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14. ABSTRACT The research involves laboratory studies utilizing xenograft models to test the hypothesis that targeting a member of the ETS transcription factor family with small molecules such as YK-4-279 may effectively treat prostate cancer. In year three, Dr. Morrissey was to evaluate the anti-tumor effect of the S-enantiomer of YK-4-279 on 4 human prostate xenograft lines subcutaneously and 2 lines intra-tibial. Dr. Uren supplied Dr. Morrissey with the required compound. The S-enantiomer of YK-4-279 had no significant effect on tumor growth on the first and second ERG+ xenograft. It had an effect on the third ERG+ xenograft line, and no effect on the control xenograft line. A gene expression analysis of the first study suggested a change in the expression of epithelial-mesenchymal transition associated genes in ERG treated tumors. However, immunohistochemical analyses of these proteins in the tumors of treated and untreated animals suggested the change in expression only occurred in a subset of cells within the tumors. Dr. Uren has met with and Skyped with Dr. Morrissey to ensure continued collaboration. We are currently completing a study on the effect of the S-enantiomer of YK-4-279 on intra-tibial tumors and have requested a no cost extension to complete the intra-tibial animal studies.									
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Introduction

A group of chromosomal translocations were recently discovered in prostate cancer that fuses the 5' region of *TMPRSS2* (a serine protease) gene to the 3' region of *ETS* transcription factor genes (1). *TMPRSS2* is an androgen responsive gene and contributes only its promoter region and usually a very short exon-1 (2, 3). This causes aberrant expression of an *ETS* transcription factor in response to androgen. The most common *ETS* member involved in prostate cancer chromosomal translocations is *ERG* but other members such as *ETV1*, *ETV4* and *ETV5* have been also observed (4, 5). The more aggressive prostate cancers often contain these translocations, thus potentially increasing their utility as both diagnostic and prognostic marker (6-8). Cell culture and transgenic animal models suggest that increased expression of *ETS* members, as a result of the chromosomal translocations, increase cell invasion without affecting the proliferative potential (9-11). However, in some xenograft models reducing expression of *TMPRSS2-ERG* protein slows down prostate cancer growth (12, 13). Therefore, *ETS* proteins emerge as potential novel targets for treatment of primary and/or metastatic disease in prostate cancer.

We developed small molecule inhibitors that target protein products of chromosomal translocations containing *ETS* transcription factors (14). We further established that our lead compound, YK-4-279, directly binds to both *ERG* and *ETV1* proteins (15). YK-4-279 inhibits *ERG* and *ETV1* mediated transcriptional activity and subsequent cellular invasive phenotype of prostate cancer cell lines. These effects were only observed in prostate cancer cell lines containing *ETS* chromosomal translocations such as VCaP and LNCaP and absent in the PC3 prostate cancer cell line that does not contain any *ETS* chromosomal translocations. Expression of *ERG* in PC3 cells from an expression vector sensitized them to YK-4-279 and inhibiting *ERG* expression in VCaP resulted in resistance to YK-4-279 effect (15). Therefore, we hypothesize that targeting *ETS* family of transcription factors by small molecules will inhibit malignant phenotypes of human prostate cancer cells.

Keywords

Prostate Cancer, *TMPRSS2-ERG*, *ERG*, patient derived xenografts, YK-4-279, Tumor growth, Proliferation, *ERG* Inhibitor, and treatment resistance.

Overall Project Summary

This research involves laboratory studies utilizing xenograft models to test the hypothesis that targeting a member of the *ETS* transcription factor family with small molecules such as YK-4-279 may effectively treat prostate cancers. In year 1 Dr. Uren screened *ETS* transcription factors to test in animal models of prostate cancer at the University of Washington. Dr. Morrissey obtained all approvals to start the animal studies in year 2, and has consistently met and Skyped with Dr. Uren to discuss the fine details of the animal studies. Dr. Uren has screened a panel of derivatives and determined that none of the derivatives were more effective at inhibiting *ERG* than YK-4-279. Therefore we moved ahead with testing YK-4-279 in the xenograft models as proposed. Dr. Morrissey has tested YK-4-279 in 4 patient derived xenograft lines as proposed. One of the *ERG* positive xenograft lines LuCaP 23.1 responded to treatment, LuCaP 86.2 had a limited response, and LuCaP 35 did not respond to treatment. The *ERG* negative line LuCaP 96 as expected did not respond to treatment. There was limited toxicity due to weight loss in the animals in response to YK-4-279 treatment. This impacted the tumor volumes, serum PSA and survival outcomes for all xenograft lines. Further, after discussion and review of previously obtained and new data we determined that using the S-enantiomer of YK-4-279 may be beneficial as the S-enantiomer bound the *ERG* protein with greater affinity and it appeared it had less toxicity associated with it based on previous *in vitro* and *in vivo*. Therefore the S-enantiomer was used as the second derivative. Dr. Morrissey has tested YK-4-279 in 4 patient derived xenograft lines as proposed. For all tissue acquired from the animals, half paraffin embedded and the remainder was flash frozen in OCT. Unfortunately the *ERG* positive xenograft lines LuCaP 23.1 and 35 did not respond to treatment (**Figure 1-2**), however, the LuCaP 86.2

treated tumors were significantly different in tumor volume to the untreated tumors ($p=0.0009$) (**Figure 3**). The ERG negative line LuCaP 96 as expected did not respond to treatment (**Figure 4**).

