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TITLE: Exploring the Mechanisms of Pathogenesis in Prostate Cancer Involving TMPRSS2-ERG (Or ETV1) Gene Rearrangement

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## ABSTRACT

Recently, gene rearrangements involving ETS family transcription factors have been identified in >50% of prostate cancer cases. To address roles of these ETS factors, especially ERG, the most frequently rearranged ETS gene in prostate cancer, as well as in normal prostate development, we planned to: 1. Generate conditional knockin mouse models of prostate cancer based on the newly identified TMPRSS2-ERG (or ETV1) gene arrangements; 2. Explore roles of Erg during development as well as in normal prostate by disrupting its expression in mouse; 3. Identify downstream target genes of ERG or ETV1 in human prostate cancer cell lines carrying these gene arrangements using the ChIP-on-Chip approach. During the first year of this award, we have made significant progress in establishing systems and reagents for all three aims mentioned above. Specifically, we have successfully created conditional knockin mice expressing truncated human ERG and ETV1 (as found in patients) from the endogenous mouse Tmprss2 locus. We have generated an Erg knockdown allele in mice, which would allow us to study its roles during both embryonic development and postnatal prostate development. We have also made biotinylated ERG and ETV1 in prostate cell lines, which would allow us to identify the downstream targets of these factors in prostate epithelial cells. Further studies using these animal and cell culture models would allow us to develop preclinical animal models, and to identify and validate novel therapeutic targets, for treating prostate cancer.

## SUBJECT TERMS
Prostate cancer, gene targeting, mouse model, gene rearrangement, oncogenesis

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Prostate cancer is among the most frequently diagnosed cancers and the second most common cause of male cancer-related death (1, 2). It arises through a progression from an initial benign stage to frank malignancy, most often correlating with androgen-dependence and then androgen-independence in the late stage (3). Among genetic changes accompanying prostate cancer, of greatest interest are genetic “hits” in prostate epithelial cells that might represent initiating events for oncogenesis or consistent somatic mutations that specifically affect these cells. Identification of and modeling these causal genetic alterations would be invaluable in providing novel therapeutic targets and prognostic markers. Recently, chromosomal rearrangements between androgen-responsive genes, such as TMPRSS2, and genes encoding the ETS family transcription factors (ERG, ETV1, ETV4, and ETV5) were identified in >50% of prostate cancer cases (4-14). Due to their high incidence in prostate cancer, these may represent the most common gene rearrangements identified in human cancers. In addition, in analogy to the role of translocations in hematological malignancies, these chromosomal rearrangements represent either the initiating event in oncogenesis or a critical and consistent set in the cancer progression. Among ETS genes involved in these rearrangements, the most common one is ERG, followed by ETV1 (15). Arrangements of coding regions of these ETS genes to control regions of androgen-responsive genes presumably lead to overexpression of these proto-oncogenes. In this study, we plan to create and characterize a series of mouse models based on TMPRSS2-ERG (or ETV1) gene rearrangements to study roles of these ETS genes, especially ERG, in development and in prostate tumorigenesis. We also plan to use biochemical approaches to identify downstream targets of ERG as well as its interaction partners. These studies would allow us to develop preclinical animal models, and to identify and validate novel therapeutic targets, for treating prostate cancer.

Body:

Specific aim 1: Establish a novel Cre/lox conditional knockin mouse model of prostate cancer based on the newly identified TMPRSS2-ERG(or ETV1) gene arrangements.

We proposed to use the Cre/lox conditional approach to model prostate cancer based on the TMPRSS2-ERG/ETV1 chromosomal rearrangements. We planned to use two different strategies to achieve this aim. In one strategy, we proposed to generate conditional knockin alleles to express the N-terminal truncated human ERG (or ETV1, as found in patients) from the mouse endogenous Tmprss2 locus. In a second strategy, we proposed to create knockin alleles of both Tmprss2 and Erg by introducing a single loxP site to each allele, which would then permit Cre-mediated interchromosomal recombination as well as intrachromosomal excision.

