ABSTRACT

Gene fusion is a common event in cancer. The fusion RNA and protein products often play causal roles in tumorigenesis and therefore represent ideal diagnostic and therapeutic targets. Formerly, fusion chimeric products in cancer were thought to be produced solely by chromosomal translocation. Here, we show that a chimeric SLC45A3-ELK4 RNA is generated in the absence of chromosomal rearrangement. We showed that it is not a product of RNA trans-splicing, but formed by cis-splicing of adjacent genes/read-through. The binding of CCCTC-binding factor (CTCF) to the insulator sequences inversely correlates with the expression of the chimera transcript. The SLC45A3-ELK4 fusion, but not wild-type, ELK4 plays important roles in regulating cell growth in both androgen-dependent and -independent prostate cancer cells. The level of the chimeric transcript correlates with disease progression, with the highest levels in prostate cancer metastases. Our results suggest that gene fusions can arise from cis-splicing of adjacent genes without corresponding DNA changes.

SIGNIFICANCE: With the absence of corresponding DNA rearrangement, chimeric fusion SLC45A3-ELK4 transcript in prostate cancer cells is generated by cis-splicing of adjacent genes/gene read-through instead of trans-splicing. SLC45A3-ELK4 controls prostate cancer cell proliferation, and the chimera level correlates with prostate cancer disease progression. Cancer Discov; 2(7); 598–607. © 2012 AACR.

INTRODUCTION

Gene fusions are common features of human tumors (1–3). Individual examples of gene fusions are usually characteristic of specific subtypes of tumors and have become important diagnostic markers over the last several decades. As has been shown by abundant experimental evidence, these chimeric products are most often causally related to the neoplastic behavior of the tumors containing them. For many years, the assumption has been that the only mechanism by which a chimeric gene fusion product can be generated is chromosomal rearrangement. Two recent studies reported the detection by reverse transcriptase PCR (RT-PCR) of a chimeric transcript joining the 5′-end of RNA from solute carrier family 45, member 3 (SLC45A3) to the 3′-end of RNA from the ETS-domain protein SRF accessory protein 1 (ELK4) in prostate cancer (4, 5). Although several forms of SLC45A3-ELK4 RNA were amplified, a major form consists of exon 1 of SLC45A3 joined to the last 4 exons of ELK4. These 2 genes are located adjacent to each other on chromosome 1 band q32. FISH, array comparative genomic hybridization (CGH), or quantitative PCR (qPCR) for copy number variance did not detect obvious deletion of the genomic DNA between the 2 genes (4, 5), leaving the possibility of reciprocal chromosomal rearrangement or posttranscriptional changes.

Authors' Affiliation: Department of Pathology, University of Virginia, Charlottesville, Virginia

Note: Supplementary data for this article are available at Cancer Discovery Online (http://cancerdiscovery.aacrjournals.org/).

Current address for M. Gong: Department of Pediatrics, University of Virginia, Charlottesville, VA 22908.

Corresponding Author: Hui Li, Department of Pathology and Cancer Center, University of Virginia, Charlottesville, VA 22908. Phone: 434-982-6680; Fax: 434-243-7244; E-mail: hli9r@virginia.edu
doi: 10.1158/2159-8290.CD-12-0042; © 2012 American Association for Cancer Research.
Here, we conducted extensive Southern blot analysis to further rule out the possibility of DNA rearrangement and showed the presence of the fusion transcript with a non–RT-PCR-based assay. We showed that the fusion transcript, but not wild-type, ELK4 played an important role in regulating prostate cancer cell proliferation, both in androgen-dependent and -independent cell lines. Importantly, the level of the fusion transcript correlates with prostate cancer disease progression. In studying the mechanism, we found that the fusion transcript is not a product of RNA trans-splicing as originally suggested (5). Instead, we observed the presence of intergenic transcript and an inverse correlation between the binding of CCCTC-binding factor (CTCF) to the gene boundary insulators and the level of the SLC45A3-ELK4 fusion transcript. Of note, CTCF had the opposite effect on trans-spliced JAZF1-JJAZ1 RNA. In summary, the data presented here provide evidence that active cis-splicing of adjacent genes (cis-SAGE) could be sufficient to generate gene fusion products without a corresponding chromosomal rearrangement at the DNA level and that such fusion products may play important functional roles in cancer cells.

