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14. ABSTRACT

Prostate cancer is the most common cancer in men, and the second-most common cause of cancer-related death in the United States. Despite recent medical advances, once this cancer manifests beyond the confines of the prostate, most commonly in the bones and spine, it is incurable and can cause significant suffering prior to death. Current investigations study the molecular and genetic basis of the disease, to identify potential new drug targets and therapies. Approximately half of all prostate cancers contain a fusion between two genes, TMPRSS2 and ERG, and a further half of these contain a deletion of the 14 genes lying between TMPRSS2 and ERG. I have now performed a screen involving inhibition of each of these 14 genes, and am currently validating whether one or more of these genes does indeed affect cellular proliferation in vitro and in vivo.

15. SUBJECT TERMS
prostate cancer, TMPRSS2-ERG gene fusion

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ERG ASSOCIATED GENOMIC LOSS OF 21Q22 MARKEDLY AFFECTS PROSTATE CANCER PROGRESSION

INTRODUCTION

Half all prostate cancers contain a fusion between two genes, TMPRSS2 and ERG, which places the androgen-dependent promoter of TMPRSS2 directly upstream of an amino-truncated ERG transcription factor, leading to dramatic upregulation of ERG, a transcription factor with oncogenic roles in other cancers such as leukemias and sarcomas (Tomlins, Rhodes et al. 2005; Turner and Watson 2008). TMPRSS2 and ERG are located only 3 megabases apart at chromosome 21q22 with 14 genes between them and there are two mechanisms which can result in TMPRSS2-ERG gene fusion: 1) interstitial deletion of the intervening region, or 2) balanced translocation with another chromosome. A critical difference between these two mechanisms is that in the case of former, there is heterozygous loss of the intervening 14 genes. Our whole genome analysis of prostate tumors reveals that approximately 50% of TMPRSS2-ERG positive tumors are interstitial deletions, with the remaining half being balanced translocations (Taylor, Schultz et al. 2010). Interestingly, however, this ratio shifts to 75-80% interstitial deletions versus 20-25% balanced translocations in cases of advanced disease, such as patients with metastatic disease, or TMPRSS2-ERG positive small-cell cancer (Mehra, Han et al. 2007; Lotan, Gupta et al. 2011). There is a second notable finding: in every single patient, there is always at least one chromosome that exhibited a wild-type 21q22 locus, no matter the fact that in some cases, there were as many as three concurrent interstitial deletions in the same cell (Attard, Clark et al. 2008). One possibility is that although 21q22 loss is associated with advanced disease as described above, at least one genomic copy of wild-type 21q22 region is required for androgen signaling and thereby, prostate cell survival i.e. there is an obligate haploinsufficiency tumor suppressor located at 21q22 (Berger and Pandolfi 2011). In this project, I am investigating the consequences of loss of these 14 intervening genes.

KEYWORDS

metastatic prostate cancer, TMPRSS2-ERG gene fusion

OVERALL PROJECT SUMMARY

Aim 1 / Milestone Task 1: Complete shRNA Screen with Prostate Organoids

*a) Derive Organoids from the R26-TetOn*Tre-ERG mice*

The first milestone task was to perform an shRNA screen, inhibiting each of the 14 intervening genes between TMPRSS2 and ERG. In a) the goal was to create the optimal cell line for either performing or validating the shRNA screen. I proposed using prostate organoids, primary murine prostate cells grown in 3D culture conditions that allow indefinite expansion. These cells remain genetically and phenotypically stable in culture, and recapitulate prostate gland architecture. Initially, I proposed deriving such prostate organoids from the R26-TetOn*Tre-ERG mouse. However, I decided against this, since these mice do not develop prostate cancer. Instead, organoids were derived from a similar mouse, PTEN$^{flox/flox}$*R26-loxp-STOP-loxp-ERG*Probasin-Cre. Although these lack doxycycline-based induction of the ERG oncogene, these organoids have the advantage of having a physiologic output i.e. they form tumors in urogenital sinus mesenchyme tissue recombination experiments. Similarly, shPTEN-infected R26-loxp-STOP-loxp-ERG organoids generated hyperplastic prostate glandular tissue in urogenital sinus mesenchyme tissue recombination experiments (*Figure 1*).

