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PRINCIPAL INVESTIGATOR: David Takeda

CONTRACTING ORGANIZATION: Dana-Farber Cancer Institute
Boston, MA 02215-5450

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14. ABSTRACT TMPRSS2-ERG is an oncogenic translocation present in approximately half of all prostate cancers. Despite being an attractive therapeutic target, transcription factors have historically been difficult to target pharmacologically. Furthermore, the exact role of ERG in mediating tumorigenesis is unknown making it difficult to develop physiologically relevant assays to measure its activity. To address these challenges, we developed a gene expression signature for ERG activity that provides a readout for ERG activity. Using a novel bead based assay to measure gene expression in a high throughput format, we used the ERG gene signature to screen a RNAi kinome library to identify kinases that modulate ERG activity. To identify novel small molecules that directly bind to and inhibit ERG, we tested 100,000 compounds using small molecule microarrays. The identified compounds were subsequently tested using our gene signature assay to discover novel compounds that inhibit ERG activity. Using our high throughput gene expression screening method, we also screened a library of commercially available and FDA approved drugs and discovered that PKC inhibitors inhibit AR mediated ERG transcription.					
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INTRODUCTION

Approximately half of all prostate cancers harbor a translocation between the transcription factor ERG and the androgen regulated gene TMPRSS2. As a result, ERG is expressed at high levels in the prostate where it is not normally expressed. Several mouse models indicate a causal role in the development of prostate cancer, however, the exact role of TMPRSS2-ERG in tumorigenesis is unclear, making it difficult to design assays to target its function therapeutically. In addition, transcription factors have been historically difficult to target pharmacologically with small molecules. To address these problems, we used a gene expression signature as a readout of ERG activity allowing us to measure activity even without a detailed mechanistic understanding of ERG mediated oncogenesis. We used a novel method to measure gene expression in a high throughput format to screen shRNAs and small molecules in prostate cancer cells for perturbations that would modulate the ERG signature. These results will provide new insights into ERG function and as well as potential leads for therapeutic intervention.

KEYWORDS

Prostate cancer, ERG, gene expression, high throughput screening, small molecule microarray, genetic screen, VCAP

ACCOMPLISHMENTS

What were the major goals of the project?

Task 1. Genetic approach to identify kinases essential for TMPRSS2-ERG activity in prostate cancer cells (months 1-28)

- 1a. Generate and titer lentiviruses expressing shRNAs targeting candidate kinases identified from preliminary results for retesting in secondary assays (months 1-3 – completed December 2013)
- 1b. Measure effect of suppressing candidate kinases on proliferation of cell lines that are positive or negative for TMPRSS2-ERG using viability and BrdU incorporation assays (months 4-9 – completed June 2014)
- 1c. Measure effect of suppressing candidate kinases on transcription of ERG and ERG targets by quantitative PCR (months 10-12 – completed October 2104)
- 1d. For kinases that affect either proliferation or transcription from 1b and 1c, measure effect on invasion using transwell invasion assay, epithelial to mesenchymal transitions (EMT) using immunofluorescence and immunoblotting, and transformation by anchorage independent growth in soft agar (months 13-24 – 75% completed)
- 1e. Bioinformatic analysis of results correlating gene expression changes with different functional outputs critical for ERG function using data mining techniques such as hierarchical, k-means, and consensus clustering (months 25-28 – completed June 2016)

Task 2. Test small molecule inhibitors that target candidate kinases identified from genome wide kinase suppression screen (months 1-18)

- 2a. Identify and collect compounds that inhibit candidate kinases identified from genome wide kinome screen (month 1 – completed October 2013)
- 2b. Measure effect of compounds on proliferation of cell lines that are positive or negative for TMPRSS2-ERG using viability and BrdU incorporation assays (months 2-4 – completed January 2014)

- 2c. Measure effect of compounds on transcription of ERG and ERG targets by quantitative PCR (months 5-7 – completed April 2014)
- 2d. For compounds that affect either proliferation or transcription from 2b and 2c, measure effect on invasion using transwell invasion assay, EMT using immunofluorescence and immunoblotting, and transformation by anchorage independent growth in soft agar (months 8-18 – 75% completed)

Task 3. Identify compounds that bind to ERG using small molecule microarrays (SMM) with lysates overexpressing ERG (months 1-12)

- 3a. Request compounds from compound management that scored from preliminary SMM screen using 293T lysates overexpressing ERG (months 1-2 – completed November 2013)
- 3b. Reprint slides with compounds from 3a for counter SMM assays (months 3-4 – completed December 2013)
- 3c. Perform counter SMM assay with 293T lysates expressing unrelated protein (months 5-10 – completed May 2014)
- 3d. Request compounds from compound management that pass counter assays from 3c for secondary assays (months 11-12 – completed June 2014)