RESULTS

The chimeric RNA SLC45A3-ELK4 can be detected at much higher levels in the prostate cancer cell lines LNCaP and PC3 than in non-neoplastic prostate epithelial cells such as RWPE-1 and PrEC (Supplementary Fig. S1). To investigate the mechanism by which the chimeric SLC45A3-ELK4 transcript is generated, we first examined the prostate cancer cell line LNCaP. Treatment of cells with the synthetic androgen R1881 resulted in an increased level of the SLC45A3-ELK4 chimera, similar to wild-type SLC45A3 (Supplementary Fig. S2A). The increase was dependent on the androgen receptor in LNCaP cells (Supplementary Fig. S2B), as knocking down androgen receptor by siRNA abolished induction of the chimeric RNA in the presence of R1881. The androgen induction and androgen receptor dependence of the chimeric RNA resembles that of SLC45A3. To rule out a possible artifact caused by RT-PCR, we tested the existence of the chimeric RNA by RNase protection using a hybridization probe, spanning the junction of the SLC45A3 and ELK4 RNA. Using this method, chimeric RNA was easily detected in LNCaP cells and at increased levels after treatment with R1881. Essentially, very little chimeric RNA was detected in RWPE-1. No signal of the chimera was detected in its derivative cell line WPE1-NB26, or in normal placenta control (Fig. 1A).

To determine whether genomic DNA rearrangements might be responsible for the chimeric RNA, we scanned the entire 60-kb region between exon 1 of SLC45A3 and exon 2 of ELK4 by Southern blot analysis (Fig. 1B). Three representative Southern blot analyses are shown in Fig. 1B. No evidence of abnormal DNA rearrangement was observed, even after induction by androgen treatment (Fig. 1B).

Recently, we showed that JAZF1-JJAZ1 fusion RNA in endometrial stromal cells is generated through a mechanism involving RNA trans-splicing (6, 7). The presence of the chimeric RNA and absence of DNA level rearrangement suggested that SLC45A3-ELK4 might be generated by trans-splicing. This possibility was also suggested by Rickman and colleagues (5). If true, the trans-splicing machinery in cells that express SLC45A3-ELK4 could then use foreign premature RNAs as substrates to generate chimeric transcripts composed of both endogenous and foreign RNA, as in the case of JAZF1-JJAZ1 (8). We thus conducted in vitro trans-splicing assays using nuclear extracts of LNCaP cells and CV-1 green monkey kidney cells. Single-nucleotide polymorphisms in the ELK4 segment allowed us to distinguish the donors. Using a monkey-specific reverse primer that anneals within the ELK4 segment, we could not detect any trans-spliced RNA product composed of monkey ELK4 (Fig. 1C), even though both LNCaP and CV-1 nuclear extracts were active as shown by PCR amplification in the presence of ATP using other sets of primers (Fig. 1C).

Because SLC45A3 and ELK4 are neighboring genes located in the same chromosomal region, we suspected that the chimeric transcript could be a product of cis-splicing of adjacent genes (cis-SAGE), where a premature RNA is transcribed across the gene boundary and exons belonging to SLC45A3 and ELK4 are spliced together. To investigate, we used various primer pairs to amplify transcripts on ELK4, SLC45A3, and intergenic regions between SLC45A3 and ELK4. No product was detected if avian myeloblastosis virus (AMV) reverse transcriptase was omitted, suggesting the absence of DNA contamination (Fig. 2A). Intergenic transcripts were detected with primer pairs 7 and 8 and were induced with the treatment of R1881, similar to the chimeric SLC45A3-ELK4 transcript (Fig. 2A).

