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TITLE: Highly Specific Targeting of the TMPRSS2/ERG Fusion Gene in Prostate Cancer Using Liposomal Nanotechnology

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14. ABSTRACT The TMPRSS2/ERG fusion gene is found in about 55 % of prostate cancer (PCa) patients. It is absolutely specific for PCa cells, since the fusion transcript is only present in these cells. There is heterogeneity in the structure of the 5' end of the mRNA transcripts of the fusion gene. Some prostate cancers express a single mRNA type, while others express multiple isoforms of the fusion gene that arise via alternative splicing of the initial fusion transcript. We seek to target the four most common and biologically active alternatively spliced fusion gene transcript isoforms using SiRNAs to obtain maximal biological activity in cancers expressing a specific isoform or a combination of isoforms. We propose to use of systemically administered nanoliposomal siRNAs specifically targeting the TMPRSS2/ERG mRNA fusion junctions in orthotopic prostate cancer model in mice. Because this fusion gene is highly specific to PCa we do not expect off-target effects in normal tissues or minimal toxicity. Our results support the efficacy of this approach in in vivo PCa models.					
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INTRODUCTION

Since its discovery use of small-interfering RNA (siRNA) has rapidly become a powerful tool for therapeutic and specific gene silencing. Recently siRNA technology has generated much excitement for possible use as a novel therapeutic modality. However, in vivo siRNA delivery has proven difficult because of lack of non-toxic and effective systemic delivery methods. We have developed neutral based nanoliposomal delivery system for in vivo therapeutic use of siRNA therapeutics.

The discovery of recurrent fusion of the androgen-regulated TMPRSS2 gene to the ERG gene in the majority of prostate cancer (PCa) lesions, has led to a paradigm shift in the study of PCa. The TMPRSS2/ERG fusion gene occurs in 15-80% of PCa lesions, depending on the clinical stage, with 40-60% of surgically treated cancers containing the gene fusion. Most studies have shown an association between the presence of the TMPRSS2/ERG fusion and aggressive disease. We have now demonstrated that the TMPRSS2/ERG fusion gene isoforms can enhance proliferation, invasion and motility of prostate epithelial cells. More importantly, knockdown of the fusion gene in a cancer cell line inhibits tumor growth in vivo in an orthotopic mouse model, indicating that the TMPRSS2/ERG fusion gene is a potential therapeutic target which is present in the majority of prostate cancers.

All reports to date indicate that there is significant heterogeneity in the structure of the 5' end of the mRNA transcripts of the fusion gene. Thus, some prostate cancers express a single mRNA type, while others express multiple isoforms of the fusion gene that arise via alternative splicing of the initial fusion transcript. We have characterized 8 fusion types in PCa (1), which have been confirmed by others. In all cases, the fusion mRNA includes the TMPRSS2 exon 1 and often exon 2, as well. The most common transcript contains the TMPRSS2 exon 1 fused to ERG exon 4, such that translation would have to arise from an internal ATG codon and give rise to a slightly truncated protein which we have designated as the Type III isoform. This variant is expressed in 86% of fusion gene expressing prostate cancers, either alone or in combination with other isoforms. Of particular interest is an isoform in which TMPRSS2 exon 2 is fused with ERG exon 4 (designated Type VI). This variant was present in 26% of our cases with fusion gene expression (1). For this isoform, translation can be initiated from the TMPRSS2 translation initiation codon and results in a true fusion protein containing the first five amino acids of the TMPRSS2 gene fused to a slightly truncated ERG protein. We found that expression of this isoform is associated with aggressive disease. Types I and II give rise to full length ERG protein arising from the native ERG ATG and are also associated with more aggressive disease. These isoforms are present in 20% and 11% of fusion gene expressing cancers respectively.

In vivo systemic siRNA delivery: The promise of specific RNA degradation has also generated much excitement for possible use as a novel therapeutic modality. However, successful application of siRNA therapeutics to clinic requires development of safe and effective delivery system. In vivo siRNA delivery has proven difficult because of lack of non-toxic and effective systemic delivery methods. We recently developed non-toxic neutrally charged 1,3-bis(sn-3'-phosphatidyl)-sn-glycero-2'-phosphoethanolamine (DOPC)-based liposomal nanovectors (mean size 65nm) that can target siRNA in vivo into tumor cells 10-fold and 30-fold more effectively than cationic lipids and naked siRNA, respectively, leading to significant and robust target gene silencing in orthotopic cancer models.