Task 4. Identify compounds that bind to ERG using small molecule microarrays with purified ERG protein (months 1-24)

- 4a. Express and purify functional ERG protein from mammalian cells for SMM assay using immunoaffinity tag (months 1-6 – completed March 2014)
- 4b. Perform SMM screen of 84,000 compounds using purified ERG protein (months 7-12 – completed June 2014)
- 4c. Request compounds from compound management that scored from 4b (months 13-14 – completed July 2014)
- 4d. Reprint slides from 4c for counter SMM assays (months 15-16 – completed September 2014)
- 4e. Perform counter SMM assays with unrelated protein and antibody control (months 17-22 – completed December 2014)

- 4f. Request compounds from compound management that pass counter assays from 4e for secondary assays (months 23-24 – February 2015)

Task 5. Measure gene expression signature of candidate SMM compounds (months 25-30)

- 5a. Perform L1000 gene signature assay on compounds identified from Task 3 and Task 4 at concentrations of 10 uM and 30 uM (months 25-27 – completed June 2015)
- 5b. Perform dose curves with L1000 on compounds that modulate the gene signature from 5a (months 28-30 – completed August 2015)

Task 6. Functional assays with compounds that bind to ERG and modulate gene signature (months 31-48)

- 6a. Perform cytotoxicity assay to eliminate nonspecific toxic drugs (months 31- 32 – completed August 2015)
- 6b. Perform proliferation assays in cell lines that are positive or negative for TMPRSS2-ERG (months 33-35 – completed August 2015)
- 6c. Perform ERG dependent differentiation assays using high throughput flow cytometry (months 36-38, 25% completed)
- 6d. Measure transcription of ERG targets by quantitative PCR (months 39-42, 75% completed)
- 6e. For compounds that affect either proliferation, differentiation, or transcription from 6a, 6b, or 6c, measure invasion using transwell invasion assay, EMT using immunofluorescence and immunoblotting, and transformation by anchorage independent growth in soft agar (months 43-48, 25% completed)

Task 7. Biophysical assays with compounds that bind to ERG and modulate gene signature (months 25-42)

- 7a. Scale up protein production and purification for biophysical assays (months 25-30, completed June 2015)

- 7b. Assay development for thermal shift assay with purified ERG protein (months 31-36, completed July 2016)
- 7b. Assay development for surface Plasmon resonance with purified ERG protein (months 31-36, completed July 2016)
- 7c. Perform thermal shift and surface Plasmon resonance on compounds and determine binding constants (months 37-42, completed July 2016)

Task 8. Identify FDA approved drugs that modulate TMPRSS2-ERG gene signature (months 1-24)

- 8a. Perform L1000 gene expression assay on panel of 1800 FDA approved drugs in ERG positive or ERG negative cell lines (months 1-6, completed December 2013)
- 8b. Rearray drugs from 8a and perform dose curve with L1000 gene expression assay in cell lines that are positive or negative for ERG to identify drugs that inhibit ERG activity (months 7-12, completed June 2014)
- 8c. Measure effect of drugs from 8b on proliferation of cell lines that are positive or negative for TMPRSS2-ERG using viability assay and BrdU incorporation (months 13-15, completed June 2014)
- 8d. Measure effect of drugs from 8b on transcription of ERG targets by quantitative PCR (months 16-18, completed December 2014)
- 8e. For compounds that affect either proliferation or transcription from 8c or 8d, measure effect on invasion using transwell invasion assay, EMT using immunofluorescence and immunoblotting, and transformation by anchorage independent growth in soft agar (months 19-24, 75% completed)

What was accomplished under these goals?

Specific Aim 1 - Genetic approach to identify kinases essential for TMPRSS2-ERG activity in prostate cancer cells.

1) Major activities:

We continued to validate the leads that were identified from the high throughput gene expression screen of the shRNA kinome library, using physiologic cell based assays of ERG activity. We made lentivirus expressing multiple shRNAs targeting each candidate

kinase and transduced VCAP cells. We developed assays to measure ERG dependent invasion and anchorage independent growth. We then tested the effect of suppression of the candidate kinases using our cellular assays.

2) Specific objectives

- Complete cell-based assays with candidate kinases
- Determine mechanism of inhibition following suppression of validated kinases

3) Results and conclusions/other achievements

As discussed in previous progress reports, the ERG gene expression signature was used to screen the TMPRSS2-ERG positive VCAP prostate cell line using a high throughput bead based platform referred to as L1000. We screened a library of shRNAs targeting 808 kinases and identified 34 kinases for further studies. The first validation was to measure the expression of two genes known to be regulated by ERG in VCAP cells, ARGHDIB and PLA1A, following suppression of each kinase individually. Using this criterion, we confirmed 12/34 kinases as modulating ERG signaling.

