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TITLE: Highly specific targeting of the TMPRSS2/ERG fusion gene in prostate cancer using liposomal nanotechnology

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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b>  The TMPRSS2/ERG fusion gene is detected in about 50% of prostate cancer (PCa) patients. It is highly specific for PCa and involved in proliferation and invasion of PCa cells. 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. The goal of the project is to target the four most common and biologically active alternatively spliced fusion gene transcript isoforms using specifically designed siRNAs to obtain maximal biological activity in cancers expressing the particular isoform or a combination of isoforms. Although siRNA offers a powerful tool to silence gene expression of drugable and undruggable targets, in vivo delivery of siRNA remains a great challenge. Therefore development of tumor-specific safe and effective nanodelivery systems is the main goal of the studies for clinical applications of siRNA-therapeutics. We propose to use nanoliposomes encapsulating siRNAs specifically targeting the TMPRSS2/ERG mRNA fusion junctions, to minimize off-target effects in normal tissues so toxicity should be minimal. Our results support the efficacy of this approach.					
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## INTRODUCTION

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.

Due to significant 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 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.

Since its discovery, use of small-interfering RNA (siRNA) has rapidly become a powerful tool to silence gene expression and has a great potential for use of molecularly targeted therapies in cancer. siRNA specifically binds target mRNA and causes its degradation, inhibiting protein expression. The promise of sequence-specific target degradation has also generated much excitement for silencing of not only drugable also "nondrugable" targets that cannot be targeted by small inhibitors. However, currently *in vivo* delivery of siRNA-based therapeutics to primary tumor and its metastases remains a great challenge. Clinical trials with siRNA was successful when administered locally. While negatively charged cell membranes prevent efficient intracellular delivery of nucleotides, cationic liposomes exhibit toxicity to mammalian cells. Therefore development of tumor-specific safe and effective nanodelivery systems is the main goal of the studies for clinical applications of siRNA-therapeutics. We recently developed non-toxic neutrally charged 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (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. In this proposal we 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 is minimal while gene knockdown results in significant inhibition of tumors growth.

## BODY

### 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 ug/kg, but this did not improve knockdown (data not

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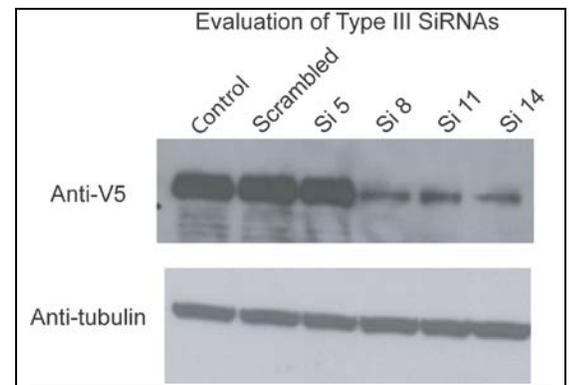


Fig 1. Western blot with anti-V5 antibody of 293T cells transfected with V5-tagged Type III fusion gene, liposomes only (control), scrambled siRNA and four targeting siRNAs. Tubulin is a loading control.