The TMPRSS2/ERG fusion gene is absolutely specific for prostate cancer cells, since the fusion transcript is only present in these cells. Unfortunately, there is heterogeneity in the structure of the 5' end of the mRNA transcripts of the fusion gene as described above. Thus, some prostate cancers express a single mRNA type, while others express multiple isoforms of the fusion gene that arise via alternative splicing of the initial fusion transcript. We seek to target the four most common and biologically active alternatively spliced fusion gene transcript isoforms, which constitute greater than 95% of all transcripts, to obtain maximal biological activity in cancers expressing a specific isoform or a combination of isoforms. In vivo knockdown of TMPRSS2/ERG fusion gene expression using liposomal nanovectors should decrease prostate cancer progression in vivo and be an effective therapeutic strategy in human prostate cancers bearing this fusion gene. Given the extremely high prevalence of this chromosomal alteration in human prostate cancer, the majority of prostate cancers may be amenable to this treatment. We propose to use siRNAs specifically targeting the TMPRSS2/ERG mRNA fusion junctions, which are present only in PCa cells, to minimize off-target effects in normal tissues so toxicity should be minimal.

BODY

As outlined in our Statement of Work a number of tasks were proposed for the first 24 months; many of these tasks have been accomplished. For the sake of clarity these tasks will be grouped under three main goals.

Goal 1. Evaluation of the efficacy of the SiRNA knockdown of the Type III fusion gene in vivo

Results:

We designed a series of 18 siRNAs spanning the fusion junction of the TMPRSS2 and ERG genes in the Type III fusion mRNA. We then tested these siRNAs systematically using transient transfection in 293T, PNT1a expressing the Type III fusion gene and VCaP cells using Western blot and/or quantitative RT-PCR. Of the 18 original siRNAs we identified three that gave strong, consistent and reproducible knockdown of the Type III TMPRSS2/ERG fusion gene. Figure 1 shows a Western blot of with anti-V5 antibody on cell extracts of 293T cells transiently transfected with V5-Tagged Type III fusion gene and several siRNAs. Control cells are liposomes only while scrambled represents a non-specific siRNA. As can be seen in Figure 1, Si8, Si11 and Si14 all give very strong knockdown of the fusion gene. These results were confirmed by quantitative RT-PCR in 293T, PNT1a with Type III fusion and VCaP cells. Based on these results we moved forward with our in vivo experiments using DOPC liposomes to deliver Si8 and Si14 in an orthotopic VCaP model. This experiment is outlined in Figure 2.

One week after orthotopic injection mice with luciferase-expressing VCaP cells, treatment was initiated with siRNAs delivered using DOPC liposomes. Mice were injected with control or twice weekly. Mouse weight was followed and tumor imaging was performed weekly using a Xenogen imaging system after luciferin injection. The experiment was terminated after 4 weeks of treatment and primary tumors weighed and submitted for histopathology and complete necropsy performed on mice. Mice were euthanized 48 hours following the last injection of siRNA. Of note, no toxicity was noted in any mouse. Tumor weights are shown in Figure 3. Both the Si8 and Si14 groups showed a significant decrease in tumor weight ($p < .001$, t-test) when compared to scrambled control. Luciferase imaging was concordant with the final tumor weight ($r^2 = .649$, $p < .0001$). Both siRNAs decreased tumor weight by approximately 50%. Our initial quantitative RT-PCR results indicate approximately 40% knockdown of fusion mRNA in both treated groups. We attempted a second experiment in which we increased the dosage of liposomes to 450 $\mu\text{g}/\text{kg}$, but this did not improve knockdown (data not shown). To determine the degree of knockdown of ERG protein in tumors we carried out Western blot analysis of tumor extracts from these experiments. As can be seen in Fig 4A, the degree of ERG knockdown was highly variable in treated tumors relative to controls. Of note, the degree of knockdown of ERG was concordant with knockdown of one of its downstream target Cyclin D1.