It has been shown previously that ERG is involved in mediating invasion *in vitro* as measured using transwell invasion assay, which measures cells' ability to invade through a matrigel membrane. In order to develop a robust measure of invasion, we ectopically expressed ERG under a tetracycline inducible promoter in PC3 cells, which do not normally express ERG. We achieved tight control of ERG expression using this method (Figure 1B). As a control we also generated a cell line expressing ERG with a mutation that prevents DNA binding. Cells were transferred to the upper chamber of the transwell plate and doxycycline added at different concentrations. After 48 hours, the cells on top above the membrane were removed with a Q-tip and the invading cells under the membrane were fixed, stained with crystal violet and images taken under a dissecting microscope. The amount of invading cells was then quantified by solubilizing the crystal violet with acetic acid and measuring absorbance at 590 nm. Using this model, we could demonstrate invasion that was dependent on the level of ERG protein (Figure 1).

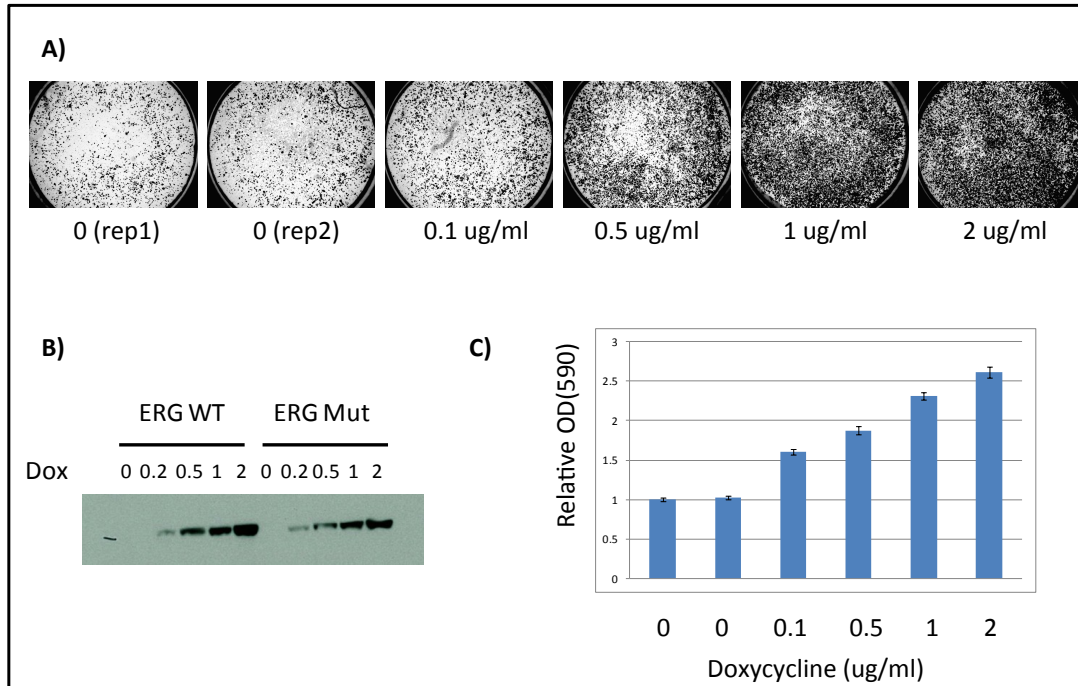


Figure 1: Transwell invasion assay to measure ERG dependent invasion. A) Images taken of invading cells fixed and stained by crystal violet after induction with indicated concentrations of doxycycline for 48 hours. B) Immunoblot showing levels of ERG protein after induction by doxycycline. C) Quantification of crystal violet staining.

Cells were transduced with lentivirus expressing shRNA targeting the candidate kinases and 72 hours after selection, cells were plated in transwell plates and doxycycline was added at 1 ug/ml based on the results shown in Figure 1, and invasion determined 48 hours later. We discovered that 3 kinases showed significant inhibition of ERG mediated invasion by the transwell invasion (Figure 2).

