Highly specific targeting of the TMPRSS2/ERG fusion gene using liposomal nanovectors

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STATEMENT OF CLINICAL RELEVANCE

The TMPRSS2/ERG (T/E) fusion gene is present in approximately 50% of all prostate cancers (PCa). Fusion of the oncogenic ERG gene with the androgen-regulated TMPRSS2 gene promoter results in androgen regulated expression of fusion mRNAs in PCa cells. The junction of the TMPRSS2 and ERG derived portions of the fusion mRNA constitutes a cancer specific target in cells containing the T/E fusion gene. We have designed and optimized siRNAs targeting the junction sequences of the most common and clinically significant alternatively spliced isoforms of T/E fusion gene and tested their specific fusion protein knockdown effect in vivo when delivered using liposomal nanovectors. In vivo gene delivery of targeted siRNAs results in tumor growth inhibition with no apparent toxicity. Thus, targeting the T/E fusion junction in vivo with specific siRNAs delivered via liposomal nanovectors is a promising therapy for men with PCa.
ABSTRACT

Purpose: The TMPRSS2/ERG (T/E) fusion gene is present in half of all prostate cancer (PCa) tumors. Fusion of the oncogenic ERG gene with the androgen-regulated TMPRSS2 gene promoter results in expression of fusion mRNAs in PCa cells. The junction of the TMPRSS2 and ERG derived portions of the fusion mRNA constitutes a cancer specific target in cells containing the T/E fusion gene. Targeting the most common alternatively spliced fusion gene mRNA junctional isoforms in vivo using siRNAs in liposomal nanovectors may potentially be a novel, low toxicity treatment for PCa.

Experimental Design: We designed and optimized siRNAs targeting the two most common T/E fusion gene mRNA junctional isoforms (Type III or Type VI). Specificity of siRNAs was assessed by transient co-transfection in vitro. To test their ability to inhibit growth of PCa cells expressing these fusion gene isoforms in vivo, specific siRNAs in liposomal nanovectors were used to treat mice bearing orthotopic or subcutaneous xenograft tumors expressing the targeted fusion isoforms.

Results: The targeting siRNAs were both potent and highly specific in vitro. In vivo they significantly inhibited tumor growth. The degree of growth inhibition was variable and was correlated with the extent of fusion gene knockdown. The growth inhibition was associated with marked inhibition of angiogenesis and, to a lesser degree, proliferation and a marked increase in apoptosis of tumor cells. No toxicity was observed.

Conclusions: Targeting the T/E fusion junction in vivo with specific siRNAs delivered via liposomal nanovectors is a promising therapy for men with PCa.
INTRODUCTION

The TMPRSS2/ERG (T/E) fusion gene is present in approximately 50% of prostate cancer (PCa) lesions (1-4) and arises by fusion of the promoter and 5’ portions of the TMPRSS2 gene with the coding sequence of the oncogenic ERG gene. Experiments in PCa cells containing the T/E fusion (1) indicate that the TMPRSS2 promoter, which contains androgen receptor (AR)-responsive promoter elements (5), can mediate the overexpression of ERG in PCa in response to androgens. The ubiquitous activity of AR in PCa cells would then result in the constitutive expression of TMPRSS2/ERG fusion transcripts in the neoplastic prostatic epithelium bearing this fusion gene.

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 (2-4, 6). Thus, some PCas 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 several fusion mRNA junctional isoforms in PCa (2), which have been confirmed by others (3-4). In all cases, the fusion mRNA junctional isoform includes the TMPRSS2 exon 1 and often exon 2, as well. The most common isoform, which we have designated as the Type III isoform, contains the TMPRSS2 exon 1 fused to ERG exon 4, such that translation is initiated from an internal ATG codon (7) and gives rise to a slightly truncated protein. This variant was expressed in 86% of fusion gene expressing PCas, 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 (2) and expression of this isoform is associated with aggressive disease.

We and others have demonstrated that the T/E fusion gene can enhance proliferation, invasion and motility of prostate epithelial cells (8). More importantly, stable knockdown of the
T/E fusion mRNA in VCaP cells, which express the Type III fusion, inhibits tumor growth in vivo, indicating that the T/E fusion gene is a potential therapeutic target which is present in the majority of prostate cancers (8-9). If one can target specifically the T/E mRNA fusion junctions (i.e. the region where the TMPRSS2 and ERG portions of the fusion mRNA join), which are present only in PCa cells, this would eliminate any specific off-target effects in normal tissues so toxicity should be minimal. It should be noted that native ERG protein is highly expressed in normal endothelial cells (10) so that targeting ERG itself may have undesirable consequences in vivo.