We did molecular profiling by RNAseq of tissues treated with the YK-4-279 compound (**Figure 5**). We determined that epithelial-mesenchymal transition (EMT) associated proteins RASAL2, VIM, NREP, and BCL11A were decreased among others. ERG has been associated with EMT in the past. To verify these changes at the protein level we analyzed additional EMT associated proteins to determine if we could identify changes in protein expression in the xenografts. A tissue microarray that was constructed last year from the study tissues was stained for Ki67, CD34, vimentin, ZEB1, CTGF, SOX4, Twist, SCUBE2, and JAG1 (**Figure 6**). We assessed proliferation however based on KI67 staining there was no significant change in proliferation between treated and untreated tumors (data not shown). For the EMT associated genes we did not see significant differences in EMT associated gene expression, but did observe high expression in cells with elongated nuclei in the tumors, suggesting the EMT-associated proteins were being expressed in a subset of cells in the tumors that may be undergoing EMT. We are further investigating the presence of these cell types in patient metastases.

In year 2, to determine if a set of genes are expressed in prostate cancer metastases and the xenografts we did an independent study of primary prostate cancer, prostate cancer metastases and the LuCaP xenografts by gene expression analysis. Using gene expression analysis on primary prostate and metastases specimens we determined that ERG was expressed in fewer patients with metastases than patients with primary prostate cancer (**Figure 7**). IHC revealed that 43% of primary prostate cancer were ERG+, 35% of the LuCaP PDXs were ERG+, and 18% of the CRPC metastases were ERG+, representing 12 of 48 patients (25%) with at least 1 ERG+ metastasis (data not shown). Additionally we identified a protein DCLK1 that was associated with ERG expression. DCLK1 was upregulated at the protein level in both ERG+ primary prostate cancer and CRPC metastases ($p=0.0013$ and $p<0.0001$, respectively) (**Figure 8**). In primary prostate cancer, ERG status or expression of targeted proteins was not associated with BCR-free survival. However, ERG+DCLK1+ patients exhibited shorter time to BCR ($p=0.06$) compared to ERG+DCLK1- patients (**Figure 9**). However, we saw no association of DCLK1 with ERG inhibition in the LuCaP xenografts. This could be for a number of reasons, as DCLK1 has been described as a putative stem cell marker and may not reside in sufficient numbers of cells for us to identify the change in DCLK1 in the xenograft models.

Currently we have injected intra-tibial tumors into animals for treatment with the S-enantiomer of YK-4-279 to determine if the compound will impact tumors in the bone. We are awaiting an application for a no cost extension to continue the intra-tibial studies.