Insulators between the neighboring genes act as boundaries to protect a gene against the encroachment of adjacent inactive condensed chromatin or against the activating influence of distal enhancers associated with other genes (9). Insulator activity is controlled mainly by CTCF. Chromatin immunoprecipitation (ChIP) combined with high-throughput sequencing has identified 2 insulator sequences existing in between the SLC45A3 and ELK4 genes (10). Using ChIP, we validated CTCF binding to these 2 insulators (Supplementary Fig. S3). If the chimeric RNA is generated by a cis-SAGE mechanism, an inverse correlation between CTCF binding to the insulators and fusion RNA expression is anticipated. While SLC45A4-ELK4 RNA level was induced by R1881 in LNCaP cells, qPCR indeed detected significantly less binding of CTCF to insulator 1 (INSUL_ZHA00174), shown in Fig. 2B. Similarly, a significant reduction in binding of CTCF to insulator 2 (INSUL_ZHA001740) was also observed.

To confirm this effect on SLC45A3-ELK4 expression, we used siRNA to silence CTCF expression. Chimeric SLC45A3-ELK4 expression was significantly upregulated by siCTCF in androgen-deprived LNCaP cells (Fig. 2C), consistent with the hypothesis that the binding of CTCF to insulators prevents basal levels of transcription through the boundary of the SLC45A3 and ELK4 genes. This effect was specific to the SLC45A3-ELK4 transcript because wild-type SLC45A3 and ELK4 transcripts were slightly downregulated by siCTCF (Supplementary Fig. S4), arguing against the possibility of a general effect on cis-splicing caused by hormone deprivation. In the presence of androgen, knocking down CTCF had no obvious effect on the expression of the chimera (Fig. 2C), presumably because the binding of CTCF to the insulators was reduced (Fig. 2B). CTCF was found to mediate interchromosomal interactions between the Igf2/H19 imprinting control...
A SLC45A3-ELK4 chimeric RNA in LNCaP cells detected by RNAse protection assay (RPA). RNAs from LNCaP cells, LNCaP cells treated with R1881, RWPE-1, WPE1-NB26, and a normal placenta were extracted and hybridized to an antisense probe spanning the fusion junction. Besides the wild-type SLC45A3 and ELK4 bands, an additional band around 150 bp was detected in LNCaP cells. The signal was stronger in LNCaP cells treated with R1881. In contrast, only wild-type transcripts were detected in RWPE-1 and WPE1-NB26 cells. No band was detected in normal placenta control because of the absence of both SLC45A3 and ELK4 transcription in placenta.

B, lack of DNA rearrangement at SLC45A3 and ELK4 loci shown by Southern blot analysis. Genomic DNAs from a normal placenta control, LNCaP cells, and LNCaP cells treated with R1881 were extracted. Representative results for probes 1, 4, and 10 are shown. Without DNA rearrangement, these probes should hybridize to DNA fragments of 6 kb (probe 1), 7.5 kb (probe 10), and 0.5 and 0.8 kb (probe 4).

C, SLC45A3-ELK4 is not a product of RNA trans-splicing. The underlined codes represent nucleotide polymorphisms between human and monkey on ELK4. In the in vitro trans-splicing assay, if trans-splicing occurred, a chimera containing monkey ELK4 would be detected with the common forward primer SLC45A3-181F and the monkey-specific ELK4 reverse primer. Human-specific reverse primer paired with SLC45A3-181F gave a stronger signal in the presence of ATP, as did the ELK4 forward and monkey-specific reverse primer pair, suggesting that the nuclear extracts from LNCaP and CV-1 were both active.