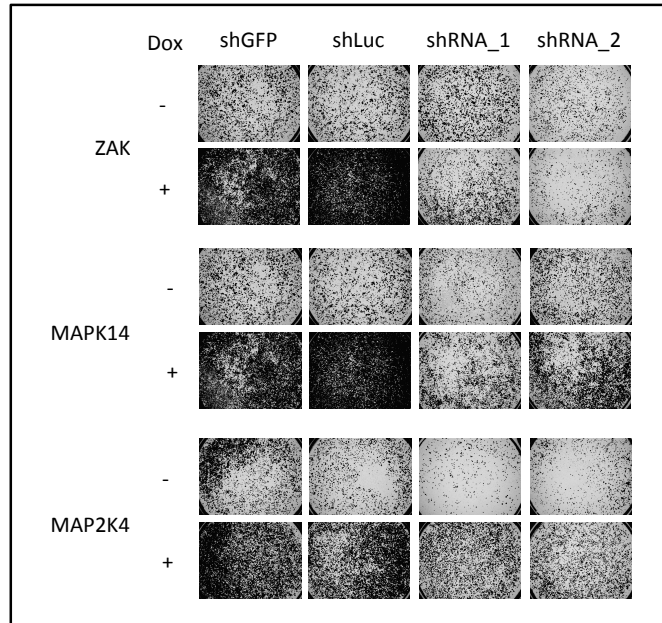


Figure 2: Suppression of ZAK, MAPK14, or MAP2K4 inhibits ERG mediated invasion. PC3 cells expressing tet-inducible ERG were transduced with lentivirus expressing the two different shRNAs targeting the indicated kinases or two control shRNAs (shGFP and shLuc). Invasion was measured by transwell assay in the absence or presence of doxycycline. Cells were harvested 48 hours after induction.

In order to determine the effect of candidate kinases on ERG oncogenic activity, we developed a soft agar assay to measure ERG dependent anchorage independent growth. We used a nontransformed prostate epithelial cell line LHSAR, which is immortalized with human telomerase and SV40 large T antigen. It is also engineered to express AR ectopically and forms tumors in nude mice in an androgen dependent manner when transduced with oncogenic factors such as Ras and SV40 small T antigen. Similar to the PC3 cell line we used for the invasion assay, we transduced LHSAR cells with lentivirus expressing tetracycline inducible ERG. After addition of doxycycline, cells are plated in soft agar in the presence of the synthetic androgen R1881. Figure 3 shows anchorage independent colony formation dependent on doxycycline (ERG induction) and R1881. We are currently using this assay to identify kinases required for transforming activity of ERG.

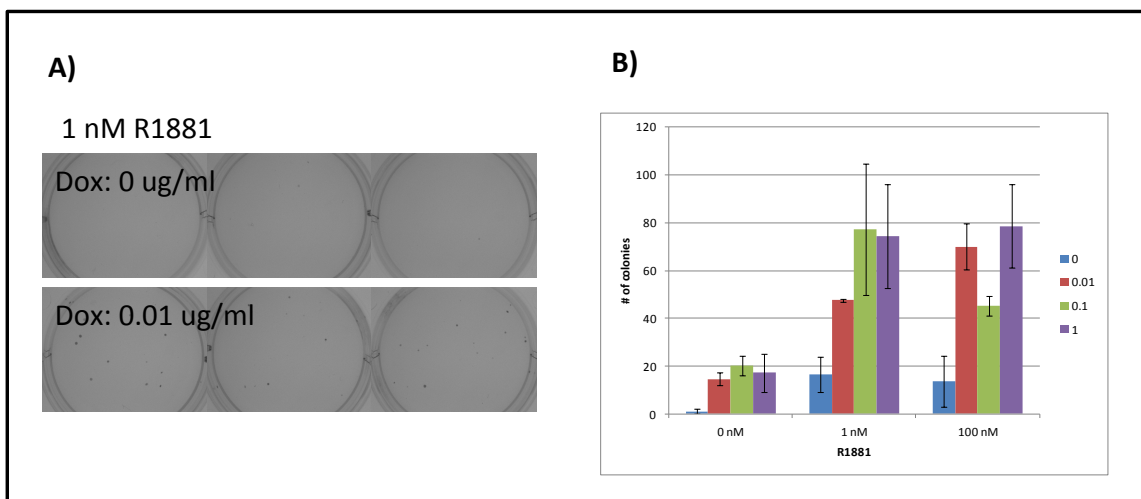


Figure 3: Soft agar assay to measure ERG dependent anchorage independent growth. A) Representative soft agar images of LHSAR cells stably expressing inducible ERG treated with indicated concentrations of doxycycline and 1 nM of R1881. B) Results of soft agar assay at different concentrations of doxycycline and R1881.

Specific Aim 2 - Chemical approach to identify small molecules that directly bind to and inhibit TMPRSS2-ERG activity.