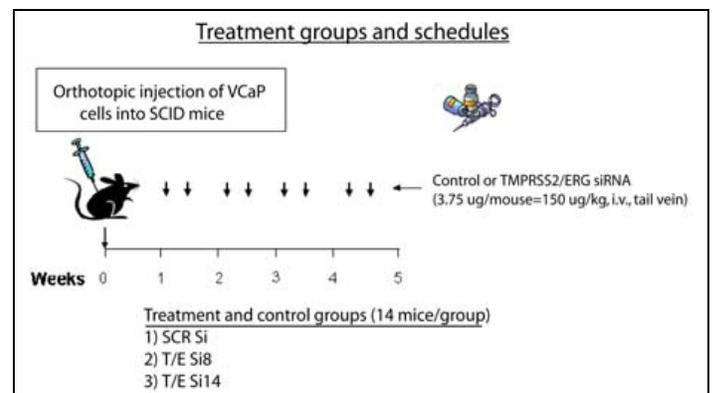
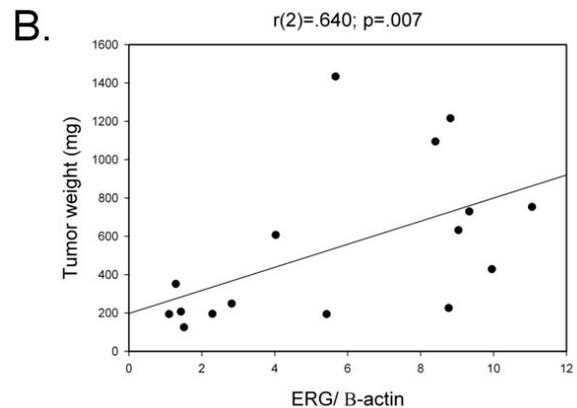
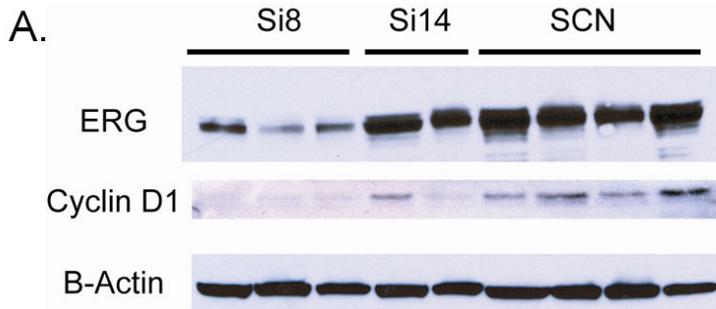
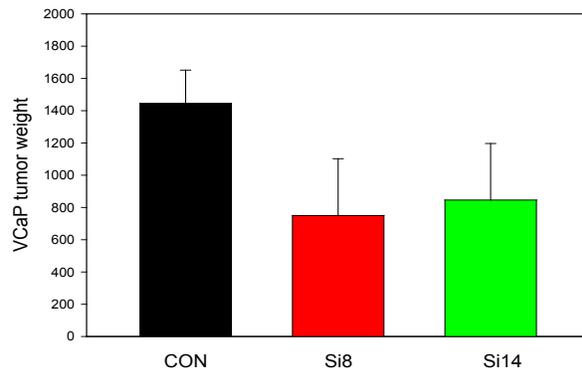


Fig 2. Treatment plan for mice following orthotopic injection of VCaP using fusion specific siRNAs. 14 mice were used per group

we increased the dosage of liposomes to 450 ug/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.

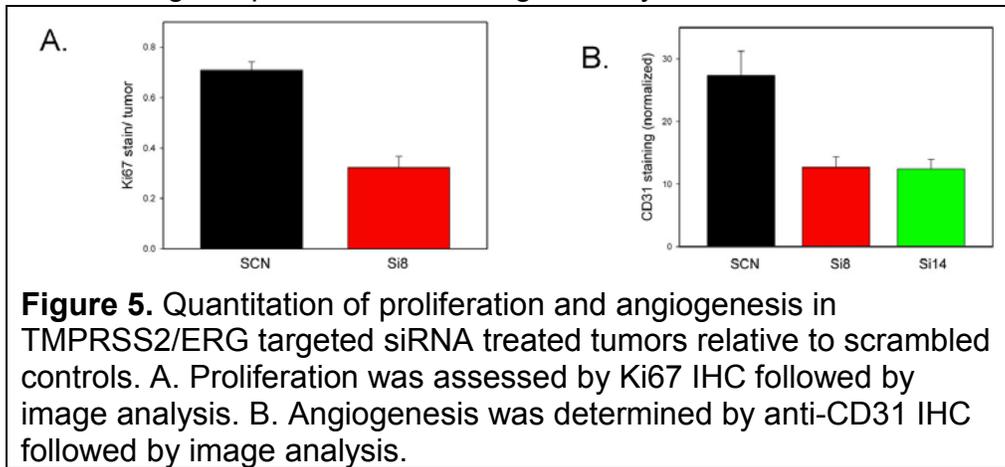
**Fig 3.** Tumor weight after treatment with Type III fusion mRNA targeting siRNAs. Tumor weight at the termination of the experiment outlined in Figure 2 is shown. Mean +/- SD.