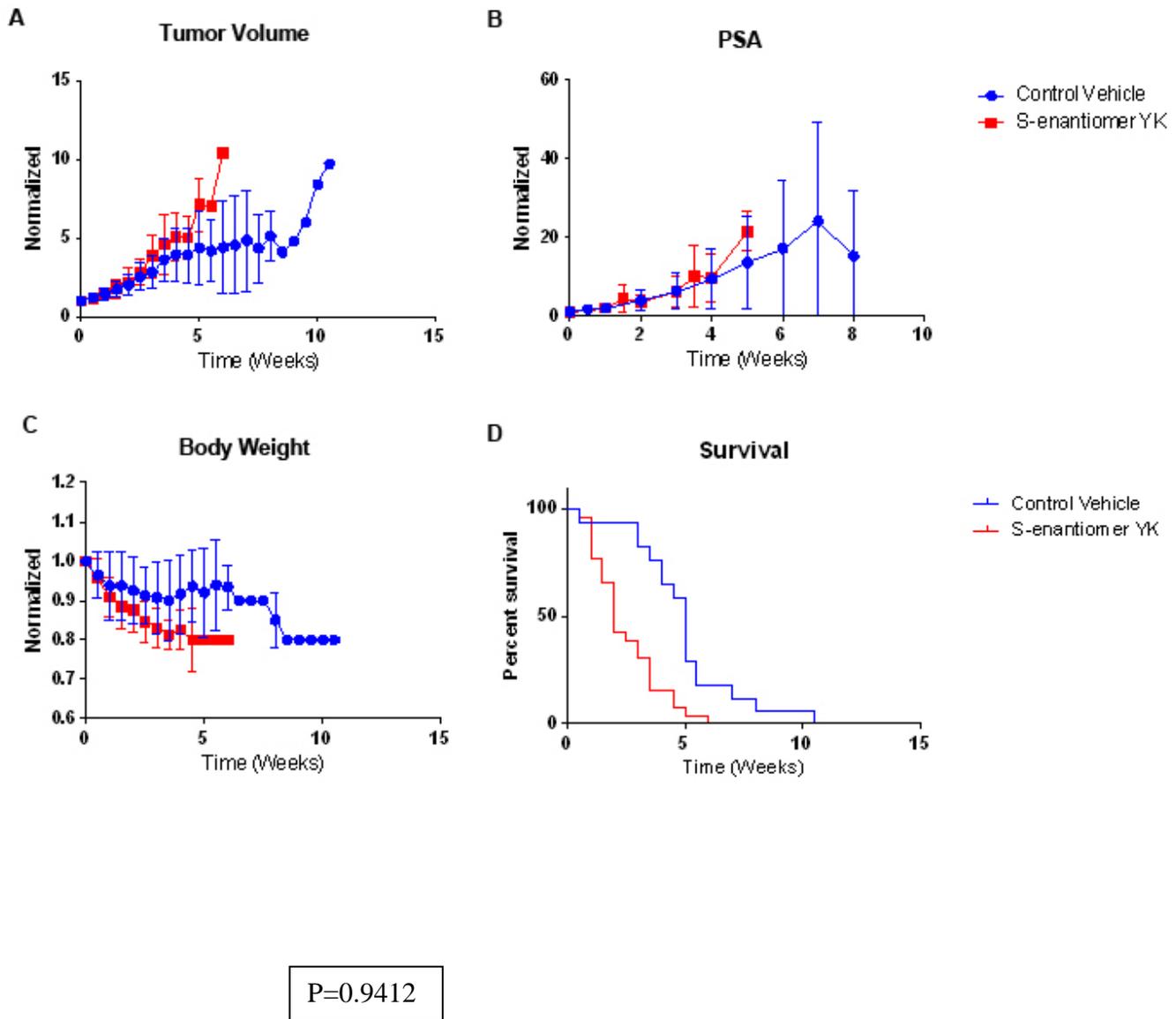


Figure 1. Response of animals bearing the ERG positive LuCaP 23.1 xenograft to the S-enantiomer of YK-4-279. (A) Tumor volume, (B) Serum PSA, (C) Body weight, and (D) Survival. The ERG positive LuCaP 23.1 xenograft did not respond to ERG inhibition using the S-enantiomer of YK-4-279.

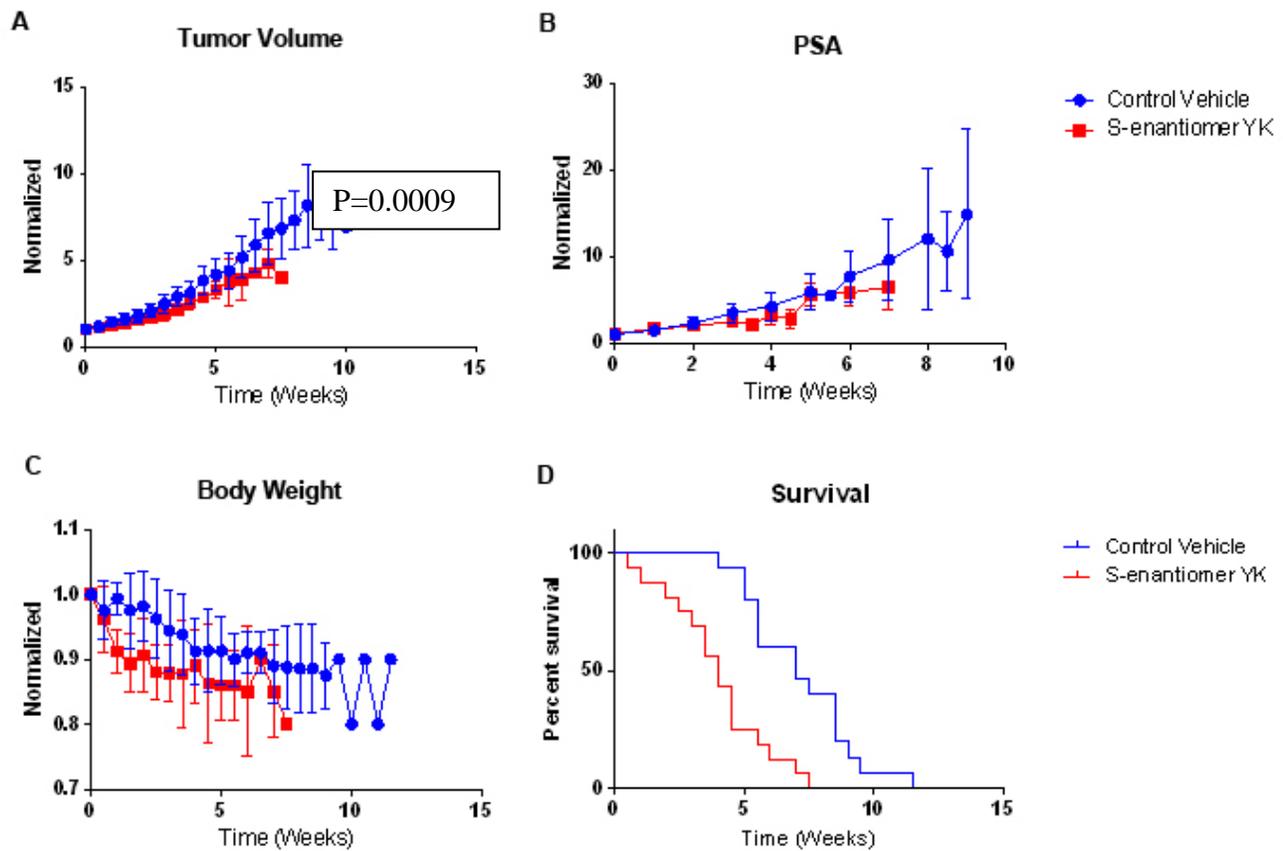


Figure 2. Response of animals bearing the ERG positive LuCaP 86.2 xenograft to the S-enantiomer of YK-4-279. (A) Tumor volume, (B) Serum PSA, (C) Body weight, and (D) Survival. Surprisingly, the ERG positive LuCaP 86.2 xenograft responded significantly to ERG inhibition.

