLUSION

We had a significant delay in initiating the specific tasks due to a transition in personnel and the need to optimize the aptamer synthesis. However, we are now moving forward rapidly and anticipate we will be right on schedule within the next 6 months. The research to date indicates that MOD-12 is the most effective AIA to diminish in vitro invasion of prostate cancer cells. Furthermore, since there is no direct effect on overall prostate cancer cell growth, any anti-metastatic effect on prostate cancer observed in in vivo models will most likely be due to an anti-invasive effect, as opposed to direct anti-tumor growth

effect. These data indicate that we are close to being able to move forward to animal studies to test the impact of MOD-12 on prostate cancer metastasis in an animal model. What is the impact of these studies? At this point, they are too preliminary to move to clinical trial, but if successful, they will provide proof of concept that we can target the metastatic phenotype through use of aptamers. Aptamers are already in clinical use, so these results could rapidly be translated to clinical trials.

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