To determine whether SLC45A3-ELK4 RNA has a biologic function in prostate cancer cells, we carried out knockdown experiments with siRNA designed to specifically reduce expression of the chimeric transcript. siRNA against the luciferase gene transcript was used as a negative control (12). Two siRNAs against SLC45A3-ELK4 RNA, siSE1 and siSE2 (Supplementary Fig. S5), were very effective in silencing the chimeric RNA, with minimal, if any, effects on the wild-type, parental transcripts of either SLC45A3 or ELK4 (Fig. 3A). LNCaP cells transfected with each of these 2 siRNAs had significantly less cell proliferation as reflected by MTT assay (Fig. 3B) and cell counting (Fig. 3C). Inhibition of cell proliferation did not occur when the LNCaP cells were in

region on chromosome 7 and the Wsb1/Nf1 gene complex on chromosome 11 (11). CTCF has also been found to play a role in bringing distal intrachromosomal and interchromosomal regions into proximity (8, 11), which prompted us to speculate on its role in facilitating trans-splicing events. Indeed, when we silenced CTCF in endometrial stromal cells, the JAZF1-JJA21 chimeric RNA level went down, unlike SLC45A3-ELK4 expression, further indicating that SLC45A3-ELK4 is generated by a mechanism different from trans-splicing (Fig. 2D).

To determine whether SLC45A3-ELK4 RNA has a biologic function in prostate cancer cells, we carried out knockdown

Downloaded from cancerdiscovery.aacrjournals.org on April 6, 2017. © 2012 American Association for Cancer Research.
androgen-depleted medium (Fig. 3B and C), indicating that the chimeric SLC45A3-ELK4 is required only for androgen receptor–dependent proliferation and not basal growth in LNCaP. This is consistent with the observation that the chimeric product is androgen-regulated in LNCaP cells. Two insulators are located 3′ of SLC45A3 and 5′ of ELK4. Insulator 1: chr1: 203893201-203895199; Insulator 2: chr1: 203866801-203869599. The right graph shows comparison of the ChIP amount of the insulator DNA between R1881-treated (red) and ethanol-treated LNCaP cells (green). The left graph shows the chimeric SLC45A3-ELK4 RNA expression in samples harvested parallel with the samples for ChIP assay. **, P < 0.05 (Student t test). A, RT-PCR demonstrated that intergenic pre-mRNA between SLC45A3 and ELK4 was present and induced by androgen. The location of the primers is shown on the right. Pairs of primer 4, 5, 6, and 9 are located on exons or introns of SLC45A3 and ELK4. Pairs of primer 7 and 8 are located between SLC45A3 and ELK4. Left, the experimental design. Placental DNA was used as positive control. The electrophoresis images are the representative results of more than 3 replicates. B, ChIP assay to investigate CTCF binding on insulators between SLC45A3 and ELK4. Two insulators are located 3′ of SLC45A3 and 5′ of ELK4. Insulator 1: chr1: 203893201-203895199; Insulator 2: chr1: 203866801-203869599. The right graph shows comparison of the ChIP amount of the insulator DNA between R1881-treated (red) and ethanol-treated LNCaP cells (green). The left graph shows the chimeric SLC45A3-ELK4 RNA expression in samples harvested parallel with the samples for ChIP assay. **, P < 0.05 (Student t test). C, SLC45A3-ELK4 upregulation by CTCF silencing. CTCF knockdown by siRNA was conducted in LNCaP cells. After 24 hours, the medium was changed to androgen-deprived medium for another 48 hours. RNA was harvested after 6 hours of R1881 or ethanol treatment. **, P < 0.05 (Student t test).
used as a diagnostic/prognostic marker. No similar correlation of wild-type SLC45A3 or ELK4 expression with the Gleason score was observed (Fig. 3F and G), indicating that the chimeric RNA is not a specific splicing product of excess wild-type transcripts.

To gain insight into the nature of SLC45A3-ELK4–mediated cell growth regulation, we conducted cell-cycle analysis following SLC45A3-ELK4 knockdown. LNCaP cells transfected with siSE1 had a significantly higher percentage of G1 phase and lower percentage of S-phase cells (Fig. 4A). Increased cell death was not obvious by propidium iodide staining. Interestingly, an siRNA targeting ELK4, which effectively silenced wild-type ELK4 without affecting SLC45A3-ELK4 (Supplementary Fig. S6), did not induce the same cell-cycle arrest (Fig. 4A). As high levels of CDKN1A (p21) have been known to cause G1–S arrest, we used real-time PCR to monitor p21 RNA levels in these cells. p21 was indeed upregulated in LNCaP cells transfected with siSE1 and siSE2, but not with siELK4 (Fig. 4B).