1) Major activities

Based on our small molecule screen as described in the previous progress report, we identified several compounds for follow up. We identified one compound that showed significant activity against ERG transcription and proliferation of prostate cancer cells. We demonstrate that the mechanism appears to be through AR dependent proliferation. We are currently doing gene expression and pulldown experiments to elucidate the mechanism of action.

2) Specific objectives

- Target confirmation of small molecule
- Determine mechanism of action of novel small molecule

3) Results and conclusions/achievements

The compound identified as DOS16 was found to inhibit the ERG signature in a dose dependent manner as measured by the L1000 gene expression assay. We confirmed the ability of DOS16 to inhibit transcription of ERG targets by QPCR. Figure 4 shows dose dependent inhibition of two ERG targets ARGHDIB and PLA1 by DOS16. Interestingly, we also found that transcription of ERG itself was also inhibited by DOS16 suggesting that it may inhibit AR dependent transcription of ERG. To confirm inhibition of AR activity, we also measured KLK3 and FKBP5, which are two known targets of AR. Transcription of both KLK3 and FKBP5 were also inhibited by DOS16 in a dose dependent manner suggesting that DOS16 inhibits the activity of AR.

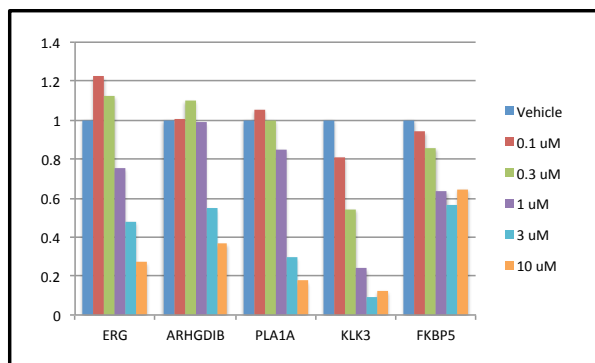


Figure 4: DOS16 inhibits AR dependent transcription of ERG. VCAP cells were treated with indicated concentrations of DOS16 and transcription measured by QPCR.

In order to determine the effect on proliferation, we treated cancer cells with different concentrations of DOS16 and measured viability by Cell Titer Glo. We discovered that the AR positive prostate cancer cells, VCAP and LNCAP, were sensitive to DOS16 with an IC50 in the high nM to low uM range. Interestingly, there was no effect on the proliferation of the AR negative prostate cancer cell lines DU145 and PC3 supporting the hypothesis that the compound specifically targets AR. In addition, DOS16 did not have any effect on the proliferation of the bladder cancer cell line RT114.

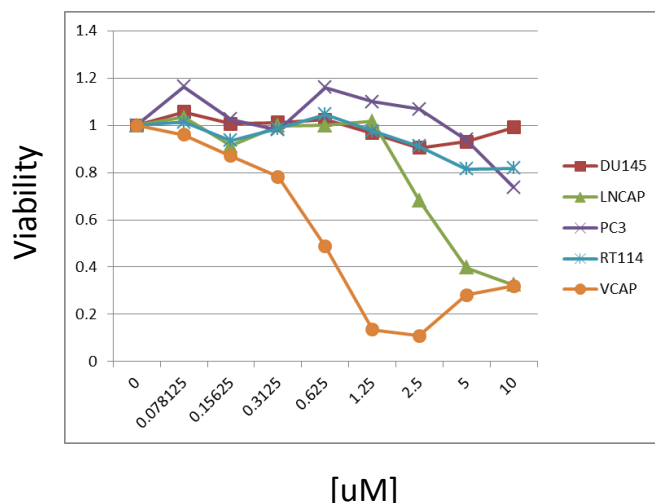


Figure 5: DOS16 inhibits proliferation of AR dependent prostate cancer cell lines. Cancer cell lines were treated with the indicated concentrations of DOS16 and proliferation measured 5 days later using cell titer glo.

The chemical structure of DOS16 contains three stereocenters. Therefore to test whether the activity of DOS16 shows any stereospecificity, we measured transcription of ERG and AR targets following treatment of all 8 possible stereoisomers. (Figure 6).

Interestingly, only two of the stereoisomers showed activity (SSS and SSR). Both active isomers contained the S stereocenter at the first positions. The stereospecificity of DOS16 suggests that it may bind a specific cellular target.