Since its discovery, use of small-interfering RNA (siRNA) has rapidly become a powerful tool in discovering protein function, gene discovery, and specific gene silencing (11). Common features of RNA silencing are production of small (21-27 nucleotide) double-stranded RNAs that cause a sequence-specific silencing of gene expression by inducing target mRNA degradation and/or decreasing protein translation. The promise of specific RNA degradation has also generated much excitement for possible use as a novel therapeutic modality especially for those targets that cannot be targeted by small inhibitors (12-13). However, in vivo siRNA delivery has proven difficult because of lack of non-toxic and effective systemic delivery methods. We have 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 ovarian and breast cancer animal models (14-15). This strategy not only enhances targeting of siRNA to tumor tissues, also increases retention of siRNA, and protect against degradation by nucleases, thus enhancing the efficacy of encapsulated siRNA. More importantly, siRNA-mediated target silencing appears to be much more potent (>100 fold difference in IC50) and efficient with long-lasting silencing compared with antisense oligonucleotides or ribozymes (16-17).
We report here the use of liposomal nanovectors to target the fusion junctions of the two most common T/E fusion gene isoforms. This targeted approach resulted in significant knockdown of T/E fusion gene protein and inhibition of tumor growth in xenograft models in vivo with no apparent toxicity. Thus this approach holds significant promise as a therapeutic modality in men with prostate cancer expressing the T/E fusion gene.

MATERIALS AND METHODS

Cell lines and tissue culture. PNT1a cells were maintained in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin (Invitrogen). 293T cells or producer cell line 293TN (System Biosciences) were grown in DMEM (Invitrogen) supplemented with 10% FBS. VCaP cells were grown in DMEM under similar conditions; luciferase expressing VCaP cells designated VCaP-Luc for in vivo studies have been described previously (8). The origins of cell lines were validated by STR DNA fingerprinting performed by University of Texas M.D Anderson Cancer Center Characterized Cell Line Core Facility.

To establish VCaP cells stably expressing the Type VI isoform, the T/E Type VI fragment containing TMPRSS2 sequence nt 12-142 (NM 005656.2) and ERG sequence 226-1572 (NM 004449) was amplified by PCR using primer pair: TMP TE6 F \textit{NheI}: 5’-CCCTATCGT gctagc GGCAGGTCAATTGACATTT -3’and TMPERG R \textit{NotI}: 5’- CCGTAGATCG gcggccgc TTAGAGTAGAAGGTGGCAGGTGGCCAGG -3’. PCR template was the previously reported pcDNA 3.1/V5-Topo-TE6 plasmid DNA (8). The amplified fragment was double digested with Nhel and NotI and subcloned into pCDH-cmv-MCS-EF1-neomycin (System Biosciences) vector to produce pCDH-cmv-MCS-EF1-TE6-neo. Lentiviral particles were generated using pCDH-cmv-MCS-EF1-TE6-neo and pPACK Lentivector packaging Kit in 293 TN cells, and infected into VCaP-Luc to generate VCaP-TE6-Luc cells, which were stabled selected with puromycin then
grown in DMEM medium supplemented with puromycin (0.5µg/ml) and G418 (200 µg/ml).

**siRNA design and evaluation.** We designed 18 siRNAs spanning the fusion junction of the T/E fusion gene in the Type III or Type VI fusion mRNA that systematically covered all possible sequences of each junction. The analyzed sequences are shown in Supplementary Table 1. The dTdT overhang was added to 3’ terminal for siRNA sequences. Control non-silencing siRNA (scramble) corresponded to sequence 5’-AATTCTCCGAACGTGTCACGT-3’. The siRNAs used in this study were purchased from Sigma Life Science. SiRNA knockdown efficiency was tested using transient transfection in 293T cells. Co- transfection of individual siRNA at concentration of 50nM with pcDNA 3.1/V5-His-Topo-TE3 plasmid DNA (6 µg) or pcDNA 3.1/V5-His-Topo-TE6 plasmid DNA (6 µg) was performed in 293T cells grown at 50-60% in 10 cm dishes using 32 µl Lipofectamine 2000 (Invitrogen) for 48-72 hr. PNT1a stably expressing T/E fusion isoforms or VCaP cells were transfected with siRNAs in a similar manner. Cell pellets were collected, proteins were resolved by electrophoresis and V5-antibody was used to probe the tagged T/E fusion protein or ERG-antibody (for VCaP). Pellets were also used for RNA extraction for quantitative RT-PCR.