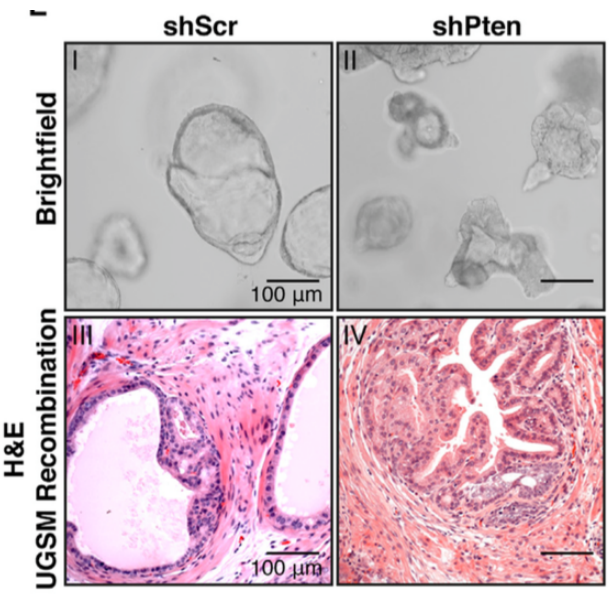


Figure 1

b) Testing of Hairpins' Inhibition of Target Expression

A subset of library shRNAs were tested to confirm adequate infectivity of the screen cells, as well as to ensure adequate knockdown of target mRNA via qPCR (*Figure 2 left panel*) and target protein levels via fluorescent immunoblot (*Figure 2 right panel*), which gives a more quantitative result than chemiluminescent immunoblot. In most cases mRNA knockdown is at least 70%, and protein level knockdown is at least 80%.

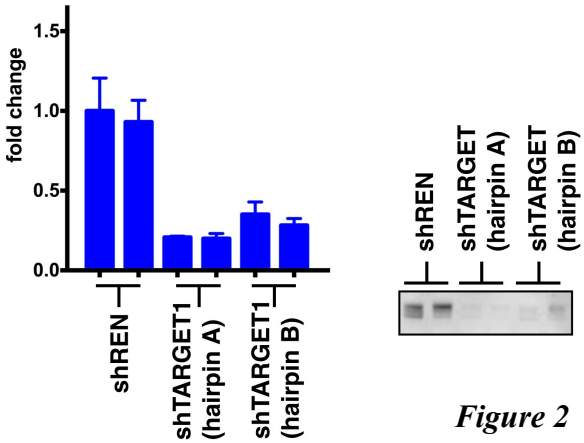


Figure 2

c) shRNA screen

A pooled shRNA screen design was used (*Figure 3*). The basic premise is as follows: a pool of shRNAs were created, some of which targeted growth promoting genes, some targeted growth inhibitory genes, and other targeted genes that had no effect on proliferation. The cells were infected with a low MOI, ensuring that each cell was infected with only one given shRNA. The shRNAs that target a growth-inhibitory gene will lead to increased growth of those particular cells, which in turn will lead to accumulation of that particular shRNA relative to the others. On the other hand, shRNAs that target a pro-growth gene will lead to decreased growth of those particular cells, or even cell death, leading to depletion of that particular shRNA relative to the others. Finally, shRNAs that target a gene with no effect on proliferation, will neither accumulate or deplete relative to other shRNAs. Therefore, by deep-sequencing the genome-integrated lentiviral shRNA and measuring the change in the relative abundance of shRNAs from the beginning to the end of the screen, the relative contribution of a gene to proliferation can be inferred.