We then conducted microarray analysis using RNA extracted from LNCaP cells transfected with si- siSE1 and siSE2. A cluster of 35 genes including p21 was found to be up or downregulated by SLC45A3-ELK4 knockdown (Fig. 4C). Ten of 12 genes/transcripts were subsequently confirmed by real-time qPCR to be significantly affected by siRNA against SLC45A3-ELK4 (Fig. 4D). Among these genes, the expression
SLC45A3-ELK4 Controls Prostate Cancer Cell Proliferation

In addition to prostate cancer cells, SLC45A3-ELK4 could also be detected in HCT116, 293T, and HeLa cells but not in the lung cancer cell lines H520 and A549 (Fig. 5A). Similar to LNCaP and PC3 cells, silencing SLC45A3-ELK4 with siSE1 slowed down cell growth in 293T cells as shown by cell counting and MTT (Fig. 5B). In contrast, no effect on cell proliferation was observed with siSE1 in HCT116 cells (Fig. 5C), suggesting cell-type–specific effects of silencing the chimeric transcript.

**DISCUSSION**

Chimeric RNAs containing exons of neighboring genes have been considered rare in mammalian cells, with only a handful of examples experimentally identified (13). Recent studies incorporating systematic *in silico* analysis and paired-end RNA sequencing have identified many chimeric RNAs involving 2 adjacent genes (14, 15). No functional role of these chimeras has yet been identified and the underlying mechanism remains elusive (13, 15). The terms “gene read-through” and “co-transcription and intergenic splicing” have been used to describe these chimeric RNAs that join neighboring gene fragments. However, “gene read-through” was originally used to describe protein translation processes that skip the stop codon. “Co-transcription and intergenic splicing” does not differentiate trans-splicing events from events involving transcription through gene boundaries that result in splicing of exons from different genes. To differentiate the latter type of event from trans-splicing, we use the term “cis-splicing of adjacent genes” in this communication. Judging by sequence alone, it is impossible to discern these 2 types of events. In fact, RNA trans-splicing may also have a higher incidence rate among genes that are close to each other (15). SLC45A3 and ELK4 are found in the same chromosomal region, 1q32, with about 60 kb separating SLC45A3 exon of p21 and S100A10 was not affected by siELK4 (Fig. 4B and data not shown).

In addition to prostate cancer cells, SLC45A3-ELK4 could also be detected in HCT116, 293T, and HeLa cells but not in the lung cancer cell lines H520 and A549 (Fig. 5A). Similar to LNCaP and PC3 cells, silencing SLC45A3-ELK4 with siSE1 slowed down cell growth in 293T cells as shown by cell counting and MTT (Fig. 5B). In contrast, no effect on cell proliferation was observed with siSE1 in HCT116 cells (Fig. 5C), suggesting cell-type–specific effects of silencing the chimeric transcript.