**Western blotting.** Protein extracts were prepared from cultured cells or VCaP primary tumors with RIPA buffer supplemented with protease inhibitor, 2 mM sodium orthovanadate, 1 mM PMSF and clarified by centrifugation. Protein concentrations were determined using a BCA protein assay kit (Thermo Scientific). Western blots were performed using primary antibodies: Anti-V5 (Cat. #R96025, Invitrogen), anti-ERG (Cat. #2805-1, Epitomics) or anti-Cyclin D1 (Cat. #2926, Cell Signaling Technology, Inc.). Monoclonal anti-α-Tubulin antibody or anti-β-actin antibody was used for protein loading control. Blot signals were visualized using enhanced chemiluminescence (Thermo) and exposed and developed with films or Bio-Rad imaging System and quantified by a densitometer using Quantity One (Version 4.5.2, Bio-Rad).
Quantitative RT-PCR. mRNA levels of the gene of interest were determined using quantitative RT-PCR using general procedures described previously (18).

Liposomal siRNA preparation. For in vivo delivery, siRNA was mixed with DOPC(1,2-dioleoyl-sn-glycero-3-phosphatidylcholine) in the presence of excess tertiary butanol at a ratio of 1:10 siRNA:DOPC (weight: weight) as described previously (19). Prior to in vivo administration, the lyophilized mixture was hydrated with saline at a concentration of 15 µg/ml to achieve the desired dose in 20 µl per i.v. injection via tail vein. The mean size of the liposomes was about 65nm by Zetasizer Nano (Malvern).

In vivo treatment of VCaP xenograft models. Nude male mice were purchased from Charles River Laboratories. Orthotopic tumors were established as described previously (8). Ten days later those mice bearing tumors (as detected by imaging) were divided into 3 treatment groups: DOPC-liposomes with scrambled siRNA, or one of two targeted siRNAs (Si8 or Si14) using 10 mice per in group. Treatments were carried twice weekly intravenously for 4 weeks. Body weights and intraprostatic tumor mass was monitored weekly. Tumor imaging was performed weekly using an IVIS imaging system (Xenogen, Alameda, CA) after intraperitoneal injection of D-Luciferin (Caliper Life Sciences) at 80 mg/kg body weight concomitant with anesthesia. Mice were euthanized 48 hours after the final injection and primary tumors were excised, weighted, and a portion of the tumor was frozen in liquid nitrogen for molecular analysis and another portion fixed and paraffin-embedded. Necropsy was also performed on mice to rule out side effects of treatment. Subcutaneous tumors were established by injecting 1X10⁶ VCaP-Luc or VCaP-Luc TE6 cells subcutaneously in the left and right flanks. The procedures for the siRNA treatments, tumor growth assessment and collection were carried on as similar as those for orthotopic animal experiments. Differences in mean tumor size and image signal were examined by t test or Mann-Whitney. All procedures were approved by the Baylor College of Medicine Institutional Animal Use and Care Committee.
Immunohistochemistry and analysis of apoptosis. Immunohistochemistry (IHC) of mouse tissues was performed using the basic procedures described previously (20). Paraffin sections (5 μM) of formalin-fixed mouse xenografts were used for H&E staining or immunostaining for different protein markers according to the manufacturer’s protocol and counterstained in Mayer’s hematoxylin. Primary antibodies were used as follows: ERG (Rb mAb, Epitomics); Ki67 (Thermo, RM-9106) and mouse anti-CD31 (MEC13.3, rat monoclonal, BD Biosciences). Chromogenic visualization of stained sections were performed as described previously (21). TUNEL was performed using an in situ cell death detection kit (Roche) according to manufacturer's instructions. Ki67, TUNEL and CD-31 staining were quantitated using image analysis as described previously (22). NFκB p65 phospho-Ser536 IHC was performed as described previously (21). Nuclear staining was quantified using a Vectra™-Inform™ image analysis system (Caliper Life Sciences, Hopkinton, MA). After segmenting nuclei, we scored IHC nuclear intensity into 4 bins as 0-3+ (0: no expression; 1+: low expression; 2+: moderate expression and 3+: high expression). inForm™ was then used to quantitate the number of nuclei with each staining intensity in each image. The percentage in each bin was then calculated. For all studies values compared using t-test or Mann-Whitney.

RESULTS

Development of siRNAs spanning the fusion junction of the Type III T/E fusion mRNA.