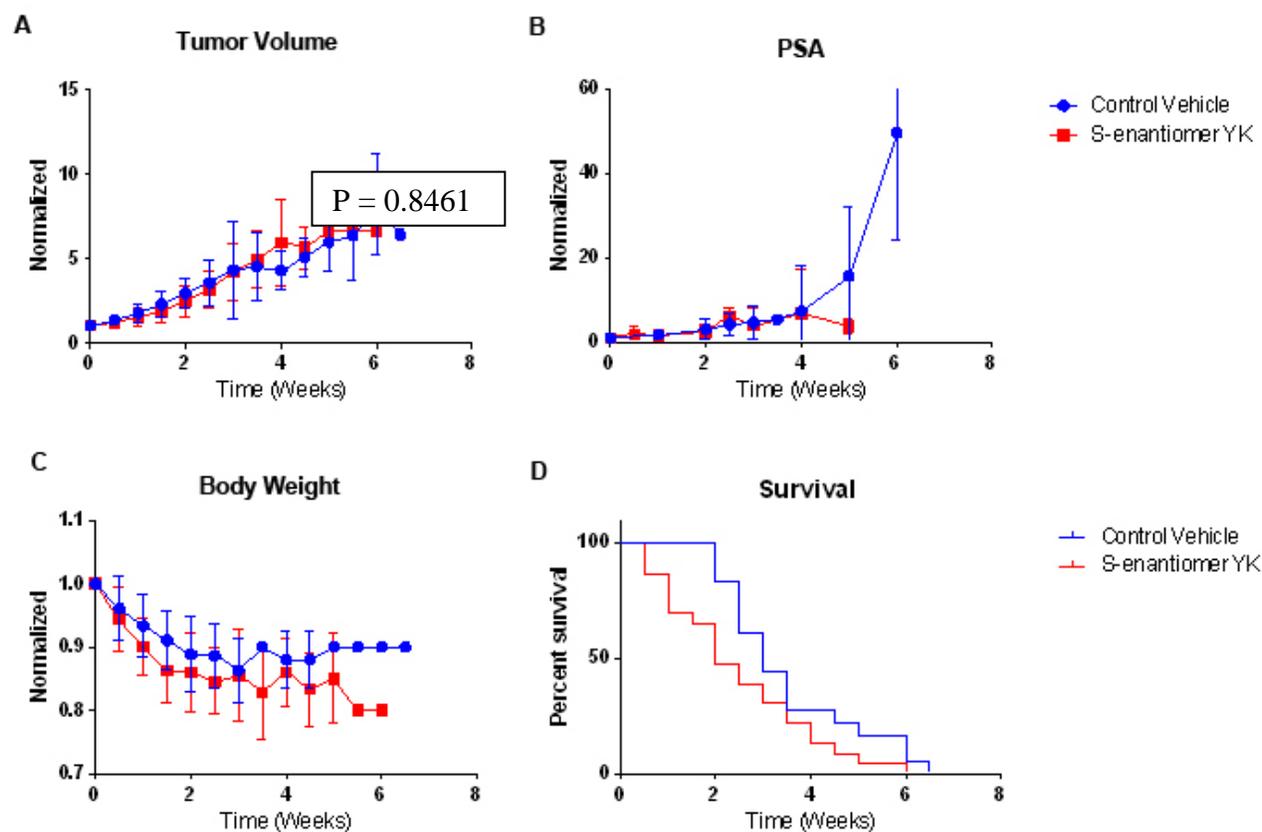
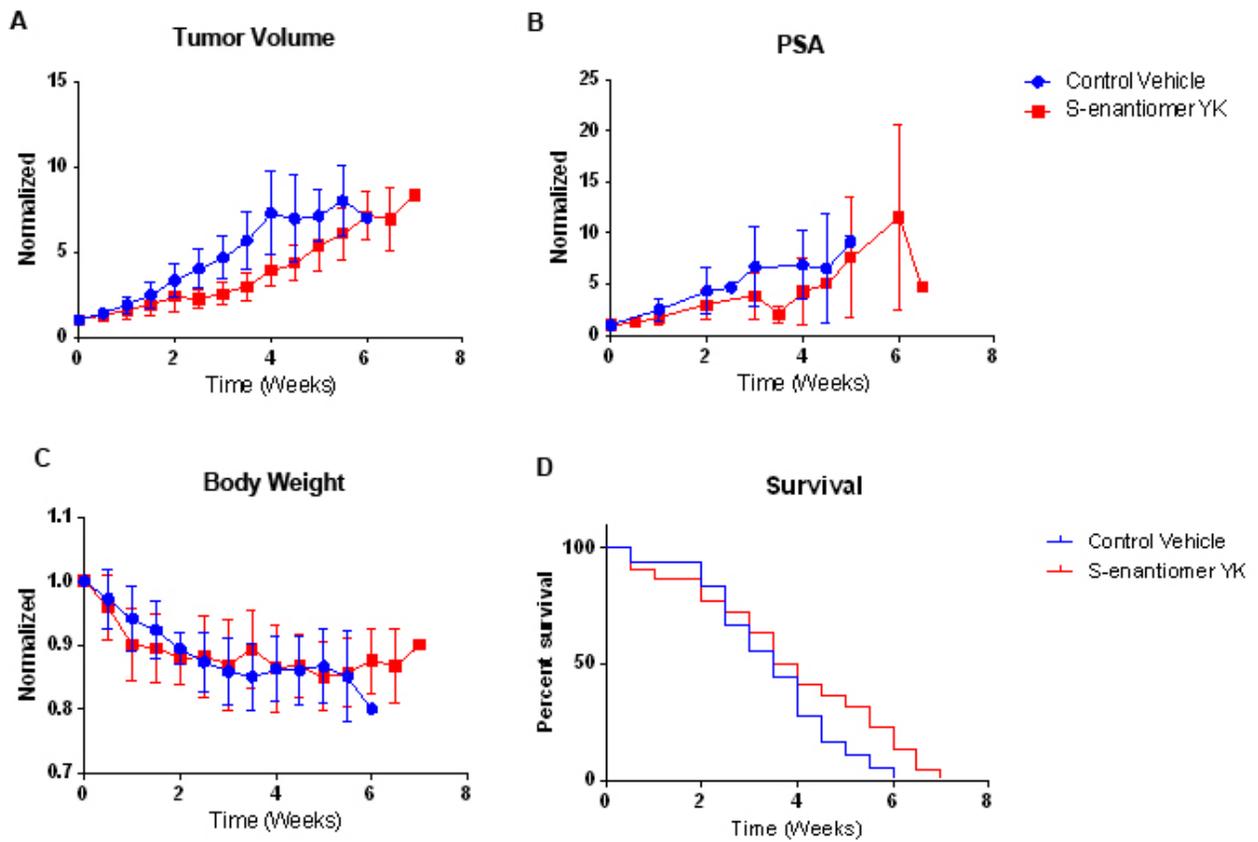


Figure 3. Response of animals bearing the ERG positive LuCaP 35 xenograft to to the S-enantiomer of YK-4-279. (A) Tumor volume, (B) Serum PSA, (C) Body weight, and (D) Survival. The ERG positive LuCaP 35 xenograft did not respond to ERG inhibition.