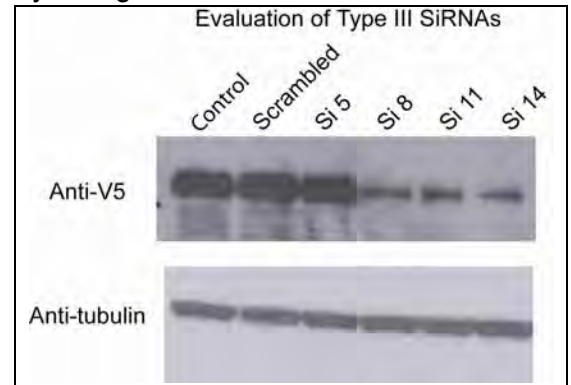


Fig 1. Western blot with anti-V5 antibody of 293T cells transfected with V5-tagged Type III fusion gene, liposomes only (control), scrambled siRNA and four targeting siRNAs. Tubulin is a loading control.

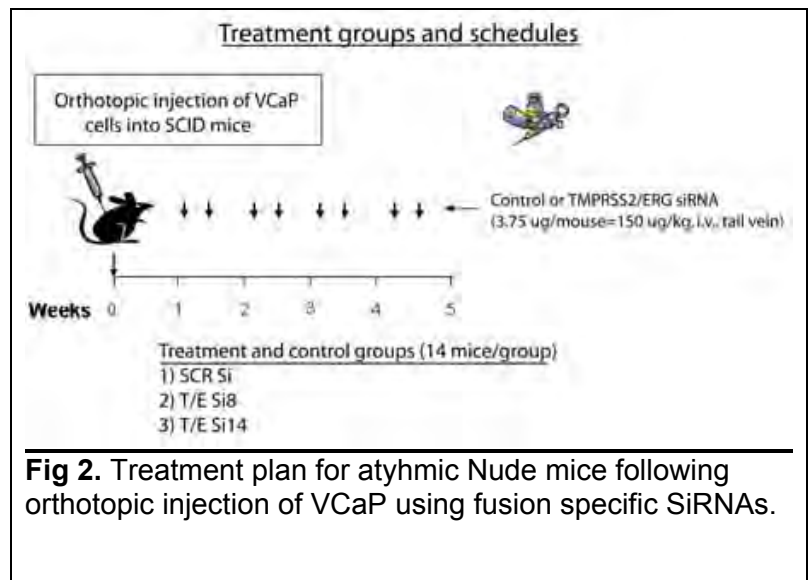


Fig 2. Treatment plan for athymic Nude mice following orthotopic injection of VCaP using fusion specific siRNAs.

After analysis of Western blots we found a strong correlation between tumor levels of ERG and final tumor weight ($r(2) = .64$, $p = .007$; Fig 4B). These results indicate that variable delivery or efficacy of siRNA due to tumor or mouse specific factors is decreasing the therapeutic efficacy of the siRNA treatments. To determine the mechanism(s) of decreased tumor growth in siRNA treated tumors we quantitated proliferation using Ki67

immunohistochemistry (IHC) and image analysis of stained sections. As shown in Fig 5A, proliferation was significantly decreased in treated tumors ($p < .001$, Mann Whitney). A similar analysis of angiogenesis was carried using IHC with anti-CD31 antibody (Fig 5B). The extent of blood vessels in Si8 and Si14 treated tumors was significantly decreased ($p < .001$) relative to scrambled control treated tumors (SCN). TUNEL analysis is pending

To further enhance delivery in order to maximize therapeutic efficacy we created Polyethylene glycol (PEG) coated liposomes since these type of liposomes have longer circulation time and considered “stealth liposomes”. We have shown significant anti-tumor effects of our T/E fusion gene targeting nanoliposomal vectors.