We designed a series of 18 siRNAs spanning the fusion junction of the TMPRSS2 and ERG genes in the Type III fusion mRNA. We tested these siRNAs systematically using transient co-transfection of each siRNA in 293T cells with a V5-tagged Type III T/E fusion constructs. Of the 18 original siRNAs, we identified three that gave strong, consistent and reproducible knockdown of the Type III T/E fusion mRNA. Figure 1A 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. Si8, Si11 and Si14 all give very strong knockdown of the fusion gene. The effectiveness of these SiRNAs was confirmed in immortalized prostatic epithelial cells (PNT1a) stably expressing the Type III fusion gene (8) and VCaP cells using Western blots. These results were confirmed by Q-RT-PCR in 293T, PNT1a with Type III fusion and VCaP cells (data not shown). The sequences targeted by Si8 and Si14 are shown in Figure 1B. The portion derived from the TMPRSS2 exon 1 is shown in green, while the portion from ERG exon 4 is shown in red.

Orthotopic and subcutaneous xenografts treated with Type III targeted siRNAs.

Based on these results, we moved forward with in vivo experiments using DOPC liposomes to deliver either Si8 or Si14 in an orthotopic VCaP model. Ten days after intraprostatic injection of luciferase-expressing VCaP cells into nude mice, siRNA treatment was initiated. Mice were injected with control scrambled siRNA, Si8 or Si14 in DOPC liposomes twice weekly at 150 ug/kg body weight intravenously via tail vein. Mice were weighed and luciferase based tumor imaging was performed weekly. The experiment was terminated after 4 weeks of treatment. Mice were euthanized 48 hours following the last injection of siRNA, and primary tumors excised, weighed and snap frozen for molecular studies or submitted for histopathology and a complete necropsy performed on mice. Of note, no toxicity or decreased body weight was noted (Fig 2A). Tumor weight and luciferase activity prior to euthanasia is shown in Figure 2A. Both the Si8 and Si14 groups showed a significant decrease in tumor luciferase flux of ~75% (p<.001, t-test) when compared to scrambled control. Tumor weight was decreased to a lesser extent (~45%) compared to luminescence, presumably due to the contribution of stroma and dead tumor cells to tumor weight (p<.001, t-test). This experiment was repeated using a higher dose of siRNAs (450 ug/kg) and very similar inhibition of tumor growth was observed, again with no toxicity. We also carried out another experiment using subcutaneous xenografts to determine whether tumor site impacted response to therapy. Two weeks after injection of tumor cells treatment was initiated with Si14 in nanoliposomes and continued for 6 weeks. As can be seen
in Fig 2B there was a marked inhibition of tumor growth and at the end of treatment tumor luminescence was decreased 74%. Final tumor weight was decreased by 50% (data not shown), similar to results in our orthotopic experiments. Thus knockdown of the T/E fusion gene with siRNAs in established tumors can significantly inhibit tumor growth in vivo.

Analysis of orthotopic tumors from mice treated with 150 ug/kg body weight of targeted siRNAs or scrambled control was performed using Ki67 immunohistochemistry (IHC) followed by quantitative image analysis and showed a statistically significant decrease in proliferation of 11-25% in treated versus control tumors (Fig 2C). TUNEL staining of treated tumors showed a significant increase in cell death by 45-192% over control. Thus the decreased tumor growth can be attributed to both decreased proliferation and increased cell death, although the latter appears to be more quantitatively important. We also performed anti-CD31 IHC to evaluate microvessel density. We observed a 71% decrease in microvessel density for both targeted siRNAs compared to controls. This marked decrease in angiogenesis was somewhat surprising and may contribute to the effects on proliferation and cell death observed. Representative images or all of the analyses are shown Supplementary Figure 1.