**Fig.4.** Variable decrease in ERG in TMPRSS2/ERG targeted siRNA treated tumors which is correlated with degree 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.

Quantitative analysis of Western blots revealed 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 out 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 showed a significant increase in apoptotic cells in treated

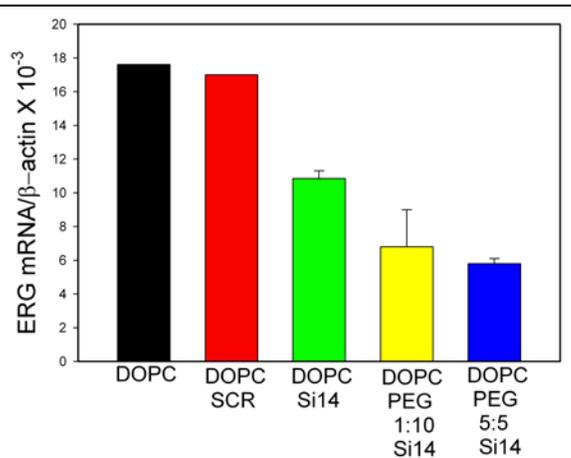
tumors from 1.8% (scrambled control) to 2.0% (Si8,  $p = .03$ ) or 3.4% (Si14,  $p = .008$ ). All of these effects are consistent with the decreased tumor growth observed. The effect on angiogenesis is unexpected since the T/E fusion gene has not been previously linked to promotion of angiogenesis. Further mechanistic studies are needed to discern the mechanism for this decrease in angiogenesis.

While we have shown significant anti-tumor effects of our T/E fusion gene targeting nanoliposomal vectors we need to further enhance delivery in order to maximize therapeutic efficacy. 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.

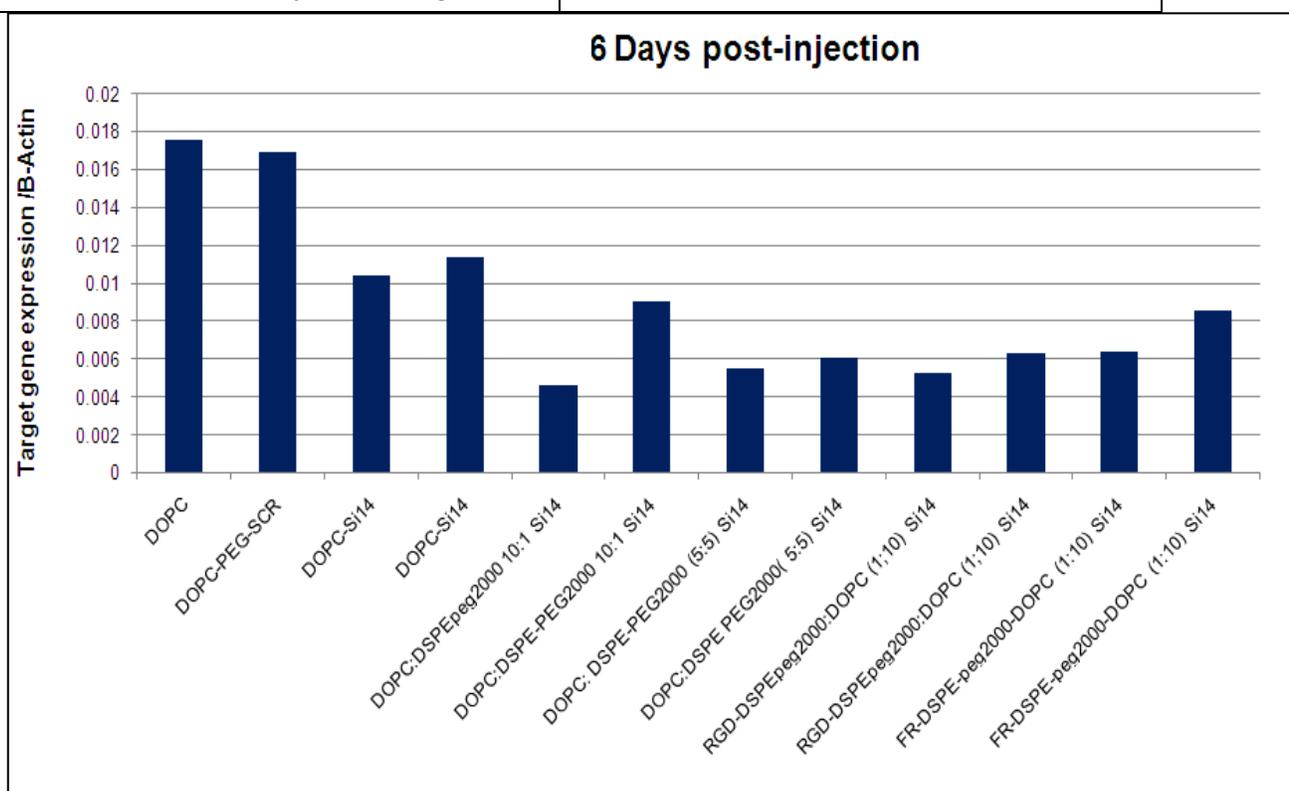
To test the potential to increase fusion gene knockdown using PEGylation we carried out a small scale experiment. Subcutaneous VCaP tumors were established in nude mice. The mice were then injected with a single dose of either DOPC liposomes, DOPC liposomes with scrambled siRNA, DOPC Si14 as shown in 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.

