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TITLE: Targeting TMPRSS2-ERG in Prostate Cancer

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Targeting TMPRSS2-ERG in Prostate Cancer

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. We applied this technique to screen genetic and chemical libraries to study ERG mediated tumorigenesis and identify novel therapeutic agents targeting ERG activity.

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

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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. The TMPRSS2-ERG fusion results in ERG being expressed at high levels in the prostate where it is not normally expressed. Several lines of evidence demonstrate a causal role for TMPRSS2-ERG in tumorigenesis making it an attractive therapeutic target, however, the mechanism remains unclear making it difficult to target its function. In this project we used a gene expression signature readout for TMPRSS2-ERG that allows us to measure ERG activity even without a detailed mechanistic understanding of the molecular pathways involved in ERG mediated oncogenesis. We generated the TMPRSS2-ERG signature using a loss of function prostate cancer cell line model and showed that the signature recapitulates the gene expression profiles observed in fusion positive human tumors. Using a novel high throughput gene expression profiling method, we identified genetic and chemical modulators of ERG activity that will aid in understanding the mechanism of TMPRSS2-ERG mediated tumorigenesis and ultimately target its activity as a treatment for prostate cancer.

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 – 75% completed)

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 – 20% 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 – not started)

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 – 25% 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 – 10% completed)

4f. Request compounds from compound management that pass counter assays from 4e for secondary assays (months 23-24 – not started)
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 – not started)
5b. Perform dose curves with L1000 on compounds that modulate the gene signature from 5a (months 28-30 – not started)

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 – not started)
6b. Perform proliferation assays in cell lines that are positive or negative for TMPRSS2-ERG (months 33-35 – not started)
6c. Perform ERG dependent differentiation assays using high throughput flow cytometry (months 36-38, not started)
6d. Measure transcription of ERG targets by quantitative PCR (months 39-42, not started)
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, not started)

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, 20% completed)
7b. Assay development for thermal shift assay with purified ERG protein (months 31-36, 25% completed)
7b. Assay development for surface Plasmon resonance with purified ERG protein (months 31-36, 25% completed)
7c. Perform thermal shift and surface Plasmon resonance on compounds and determine binding constants (months 37-42 – not started)

**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. Rearrange 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, 75% completed)

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, not started)

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 developed and validated a gene expression signature of ERG activity that could subsequently be adapted to a high throughput format using a novel bead based gene expression method. We used the high throughput gene expression method to screen a shRNA library of 808 kinases to identify kinases that modulate ERG activity.

2) Specific objectives
- Validate gene expression signature for ERG activity
• Screen shRNA kinome library to identify modulators of ERG activity

3) Results and conclusions/other achievements

To define an ERG expression signature, we used the VCaP cell line, which was isolated from a vertebral body metastatic lesion and harbors the TMPRSS2-ERG translocation. We suppressed ERG expression in VCaP cells using lentiviral mediated delivery of shRNAs specific to ERG. To identify differentially regulated genes after ERG suppression, we used a recently developed method for measuring gene expression in a high throughput format referred to as L1000.

Being able to accurately measure ERG activity in a cell line does not necessarily imply that the signature will be relevant to the biology of actual human tumors. For our signature to be useful in ultimately identifying novel therapeutics, our model should recapitulate the biology observed in tumors. Therefore our benchmark for evaluating the performance of the gene signatures is its ability to distinguish TMPRSS2-ERG positive tumors from negative tumors implying that we are capturing physiologically relevant ERG-related pathways. We compared the loss of function ERG signature to a large published tumor dataset of 455 tumors whose TMPRSS2-ERG status was annotated based on fluorescence in situ hybridization. We projected the signatures onto the expression profile of each tumor sample and ranked tumors based on signature enrichment. We find that the ERG signature enriches for TMPRSS2-ERG positive tumors (Figure 1).