**Figure 4.** A, cell-cycle analysis. LNCaP cells were transfected with si-, siSE1, and siELK4. Five days after transfection, cells were harvested, fixed, and stained by propidium iodide for cell-cycle analysis. B, p21 was upregulated with the silencing of SLC45A3-ELK4, not by ELK4 silencing. C, microarray analysis on si-, siSE1, and siSE2 in LNCaP cells. Human Genome 1.0 ST Array (Affymetrix) was used for microarray analysis. Hierarchical clustering of a list of genes compiled using the criteria of at least 2-fold change compared with control (si-). D, genes with high expression differences were chosen to be reexamined by real-time PCR. Fold change is described by the gene expression relative to si- control. Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).
1 and ELK4 exon 2. One possible mechanism to generate the fusion is through chromosomal deletion, as in the case of TMPRSS2-ERG (16). Two groups that identified the SLC45A3-ELK4 fusion failed to find such a deletion (4, 5). However, the approaches used previously were not sufficient to detect reciprocal chromosomal translocations. In addition, the detection of the fusion transcript was solely based on RT-PCR, which is known to cause artifacts. In fact, recent studies have attributed many chimeras to template switching by reverse transcriptase during cDNA preparation in vitro (17, 18). Here, with extensive Southern blot analysis, we confirmed the absence of DNA rearrangement in LNCaP cells. We also confirmed the presence of the fusion transcript by RNAase protection. We then carried out a series of experiments to study the mechanism of SLC45A3-ELK4 chimeric RNA generation: (i) An in vitro trans-splicing assay did not detect chimeric RNAs fusing human and monkey SLC45A3 and ELK4 transcripts, (ii) RNAs were transcribed from intergenic regions, (iii) CTCF binds to the insulators between the 2 genes and the binding was reduced in the presence of androgen, and (iv) knocking down CTCF induced the expression of the chimeric SLC45A3-ELK4 RNA. It is also interesting to note that knockdown of CTCF caused the opposite effect on the level of trans-spliced JAZF1-JJAZ1. On the basis of these data, we conclude that the chimeric transcript is a product of cis-SAGe/gene read-through as suggested previously (4), but not trans-splicing as suggested by a later study (5).

Chimeric SLC45A3-ELK4 RNA is translated into wild-type ELK4 protein. The 5'-untranslated region of SLC45A3 in the chimera relates to the induced control of SLC45A3 by androgen. In addition, SLC45A3-ELK4 expression seems to

---

Figure 5. A, real-time PCR assay using TaqMan probe to detect the expression of SLC45A3-ELK4 in LNCaP, HCT116, 293T, HeLa, H520, and A549 cell lines. Transcript amounts were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The level of SLC45A3-ELK4 in LNCaP was set to 1. B, in 293T cells, siSE1 silenced SLC45A3-ELK4 and slowed down cell growth as measured by cell counting and MTT. C, in HCT116 cells, siSE1 silenced SLC45A3-ELK4, but resulted in no obvious growth inhibition as measured by cell counting and MTT.
have unique consequences different from wild-type ELK4: (i) proliferation was inhibited by SLC45A3-ELK4 silencing, but not by ELK4 silencing; (ii) the growth-inhibitory effect could be rescued by transfection of an SLC45A3-ELK4-expressing construct, but not an ELK4 construct; (iii) cell-cycle arrest was only observed with SLC45A3-ELK4 silencing; (iv) p21 and S100A1 levels were affected by silencing of SLC45A3-ELK4, but not ELK4, and (v) SLC45A3-ELK4, not ELK4, RNA level correlates with clinical prostate cancer progression.

Currently, the mechanism of SLC45A3-ELK4 overexpression in malignant prostate cancer is not known. Poly(A) signal mutation has been reported to disrupt the normal site of transcriptional termination and block mRNA polyadenylation (19). In mild thalassemia, a point mutation in the canonical poly(A) signal (AAUAAA) of the HBB gene caused a larger transcript to be produced (20). To investigate whether any point mutation in the poly(A) site could explain the continuation of the SLC45A3 transcript, we sequenced the last exon of the gene in LNCaP, RWPE, and PC3 cells and a normal placenta control. No mutation was found at the predicted poly(A) signal, in this case AUUAAA, or the G/U-rich region downstream (data not shown). We also sequenced the insulators in LNCaP cells and found no abnormality (data not shown). As CTCF is a ubiquitous factor, its effect on the chimeric RNA is unlikely to be due to differential expression. Indeed, we did not observe any changes of CTCF expression level when LNCaP cells were treated with androgen (Fig. 2C and data not shown).

SLC45A3-ELK4 is also expressed in PC3 cells, which have no androgen receptor, indicating that a downstream target of androgen receptor or an androgen receptor-independent pathway might regulate the chimera overexpression in these cells. This is likely to be the case in other cancer types (Fig. 5A). It has been shown that growth factors can facilitate translocation of CK2 into the nucleus (21), which then phosphorylates CTCF and facilitates a switch of CTCF from a transcriptional repressor to an activator (22, 23). Silencing the chimeric transcript reduced cell proliferation in both LNCaP and PC3 cells, suggesting a more immediate role of the chimera in controlling cell growth than androgen receptor.