P = 0.3868

Figure 4. Response of animals bearing the ERG positive LuCaP 96 xenograft to the S-enantiomer of YK-4-279. (A) Tumor volume, (B) Serum PSA, (C) Body weight, and (D) Survival. The ERG negative LuCaP 96 xenograft did not respond to ERG inhibition.

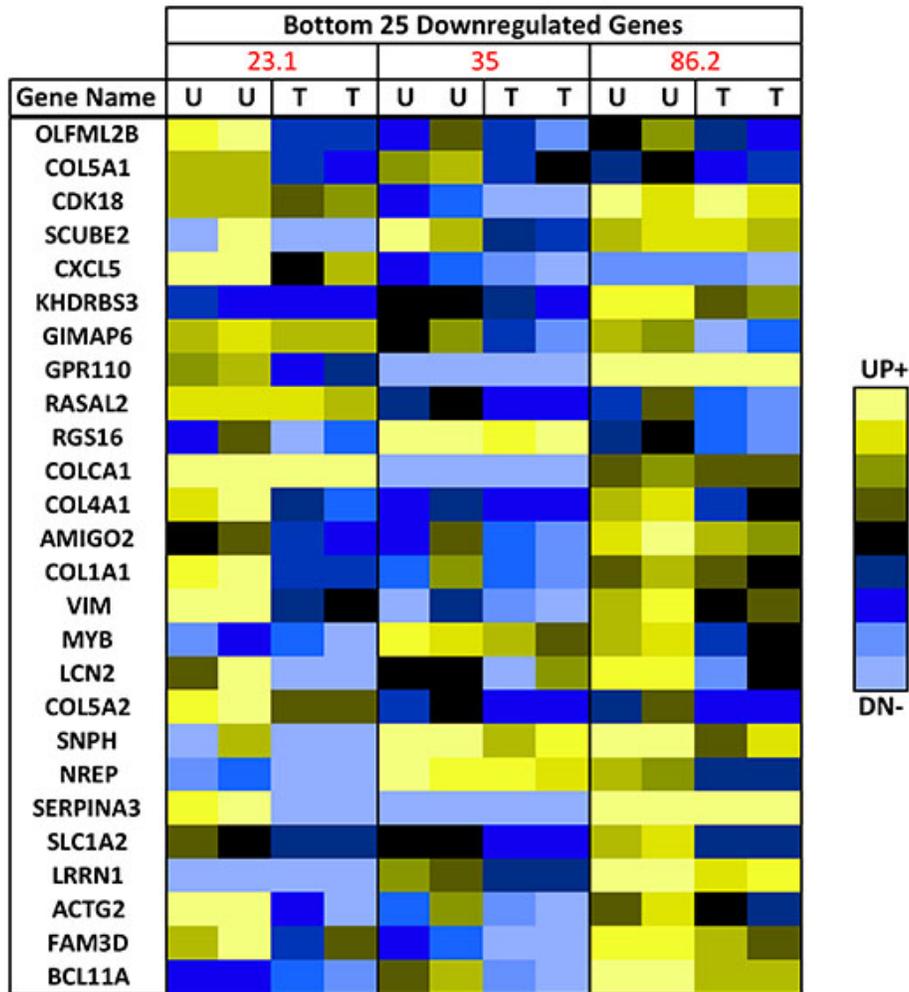


Figure 5. Genes decreased in expression after YK-4-279 treatment in ERG positive LuCaP 23.1, 35 and 86.2 xenografts. RNASeq was performed to identify genes that were suppressed by YK-4-279. Epithelial-mesenchymal transition associated proteins were downregulated in the expression arrays. Note that differences in expression are significantly different between lines. U= Untreated; T = Treated.