For the first strategy, we knocked in the N-terminus-truncated human ERG or ETV1 cDNA together with an ires-Gfp marker into the exon 2 of mouse Tmprss2 locus. We placed a transcriptional stopper cassette together with Neo (Neo-ST) upstream of the knockin cDNA. The Neo-ST cassette is flanked by loxP sites. Cre-mediated excision would remove this cassette and activate the Tmprss2-ERG or ETV1 fusion genes. Several correctly targeted independent ES clones from either ERG or ETV1 knockins (Tmprss2-Neo-ST-ERG/ETV1) were injected into mouse blastocysts and a colony of germline-transmitted heterozygous and homozygous knockin mice was established. These knockin mice have been bred to the prostate-specific PB-Cre4 transgenic mouse (16) to generate PB-Cre4;Tmprss2-Neo-ST-ERG/ETV1 double transgenic males. In addition, we also generated several stopper-removed knockin Tmprss2-ERG ES cell lines in vitro and made chimeric male mice from them by blastocysts injection. From these male chimeras, we have also established a colony of stopper-removed knockin Tmprss2-ERG heterozygous (one copy of Tmprss2-ERG) and homozygous (two copies of Tmprss2-ERG) mice. In these animals, since the Neo-ST cassette has already been removed, ERG expression is activated and under the control of the Tmprss2 promoter. In fact, by RT-PCR, we confirmed that the Tmprss2-ERG fusion transcript was expressed from the endogenous Tmprss2 locus from prostate tissues of the Tmprss2-ERG chimeric males (Figure 1), suggesting the human ERG cDNA was indeed correctly targeted to the mouse Tmprss2 locus.

We have followed the Tmprss2-ERG heterozygous and homozygous males for up to eight months and have not observed any abnormality in their prostates, suggesting ectopic expression of ERG alone in prostate is not sufficient to initiate prostate tumorigenesis and additional mutation(s) may be needed to cooperate with ERG for the development of prostate cancer. Recently, several groups reported prostate intraepithelial neoplasia (PIN) lesions in prostates from transgenic male mice overexpressing ERG from the probasin promoter (PB-ERG), although the reported phenotype is very weak and apparently ERG overexpression alone is not
sufficient to drive prostate cancer development (17, 18). Genetically, the major difference between our knockin model and the reported transgenic models is that in our model, similar to what occurs in human patients, one or both copies of the endogenous Tmprss2 gene is disrupted, whereas in PB-ERG transgenic mice, both alleles of Tmprss2 are not affected. Tmprss2 is an androgen regulated transmembrane serine protease that belongs to the type II transmembrane protease (TTSP) family (19). It is overexpressed in both prostatic hyperplasia and prostate cancers (20, 21). It is also mislocalized (cytoplasm compared to its normal location in the cell membrane) in most high-grade prostate tumors (20). These suggest that Tmprss2 may also play a role in prostate tumorigenesis. If this is the case, overexpression of ERG and disruption of Tmprss2 may have opposite effects on prostate tumorigenesis and they may cancel each other in our knockin model. Thus, future study may need to focus on assessing the role of Tmprss2 on prostate tumorigenesis as well.

For the second strategy, we aimed to introduce a single loxP site to both the Tmprss2 and the Erg loci, respectively. If the two knockin loxP sites are located on the same chromosome (chromosome 16 for mice), Cre-mediated recombination would delete the ~3Mb region between them, and generate the Tmprss2-Erg fusion gene following deletion. If the two loxP sites are knocked into two different chromosome 16s, then Cre-mediated recombination would lead to chromosomal translocation. The Tmprss2-Erg fusion gene would be created following translocation. To generate such a model, we first introduced the above-described floxed Neo-ST cassette into the Erg locus in the intron upstream of its exon 4. This immediately gave us a knockdown allele for Erg, which would allow us to study the role of Erg in normal development (will discuss in the progress for specific aim 2). In ES cells carrying this Erg knockdown (kd) allele, we expressed Cre transiently in vitro to remove the floxed Neo-ST cassette. After excision, only a single loxP site remains. We then performed sequential targeting from these ES cells to introduce the second loxP site into the Tmprss2 locus. This was also achieved through the introduction of the floxed Neo-ST cassette. We initially planned to remove the Neo-ST cassette by Cre-mediated recombination so that only one loxP site would remain in the Tmprss2 locus. However, transient transfection of sequentially targeted ES cells by Cre efficiently excised both the Neo-ST cassette and the 3Mb region between Erg and Tmprss2. The correct Tmprss2-Erg recombination was confirmed by PCR and sequencing (Figure 2).