Other fusion products involving ETS family members have been shown to promote cell migration, with no obvious effect on cell proliferation (24, 25). In SLC45A3-ELK4 gain- and loss-of-function systems, we did not observe significant difference of cell migration ability, at least in LNCaP cells (data not shown). Interestingly, we found that SLC45A3-ELK4 was also present in a few cell lines of other cancer types, but not in lung cancer cell lines (Fig. 5A). Silencing the chimeric transcript yielded a similar result to LNCaP cells in 293T cells, but not in HCT116 cells (Fig. 5B and C), suggesting that the chimeric transcript may be more general and may regulate cell proliferation at least in some non-prostate cancer cells.

In conclusion, our results suggest that cis-SAGe could be an alternative pathway to generate gene fusions in the absence of DNA rearrangement. Given that most chimeric gene fusions play causal roles in tumorigenesis, it is conceivable that abnormal cis-SAGe might be a novel epigenetic way to drive cancer.

**METHODS**

**Cell Culture and Infection**

Prostate cancer cell lines, LNCaP, PC3, RWPE-1, and WPE1-NB26, cells were acquired from Dr. Anindya Dutta (University of Virginia). PrEC cells were obtained from Dr. William Hahn (Harvard University). 293T, CV-1 and HCT116 cells were purchased from American Type Culture Collection. The cell lines have not been further tested and authenticated. They were maintained in RPMI-1640 medium containing 10% FBS, 1% pen/strep, and 1% l-glutamine. Monkey kidney CV-1 cell line was maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% FBS, 1% pen/strep, and 1% l-glutamine. When androgen-deprived, cells were cultured in phenol-free RPMI medium supplemented with 5% charcoal-stripped serum (Invitrogen). For androgen treatment, cells were androgen-deprived for 2 days before treatment with 1 nmol/L R1881. Controls were treated with the same volume of ethanol. Cells were transfected with Lipofectamine RNAiMAX (Invitrogen) according to manufacturer’s protocol. Infection was conducted by using 8 μg/mL polybrene and 293T cell packaged SLC45A3-ELK4 or ELK4 expressing retrovirus.

**Clinical Samples**

Zinc formalin-fixed, paraffin-embedded samples of normal prostate and prostate cancer were selected for analysis. Care was taken to ensure that the specimens consisted predominantly of normal prostate epithelium or, alternatively, prostate cancer epithelium.

**PCR and Real-time PCR**

RNA was extracted using TRIzol reagent (Invitrogen) and quantified with Nanodrop (Thermo). cDNA was generated by AMV-RT kit (NEB) and random hexamer primer. Real-time qPCR was carried out on StepOne Plus system from Applied Biosystems. All the other gene expression levels were detected by SYBR mix (Thermo). Primers and the annealing temperature used in our study are listed in Supplementary Table S1. To detect the combination of human and monkey chimera in the in vitro trans-splicing assay, Platinum Taq from Invitrogen with primers SLC45A3-181F, human reverse primer: GTGGTGCGCAAAAAGCATGCA and monkey reverse primer: TGGTGGGGGCAAAAACAGTTG were used. ELK4-1201F was applied with monkey reverse primer to test the existence of the monkey ELK4 transcript. To improve specificity and sensitivity, the above amplifications were conducted with a touchdown protocol consisting of 2 cycles with annealing temperature of 66°C, 6 cycles with 64°C, and 8 cycles of 62°C, followed by 24 cycles of 60°C annealing amplification. Pairs of primers 4 to 9 used to investigate pre-precursor mRNA are the same as the primers used for probe synthesis in Southern blot analysis and are listed in Supplementary Table S1. Statistical analysis was conducted with Excel (Microsoft) and P values were calculated with the Student t test or nonparametric test (Mann–Whitney U test).