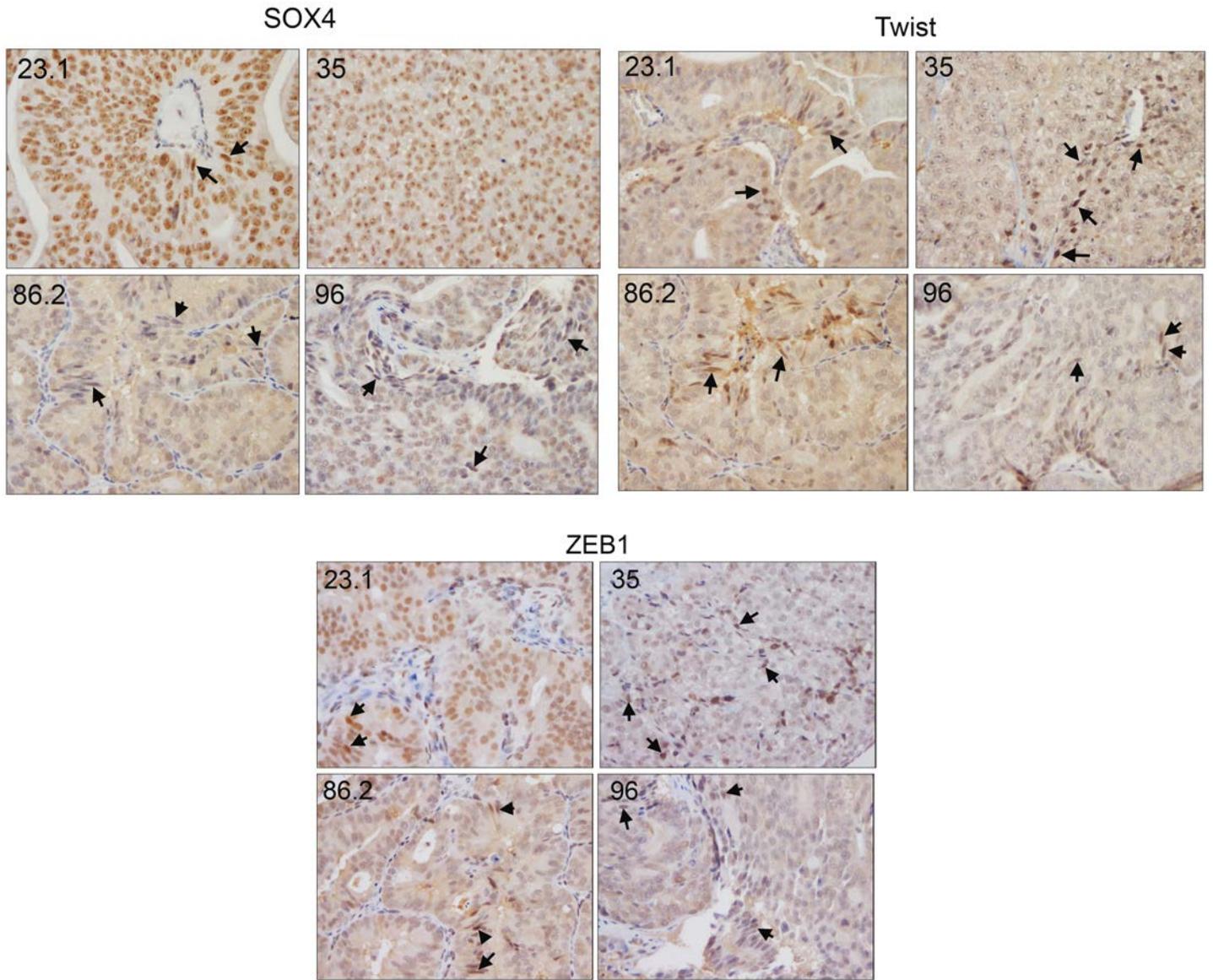


Figure 6. SOX4, Twist, and ZEB1 expression in LuCaP 23.1, 35, 86.2, and 96. While no difference was observed in staining between YK-4-279 treated and untreated xenografts, differences in the presence of cells with elongated nuclei that stained for SOX4, Twist and ZEB1 were observed. Arrows highlight nuclear staining of epithelial cells with elongated nuclei.

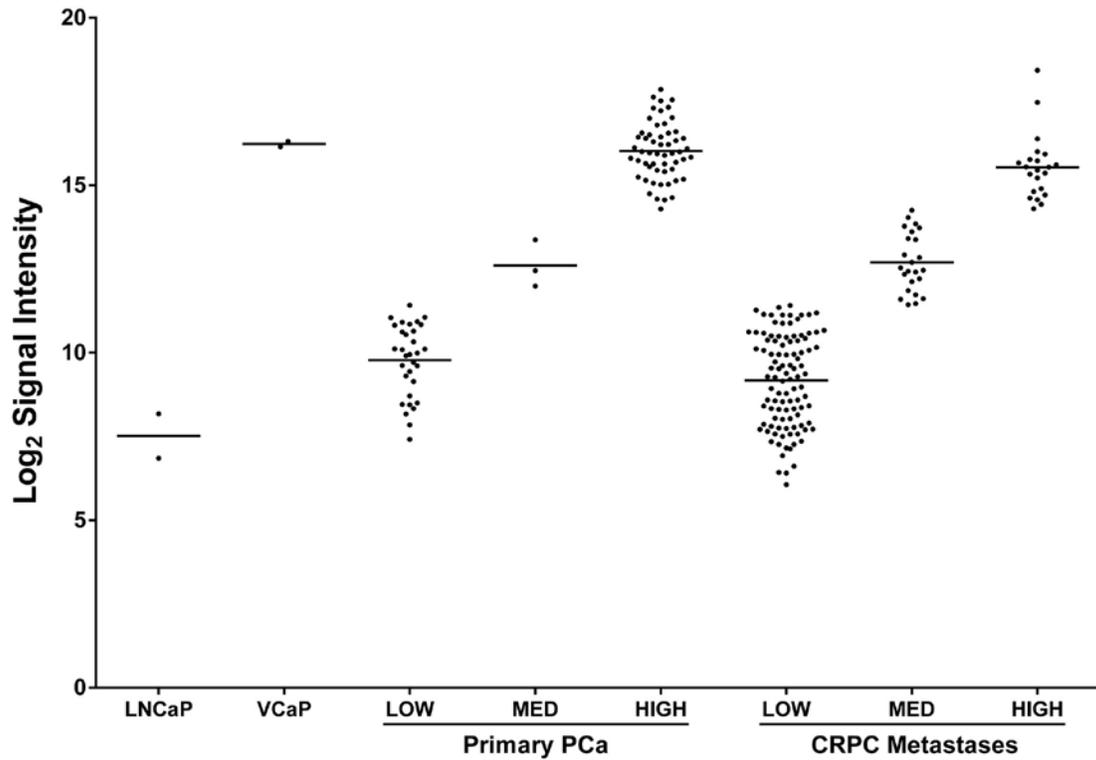


Figure 7. Figure 1: ERG gene expression in primary prostate cancer and CRPC metastases. Agilent gene expression array Log₂ signal intensities of LNCaP (ERG-) and VCaP (ERG+) cells, primary prostate cancer, and CRPC metastases. Based on ERG microarray expression Cy3 values primary prostate cancer specimens were divided into those with low (<2750), medium (>2750 <20,000) and high (>20,000) levels of ERG expression. Of note, the CRPC metastases group contains a large cohort of medium ERG expressors.