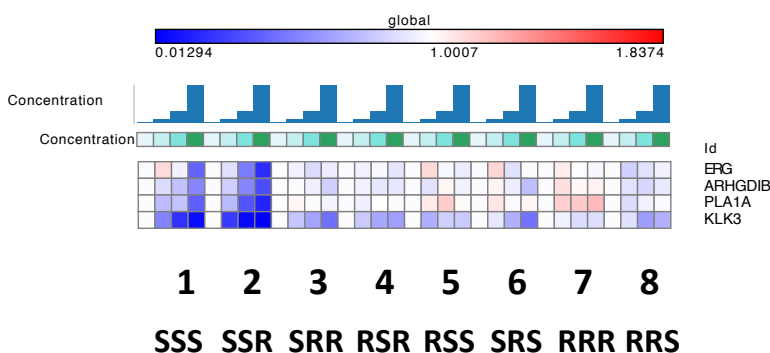


Figure 6: DOS16 shows stereospecific activity. VCAP cells were treated with eight possible stereoisomers of DOS16 at multiple concentrations and transcription measured by QPCR.

We also tested analogs of DOS16 with different substituents that were taken from the DOS library (Figure 7). We found that substitution of the R1 position with any of the substituents tested resulted in loss of activity. However, substitution at the R2 position had no effect on activity. These results suggest that the R1 substituent is critical for activity.

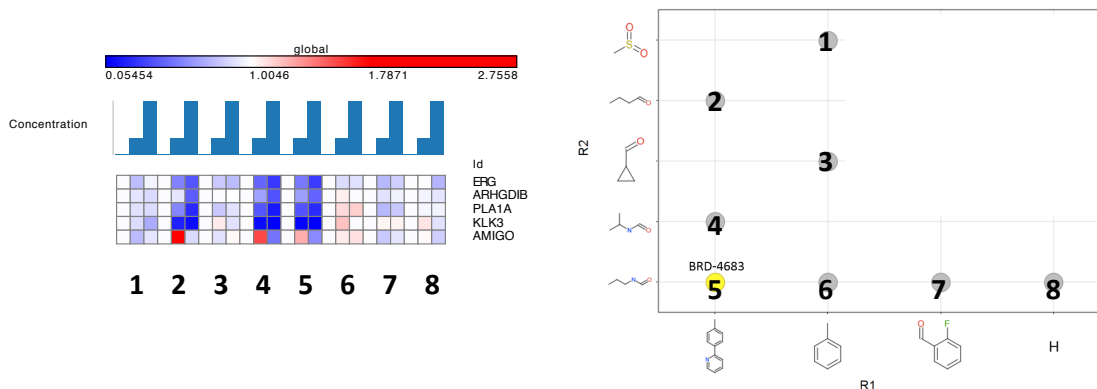


Figure 7: R1 substituent is important for activity of DOS16 . VCAP cells were treated with DOS16 analogs and transcription of ERG and AR targets measured by QPCR.

We are currently attempting to confirm the target of DOS16 by conjugating the compound to agarose beads to do pulldown experiments. Since the R2 position appears to be dispensable for activity (Figure 7), we are using the R2 position to attach a chemical linker for subsequent conjugation to beads. In addition we are doing gene expression studies using DOS16 as a complimentary approach to determine the mechanism of action

Specific Aim 3. Repurpose FDA approved drugs that inhibit TMPRSS2-ERG activity.

1) Major Activities

We previously identified PKCi as modulating the ERG signature and validated their effect using cell based assays. We have focused on determining the mechanism of by

which PCKi inhibits ERG activity using gene expression profiling and chromatin immunoprecipitation followed by next generation sequencing (ChIP-seq).

2) Specific objectives

- Determine the mechanism by which PKC inhibitors (PKCi's) inhibit ERG activity
- Identify AR cofactors that are affected by PKCi treatment

3) Results and conclusions/achievements

As discussed in the last progress report, we discovered that treatment with several PKC inhibitors modulated ERG activity. We confirmed that the PKC inhibitors identified inhibit transcription of ERG in VCAP cells and decreased proliferation of VCAP cells versus an ERG negative cell line. We went on to demonstrate that PKCi inhibited the upregulation of a subset of AR regulated genes including TMPRSS2.

To determine which subset of genes are regulated by PKCi, we performed global gene expression profiling by Affymetrix microarrays in LNCAP prostate cancer cells. As a positive control for AR regulated genes we used the AR antagonist MDV3100. Figure 8 shows that as expected MDV3100 reverses the effect of R1881 (compare columns 2 and 3). Interestingly, PKCi inhibited a subset of androgen induced genes (column 4). Furthermore PKCi treatment had no effect on androgen suppressed genes.

