Highly Specific Targeting of the TMPRSS2/ERG Fusion Gene Using Liposomal Nanovectors

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Abstract

Purpose: The TMPRSS2/ERG (T/E) fusion gene is present in half of all prostate cancer tumors. Fusion of the oncogenic ERG gene with the androgen-regulated TMPRSS2 gene promoter results in expression of fusion mRNAs in prostate cancer 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 prostate cancer.

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 prostate cancer 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 prostate cancer.

Introduction

The TMPRSS2/ERG (T/E) fusion gene is present in approximately 50% of prostate cancer 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 prostate cancer cells containing the T/E fusion (1) indicate that the TMPRSS2 promoter, which contains androgen receptor–responsive promoter elements (5), can mediate the overexpression of ERG in prostate cancer in response to androgens. The ubiquitous activity of androgen receptor in prostate cancer cells would 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 prostate cancers express a single mRNA type, whereas 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 prostate cancer (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 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 (2), and expression of this isoform is associated with aggressive disease.

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systemic delivery methods. We have recently developed inhibitors (12, 13). However, for possible use as a novel therapeutic modality of gene expression by inducing target mRNA degradation. The promise of double-stranded RNAs that cause a sequence-specific silencing 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 prostate cancer.

We and others have shown 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 prostate cancer 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 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 nontoxic and effective systemic delivery methods. We have recently developed nontoxic neutrally charged 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC)-based liposomal nanovectors (mean size, 65 nm) that can target siRNA in vivo into tumor cells 10- 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 but also increases retention of siRNA and protects 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 2 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% FBS (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). 293T cells or producer cell line 293TN (System Biosciences) were grown in Dulbecco’s Modified Eagles’ Medium (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 conducted by University of Texas M.D Anderson Cancer Center Characterized Cell Line Core Facility (Houston, TX).

To establish VCaP cells stably expressing the type VI isoform, the T/E type VI fragment containing TMPRSS2 sequence 12 to 142 nt (NM 005656.2) and ERG sequence 226 to 1,572 (NM 004449) was amplified by PCR using primer pair: TMP TE6 F Nhel: 5’-CCCTATCGTGctagc GGCAAGGTCATATTGAACATT-3’ and TMPERG R NotI: 5’-CCGTAGATCGgccgcggc TTATGCTAAGTGCCCGCATGACTG-3’. PCR template was the previously reported pcDNA 3.1/V5-His-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 stably selected with puromycin and then grown in DMEM supplemented with puromycin (0.5 µg/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 S1. The dTdT overhang was added to 3’-terminal for siRNA sequences. Control nonsilencing siRNA

Translational Relevance

The TMPRSS2/ERG (T/E) fusion gene is present in approximately 50% of all prostate cancers. Fusion of the oncogenic ERG gene with the androgen-regulated TMPRSS2 gene promoter results in androgen-regulated expression of fusion mRNAs in prostate cancer cells. The junction of theTMPRSS2- 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.

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(scramble) corresponded to sequence 5’-AATCTCC-GAACGTGTCAGGT-3’. The siRNAs used in this study were purchased from Sigma Life Science. siRNA knockdown efficiency was tested using transient transfection in 293T cells. Cotransfection of individual siRNA at concentration of 50 nmol/L 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 conducted in 293T cells grown at 50% to 60% in 10-cm dishes using 32 µL Lipofectamine 2000 (Invitrogen) for 48 to 72 hours. 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 real-time PCR (qRT-PCR).

Western blotting
Protein extracts were prepared from cultured cells or VCaP primary tumors with radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitor, 2 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonylfluoride and clarified by centrifugation. Protein concentrations were determined using a BCA protein assay kit (Thermo Scientific). Western blot analyses were conducted 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 qRT-PCR using general procedures described previously (18).