We next evaluated changes in ERG expression in tumors by IHC. Variable and heterogeneous loss of ERG protein was noted in VCaP tumor cells treated with targeted siRNAs compared to scrambled controls. An example of a potent knockdown is shown in Figure 3A. It should be noted that we increased the antibody dilution from our normal protocol to observe more subtle differences in ERG expression. It is known that ERG is highly expressed in endothelial cells and strong staining is maintained even at the higher antibody dilution in tumors treated with either scrambled or targeted siRNAs. This observation argues that the observed effects on angiogenesis are not related to direct downregulation of endothelial ERG by the targeted siRNAs. Marked knockdown of ERG expression, as illustrated in Figure 3A, was noted in some tumors treated with targeted siRNAs, but in other cases tumors showed variable
knockdown within different tumor regions and even between adjacent cells. We therefore analyzed ERG protein expression in treated versus untreated tumors using quantitative Western blotting. As shown in Figure 3B, there was a significant variability in the extent of fusion protein knockdown in individual treated tumors. There was also decreased expression of the cell cycle protein cyclin D1 that was proportional to the decreased expression of ERG. We therefore examined the relationship between the amount of ERG protein in tumors treated with targeting siRNAs as determined by quantitative Western blotting and final tumor weight. There is a highly significant relationship between the level of ERG protein in tumors and final tumor weight ($r^2 = .64$, $p = .007$, Pearson Product Moment, Fig 3C). Of note, while there was variability both in ERG expression and tumor weight in mice treated with scrambled control SiRNA, there was no significant correlation between these two parameters ($p = .569$, Pearson Product Moment) consistent with the idea that the efficiency of knockdown is driving the correlation in treated mice rather than a factor intrinsic in the mice (for example androgen levels) which may impact ERG expression. These findings together argue that stable tumor or animal specific factors impede the effectiveness of fusion gene knockdown in some treated mice. Overall, the data indicates that the DOPC liposomes partially knock down T/E fusion protein to a variable extent and suggest that enhancing T/E fusion gene knockdown can further improve the therapeutic efficacy of this targeted therapy.

To determine if downstream targets of ERG were downregulated in treated mice we analyzed expression of NFκB p65 phospho-Ser536. Our group has previously shown that ERG significantly increases phosphorylation of p65 Ser536, which results in increased NFκB activity, and that ERG expression is significantly correlated with levels of phospho-Ser536 in human PCa tissues (21). We therefore carried out IHC using anti-p65 phospho-Ser536 antibody followed by image analysis of VCaP tumors. There was significant decrease in phospho-Ser536 staining in tumors from mice treated with either targeted SiRNA compared to scrambled control
(Supplementary Figure 2). Using Inform software we quantitated the percentage of nuclei staining at for different pre-set levels: 0, 1+, 2+ and 3+ corresponding to no, weak, moderate and strong staining, respectively. Results are shown in Fig 3D. In tumors treated with control scrambled RNAs, 66% of tumor cells had moderate to strong staining. This was decreased to less than 8% in both groups treated with targeted SiRNAs. This difference was highly statistically significant for both targeted groups (p<.001, t-test). Of note, cyclin D1 is a known target of NFκB in PCa (23), so the downregulation of Cyclin D1 (Fig 3B) may be due to decreased NFκB activity in tumors treated with targeted SiRNAs. Thus we were able to achieve significant downregulation of a validated ERG target in VCaP tumors treated with targeted SiRNAs.

Development of fusion junction spanning SiRNAs for the Type VI fusion mRNA.

In a manner similar to the procedure for the Type III fusion gene, 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 by transient co-transfection in a manner similar to our analysis of the Type III targeting siRNAs. Of the 18 original siRNAs we identified four that gave strong, consistent and reproducible knockdown of the Type VI TMPRSS2/ERG fusion gene. Figure 4 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 4, TE6 Si1, TE6 Si8, TE6 Si14 and TE6 Si15 all give very strong knockdown of the fusion gene. The TE6 designation distinguishes the Type VI targeting siRNAs from the Type III targeting siRNAs. These results were confirmed by quantitative RT-PCR in 293T and in PNT1a cells with stable expression of the Type VI fusion gene (8) transfected with the best siRNAs. In multiple experiments TE6 Si14 gave the best consistent knockdown and its sequence is shown in Fig 4B. It is interesting to note that the TE6 Si14 siRNA, which gave the best knockdown
efficiency, has the targets the same 15 nucleotides in ERG exon 4 as the Type III Si14 (Figure 1B) but targets 6 nucleotides from exon 2 of TMPRSS2 exon 2 rather than exon 1.

**Specificity of siRNAs targeting the T/E Type III or Type VI isoforms.**

To evaluate possible off-target effects of the siRNAs targeting the Type III and Type VI fusion genes, 293T cells were co-transfected with plasmids encoding native ERG, the Type III fusion gene or the Type VI fusion gene and Si14 (targeting T/E Type III) or TE6 Si14 (targeting the Type VI junction). Scrambled siRNA was used as control. It should be noted that the two fusion proteins isoforms and wild-type ERG are completely identical downstream of methionine 40 of the wild-type ERG and differ only in the 5’ region (7). As can be seen in Figure 5, the specific siRNAs for each isoform show strong knockdown of their specific targeted isoform without any effect on the other isoform or the native ERG protein. This result indicates that the siRNAs are isoform specific and do not target the ERG protein except in the context of the appropriate fusion.