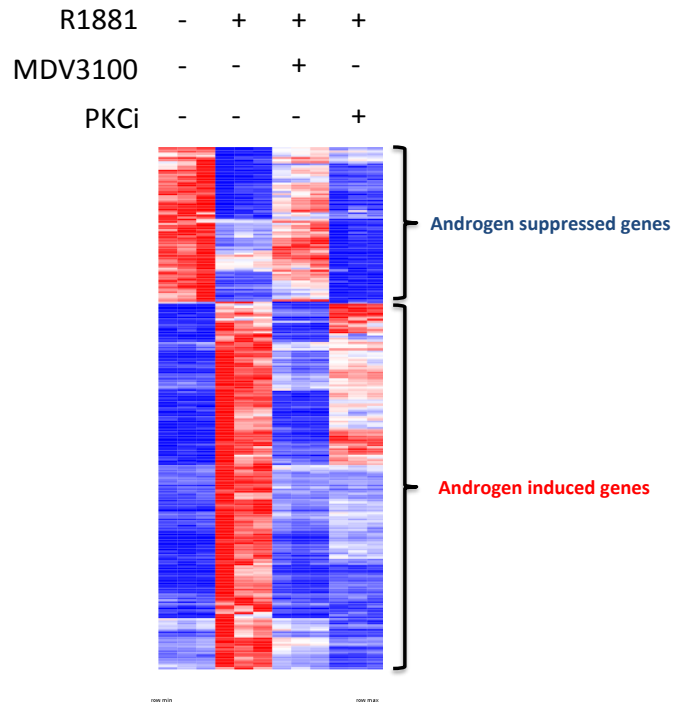
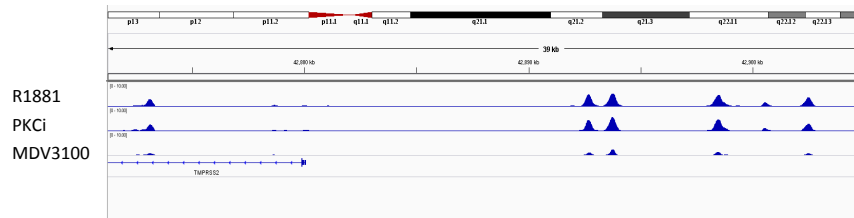


Figure 8: PKCi inhibits a subset of androgen induced genes. LNCAP cells were treated with the indicated compounds and expression profile measured by Affymetrix microarray.

In order to further elucidate the mechanism of PKC inhibition on AR activity, we performed AR chromatin immunoprecipitation followed by next generation sequencing (ChIP-seq) to determine if AR binding is affected by PKC inhibition. We discovered that PKCi had no effect on genomewide binding by AR (Figure 9). AR is still able to bind to chromatin suggesting that the mechanism of PKCi occurs after AR binding to chromatin.

TMPRSS2 Enhancer



PSA Enhancer

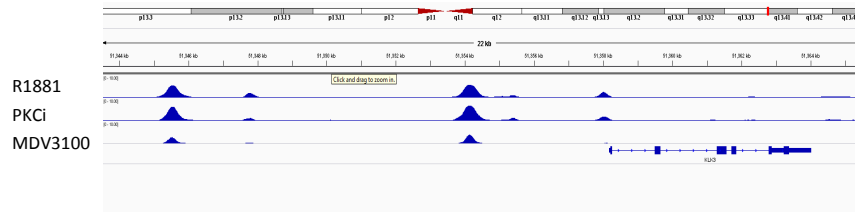


Figure 9: Treatment with PKCi does not affect AR binding. ChIP-seq was performed after treatment with PKCi. Treatment with MDV3100 was used as a positive control. Example tracks at well characterized AR binding loci showing no effect on AR binding peaks with PKCi treatment.

These results suggest that PKCi inhibits the action of a cofactor of AR that is recruited to chromatin after AR binding. Efforts are ongoing to determine the identification of this cofactor by treating cells with PKCi and measuring changes in AR complexes.

What opportunities for training and professional development has the project provided?

I continued to maintain a clinic one morning a week seeing prostate cancer patients. By being involved in taking care of patients, enrolling patients on clinical trials, and participating in our department's protocol development meetings, I have further enhanced my understanding of translational medicine. Interacting with clinicians will be important moving forward in understanding how to translate my results in the laboratory to meaningful treatments for patients.

During this past year I had the opportunity to present my preliminary results at the Broad Institute's weekly cancer meeting where unpublished data are presented in an

informal lab meeting discussion oriented format. I was able to present to a diverse group of scientists in the Broad cancer program and received critical feedback and evaluation.

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

Specific Aim 1 - Genetic approach to identify kinases essential for TMPRSS2-ERG activity in prostate cancer cells.

We will complete the cellular assays, in particular anchorage independent growth, to validate the candidate kinases that modulate the ERG signature. In addition we will determine the mechanism by which these kinases exert their effects on ERG activity.

Specific Aim 2 - Chemical approach to identify small molecules that directly bind to and inhibit TMPRSS2-ERG activity.