To increase tissue specific delivery we also tested target gene silencing single injection of nanoliposomal siRNA (4ug/mouse) with variety of liposomes decorated with folate or RGD (PEG2000-folate or PEG2000-RGD0). As shown in Figure 6B when compared with the inhibition of expression with those induced by DOPC-siRNA PEG liposomes which led to target gene expression for 6 days (2 mice /group) we did not see additional increase by folate or RGD coated liposomes..

**Fig 6.** A.T/E fusion gene knockdown is enhanced by PEGylation. Subcutaneous VCaP tumors were established in nude mice. The mice were then injected with a single dose of either DOPC liposomes (n=1), DOPC liposomes with scrambled SiRNA (n=1), DOPC Si14 (n=2), DOPC liposomes with PEG 2000 (1:10 ratio) and Si14 (n=2) or DOPC liposomes with PEG 2000 (5:5 ratio) with Si14 (n=2). After 6 days tumors were harvested and fusion gene mRNA measured by Q-RT-PCR. Mean +/- range shown.



**B.** Single injection of nanolipsosomal siRNA (4ug/mouse) with variety of liposomes decorated with PEG2000 or PEG2000-folate or PEG2000-RGD leads to target gene expression for 6 days(2 mice /group).

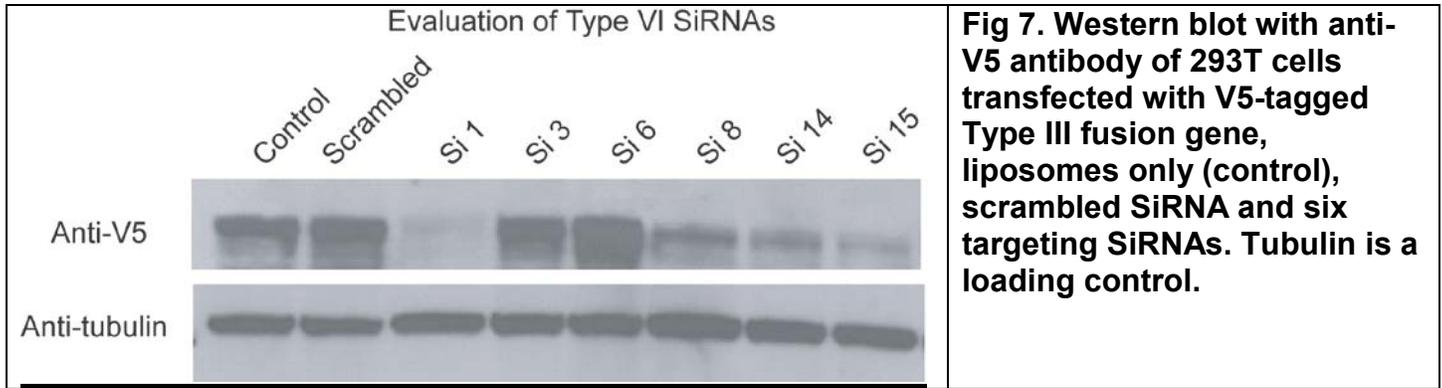