**In vivo efficacy of the Type VI targeting SiRNA.**

We then established Type VI stably expressing VCaP-Luc cells, designated as VCaP-Luc TE6, using lentivirus infection. Expression of the Type VI protein was similar to the level of native VCaP Type III protein (Fig 6A). Analysis of cell proliferation in vitro revealed that these cells proliferated approximately 20% faster than vector controls (p<.001, t-test; data not shown). Subcutaneous xenograft in which male nude mice were injected subcutaneously with VCaP-Luc or VCaP-Luc TE 6 were established and after 10 days treated with siRNA targeting the Type VI fusion gene (TE6 Si14) or scrambled control siRNA for 4 weeks. Not surprisingly, as shown in Fig 6B, the VCaP cells expressing the Type VI fusion grew significantly faster than VCaP controls when treated with scrambled control siRNA (p=.034, t-test). 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-Luc control cells treated scrambled control (p=.96) or TE6 Si14 SiRNA (p=.75), indicating that the growth promoting activity of the Type VI fusion was completely abrogated by the specific siRNA targeting this isoform.

**DISCUSSION**

Our results demonstrate that the T/E fusion gene plays an important role in prostate tumor growth and progression and is a potential therapeutic target which is present in the majority of PCas. Prior studies from our group and others (8-9) have shown decreased tumor growth in vivo using VCaP cells with stable knockdown of the T/E fusion gene protein but such studies cannot exclude a potential impact of the T/E fusion gene on establishing tumors after injection, which could ultimately affect final tumor size. In these studies we show decreased tumor growth after fusion gene protein knockdown in established tumors, which more closely mimics the situation in the therapy of human PCa.

The siRNAs targeting the fusion gene mRNA isoforms were designed to span the junction of the fusion mRNAs to avoid targeting the native ERG protein since the fusion mRNAs are only present in cancer cells. This strategy was successful and we found no evidence of downregulation of the native ERG protein. There was also specific targeting of each T/E fusion isoform as well. No toxicity was detected from the siRNA treatment. Thus, delivery of junction spanning siRNAs to fusion gene expressing PCa is a potential efficacious treatment with low toxicity for men with PCa.

Optimally, T/E fusion targeted treatment would target the specific fusion gene isoforms expressed by an individual patient’s tumor. Based on our prior results (8), 49% of PCa tumors expressing the fusion gene express the Type III fusion isoform alone, which could be targeted with a Type III targeting siRNA. Another 20% of T/E fusion gene positive tumors express only the Type III and VI isoforms and could derive further benefit from targeting both these two isoforms simultaneously. Another 11% of fusion gene expressing tumors express one or both of
the other common isoforms (Type 1 and Type II, see ref (8)) with or without the Type III fusion isoform and again could be treated with combinations of the relevant junction spanning siRNAs. Of note, we have already identified potent junction targeted siRNAs for the Type I and II isoforms (data not shown). Overall, 80% of PCas can potentially have all of their detectable fusion isoforms targeted using siRNAs targeting the four common isoforms (singly or in combination). Almost all the rest of the T/E fusion gene expressing cancers can be at least partially targeted using one or more of these four targeted siRNAs, with some residual levels of rarer fusion gene isoform variants that are not targeted.