A) Primary PCa

B) Metastatic CRPC

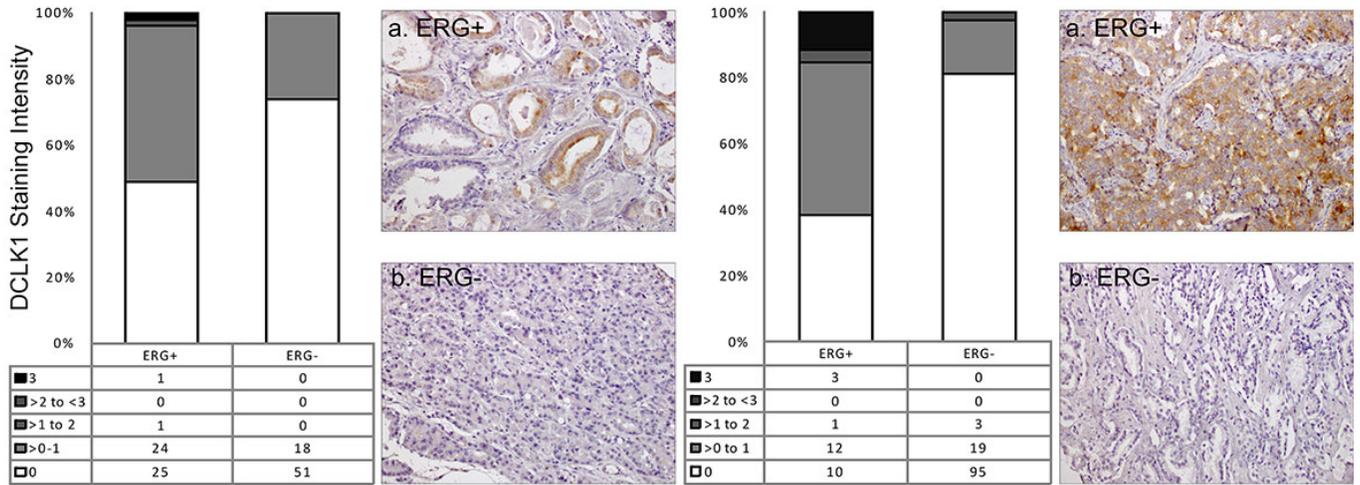


Figure 8. DCLK1 protein expression in ERG+ and ERG- primary prostate cancer and CRPC. A. DCLK1+ was found in 51% of ERG+ primary prostate cancer revealing significant correlation with ERG+ ($r=0.290$, $p=0.001$). Representative IHC images of DCLK1+ in ERG+ (a) and ERG- (b) primary prostate cancer are shown. **B.** DCLK1 was present in 62% of ERG+ CRPC specimens revealing significant correlation with ERG+ ($r=0.391$, $p<0.0001$). Representative IHC images of DCLK1+ cells in ERG+ (a) and ERG- (b) CRPC specimens are shown.