## **Goal 2: Evaluation of the efficacy of the SiRNA knockdown of the Type VI fusion gene in vivo**

### **Results:**

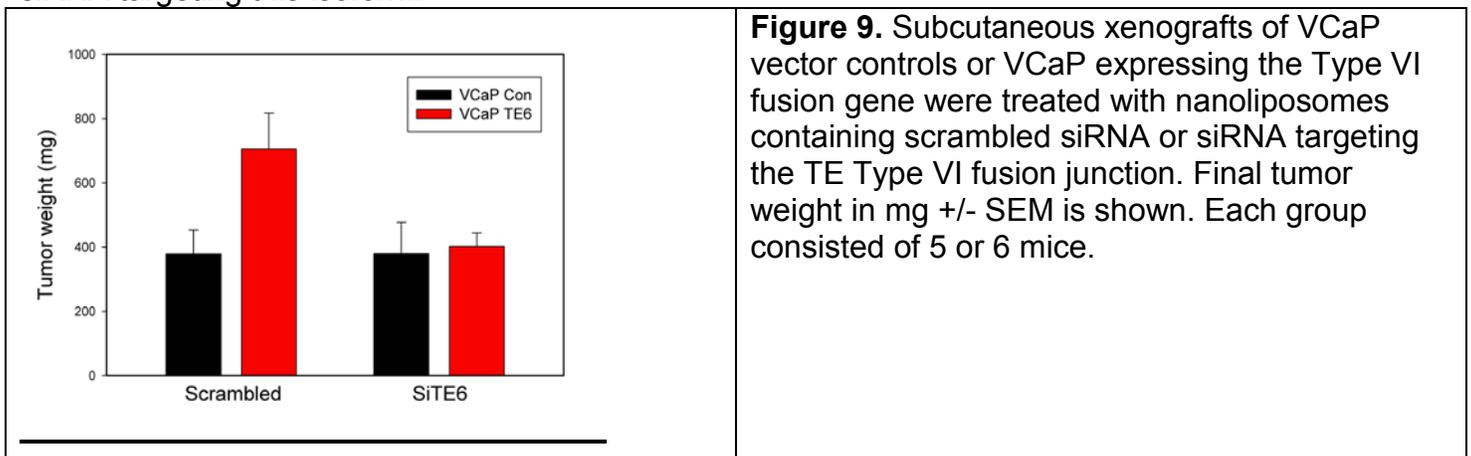
We designed a series of 18 siRNAs spanning the fusion junction of the TMPRSS2 and ERG genes in the Type VI fusion mRNA. We then tested these siRNAs systematically using transient transfection in 293T, PNT1a expressing the Type VI fusion using Western blot and/or quantitative RT-PCR. Of the 18 original siRNAs we identified four that gave strong, 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. In several experiments Si14 gave the best consistent knockdown.



We then evaluated the specificity of the siRNAs targeting the Type III and Type VI fusion genes. 293T cells were transfected with plasmids encoding Type III or Type VI fusion gene and siRNAs specifically targeting the Type III or Type VI junctions. It should be noted that the two plasmids differ by only 5 amino acids in their encoded proteins and contain almost the entire ERG protein. We showed (Dr. Ittmann) the specific siRNAs for each isoform show strong knockdown of their specific targeted isoform without any effect on the other isoform.