**RNase Protection Assay**

To generate the probe, the RT-PCR product of SLC45A3-ELK4 RNA was amplified by primer SLC45A3-181F and ELK4-exon2-R1 and cloned into the pBluescript vector. The antisense RNA probe for SLC45A3-ELK4 RNA was transcribed in vitro by the MAXIscript T7 Kit (Ambion). The ribonuclease protection assay was conducted by the Ambion RPA III Kit and 10 μg of cellular RNA.

**Southern Blot Hybridization**

Procedures for Southern blot hybridization have been described (6, 26). In Southern blot analysis, genomic DNA was digested with BamHI or BamHI + KpnI (for probes 6 and 7). Pairs of primers 1 to 10 designed for synthesizing probe 1 to 10 are listed in Supplementary Table S1.
**Nuclear Extraction and In Vitro Trans-Splicing**

Methods of nuclear extract and in vitro trans-splicing were reported in our previous work (8).

**Chromatin Immunoprecipitation**

ChiP was conducted according to supplier protocol (Millipore). CTCF antibody (Cell Signaling), rabbit IgG, protein G agarose beads were used. HS5 CTCF-binding site was used as positive control. MLH1 exon 19 was used as reference. Binding of the CTCF with insulators was detected by real-time PCR with SYBR mix. Pulling down of insulator 1, insulator 2, HS5, and MLH1 were detected by 64°C annealing temperature. Primers designed to detect the amount of insulator 1 (INSUL_ZHAO01741), insulator 2 (INSUL_ZHAO01740), MLH1, and HS5 are listed in Supplementary Table S1.

**RNA Interference**

Targets of GL2, siSE1, siSE2, siELK4, androgen receptor (AR), and CTCF are listed in Supplementary Table S1; siRNAs for androgen receptor and CTCF have been published before (27, 28).

**MTT**

LNCap and PC3 cells were plated on 96-well plates with 1,000 cells per well and silenced by si-, siSE1, and siSE2 or siELK4. For the androgen-deprived group of cells, medium was changed to androgen-deprived medium 24 hours after siRNA transfection. Seventy-two hours after transfection, cell viability was measured by MTT (Sigma).

**Flow Cytometry**

LNCap and PC3 cells were plated on 6-well plates and silenced by si-, siSE1, and siSE2 or siELK4 1 day later. Seventy-two hours after siRNA transfection, cells were harvested, washed by PBS, and fixed by cold 80% ethanol (prepared by PBS) overnight in −20°C. Cell-cycle analysis was conducted by propidium iodide staining to evaluate DNA content in cell cycle.

**Microarray Analysis**

Oligonucleotide microarray analysis was conducted using the Affymetrix Human Gene 1.0 ST Arrays by the University of Virginia (Charlottesville, VA) Microarray Core Facility. A list of genes of interest was compiled using the criteria of at least 2-fold change compared with control siRNA. Hierarchical clustering was conducted using the BRB ArrayTools developed by Dr. Richard Simon (National Cancer Institute) and BRB-ArrayTools Development Team. Primers used for validating by real-time PCR are listed in Supplementary Table S1. Raw and processed data have been deposited into GEO with accession number GSE37410.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interests were disclosed.

**Acknowledgments**

The authors thank Dan Gioeli and John DaSilva for donating siRNA against AR and real-time qPCR primers for AR; Anna Lee for technical assistance; and Drs. Jeffrey Sklar, Michael Weber, Sally Parsons, Dennis Templeton, Ann Beyer, and Benjamin Purow for reviewing the manuscript.

**Grant Support**

H. Li was supported by a Stand Up To Cancer Innovative Research Grant, a Program of the Entertainment Industry Foundation (SU2C-AACR-IRG0409); V Scholarship; American Cancer Society Institutional grant, and Funds for Excellence in Science and Technology.

**References**


Chimeric Transcript Generated by cis-Splicing of Adjacent Genes Regulates Prostate Cancer Cell Proliferation

Yanmei Zhang, Mei Gong, Huiling Yuan, et al.