![Figure 1: Loss of function signature correctly predicts TMPRSS2-ERG positive tumors.](image)

Single sample GSEA was performed on the Setlur et al. tumor dataset using the ERG loss of function gene signature. Each column represents one tumor sample that is colored
based on fusion status and ordered according to enrichment score from the ssGSEA analysis (bottom row). P-values were calculated using the signal to noise statistic for how well the gene signature differentiates the two classes.

Therefore the gene signature that we derived from VCaP cells after suppression of ERG may represent an accurate model for TMPRSS2-ERG prostate cancers.

Having confidence that our signature models what is observed in human tumors, we used this signature as a readout for ERG activity. We hypothesized that perturbagens that inhibit ERG signaling should recapitulate the ERG suppressed gene signature. We performed a screen in VCaP cells where each kinase in the genome was suppressed by shRNA. VCaP cells were infected with lentiviruses targeting 808 kinases with an average of 8 shRNAs per kinase and gene expression was measured using the L1000 assay (Figure 2).

![Figure 2: Distribution of summed score metric from kinome screen](image)

Summed score metric was calculated for each expression profile and plotted. The negative control wells are shown in red and the positive controls in dark blue. Candidate shRNA are located in the tail of the distribution amongst the positive controls.

Positive hits were called using 5 different metrics referred to as summed score, weighted summed score, naïve bayes, K-nearest neighbor, and support vector machine which have been used previously for gene expression high throughput screening. Based on previous gene expression high throughput screening, true positives tend to score by multiple metrics. Using a cutoff of 2 or more metrics, we identified 34 kinases for further study.
Specific Aim 2 - Chemical approach to identify small molecules that directly bind to and inhibit TMPRSS2-ERG activity.

1) Major activities
To test the small molecules identified from small molecule microarrays, we are developing ERG binding assays such as surface plasmon resonance and thermal shift assays. As these assays require significant amounts of purified protein, a significant amount of effort was dedicated to optimizing the synthesis of purified ERG. The small molecules were also tested for in vivo activity against TMPRSS2-ERG using the high throughput gene expression assay developed in specific aim 1.

2) Specific objectives
• Screen small molecule microarrays for candidate compounds that directly interact with ERG
• Develop direct binding assays with ERG
• Purification of recombinant ERG protein
• Determine effect of candidate small molecules on ERG activity in prostate cancer cells

3) Results and conclusions/achievements
In order to identify direct small molecule inhibitors of ERG we took advantage of the small molecule array (SMM) platform developed at the Broad Institute. Small molecules are covalently printed onto glass slides and the protein of interest is flowed over the slide and allowed to bind to the immobilized small molecules. SMMs allow one to screen against many compounds without needing large quantities of compounds or protein. In addition, the library screened is designed to incorporate analogues allowing one to quickly perform stereochemical structure activity relationship (SSAR) analyses using informatics tools developed at the Broad Institute.

We successfully screened 100K compounds from the Broad Institute’s DOS collection using both cell lysates from 293T cells overexpressing ERG as well as purified ERG protein. We hypothesized that using cell lysates might identify compounds that bind to physiologic ERG complexes, while using purified ERG would primarily identify
direct interactors. We identified 742 compounds in the lysate screen and 897 compounds in the purified ERG screen. There were 182 compounds that scored in both screens, which is more than expected by chance. As a counter screen we used an unrelated transcription factor, which showed minimal overlap between these compounds.

In order to confirm these interactions and measure quantitative binding, we designed biophysical binding assays including thermal shift and surface plasmon resonance. However, these experiments required significant quantities of purified ERG protein. Although the protein we generated from 293T cells was sufficient for SMMs, we needed larger quantities for the planned biophysical assays. Transcription factors such as ERG have historically been difficult to purify as they tend to be unstructured and subject to either inclusion body formation or degradation. After screening several constructs in bacteria, we identified a nearly full length version of ERG that could be expressed at high levels and purity (Figure 3).