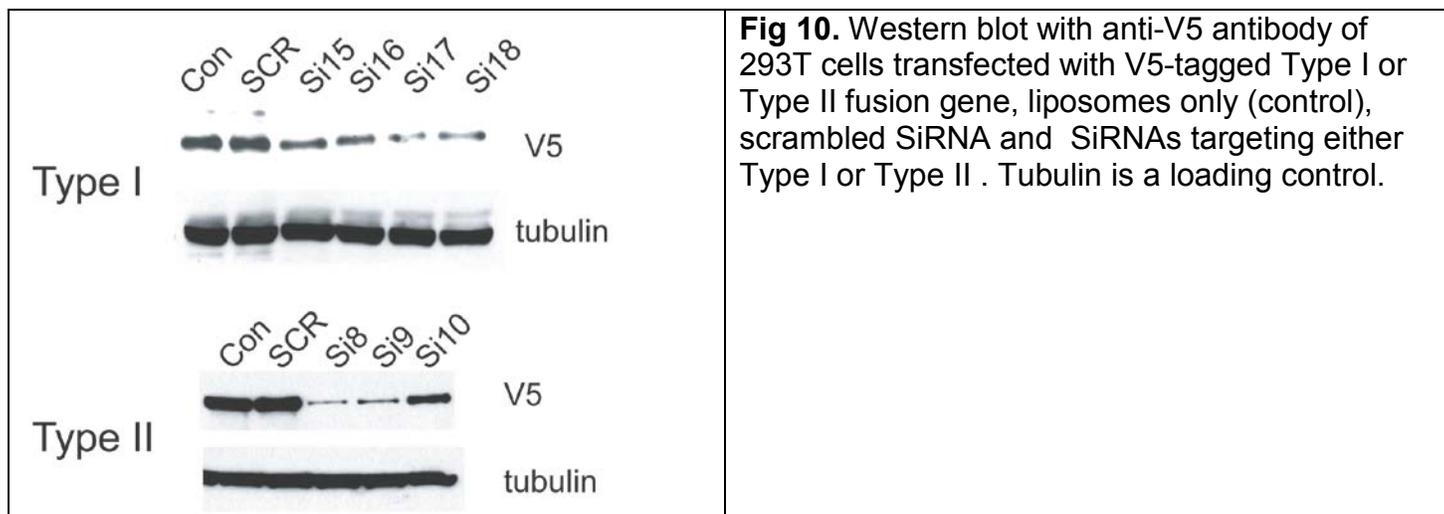
We then established Type VI expressing VCaP cells using a lentivirus. Nude mice were injected subcutaneously with VCaP vector control cells or VCaP expressing the Type VI fusion gene (VCaP TE6). After two weeks we then treated mice with siRNA targeting the Type VI fusion gene (Si14, Fig 7) or scrambled control siRNA for 4 weeks. Not surprisingly, as shown in Fig 9, the VCaP cells expressing the Type VI fusion grew significantly faster than VCaP controls when treated with scrambled control RNA ( $p=.04$ ). However, when Type VI expressing VCaP were treated with siRNA targeting the Type VI fusion they were significantly growth inhibited compared to the same cells treated with scrambled control ( $p=.035$ ) and had similar tumor weights to VCaP control cells indicating that the growth promoting activity of the Type VI fusion was completely abrogated by the specific siRNA targeting this isoform.



### **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 10 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. We plan to carry out these experiments this summer using unrestricted funds available to us.

### **Summary**

We have developed highly effective siRNAs targeting all the most common TMPRSS2/ERG fusion gene isoforms. Furthermore, we have shown efficacy in vivo with long-acting Pegylated nanoliposomes containing siRNAs in an orthotopic and subcutaneous models 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.

### **KEY RESEARCH ACCOMPLISHMENTS**

- 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 I and II fusion gene mRNAs.
- Showed efficacy and lack of toxicity in vivo of siRNAs delivered via DOPC liposomes using orthotopic and subcutaneous VCaP models.
- Developed long-acting PEGylated nanoliposomes containing siRNAs for longer target gene downregulation in in vivo Pca tumor models
- Developed VCaP cells expressing Type I, II and VI fusion gene mRNAs for evaluation of DOPC liposomes with siRNAs targeting these variants in vivo.

### **REPORTABLE OUTCOMES**

- Identification of junction specific siRNAs targeting all the most common isoforms of the TMPRSS2/ERG fusion gene
- Using DOPC liposomes to deliver specific siRNAs targeting the Type III and VI fusion gene isoforms we have demonstrated statistically significant downregulation of tumor progression in vivo.
- 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 in vivo using siRNAs and that this results in decreased tumor progression. A manuscript reporting these results is currently in preparation for submission to *Clinical Cancer Research*.

## **REFERENCES**

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