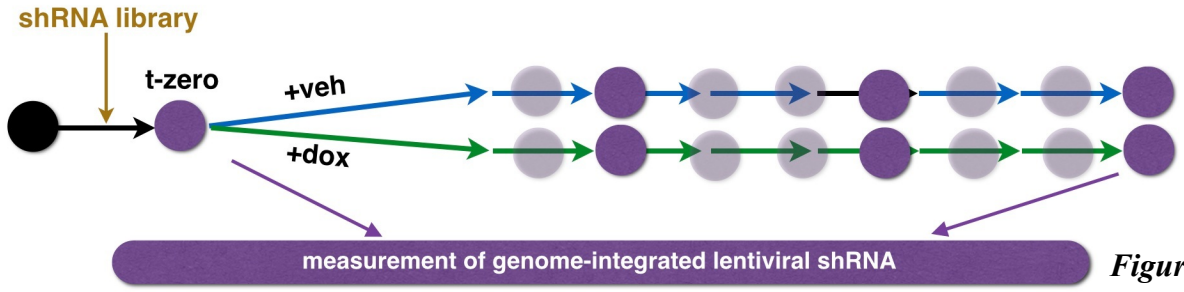


Figure 3

Specifically, cells were infected with lentivirus containing a shRNA library containing 4-5 shRNAs per gene, mostly targeting the 14 intervening genes between TMPRSS2 and ERG. The time zero ("t-zero") genomic DNA containing the integrated lentiviral shRNA was isolated from cells two days after infection, and 3 subsequent data points were collected over the next two months. The genomic DNA for each of the data points was subjected to PCR in order to amplify the integrated lentiviral element, and add the flanking sequences necessary for deep sequencing.

d) siRNA validation

Validation was performed via a mixing assay with shRNAs distinct from the screen, rather than siRNAs, thereby ensuring stable protein knockdown (*Figure 4*). In all cases, cells infected with an mCherry-vector containing a non-targeting shRNA (shNT) were mixed with cells infected with a GFP vector containing an shRNA to the validation target of interest (shTARGET1). The relative

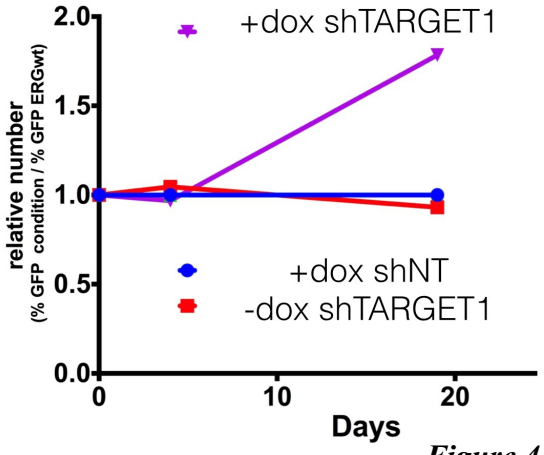


Figure 4

number of GFP to mCherry containing cells was assessed via flow cytometry. As in the actual example provided in *Figure 4*, the GFP-shRNA targeting the putative growth-inhibitory gene, was indeed enriched, relative to the mCherry-shNT.

e) Doxycycline screen for ERG cooperativity

The cells used in the screen were designed to contain a second lentiviral construct, this one with a doxycycline-inducible shRNA targeting ERG. Thus, the shRNA screen and validation steps were also performed in the absence vs presence of doxycycline to test ERG cooperativity (*Figures 3,4*).

Aim 2 / Milestone Task 2: Mechanism of Screen Hits to Suppress Tumor Formation and Regulate Androgen Signaling

a) Screen Hits shRNA generation

The second milestone task was to determine the mechanism by which screen hits can potentially suppress tumor formation and regulate androgen signaling. The shRNAs targeting screen hits were generated and cloned into a superior, next-generation vector for further experiments (*Figures 5*). The major advantage of this miR-E vector over prior versions, was the correction of a synthetically added EcoRI site, which inadvertently led to lower shRNA expression and reduced knockdown. The offending nucleotides were removed in the miR-E vector, restoring full endogenous miR activity and thereby ultimately leading to maximum knockdown. These miR-E vectors also contained the highly active SFFV promoter, which is silenced less frequently in mammalian cells than the more common CMV promoter. Furthermore, GFP expression is under the same promoter as the shRNA, and positioned upstream of the shRNA. Together, this ensured that all cells containing the shRNA also contained GFP fluorescence. There was also a puromycin selection cassette for quick antibiotic selection

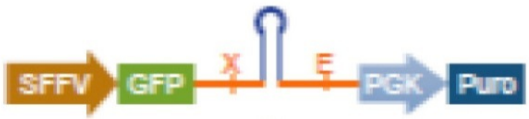


Figure 5

b) Microarray and data analysis of screen hits to examine interplay with androgen signaling

Given the critical nature of the androgen receptor signaling in prostate cancer, it was inferred that any screen hit that modulates proliferation, may do so by modulating androgen signaling. First, the androgen-regulated transcriptome needed to be defined in the screen cells. This was achieved by treated the cells with 16hrs of dihydrotestosterone or vehicle, then harvesting mRNA, and performing differential expression analysis between the two conditions via RNA-Seq; this demonstrated that hundreds of genes are under the control of the androgen receptor. (*Figure 6*).