We will couple the hit compound to agarose beads to perform pulldown experiments to identify the molecular target and provide insight into the mechanism of action.

Specific Aim 3. Repurpose FDA approved drugs that inhibit TMPRSS2-ERG activity.

We will identify cofactors of AR that are inhibited by treatment with PKCi to determine the mechanism of PKC inhibition.

IMPACT

What was the impact on the development of the principal discipline(s) of the project?

About half of all prostate cancers are known to harbor a genetic mutation that fuses a gene known as ERG to the regulatory region of the gene TMPRSS2. The TMPRSS2-ERG fusion results in ERG becoming aberrantly activated in prostate cells, which contributes to the development of cancer. However, despite being an attractive and logical therapeutic target, there are currently no drugs that target ERG activity. ERG belongs to a group of proteins known as transcription factors, which have been historically difficult for drug development because they lack the well characterized active sites of enzymes in which to fit small molecule inhibitors. To address these challenges, we developed a method to measure gene expression patterns in a high throughput format and generated a gene “signature” that differentiates between cells that have active TMPRSS2-ERG activity versus cells in which its activity is suppressed. By using a gene signature as a surrogate for biological activity, we have developed an accurate readout for TMPRSS2-ERG activity. Using this method, we can then use novel techniques in genetics and chemistry to target TMPRSS2-ERG in prostate cancer.

We inhibited the activity of 800 kinases, molecules commonly involved in signaling pathways, and measured its effect on our ERG signature. Unlike transcription factors, kinases are more amenable to drug development as they have well defined activities and active sites. We identified kinases that regulate ERG activity and show using cellular assays kinases that they inhibit the ability of ERG to cause invasion of prostate cancer cells. We are currently determining the role of these kinases in other cellular assays important for ERG activity

To identify novel small molecules that directly bind to and inhibit ERG activity, we screened a small molecule library of novel compounds using our high-throughput gene expression method. We identified a novel compound which appears to function by inhibiting AR dependent ERG transcription. Consistent with a role in AR regulation, the compound selectively inhibits the proliferation of AR dependent prostate cancer cells. Interestingly, the compound shows structural stereospecificity suggesting interaction with a specific cellular target. Ongoing efforts are aimed at determining the molecular target

of this novel compound, which could serve as a lead for the development of a novel class of drugs.

The process of transitioning a drug from the laboratory to FDA approval is a long and costly process typically taking years and costing an estimated \$1.8 billion per drug. Therefore there has been great interest in repurposing approved drugs for new indications. We have assembled and tested a panel of FDA approved drugs in our gene signature assay and identified multiple drugs that can modulate the ERG signature. We discovered that PKC inhibitors (PKCi), which are commercially available and have been used in clinical trials modulates the ERG signature. Using global gene expression profiling, we find that PKCi appear to inhibit a subset of AR upregulated genes including TMPRSS2. Using chromatin immunoprecipitation followed by next generation sequencing (ChIP-seq) we found that AR binding is unaffected. Current efforts are aimed at determining the mechanism by which PKCi inhibit AR mediated transcription.

What was the impact on other disciplines?

Our method is potentially generalizable to the study of any molecular process that can be characterized by a gene expression signature. The signature can be applied to identify either genes or drugs that modify a particular phenotype. These types of functional genomic studies will be important for determining the clinical relevance of the genetic alterations discovered by genomic sequencing studies.

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report.

Changes that had a significant impact on expenditures

Nothing to report.

**Significant changes in use or care of human subjects, vertebrate animals,
biohazards, and/or select agents**

Nothing to report.

PRODUCTS

Publications, conference papers, and presentations

Nothing to report.

Journal publications

Nothing to report.

Books or other non-periodical, one-time publications

Nothing to report.

Other publications, conference papers, and presentations.

Nothing to report.

Websites or other internet sites

Nothing to report

Technologies or techniques

Nothing to report

Inventions, patent applications, and/or licenses

Nothing to report

Other products

Nothing to report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	<i>David Takeda</i>
Project Role:	<i>PI</i>
Researcher Identifier (e.g. ORCID ID):	<i>PI</i>
Nearest person month worked:	<i>12</i>
Contribution to Project:	<i>Performed experimental work and data analysis.</i>
Funding Support:	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Organization Name: Broad Institute

Location of Organization: Cambridge, MA

Partner's contribution to the project (identify one or more)

- Facilities: RNAi screening libraries, chemical screening libraries, screening platform
- Data analysis: Computational support with data analysis, chemical biology with assistance in medicinal chemistry

SPECIAL REPORTING REQUIREMENTS

Collaborative Awards:

Nothing to report

Quad Charts:

Nothing to report

APPENDICES

Nothing to report