Liposomal siRNA preparation
For in vivo delivery, siRNA was mixed with DOPC in the presence of excess tertiary butanol at a ratio of 1:10 siRNA: DOPC (weight:weight) as described previously (19). Before 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 65 nm 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 1 of 2 targeted siRNAs (Si8 or Si14) using 10 mice per group. Treatments were carried twice weekly intravenously for 4 weeks. Body weights and intraprostatic tumor mass were monitored weekly. Tumor imaging was conducted weekly using an IVIS imaging system (Xenogen) after intraperitoneal injection of α-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, weighed, and a portion of the tumor was frozen in liquid nitrogen for molecular analysis and another portion fixed and paraffin-embedded. Necropsy was also conducted on mice to rule out side effects of treatment. Subcutaneous tumors were established by injecting 1 × 10⁶ 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 (Houston, TX).

Immunohistochemistry and analysis of apoptosis
Immunohistochemistry (IHC) of mouse tissues was conducted using the basic procedures described previously (20). Paraffin sections (5 µmol/L) of formalin-fixed mouse xenografts were used for hematoxylin and eosin (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 was conducted as described previously (21). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was conducted using an In Situ Cell Death Detection Kit (Roche) according to manufacturer’s instructions. Ki67, TUNEL, and CD31 staining were quantitated using image analysis as described previously (22). NF-kB p65 phospho-Ser536 IHC was conducted as described previously (21). Nuclear staining was quantitated using a Vectra-Inform image analysis system (Caliper Life Sciences). 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 staining intensity in each image. The percentage in each bin was then calculated. For all studies, values were compared using t or Mann–Whitney tests.

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 cotransfection of each siRNA in 293T cells with a V5-tagged type III T/E fusion constructs. Of the 18 original siRNAs, we identified 3 that gave strong, consistent, and...
injected with control scrambled siRNA, Si8, or Si14 into nude mice. Mice were intraprostatic injection of luciferase-expressing VCaP cells Si8 or Si14 in an orthotopic VCaP model. Ten days after in vivo experiments using DOPC liposomes to deliver either type III targeted siRNAs Orthotopic and subcutaneous xenografts treated with control cells are liposomes only, whereas scrambled control. Tumor weight was decreased to a lesser extent (~45%) compared with luminescence, presumably due to the contribution of stroma and dead tumor cells to tumor weight (P < 0.001, t test). This experiment was repeated using a higher dose of siRNAs (450 μg/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 by 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 μg/kg body weight of targeted siRNAs or scrambled control was conducted using Ki67 IHC followed by quantitative image analysis and showed a statistically significant decrease in proliferation of 11% to 25% in treated versus control tumors (Fig. 2C). TUNEL staining of treated tumors showed a significant increase in cell death by 45% to 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 conducted anti-CD31 IHC to evaluate microvessel density. We observed a 71% decrease in microvessel density for both targeted siRNAs compared with controls. This marked decrease in angiogenesis was somewhat surprising and may contribute to the effects on proliferation and cell death observed. Representative images of all of the analyses are shown in Supplementary Fig. S1.

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 with scrambled controls. An example of a potent knockdown is shown in Fig. 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

reproducible knockdown of the type III T/E fusion mRNA. Figure 1A shows a Western blot analysis of with anti-V5 antibody on cell extracts of 293T cells transiently transfected with V5-tagged type III fusion gene and liposomes only (control), scrambled siRNA in liposomes, or 1 of 4 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, whereas the portion from ERG exon 4 is shown in red.

Orthotopic and subcutaneous xenografts treated with type III targeted siRNAs

On the basis of 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 μg/kg body weight intravenously via tail vein. Mice were weighed, and luciferase-based tumor imaging was conducted 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 conducted on mice. Of note, no toxicity or decreased body weight was noted (Fig. 2A). Tumor weight and luciferase activity before euthanasia are shown in Fig. 2A. Both the Si8 and Si14 groups showed a significant decrease in tumor luciferase flux of approximately 75% (P < 0.001, t test) when compared with scrambled control. Tumor weight was decreased to a lesser extent (~45%) compared with luminescence, presumably due to the contribution of stroma and dead tumor cells to tumor weight (P < 0.001, t test). This experiment was repeated using a higher dose of siRNAs (450 μg/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 by 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.