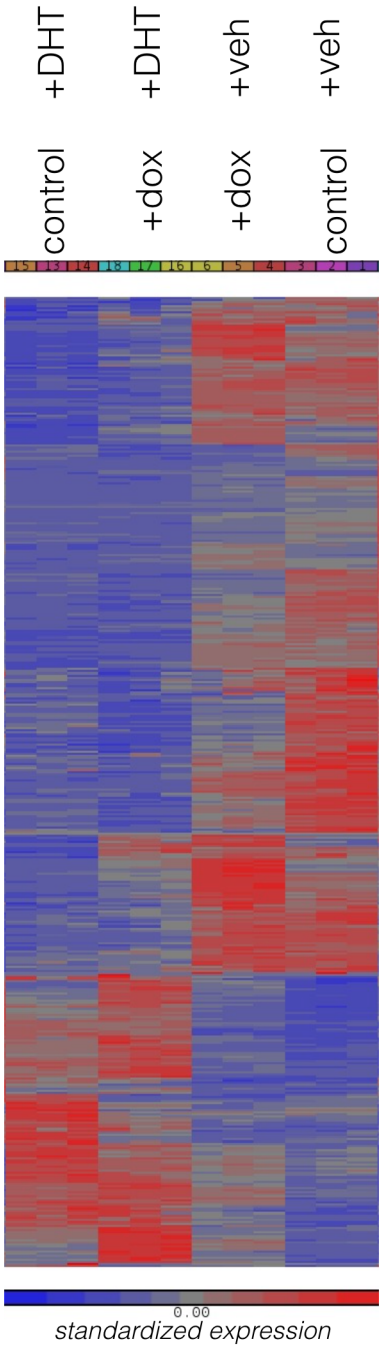


Figure 6

Next, these cells were infected with miR-E vectors containing the hit shRNAs, and the cells were treated with or without androgen. Unfortunately, targeting the genes located between TMPRSS2 and ERG, such as ETS2, had modest effects at best, but other shRNAs that demonstrated ERG cooperativity, did indeed show proliferative effects. One such ETS2 related protein did indeed show activation of many androgen regulated genes when inhibited. Preliminary effects of the knockdown on androgen signaling was initially observed by qPCR (*Figure 7*). Subsequently, differential expression analysis was performed via RNA-seq. Together, these confirmed that ERG cooperative genes do have substantial effects on androgen signaling.

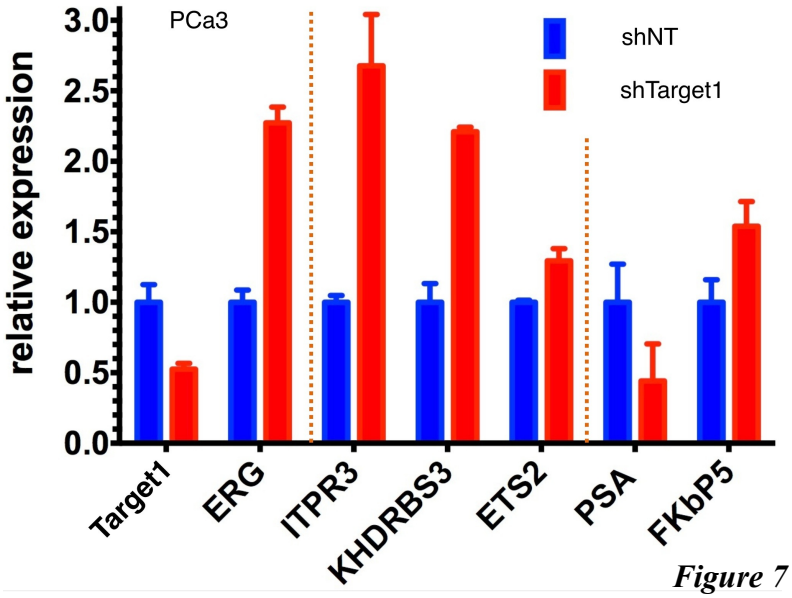


Figure 7

c) ChIP assay and data analysis of screen hits to examine interplay with ETS binding sites

Given the known association between ETS binding sites and androgen receptor binding sites near androgen-regulated genes, ETS ChIP was performed. Preliminary ChIP-qPCR confirmed that the ChIP was successful (*Figure 8*). Subsequently, ChIP-Seq was performed (*Figure 9*), confirming considerable overlap between androgen receptor and ETS bindings sites.