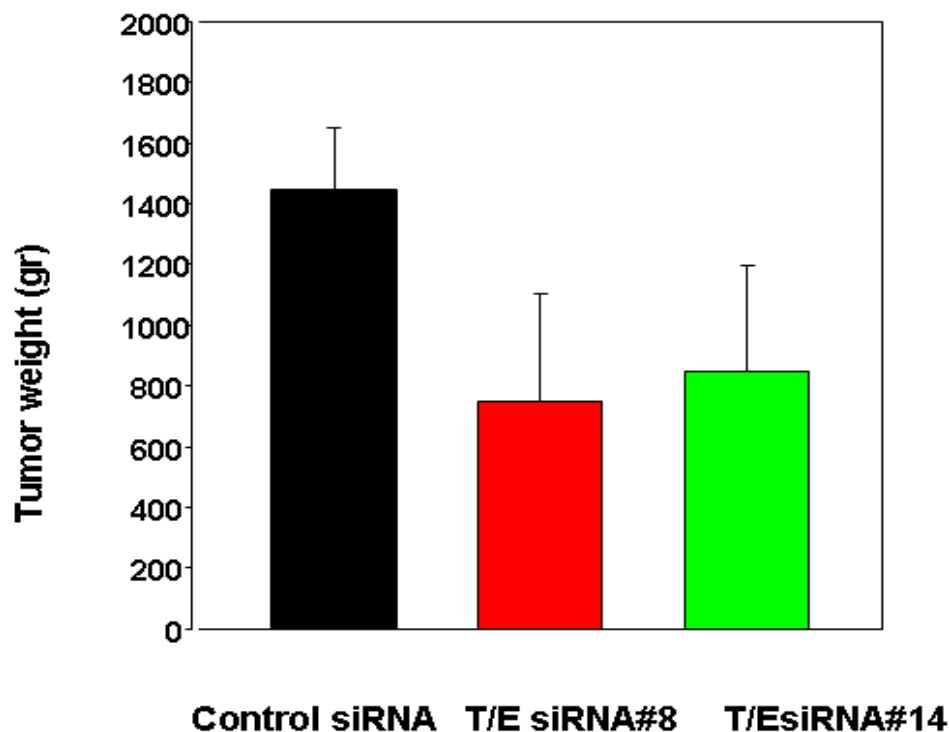
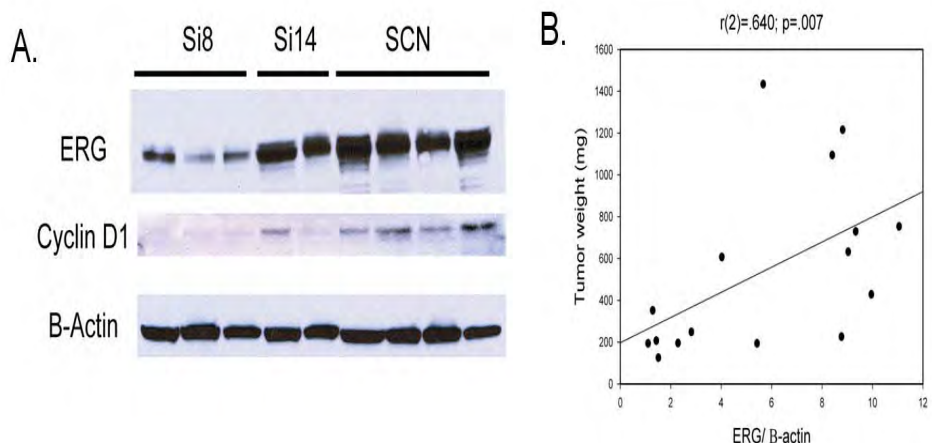


Fig 3. In vivo therapeutic targeting of Type III fusion gene by liposomal siRNA inhibits tumor growth of prostate cancer.

Liposomal non-silencing control siRNA, siRNA #8, or siRNA#14 were used as treatment groups. Mice ($n=14$) received 2 injections (150ug/kg or ~ 4 ug/mouse) every week and treatment was continued for 4 weeks. Tumors were removed and weighed at the termination of the experiment. Mice did not have any side effects. Standard error of the mean was calculated by Excell software.