It is important to note that the final tumor weight and the level of expression of the ERG were highly correlated. The fact that the final ERG level correlated with tumor weight that results from growth over four weeks implies that there are stable factors in each mouse or tumor that impact the efficacy of fusion gene knockdown. The nature of these factors is unclear. One possibility is that some mice may clear the DOPC liposomes from the circulation more rapidly, perhaps due to enhanced activity of the reticuloendothelial system in such mice. Alternatively, some tumors may have leakier tumor vasculature enhancing uptake of liposomes. Tumor cell intrinsic mechanisms are also a possibility. For example, decreased uptake or enhanced degradation of siRNAs could occur in a subgroup of tumors cells and such cells would preferentially grow and ultimately predominate in tumors from mice treated with targeted siRNAs. It should be noted that variable delivery of agents into tumors by liposomes has been observed for other types of liposomes (24), so this phenomenon does not appear to be limited to DOPC liposomes. Thus the therapeutic impact of fusion knockdown may be able to be significantly enhanced by increasing the level of knockdown by improved delivery of targeted siRNAs into tumor cells via modification of the liposomal particles or the mode of the delivery or both.
We have shown previously that the T/E fusion gene expression can significantly enhance invasion of prostate epithelial cells in vitro but has modest effects on net cell proliferation in vitro (8). Inhibition of invasion by T/E fusion gene protein knockdown may inhibit tumor growth in vivo by decreasing the ability of the tumor to invade into the surrounding tissue to increase its mass. The impact of decreased T/E fusion gene protein on net cell growth that we have observed in vivo is more pronounced than in vitro and appears to be mediated predominantly by increased cell death. The reason for the difference between in vitro and in vivo effects is not known but may be related to the surprisingly large effects on angiogenesis that we observed, that may inhibit proliferation and promote apoptosis. There are several potential mechanisms for the observed decrease in angiogenesis. Tomlins et al (25) have shown that both uPA and MMP9 are direct ERG targets in PCa. Both of these proteases are known to enhance angiogenesis, presumably by releasing bound pro-angiogenic growth factors and cytokines from the extracellular matrix as well as altering the structure and function of other extracellular matrix factors (26-27). In addition, we have shown that the T/E fusion gene enhances NFκB activity by promoting phosphorylation of p65 at Ser536 (21) and that this results in increased expression of CCL2, which is known to promote angiogenesis (28). Increased NFκB activity can also enhance expression of other angiogenic proteins such as VEGF and IL8 (23). While further studies are needed, our findings show for the first time that knockdown of the T/E fusion gene protein in PCa cells has important effects on angiogenesis, which may account for some of the disparity between the magnitude of observed in vitro and in vivo effects of fusion gene knockdown on cell growth.

In summary, siRNAs targeting the TMPRS2/ERG fusion junctions delivered via DOPC liposomes represent a potent novel therapy that can inhibit tumor growth with low toxicity. Additional studies are needed to enhance siRNA uptake by tumor cells to increase the efficacy of this targeted therapy. The challenges of effectively delivering RNA interference-based
therapies to cancer cells in vivo has recently been reviewed (29-30). Three approaches merit consideration in our delivery system. First, chemical modification of the DOPC liposome by PEGylation should decrease clearance of particles by the reticuloendothelial system, prolong circulation time and potentially increase delivery to tumors. Second, addition of molecules that enhance targeting to tumor cells can significantly enhance siRNA delivery. Finally, the enhanced permeability and retention effect can be manipulated pharmacologically to enhance nanoliposomal delivery. Ultimately the goal is to move the most efficacious method of delivering the targeted siRNAs to tumors in vivo into the clinical to improve outcomes for men with PCa.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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FIGURE LEGENDS

Figure 1. Evaluation of siRNAs targeting the Type III fusion mRNA junctional sequence.
A. Western blot with anti-V5 antibody of 293T cells transfected with V5-tagged Type III fusion 
gene and, liposomes only (control), scrambled SiRNA in liposomes or one of four targeted 
siRNAs in liposomes. Tubulin is a loading control. B. Sequences of Type III targeted siRNAs are 
shown. The portion derived from the TMPRSS2 exon 1 is shown in green, while the portion from 
ERG exon 4 is shown in red.

Figure 2. SiRNAs targeting the Type III T/E fusion mRNA inhibit tumor progression in 
vivo. A. Nude mice were injected orthotopically with VCaP expressing luciferase. After 10 days 
mice were treated with DOPC liposomes containing scrambled (control) or targeted siRNAs (Si8 
or Si14) twice weekly for 4 weeks (150 ug/kg). Luciferase flux of tumors prior to euthanasia and 
tumor and body weights at termination of treatment are shown. The control group contained 9 
mice while each targeted group contained 11 mice. B. Nude mice were injected subcutaneously 
with VCaP expressing luciferase (time 0). After two weeks mice were treated with DOPC 
liposomes containing scrambled (control) or targeted Si14 twice weekly for 6 weeks. Luciferase 
flux of tumors at indicated time is shown. The control group had 7 mice and the Si14 treated 
group had 8 mice. C. Mean percent nuclei stained with Ki67 or TUNEL or mean tumor area 
stained with anti-CD31 in treated and control tumors. Values are mean +/- SEM normalized to 
control (100%). Asterisks indicate statistically significant differences between targeted siRNA 
and scrambled control.