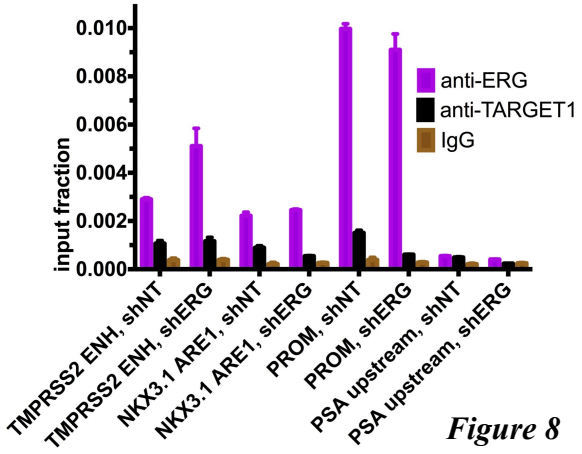
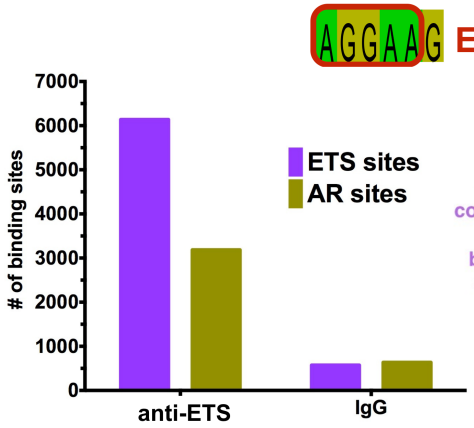


Figure 8



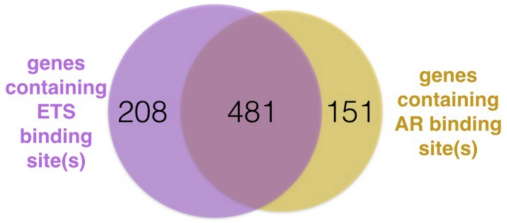


Figure 9

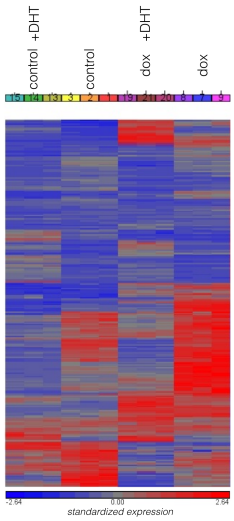


Figure 10

The relationship between ETS binding and androgen binding sites was next confirmed by inhibiting the ERG oncogene via doxycycline administration, followed by androgen transcriptome analysis (*figures 6, 10*). This, too, confirmed that inhibition of a key ETS transcription factor has profound effects on the androgen transcriptome.

d) RAS/MAPK pathway analysis

The interplay between RAS/MAPK pathway and screen hits via drugs such as MEK inhibitor PD98059 is potentially interesting, but such pathway analysis has not been performed to date.

Aim 3 / Milestone Task 3: Crossing of ERG Overexpressing Mice with Screen Hit Knockdown Mice

b) Isolation of organoids derived from PTEN-/- mice, with and without the ERG transgene for pending candidate shRNA infection