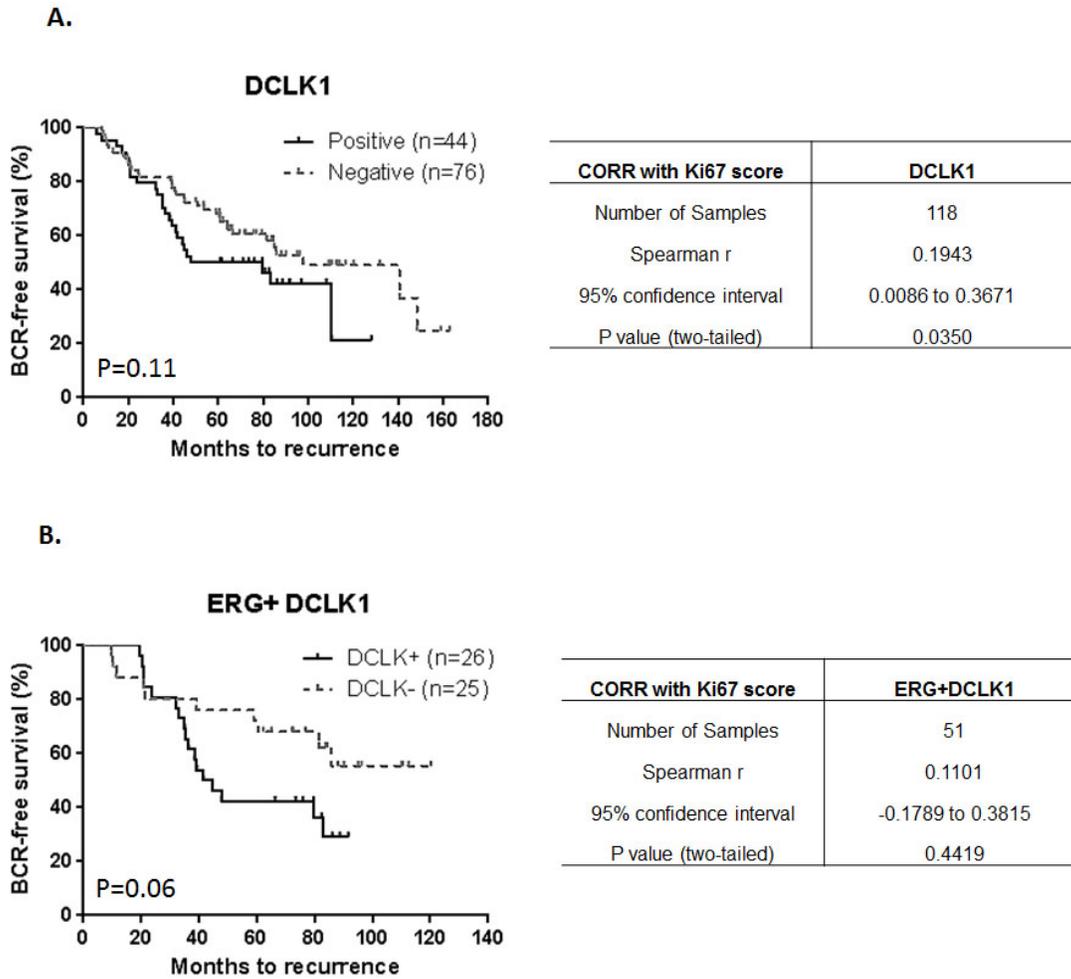


Figure 9. Primary prostate cancer BCR-free survival and proliferation relative to DCLK1 status. A. Kaplan-Meier analysis of DCLK1+ vs. DCLK1- status did not correlate with BCR-free survival overall ($p=0.11$). There was a positive correlation with Ki-67 proliferation relative to DCLK1 status ($r=0.194$, $p=0.0350$). **B.** In ERG+ samples, DCLK1+ vs. DCLK1- status revealed a trend towards worsened BCR-free survival ($p=0.06$). DCLK1 did not correlate significantly with Ki-67 proliferation in this grouping ($r=0.110$, $p=0.4419$).

Key Research Accomplishments

1. The ERG inhibitor has an impact on tumor volume in patient derived xenograft models of prostate cancer.
2. Not all ERG positive tumors respond to therapy.
3. The effects of the ERG inhibitors are not non-specific as no effect was observed in an ERG negative xenograft line.
4. The S-enantiomer of YK-4-279 has limited impact on tumor volume in patient derived xenograft models of prostate cancer.
5. The S-enantiomer of YK-4-279 is the active component but is no less toxic than the racemic mixture *in vivo*.
6. ERG regulated genes in prostate cancer metastases may be different to ERG regulated genes in primary prostate cancer.
7. We uncovered DCLK1 is a novel protein that may be downstream of ERG in prostate cancer.

Conclusion

This data suggests ERG inhibitors can impact tumor volume in some but not all ERG positive prostate cancer tumors. It also reveals that the YK-4-279 racemic mixture has some toxicity which limits its efficacy in these studies. We determined the S-enantiomer does not have significantly less toxicity than the racemic mixture. Additionally, we determined that the decrease in tumor volume and serum PSA was not only due to a decrease in proliferation. We determined that fewer patients with prostate cancer metastases are ERG positive than patients with primary prostate cancer. Furthermore, we used RNASeq data and immunohistochemistry to identify ERG regulated genes impacted by ERG inhibition and identified a novel ERG regulated protein DCLK1 in prostate cancer. We did not identify mechanisms of resistance to ERG inhibition.

Publications, Abstracts, and Presentations

Identifying common molecular features of ERG positive tumors in primary and castration resistant prostate cancer. Colm Morrissey, Martine Roudier, Ilsa Coleman, Xiaotun Zhang, Hung-Ming Lam, Roger Coleman, Lisy Chéry, Celestia Higano, Lawrence D. True, Paul H. Lange, Eva Corey, Shiv Srivastava, Aykut Üren, Linda Snyder, Robert L. Vessella, Peter S. Nelson [Abstract of a poster presentation at the Prostate Cancer Foundation, Washington DC. October 2013].

Pacific Northwest SPORE Presentation - Identifying Common Molecular Features of ERG Positive Tumors in Primary and Castration Resistant Prostate Cancer, October 2013.

YK-4-279 is a small molecule inhibitor of ETV1 and inhibits metastasis in a mouse model. Said Rahim, Sarah Justvig, Sung-Hyeok Hong, Perrin Tosso, Haydar Celik, Yasemin Sayedigar-Kont, Milton Brown, Colm Morrissey, Jeffrey Toretsky, Aykut Üren. [Abstract of a poster presentation at the American Association of Cancer Research, San Diego CA. April 2014].

Prostate Cancer: Basic Research V. Inhibition Of ERG Activity In Patient Derived Prostate Cancer Xenografts Using The Small Molecule Inhibitor YK-4-279. Brian Winters, Lisha Brown, Ilsa Coleman, Tsion Minas, Xiaotun Zhang, Lori Kollath, Holly Nguyen, Peter Nelson, Eva Corey, Aykut Uren, Colm Morrissey. [Abstract of a poster presentation at the American Urological Association, New Orleans. May 2015].

Papers in Submission:

Characterizing the molecular features of ERG-positive tumors in primary and castration resistant prostate cancer. *Martine P Roudier, *Brian R Winters, Ilsa Coleman, Hung-Ming Lam, Xiaotun Zhang, Roger Coleman, Lisy Chéry, Lawrence D. True, Celestia S. Higano, Bruce Montgomery, Paul H. Lange, Linda A. Snyder, Shiv Srivastava, Eva Corey, Robert L. Vessella, Peter S. Nelson, Aykut Üren, and Colm Morrissey. The Prostate 2015.

Reportable Outcomes

This work so far has shown that ERG inhibition can result in a decrease in tumor growth, but that not all ERG positive tumors will respond to ERG inhibition.

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