This PCR strategy also allowed us to determine whether two targeting events occurred on the same chromosome 16, or two different 16s in sequentially targeted ES clones. Overall, we obtained two ES clones with loxP sites on the same chromosome 16 and one ES clone targeted to two different chromosome 16s. We have generated germline-transmitted mice from these ES clones and are crossing them with PB-Cre4 mice to achieve prostate-specific recombination between the Erg and Tmprss2 loci. TMPRSS2-ERG fusion with interstitial deletion is the most common gene fusion subtype identified in prostate cancer patients and more importantly, a recent study found in metastatic prostate tumors, if they carry the TMPRSS2-ERG fusion, it is always the subtype with deletion (22). This suggests haploinsufficiency of gene(s) deleted between Tmprss2 and ERG may play a role in promoting metastasis. The mice (PB-Cre4; Tmprss2-loxP-3Mb-loxP-Erg) we generated with prostate-specific excision of the equivalent region between Tmprss2 and Erg on mouse chromosome 16 would allow us to study this (Figure 2).

Since overexpression of ERG alone is not sufficient to initiate prostate cancer development, we also started to test a potential synergy between Tmprss2-ERG or ETV1 fusion models and other mouse models of prostate cancer. We acquired Pten conditional knockout mice (23) and have started to breed this strain to our Tmprss2-ERG knockin mice. Both the Pten conditional allele and the Tmprss2-ERG conditional allele would be activated by breeding in the prostate-specific PB-Cre4 transgene. This ongoing experiment would eventually allow us to test if ERG can cooperate with Pten deficiency for driving prostate cancer development.

In summary, in this specific aim, we have created knockin mouse models to model gene fusions found in >50% of human prostate cancer cases as we initially designed. Initial characterization of one of these models (ERG cDNA knockin model) suggests ectopic expression of ERG in prostate alone is not sufficient to initiate prostate tumorigenesis, additional mutation(s) (e.g., Pten loss) may be needed to cooperate with ERG. Nevertheless, these models may serve as invaluable tools to dissect contributions of various genetic changes (e.g., ERG overexpression, Tmprss2 loss, or haploinsufficiency of genes deleted between Tmprss2 and ERG) to the development of prostate cancer with gene fusions in human.

**Specific aim 2: Explore roles of the ETS family transcription factor ERG in development and in normal prostate by making a conditional knockout allele of this gene in mouse.**

As mentioned above, we generated an Erg knockdown allele (Erg$^{kd}$) in mice by placing a transcriptional stopper cassette into the Erg locus. Mice homozygous for this knockdown allele died before E11.5 during embryonic
development. At E10.5, homozygous embryos (Erg<sup>kd/kd</sup>) appeared pale and there was only a trace amount of blood running in major vessels, suggesting a possible endothelial cell defect, or hematopoietic defect, or both (Figure 3). Since the stopper cassette we introduced is flanked by loxP site, Cre-mediated excision of this stopper cassette would allow us to restore Erg expression. We first restored Erg expression in both endothelial and blood cells during development by breeding with Tie2-Cre transgenic mice (24). This led to a complete rescue of the early lethality phenotype. We then restored Erg expression only in blood cells during development by breeding with the hematopoietic specific Vav-Cre transgenic mice. Surprisingly, this also led to rescue of the early lethal phenotype. These experiments suggest that during early development, Erg mainly plays a critical role in hematopoiesis, a finding similar to another recent study using a mutant loss-of-function allele for Erg generated from a forward genetic screen (25). Since both Tie2-Cre and Vav-Cre transgenes rescued the early lethal phenotype and would allow homozygous Erg<sup>kd/kd</sup> mice to survive to adulthood, it would allow us to study the role of Erg in prostate development, since Tie2-Cre or Vav-Cre only restores Erg expression in endothelial and hematopoietic cells, not in prostate epithelial cells.