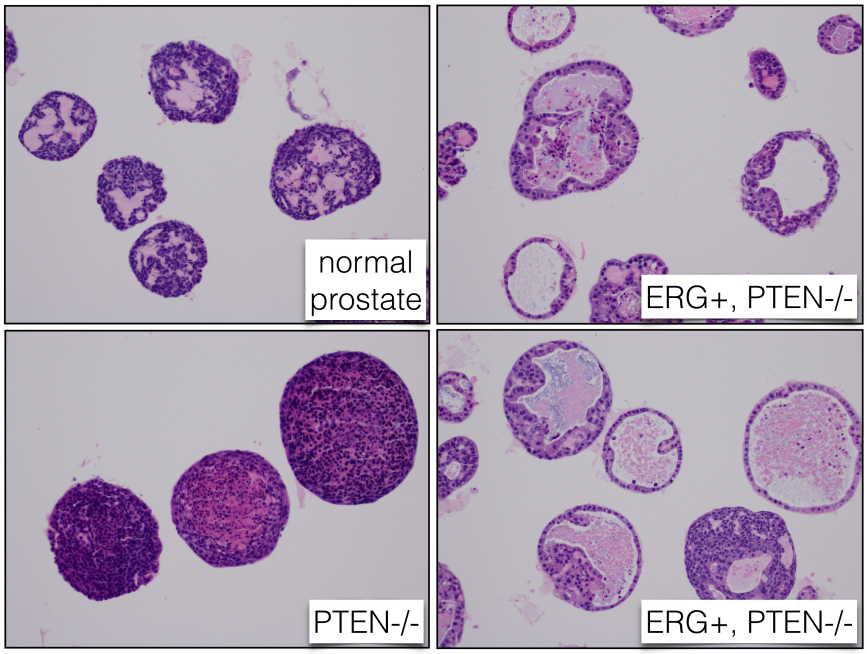


Figure 11

To speed analysis, shRNA knockdown in prostate organoids derived from mice followed by subcutaneous graft injection would be used instead of mouse knockouts. To establish murine prostate organoid cultures, dissociated cells of $PTEN^{flox/flox}$*Probasin-Cre as well as $PTEN^{flox/flox}$ mice, were embedded in Matrigel and organoid media containing EGF, Noggin, R-spondin1, A83-01 TGFb inhibitor, to prevent a proliferative block. These organoids form cystic structures composed of a basal outer layer exclusively expressing typical basal prostate markers, such as p63 and CK5, as well as a luminal inner layer exclusively expressing CK8, thereby retaining the architecture that resembles prostate glands in vivo (*Figure 11, left panels*). Such organoids retain robust expression of the androgen receptor and maintain intact androgen signaling. A similar process was carried out with the ERG transgene (*Figure 11, right panels*).

a) *Infection of mouse organoids with shRNA targeting candidate genes*

To speed analysis, shRNA knockdown in prostate organoids derived from mice were used instead of mouse knockouts. Organoids were infected with shRNA constructs similar to the miR-E vector described in Aim 2, except that they expressed mCherry rather than GFP. To improve the degree of knockdown by ensuring high vector level, the infected organoids were selected by FACS for high mCherry expression (*Figure 12*).

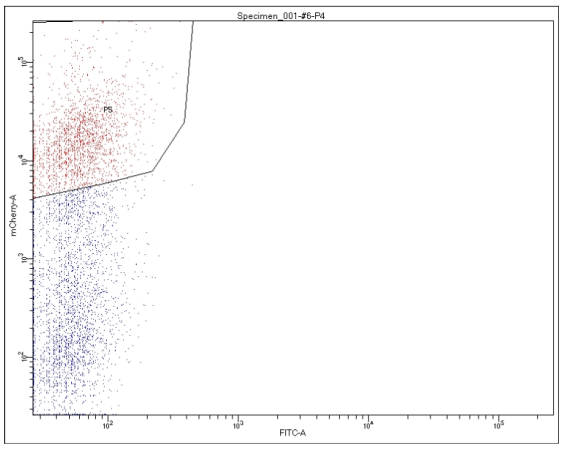


Figure 12

c) *Tumor growth assay*

Once the screen hit shRNAs have been infected into the mouse organoids, the cells can be grafted subcutaneously into mice to evaluate the screen hits for in vivo growth. These in vivo studies have not been completed to date, however it has been verified that such organoid grafts are sensitive to castration (Cast), as well as enzalutamide (Enz: an androgen-receptor antagonist), confirm that androgen signaling is a necessary element of tumor growth (*Figure 13*).

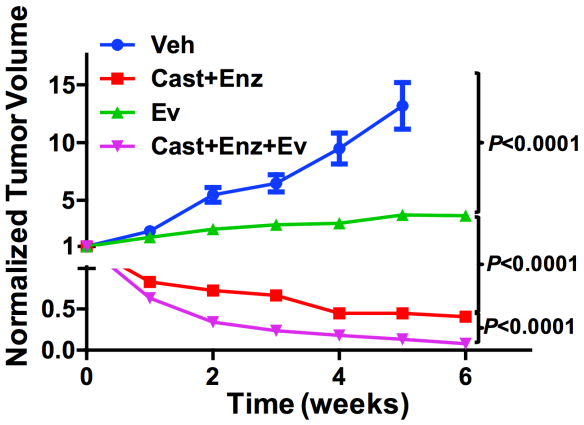


Figure 13

d) Biochemical analysis of tumors

Since the in vivo growth assays are ongoing, the biochemical analysis of the tumors has not been completed to date. However, we have verified that the organoids isolated and cultured in b) contain the ERG transgene (*Figure 14*).