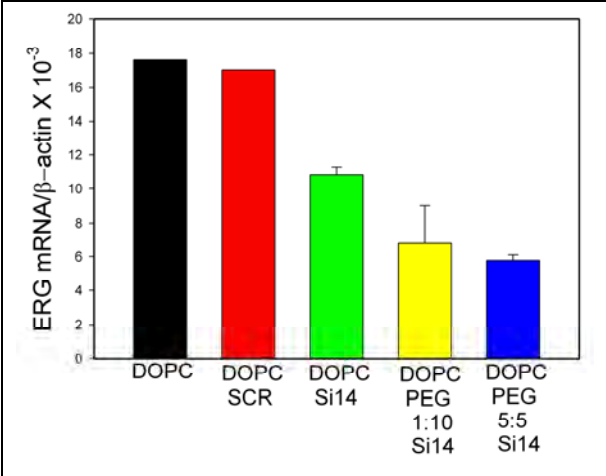
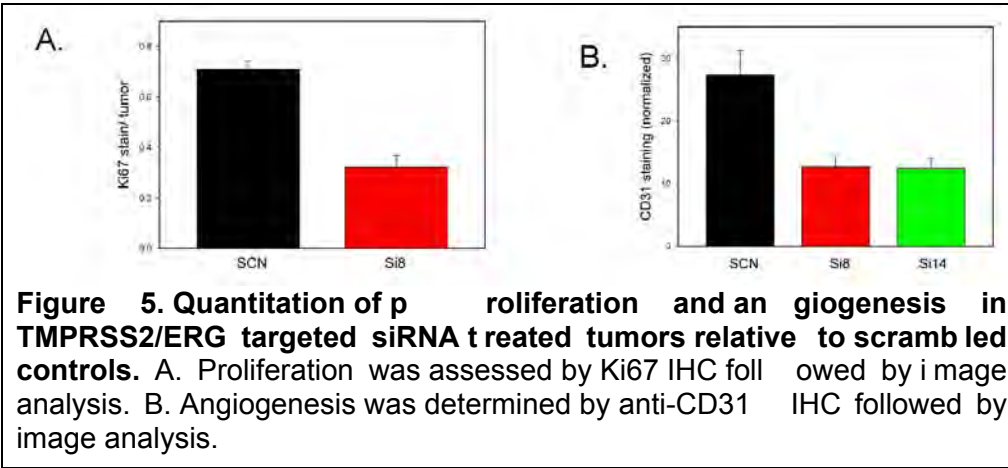
Figure 4. Knockdown of ERG in TMPRSS2/ERG targeted siRNA treated tumors correlates with of tumor growth inhibition. A. Western blot of tumor extracts from tumors using antibodies to ERG, Cyclin D1 and beta-actin. B. Western blots were quantitated using image analysis and the ERG/beta actin ratio for each tumor determined. Linear correlation was determined by the Pearson Product Moment test for Si8 and Si14 treated tumors.



Although liposomes have demonstrated one of the best established nanoplatforms with several-FDA approved formulations for cancer treatment, unmodified liposomes are limited by their short blood circulation time due to elimination by reticuloendothelial system. To increase stability and blood circulation half-life coating nanoparticles with polymers such as polyethyglycol (PEGylation) is commonly used. PEGylated liposomes have longer circulation times, increased accumulation in tumor tissues and enhanced therapeutic efficacy. PEGylated liposomes evade detection and destruction by phagocytes and are not immunogenic. More importantly, PEGylated carriers are safe and have received FDA approval.

To test the potential to increase fusion gene knockdown using PEGylation we carried out a small scale experiment. Subcutaneous VCaP tumors were established in nude mice. The mice were then injected with a single dose of either DOPC liposomes, DOPC liposomes with scrambled SiRNA, DOPC Si14 as shown in Fig 4, above, DOPC liposomes with PEG 2000 (1:10 ratio) and Si14 or DOPC liposomes with PEG 2000 (5:5 ratio) with Si14. The ratio is the ratio of DOPC to the DSPE linker lipid. After 6 days tumors were harvested and T/E

fusion gene mRNA measured by Q-RT-PCR. As can be seen in Fig 6, PEGylation increased fusion gene knockdown by ~30-40%. While preliminary, this data indicates that PEGylation can significantly enhance fusion gene knockdown even up to six days after a single treatment.



Specific Tasks related to this goal.

Months 1-2

1. Submit animal protocols and obtain approvals
2. Design and obtain siRNAs for Type III isoform and begin in vitro testing
3. Establish VCaP cells expressing V5-tagged Type III fusion isoform and V5-tagged ERG

Months 2-5

1. Evaluate Type III candidate siRNAs for knockdown efficacy of fusion gene by Western blot and quantitative RT-PCR using PNT1a cell lines expressing Type III isoform and control cell lines (V5-tagged wild type ERG and TMPRSS2 or control vector transfected cells).
2. Evaluate most effective candidate Type III candidate siRNAs for knockdown efficacy of fusion gene by Western blot and quantitative RT-PCR using VCaP cell lines expressing V5-tagged Type III isoform and control cell lines (V5-tagged wild type ERG and TMPRSS2 or control vector transfected cells).