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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 Fig. 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 Fig. 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 \( (r^2 = 0.64, P = 0.007, \text{Pearson product moment}) \) consistent with the idea that the efficiency of knockdown is driving the correlation in treated mice rather than a factor intrinsic in the mice (e.g., 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 indicate 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 whether downstream targets of ERG were downregulated in treated mice, we analyzed expression of NF-κB p65 phospho-Ser536. Our group has previously shown that ERG significantly increases phosphorylation of p65-Ser536, which results in increased NF-κB activity, and that ERG expression is significantly correlated with levels of phospho-Ser536 in human prostate cancer 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 with scrambled control (Supplementary Fig. S2). Using Inform software, we quantitated the percentage of

Figure 2. The 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 μg/kg). Luciferase flux of tumors before euthanasia and tumor and body weights at termination of treatment are shown. The control group contained 9 mice, whereas each targeted group contained 11 mice. B, nude mice were injected subcutaneously with VCaP expressing luciferase (time 0). After 2 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 percentage of 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%), * statistically significant differences between targeted siRNA and scrambled control.
nuclei staining at 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 < 0.001, t test). Of note, cyclin D1 is a known target of NF-κB in prostate cancer (23), so the downregulation of cyclin D1 (Fig. 3B) may be due to decreased NF-κB 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 cotransfection in a manner similar to our analysis of the type III targeting siRNAs. Of the 18 original siRNAs, we identified 4 that gave strong, consistent, and reproducible knockdown of the type VI TMPRSS2/ERG fusion gene. Figure 4 shows a Western blot analysis of 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, whereas scrambled represents a nonspecific siRNA. As can be seen in Fig. 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 qRT-PCR in 293T and in PNT1a cells with stable expression of the type VI fusion gene.
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, targets the same 15 nucleotides in ERG exon 4 as the type III Si14 (Fig. 1B) but targets 6 nucleotides from exon 2 of TMPRSS2 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 cotransfected 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 2 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 Fig. 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 < 0.001, t test; data not shown). Subcutaneous xenograft in which male nude mice were injected subcutaneously with VCaP-Luc or VCaP-Luc TE6 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 = 0.034, t test). However, when type VI expressing VCaP were treated with siRNA targeting the type VI fusion, they were significantly growth inhibited compared with the same cells treated with scrambled control (P = 0.035) and had similar tumor weights to VCaP-Luc control cells–treated scrambled control (P = 0.96) or TE6 Si14 siRNA (P = 0.75), indicating that the growth-promoting activity of the type VI fusion was completely abrogated by the specific siRNA targeting this isoform.

**Figure 6.** The siRNA targeting the type VI T/E fusion mRNA inhibits tumor progression in vivo. A, expression of the type VI fusion protein in VCAP TE6 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, whereas 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. *, a statistically significant difference between different cell types treated with control siRNA; **, a significant difference from same cell type treated with scrambled control siRNA versus targeted siRNA.
Discussion

Our results show 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 prostate cancers. 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 prostate cancer.

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, as 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 prostate cancer is a potential efficacious treatment with low toxicity for men with prostate cancer.

Optimally, T/E fusion targeted treatment would target the specific fusion gene isoforms expressed by an individual patient’s tumor. On the basis of our prior results (8), 49% of prostate cancer 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 2 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 prostate cancer s can potentially have all of their detectable fusion isoforms targeted using siRNAs targeting the 4 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 1 or more of these 4 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 4 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 target 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 vivo (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 and colleagues (25) have shown that both uPA and MMP9 are direct ERG targets in prostate cancer. 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 NF-kB 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 NF-kB activity can also enhance expression of other angiogenic proteins such as VEGF and interleukin (IL)-8 (23). While further studies are needed, our findings show for the first time that knockdown of the T/E fusion gene protein in prostate cancer 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 TMPRSS2/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 PEylation 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 prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Shao, I. Tekedereli, G. Lopez-Berstein, B. Ozpolat

References


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