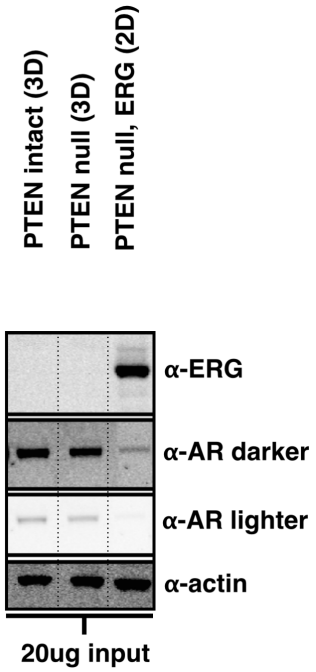


Figure 14

KEY RESEARCH ACCOMPLISHMENTS

- Development of shRNA screening capability in prostate cancer cells
- Better definition of the androgen transcriptome in prostate cancer
- Marked improvement in understanding of the relationship between the TMPRSS2-ERG upregulation and androgen signaling.
- Understanding of how to use prostate organoid technology to investigate these issues in vitro and in vivo

CONCLUSION

Virtually all current treatments for metastatic prostate cancer, the second leading cause of cancer death among men in the United States, involve inhibition of androgen (e.g. testosterone) signaling in the cancer cells. My completed research has improved our understanding of the nature of androgen signaling in ERG+ tumors (50% of all prostate cancers), which will lead to improved treatments in the future. Furthermore, by developing screening expertise, it lays the foundation for future investigations to identify novel drug targets.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

Presentations include:

- Medical Oncology Grand Rounds, Princess Margaret Cancer Centre, Oct 2014
- P01 Meeting, Memorial Sloan Kettering Cancer Center, July 2015
- Human Oncology and Pathogenesis Program / Cancer Biology and Genetics Joint Talk, Memorial Sloan Kettering Cancer, Oct 2015

INVENTIONS, PATENTS AND LICENSES

Nothing to report.

REPORTABLE OUTCOMES

Nothing to report.

OTHER ACHIEVEMENTS

Nothing to report

TRAINING AND PROFESSIONAL DEVELOPMENT

My training plan here at MSKCC is extensive and entirely cancer-related. I see patients in clinic for 15% of my weekday, particularly those with treatment-related decisions and those eligible for experimental protocols, giving me a unique and unparalleled clinical experience. The remainder of my time is spent on basic lab research. Coursework includes a clinical trials course and a biostatistics course. There are also one-time instructional courses for mouse work, microscopy etc. all of which I attend for each technique that I use. We have multiple seminars, including clinical teaching sessions twice a week that precede grand rounds, a weekly translational prostate cancer meeting, a weekly 'President's Seminar' featuring a pre-eminent scientist and a weekly Science club presentation given among members of the department. With regard to conferences, there is an annual retreat for the department, multiple workshops each year and annual conferences such as ASCO and the multi-institutional prostate cancer meetings.

My mentor, Dr. Charles Sawyers, is one of the pre-eminent physician-scientists in prostate cancer research. His prior work includes the key demonstration that tumors that progress in the castrate setting are still sensitive to androgen-receptor inhibition. He then demonstrated that the compounds enzalutamide and ARN509, which bind and inhibit the androgen receptor to a degree many fold higher than older drugs such as bicalutamide, could inhibit the growth of tumors in pre-clinical studies. Together with my clinical teacher Dr. Howard Scher, they have shown that compounds such as enzalutamide lead to a significant increase in overall survival in patients with castrate-resistant disease. Furthermore, Dr. Sawyers' lab has extensive experience with prior shRNA screens, and murine models of prostate cancer, all of which I utilize in my research proposal. He has earned numerous awards including the 2009 Lasker Award, and has several grants, including Howard Hughes funding, and a Prostate SPORE grant that he co-authored with Dr. Scher.

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