In addition, we are in the process of making an Erg conditional knockout allele by flanking its last coding exon with loxP sites. This last exon encodes the DNA binding domain of Erg that is critical for its function. For the first targeting vector we generated based on this design, we were not able to get any correctly targeted ES clones after screened several hundred clones, suggesting a genomic region (3' UTR) very difficult to target. We then modified this original targeting vector by extending its 3' homologous arm. After screening another several hundred ES clones, recently we obtained one clone that appeared correctly targeted at the 5' arm as determined by southern blot. Currently we are working on confirming this potential clone by testing its 3' arm by southern blot.

In summary, in this specific aim, we have not been able to create a conditional floxed Erg knockout allele as we initially designed, due to an unexpected technical difficulty (other people had described the same difficulty in generating such allele using a similar approach). However, we were able to use a conditionally resuable Erg knockdown allele to study the role of Erg during early development. Rescued Erg knockdown mice would also allow us to study the role of Erg in adult tissues, especially in prostate.

**Specific aim 3: Identify downstream target genes of ERG or ETV1 in human prostate cancer cell lines carrying these gene arrangements using the ChIP-on-Chip approach.**

To identify new ERG and ETV1 target genes and collaborating factors in prostate development and disease, we used LNCaP and VCaP prostate cancer cell lines and RWPE immortalized normal prostate cell line (26). LNCaP cells overexpress ETV1 under the HNRPA2B1 prostate-specific promoter (14), whereas VCaP cells are characterized by the presence of the TMPRSS2-ERG fusion protein (9). Both prostate cancer cell lines displayed high ERG (VCaP) and ETV1 (LNCaP) levels that increased after androgen stimulation.

Expressing profiling by microarrays has become a standard method to study cancer. To analyze further the role of ERG and ETV1 in the prostate, we have performed siRNA-silencing experiments. Eight and 48 hours after transient transfection with ERG and ETV1 specific siRNA from Dharmacon, LNCaP and VCaP cells were harvested. We hybridized total RNA prepared from androgen stimulated ERG and ETV1 siRNA silenced experiments onto Affymetrix human whole genome expression array U133Plus2.0 in the DFCI Gene Expression Core. From the recently obtained array data, we found ERG knockdown led to alterations in expression of genes for cell cycle & cell adhesion molecules, suggesting ectopic expression of ERG in prostate epithelial cells may alter their cell cycle status as well as their invasiveness.

Moreover, to identify potential target genes of ERG and ETV1 in prostate cancer, we planned to perform genome wide location analysis for mapping the in vivo ERG and ETV1 binding (or target) sites in normal and cancer cell lines. A key requirement for the success of this analysis is a reliable antibody that recognizes the protein of interest specifically in an immunoprecipitation assay. We tested several commercially available antibodies against human ERG and ETV1, and unfortunately they are not good for the process. Thus, to identify ERG and ETV1 binding sites, we utilized a metabolic biotin labeling approach (27, 28), in which the ERG and ETV1 cDNA were engineered to contain an amino-terminal Flag epitope and a short peptide tag that serves as a substrate for in vivo biotinylation. This cDNA was then expressed in RWPE immortalized normal cells and in LNCaP, and VCaP cells (which were isolated from metastatic prostate cancer) previously engineered to ectopically express the *Escherichia coli* biotin ligase BirA. The exceptionally strong interaction between biotin and streptavidin allows for the efficient recovery of biotinylated protein with cross-linked DNA, as in conventional chromatin immunoprecipitation (ChIP) experiments. Recently, we generated LNCaP, VCaP and RWPE cells that contain
the bioERG and bioETV1, joined to biotin ligase BirA by retroviral infection. We also started the initial "bio-ChIP" experiment to test enrichment of several known ERG or ETV1 targets, as a proof of principle. In the near future, material recovered in this "bio-ChIP" method will be hybridized to Affymetrix promoter arrays to identify putative target genes of ERG or ETV1 globally. This approach (bio-ChIP-on-Chip) has been successfully utilized in our lab to identify target genes of pluripotency genes in embryonic stem cells (27, 28). By coupling ChIP-on-Chip information with the transcriptome analysis, we will identify candidate target genes of ERG and ETV1, which will be then validated by RT-PCR and ChIP assays. In addition, data comparing prostate normal and cancer cells will allow us to identify genetic/epigenetic changes involved in cancer initiation and progression.