Months 6-9

1. Evaluate in vivo efficacy of best candidate Type III fusion gene specific siRNA by treatment of mice bearing VCaP orthotopic tumors with siRNA incorporated into DOPC liposomes and controls (total of 80 mice; see Proposal). Observe mice for non-specific toxicities during treatment. Euthanize mice after 3 weeks of treatment and weigh and collect snap frozen and formalin fixed tumor and perform full necropsy.
2. Perform histopathological analysis of tumors and all organs from mice (Ittmann)
3. Perform Ki-67 and CD31 immunohistochemistry and TUNEL on all tumors and quantitate.
4. Evaluate expression of fusion gene and total ERG by Western blotting and quantitative RT-PCR using protein extracts and RNAs from tumors.

Goal 2: Evaluation of the efficacy of the SiRNA knockdown of the Type VI fusion gene in vivo

To identify siRNA that can effectively knock down the target T/E gene we designed 18 siRNAs spanning the fusion junction of the TMPRSS2 and ERG genes in the Type VI fusion mRNA. We then tested these siRNAs systematically using transient transfection in 293T, PNT1a expressing the Type VI fusion using Western blot and/or quantitative RT-PCR. We identified four siRNA that provided consistent and reproducible knockdown of the Type VI TMPRSS2/ERG fusion gene. Figure 7 shows a Western blot of with anti-V5 antibody on cell extracts of 293T cells transiently transfected with V5-Tagged Type VI fusion gene and several siRNAs. Control cells are liposomes only while scrambled represents a non-specific siRNA. As can be seen in Figure 7, Si1, Si8, Si14 and Si15 all give very strong knockdown of the fusion gene. These results were confirmed by quantitative RT-PCR in 293T and PNT1a with Type VI fusion. We have established Type VI expressing VCaP cells using a lentivirus and these cells are ready for in vivo experiments.

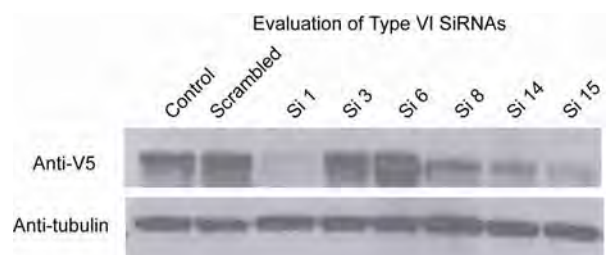


Fig 7. Western blot with anti-V5 antibody of 293T cells transfected with V5-tagged Type III fusion gene, liposomes only (control), scrambled siRNA and six targeting siRNAs. Tubulin is a loading

In order to optimize the efficacy of knockdown we are currently using several approaches (see Goal 1 above and Future Plans, below).

Specific Tasks related to this goal.

Months 1-2

1. Submit animal protocols and obtain approvals

Months 6-9

1. Establish VCaP cell lines expressing V5-tagged and VI fusion isoforms.
2. Design and obtain siRNAs for Type VI isoform

Months 10-12

1. Evaluate Type VI candidate siRNAs for knockdown efficacy of fusion gene by Western blot and quantitative RT-PCR using PNT1a cell lines expressing Type VI isoform and control cell lines (V5-tagged wild type ERG and TMPRSS2 or control vector transfected cells). The goal is to obtain a siRNA targeting a junctional sequence which will knockdown the fusion gene by 90-95% (at the protein and/or mRNA level) without affecting wild type ERG or TMPRSS2.
2. Evaluate the most effective Type VI candidate siRNAs for knockdown efficacy of fusion gene by Western blot and quantitative RT-PCR using VCaP cell lines expressing V5-tagged Type VI isoform and control cell lines (V5-tagged wild type ERG and TMPRSS2 or control vector transfected cells).