![Figure 3: Purification of recombinant ERG protein](image)

His tagged ERG protein was expressed in E. coli and purified using Ni-NTA agarose. Eluted protein was analyzed by SDS-PAGE and Coomassie Blue staining. Left lane shows molecular weight markers and right lane shows purified ERG.

Using this protein we are working to develop both a thermal shift assay and surface plasmon resonance assay that we validated using binding to DNA. We are currently testing all the compounds from the SMMs in these assays to validate binding.
In addition to biophysical assays, we are also testing these compounds in VCaP cells and measuring gene expression using L1000. We hypothesized that compounds that inhibit ERG should also modulate the ERG signature we defined in specific aim 1. We have screened several hundred compounds thus far and have identified compounds that modulate some portion of the ERG signature. We are currently retesting these compounds at dose to measure IC50 as well as in lower throughput cell based assays that measure invasion, proliferation, and transformation. Once these compounds are validated we plan to collaborate with the medicinal chemistry group at the Broad Institute for optimizing chemical structures to increase potency and efficacy. Having both a cell based gene signature assay and biophysical binding assays specific for ERG will expedite the optimization of small molecule inhibitors.

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

1) Major Activities
Using an assembled library of approximately 1800 bioactive drugs, we performed high throughput gene expression screening to identify molecules that modulate ERG activity. As these molecules have previously identified mechanisms of action, we are validating these mechanisms in prostate cancer cells and its role in ERG function.

2) Specific objectives
- Screen bioactive library by high throughput gene expression profiling to find compounds that modulate ERG gene signature
- Validate compounds in vivo in prostate cancer cells lines

3) Results and conclusions/achievements
We used a previously assembled library of approximately 1800 bioactive drugs consisting of commercially available compounds, FDA approved drugs, and drugs in clinical trials, to identify small molecules that inhibit ERG activity. We generated 10,000 profiles of VCaP cells treated with different compounds as measured by the L1000 assay. For this analysis we could not use the same analytics we did for the kinome screen, which
required positive controls to build a classification model, as there is no known small molecule inhibitor of ERG. In addition, we could not use shRNAs targeting ERG as positive controls for technical reasons as cells are typically treated for 24 hours with drug whereas virus infection requires several days. Therefore for this analysis we used our gene signature to calculate connectivity scores. The connectivity score determines the enrichment of a signature in the upregulated and downregulated genes in an expression profile. In addition to a connectivity score, we also calculated a specificity score for each signature. The specificity score identifies and attempts to eliminate compounds that have global effects on gene expression and as a result connect with many different signatures.

After ranking compounds based on connectivity score we discovered that several of the top hits represented inhibitors of protein kinase C (PKC) (Figure 4).

![Figure 4: Small molecules that connect with suppressed TMPRSS2-ERG signature](image)

The connectivity score for 10,841 compounds to the TMPRSS2-ERG signature was determined and ranked. The bar graph in A) represents the ranked list of compound profiles with those that had a significant positive connection in red and those with a significant negative connection in blue. Shown in black are the positions of the PKC inhibitors (PKCi’s) in the ranked list. The names, ranks, and scores of the PKC inhibitors are depicted in B).
Six of the top 15 compounds were different protein kinase C inhibitors. If we locate the instances of all protein kinase C inhibitors in our ranked list, we find all 9 instances had high positive scores. There are nine different protein kinase C isoforms, and these compounds inhibit the different isoforms to different extents. However, the screen was performed at one concentration of compound, 10 μM, which is significantly higher than the IC50s and hence these inhibitors are likely not specific for one isoform at the concentration tested.

While performing validation studies, we discovered that PKC inhibitors decreased levels of ERG protein in VCaP cells (Figure 5).

**Figure 5: PKCi inhibit ERG levels.**
VCAP cells were treated with increasing concentrations of PKC inhibitors (GF109, Enza), nilotinib, or vehicle control for 24 hours. Cell lysates were analyzed by immunoblotting with ERG. Actin was used as a loading control.