Figure 3. Knockdown of ERG protein in tumors treated with targeted siRNAs.
A. Immunohistochemistry with anti-ERG antibody of orthotopic VCaP tumors treated with 
scrambled control siRNA or Si8. VCaP tumors are to the left; blood vessels adjacent to tumors 
with endothelial cells expressing ERG are indicated by arrows. A residual normal gland is
marked with “P” in the scrambled control (400X). Original magnification: 400X or 600X. B. Representative Western blot of VCaP tumor extracts with antibodies against ERG or Cyclin D1. β-actin is a loading control. C. Correlation between ERG protein level by quantitative Western blotting, normalized to β-actin, and final tumor weight. D. Percentage of nuclei expressing the ERG target NFκB p65 phospho-Ser536 as evaluated my Inform image analysis in tumors treated with control or targeted SiRNAs. Asterisks indicate statistically significant differences in the percentage of moderate and strong (2+ and 3+) between control and targeted groups by t-test (p<.001).

**Figure 4. Evaluation of siRNAs targeting the Type VI fusion mRNA junctional sequence.**
A. Western blot with anti-V5 antibody of 293T cells transfected with V5-tagged Type VI fusion gene and liposomes only (control), scrambled SiRNA in liposomes or one of six targeted siRNAs in liposomes. Tubulin is a loading control. B. Sequences of Type VI targeted siRNA used for in vivo studies. The portion derived from the TMPRSS2 exon 2 is shown in green, while the portion from ERG exon 4 is shown in red.

**Figure 5. Specificity of Type III and Type VI targeted siRNAs.** A. Western blot with anti-ERG antibody of 293T cells co-transfected with plasmids encoding native ERG, the Type III fusion gene or the Type VI fusion gene and Si14 (targeting T/E Type III) or TE6 Si14 (targeting the Type VI junction). Scrambled siRNA was used as control. Tubulin is a loading control. B. ERG and T/E Type III plasmid transfected extracts rerun on new gels and shorter exposures obtained that allow visualization of specifically knocked down targets without overexposing the unaffected bands.

**Figure 6. SiRNA targeting the Type VI T/E fusion mRNA inhibits tumor progression in vivo.** A. Expression of the Type VI fusion protein in VCAP TE 6 cells engineered to express this protein detected with anti-ERG antibody. The Type VI fusion gene isoform protein is slightly larger than the Type III fusion gene protein, which is translated beginning at methionine 40,
while the Type VI protein is translated from the in-frame TMPRSS2 translation initiation codon. β-actin is a loading control. B. Subcutaneous xenografts of VCaP vector controls or VCaP expressing the Type VI fusion gene were treated with nanoliposomes containing scrambled siRNA or siRNA targeting the TE Type VI fusion junction. Final tumor weight in mg +/- SEM is shown. Six mice were used in the scrambled control groups and 8 in the SiTE6 groups. The single asterisk indicates a statistically significant difference between different cell types treated with control siRNA; double asterisks indicate a significant difference from same cell type treated with scrambled control siRNA versus targeted siRNA.
Figure 1

A

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<tr>
<td>Anti-tubulin</td>
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</tbody>
</table>

B

siRNA 8 (12+9)

\[
\text{siRNA antisense: uaggccuuaccucgccgccc}-dTdT
\]

siRNA 14 (6+15)

\[
\text{siRNA antisense: aacugauaaggccuaccgcg}-dTdT
\]
Figure 4

A

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<th>Control</th>
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<th>Si 3</th>
<th>Si 6</th>
<th>Si 8</th>
<th>Si 14</th>
<th>Si 15</th>
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</table>

Anti-V5

Anti-tubulin

B

T/E 6 siRNA 14(6+15)

TMP(6 nts)  | ERG(15 nts)

AACTCA       | GAAGCCTTATCAGTT

siRNA antisense: aacugauaaggcuucugaguu-dTdT
**A**

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**anti-ERG**

**anti-tubulin**

**B**

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<tr>
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<th>SiRNA Type</th>
<th>ERG</th>
<th>T/E Type III</th>
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<tbody>
<tr>
<td></td>
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**anti-ERG**

**anti-tubulin**
Figure 6

A.

<table>
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<tr>
<th>VCap Con</th>
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<tr>
<td>B-Actin</td>
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</tbody>
</table>

B.

![Tumor weight graph](image)

- **Scrambled**
  - VCap Con: 400 mg
  - VCap TE6: 800 mg

- **SiTE6**
  - VCap Con: 400 mg
  - VCap TE6: 600 mg

* * *
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Long-jiang Shao, Ibrahim Tekedereli, Jianghua Wang, et al.

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