Goal 3: Evaluation of the efficacy of the SiRNA knockdown of the Type I and II fusion gene in vivo

We designed a series of 18 siRNAs spanning the fusion junction of the TMPRSS2 and ERG genes in the Type I and Type II fusion mRNAs. We then tested these siRNAs systematically using transient transfection in 293T, PNT1a expressing the Type I or Type II fusion using Western blot and/or quantitative RT-PCR. Of the 18 original siRNAs we identified several that gave strong, consistent and reproducible knockdown of the Type I or Type II TMPRSS2/ERG fusion gene. Figure 8 shows a Western blot of with anti-V5 antibody on cell extracts of 293T cells transiently transfected with V5-Tagged Type I or Type II fusion gene and several siRNAs. Control cells are liposomes only while scrambled represents a non-specific siRNA. As can be seen in Figure 8, Si17 and Si18, both give strong knockdown of the Type I fusion gene while Si8 and Si9 gave very strong knockdown of Type II siRNA. These results were confirmed by quantitative RT-PCR in 293T cells.

We have established Type I and Type II fusion gene expressing VCaP cells using a lentivirus and these cells are ready for in vivo experiments. However, we have delayed these experiments in order to optimize the efficacy of knockdown using several approaches (see Goal 1 above and Future Plans, below) as for Type VI.

Months 13-15

1. Design and obtain siRNAs for Type I and II isoforms

Months 16-19

1. Evaluate Type I and II candidate siRNAs for knockdown efficacy of fusion gene by Western blot and quantitative RT-PCR using PNT1a cell lines expressing Types I or II isoform and control cell lines (V5-tagged wild type ERG and TMPRSS2 or control vector transfected cells).
2. Evaluate most effective candidate Type I and II candidate siRNAs for knockdown efficacy of fusion gene by Western blot and quantitative RT-PCR using VCaP cell lines expressing V5-tagged Type I or II isoform and control cell lines (V5-tagged wild type ERG and TMPRSS2 or control vector transfected cells).

Goals for next 12 months

We have developed highly effective siRNAs targeting all the most common TMPRSS2/ERG fusion gene isoforms. Furthermore, we have shown efficacy in vivo with DOPC liposomes containing siRNAs in an orthotopic model with no toxicity. However, to improve efficacy we have had to further optimize the DOPC liposome by PEGylation, which appears to significantly enhance gene knockdown. While the evaluation of PEGylation delayed moving forward with in vivo experiments, we believe it is better to optimize systems prior to performing large scale experiments. We have in hand VCaP cells expressing Type I, II and VI variant isoforms. Thus we are now in position to rather rapidly complete the in vivo experiments using PEGylated liposomes. We also have optimized methodologies for analysis of tumors so this should also proceed rapidly.

KEY RESEARCH ACCOMPLISHMENTS

- Showed efficacy and lack of toxicity in vivo of SiRNAs delivered via DOPC liposomes using an orthotopic VCaP model.
- Developed VCaP cells expressing Type I, II and VI fusion gene mRNAs for evaluation of DOPC liposomes with siRNAs targeting these variants in vivo.
- Developed high efficiency SiRNAs targeting the Type III fusion gene mRNA.
- Developed high efficiency SiRNAs targeting the Type VI fusion gene mRNA.
- Developed high efficiency SiRNAs targeting the Type VI fusion gene mRNA.

REPORTABLE OUTCOMES

- Using DOPC liposomes to deliver specific SiRNAs targeting the Type III fusion gene isoform we have demonstrated statistically significant downregulation of tumor progression in vivo.
- Identification of junction specific SiRNAs targeting all the most common isoforms of the TMPRSS2/ERG fusion gene
- We have shown that PEGylated DOPC liposomes are more effective than unmodified liposomes in gene knockdown in vivo.

CONCLUSION

Our results strongly support the concept that we can specifically target the TMPRSS2/ERG fusion gene and the efficacy/ tumor growth inhibition in vivo orthotopic PCa animal models using nanolipsomal SiRNAs. We will need to further optimize this system to maximize potential therapeutic benefit.

REFERENCES

1. Wang J, Cai Y, Ren C, Ittmann M. (2006) Expression of variant TMPRSS2/ERG fusion messenger RNAs is associated with aggressive prostate cancer. *Cancer Res* **66**, 8347-8351.