Key research accomplishments:

- Successfully generated Tmprss2-ERG and ETV1 knockin mice expressing truncated human ERG and ETV1 from the endogenous mouse Tmprss2 locus.
- Successfully generated another mouse model for TMPRSS2-ERG fusion with both Tmprss2 and Erg loci tagged by loxP sites.
- In vivo study suggests ectopic expression of ERG in mouse prostates alone is insufficient to initiate prostate cancer development.
- Generated a conditionally rescuable Erg knockdown allele in mice that would allow us to study roles of Erg during embryonic development and in prostate.
- Characterization of the above Erg knockdown allele suggests Erg plays a critical role in hematopoiesis during development.
- Analysis in prostate cancer cell lines suggests Erg knockdown led to alterations in expression of genes for cell cycle & cell adhesion molecules
- Generated Flag-tagged biotinylated ERG or ETV1 (bioERG and bioETV1) for biochemical studies.

Reportable outcomes:

2008 Presentation at the Dana Faber / Harvard Cancer Center Prostate SPORE program meeting: “Probing the mechanism of pathogenesis in prostate cancer with TMPRSS2-ERG gene rearrangement using preclinical mouse models”.

Animal models: Tmprss2-ERG knockin model, Tmprss2-loxP-3Mb-loxP-Erg sequential targeting model, Erg knockdown model.

Cell lines: LNCaP-BirA, LNCaP-BirA/bioETV1, RWPE-BirA, RWPE-BirA/bioERG, RWPE-BirA/bioETV1.

List of personnel (not salaries) receiving pay from the research effort: Esther Baena, PhD; Zhe Li, PhD.

Conclusion:

We planned to use both a series of mouse models and biochemical approaches to study the mechanisms of pathogenesis in human prostate cancer with TMPRSS2-ERG (or ETV1) gene fusions. During the two-year award period, we have made significant progress, including successfully generating the conditional knockin mice carrying the Tmprss2-ERG (or ETV1) fusion allele, as well as those carrying the Tmprss2-loxP-3Mb-loxP-Erg allele through sequential targeting; establishing that ectopic expression of ERG alone in prostate is not sufficient
to initiate prostate cancer; generation and initial characterization of an Erg knockdown allele; establishing the
critical role of Erg in blood cell development; establishing the role of ERG in controlling cell cycle and cell
adhesion molecules; and establishment of a biotinylation system for both ERG and ETV1 in human prostate
cancer cell lines for biochemical studies. Further studies using these animal and cell culture models would allow
us to develop preclinical animal models, as well as to identify and validate novel therapeutic targets, for treating
prostate cancer.

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Appendices:

None
Supporting Data:

Figure 1. Expression of the *Tmprss2-ERG* fusion transcript from the Stopper-excised *Tmprss2-ERG* knockin allele (thus activated) determined by RT-PCR. PCR primers used to detect the endogenous *Tmprss2* transcript and the *Tmprss2-ERG* fusion transcript are shown in the schematic drawing (red arrows). PCR primers used to detect the endogenous mouse *Erg* are located in exons further downstream (not shown).
Figure 2. The upper panel shows the schematic diagram of the Erg and Tmprss2 loci tagged by loxP sites on the same mouse chromosome 16 (Tmprss2-loxP-3Mb-loxP-Erg). Cre-mediated recombination deletes the ~3Mb region between the first loxP site at the Tmprss2 locus and the single loxP at the Erg locus. Forward and reverse primers designed to amplify the region surrounding the single remaining loxP site after excision are indicated by red arrows. The lower left panel shows PCR results using these two primers for three ES clones (#74, 84, 87) from sequential targeting, after transient transfection with a Cre-expressing plasmid. T: control DNA without Cre. The lower right panel shows the sequencing result for the PCR band showed on the left panel confirming correct recombination between the Tmprss2 and Erg loci.
Figure 3. Erg knockdown allele. Mouse embryos homozygous for this Erg knockdown (kd) allele die before E11.5, possibly due to a hematopoietic defect, or vascular defect, or both. Dramatic reduction of Erg expression was detected by RT-PCR in hematopoietic cells derived from E9.5 yolk sac progenitors. Erg expression can be restored by Cre-mediated excision of the Neo-Stopper (ST) cassette.