In addition PKC inhibitors significantly decreased proliferation of VCaP cells versus an ERG negative cell line (Figure 6). We are currently performing experiments to identify the relevant PKC isoform and determine the mechanism of transcription inhibition.
**Figure 6: PKCi decrease proliferation of ERG positive cell line.**

VCAP cells (ERG+) and PC3 cells (ERG-) were treated with increasing concentrations of the PKC inhibitor enzastaurin. Cell number was determined by counting using a ViCell counter at the indicated time points to generate growth curves.

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

I completed my master’s degree in biomedical informatics at Harvard Medical School. These skills were directly applied to designing and implementing the analytics required for the high throughput gene expression assay that is integral the success of this project. I had the opportunity to learn from and work closely with the computational biology group at the Broad Institute.

I gained clinical experience by joining the genitourinary oncology group where I dedicated 10% of my effort to clinical work. By working alongside clinicians involved in clinical trials, I gained a first had perspective into how science in the laboratory is translated into potential treatments for patients. These interactions also provide opportunities for potential future collaborations such as testing tumor samples from patients for the kinases and pathways identified in my proposal.

**How were the results disseminated to communities of interest?**

These results were presented at the Broad Institute Cancer program meeting that is open to all members of the Broad Community which includes labs from Harvard, Dana Farber Cancer Institute, and Mass General Hospital. The were also presented in poster form at...
the tri-institutional prostate cancer meeting involving Harvard, Johns Hopkins, and University of Michigan.

**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 plan to validate the shRNAs targeting the kinases identified by the kinome screen by QPCR. Confirmed kinases will then me moved to cell based assays in prostate cancer cells.

**Specific Aim 2** - Chemical approach to identify small molecules that directly bind to and inhibit TMPRSS2-ERG activity.
We will confirm that the biophysical assays are able to measuring binding to ERG protein using DNA as a positive control. We will test the compounds identified from the SMM screen in the biophysical assays. To validate compounds we will measure their ability to modulate ERG activity using our gene expression signature assays. Active compounds will then be subjected to stereochemical analyses for optimization, and proteomics to identify the cellular target.

**Specific Aim 3. Repurpose FDA approved drugs that inhibit TMPRSS2-ERG activity.**
We plan to identify the relevant PKC isoforms using genetic manipulation by RNAi. We will measure the activity of PKC inhibitors using cell based assays and perform experiments to identify the role of PKC in ERG activity.
IMPACT

What was the impact on the development of the principle discipline 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 are currently testing the role of these kinases in regulating ERG activity both to understand ERG biology and as leads for potential therapeutics. To identify novel small molecules that directly bind to and inhibit ERG activity, we tested 100,000 compounds using a recently developed technique of drug screening called small molecule microarrays. We are in the process of combining biophysical assays that quantify ERG binding to small molecules, together with our gene expression activity assay to confirm and further optimize these small molecules.

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 are attempting to identify drugs that can modulate the ERG signature, and may serve as potential leads for therapeutic development.

**What was the impact on other disciplines?**

As genomic sequencing studies continue to identify mutated and aberrantly expressed genes, we will require methods to target these genes. Our approach to understanding and targeting the transcription factor ERG is generalizable to any cellular factor in the cell that causes changes in gene expression, allowing one to rapidly elucidate the mechanism of a novel gene, and identify potential therapeutics.

**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?

<table>
<thead>
<tr>
<th>Name:</th>
<th>David Takeda</th>
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<tr>
<td>Project Role:</td>
<td>PI</td>
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<td>Researcher Identifier (e.g. ORCID ID):</td>
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<td>Nearest person month worked:</td>
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<tr>
<td>Contribution to Project:</td>
<td>Performed experimental work and data analysis.</td>
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<td>Funding Support:</td>
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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
SPECIAL REPORTING REQUIREMENTS

Collaborative Awards:
Nothing to report

Quad Charts:
Nothing to report

APPENDICES
Nothing to report