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TITLE: Selective Gene Regulation by Androgen Receptor in Prostate Cancer

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Androgen binds to the androgen receptor (AR) and is required for prostate cancer initiation and progression. Although androgen ablation is initially an effective prostate cancer therapy, patients eventually develop resistance, but disease remains driven by AR. There is a need to identify AR gene programs that promote proliferation versus differentiation to design better treatments. Using mutant ARs as models of aberrant AR activity, I generated cell lines harboring wild-type and mutant ARs and analyzed proliferation, anchorage-independent cell growth and alterations in AR target genes. I also generated a multiplexed promoter assay for high-throughput screening (HTS) to identify compounds that selectively regulate AR. After assay optimization, the primary HTS was performed with 2,5000 compounds. A dose response assay identified several compounds that selectively regulate AR and will be further studied in cell-based assays.
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Selective Gene Regulation by Androgen Receptor in Prostate Cancer

Introduction

Androgens work via the androgen receptor (AR) to play a key role in neoplastic as well as normal prostate growth and therefore the androgen signaling pathway remains a high impact therapeutic target. Antiandrogen therapy is initially successful but tumors ultimately progress to hormone resistance. In these castration-recurrent tumors, AR levels remain high and AR signaling persists, indicating that disease remains dependent on AR (1). Mechanisms underlying this AR-dependent, yet androgen-independent, state include gene amplification or somatic mutation of the AR, alterations in coregulatory proteins and ligand independent activation of AR by intracellular signaling pathways (1).

A goal of many studies has been to identify key genes involved in prostate cancer. Gene expression studies have provided global views of AR action in prostate cancer and identified a few key genes involved in growth and survival. However, the major pathways that promote disease progression remain largely unknown. A practical application of dissecting the opposing actions of AR is that it may reveal novel ways to block only a subset of AR’s functions. As opposed to complete androgen ablation, it may be beneficial to retain some AR activity to enhance a normal differentiated phenotype. Identifying compounds that control AR activity at certain promoters may allow more precision over its cellular responses. Many AR mutations in prostate cancer are frequently gain of function and may highlight gene pathways involved in tumor progression rather than normal cell differentiation. We used human and mouse tumor samples to show that AR mutations arise in response to therapy and elicit differential activity (2, 3). These AR mutations are useful tools in elucidating how AR function changes during disease progression. This proposal aims to connect differential AR activities to distinct gene expression programs and biological phenotypes. To accomplish this, we will study one mutation in the LBD (AR-R753Q) and one in the NTD (AR-E255K) because they rely on distinct mechanisms to alter AR activation (3). Combined analysis of these mutants may highlight common pathways that AR uses to drive oncogenic proliferation. Their locations in distinct AR domains may reveal ways to block both androgen-dependent and -independent AR activities by targeting the NTD as well as the LBD. Furthermore, distinguishing the cancer-promoting versus differentiative actions of AR may highlight gene subsets to be selectively repressed by novel compounds.

Body

Task 1: Generate stable cell lines that simultaneously express the mutant or wild-type ARs and suppress the endogenous AR.

Generate Stable Cell Lines. To express the mutant ARs and suppress endogenous AR, I generated two lentiviral vectors that will be co-infected into RWPE and VCaP cells. The first vector contains either previously validated shRNA to suppress endogenous AR or a non-targeting sequence as a control (4,5). The second vector contains mutant or wt ARs with a Flag epitope tag to distinguish transfected AR from endogenous AR that has escaped knockdown. Silent base changes engineered into the mutant ARs have made
them shRNA resistant. Two cell lines will be used that mimic early and late stage disease. AR-negative RWPE-1 cells are derived from normal prostate epithelia (6) and AR-positive VCaP cells are derived from a bone metastasis and express the TMPRSS2-ERG fusion (7). Virus was produced in HEK293T cells, and RWPE were transduced with a lentiviral vector containing wild-type AR (wt AR), AR-E255K or AR-R753Q. Stable cell lines were selected with hygromycin, and equivalent protein expression was confirmed by Western blotting (Fig. 1A). To confirm receptors respond to hormone, normal RWPE, empty vector, wt AR, AR-E255K and AR-R753Q cells were transfected with an ARE-responsive reporter and renilla as control. Cells were treated with 0 or 1 nM R1881 for 24 hours and lysed for luciferase assay. As expected, the ARs were responsive to hormone (Fig. 1B).

I have transduced cells with a lentiviral vector containing shRNA targeted towards AR, or scrambled control, to suppress AR. These cells are currently being transduced with lentiviral vectors containing the ARs.

**Task 2. Examine the biological effects of the mutant ARs using cell-based assays and xenografts.**

The biological effects of AR-E255K and AR-R753Q were first examined using the crystal violet assay to assess cell growth in response to hormone. Each RWPE cell line was plated into two sets of 96-well plates. The first set was assayed for crystal violet on Day 1 to serve as a baseline control. The second set was treated with 1 nM R1881 on Day 1 and assayed for crystal violet on Day 5. AR-E255K and AR-R753Q grew faster than wt AR (Fig 2). Anchorage independent growth is considered the “gold standard” predictor of in vivo tumorigenicity. To assess this, cells were embedded in agar and treated with 0, 0.1 nM or 1 nM R1881 for 14 days. Colonies were stained with MTT, photographed and enumerated (Fig 3). AR-E255K showed dose-dependent colony formation in response to hormone, emphasizing
the oncogenic potential of this mutant AR. Interestingly, the mouse AR-E231G, analogous to human AR-E255K is oncogenic as a transgene (8). Xenograft models will examine the tumorigenic capacity of the mutant ARs and validate *in vitro* results. Tumor samples will be valuable for examining the expression of known AR target genes in addition to validating genes identified through future microarray.

*Task 3: Compare the gene expression programs differentially regulated by the mutant ARs.*

**Mutant AR Gene Regulation.** Our ultimate goal is to use microarray and bioinformatic tools to identify gene pathways/networks differentially regulated by the mutant ARs. This should reveal common ways to target AR regardless of mutation. The use of both normal and carcinoma cells will reveal changes in gene expression as tumors progress. These data will allow us to connect mutant AR-regulated gene expression to their biological profiles determined in Task 2. Additionally, target genes will be used to validate compounds of interest identified in our drug screen in Task 4. Prior to microarray, alterations in known AR target genes, upregulated or suppressed by hormone, were analyzed in RWPE cells containing empty vector, wt AR, AR-E255K and AR-R753Q. Cells were treated with 0 or 1 nM R1881 for 24 hrs. RNA was collected and reverse transcribed and gene expression was analyzed using Real Time PCR. Three genes of interest were membrane metallo-endopeptidase (MME), an inhibitor of prostate cancer migration, aquaporin 3, which reduces the effectiveness of chemotherapy in prostate cancer, and follistatin, a promoter of prostate cancer growth. Results reveal MME is induced by wt AR to a greater extent compared to the mutant ARs. Aquaporin 3 is induced similarly by wt AR and AR-E255K but shows minimal induction by AR-R753Q. Follistatin shows most suppression by wt AR (Figure 3). Collectively, these data confirm that introduction of ARs into RWPE cells results in AR-target gene alterations and reveal differential activation by the various ARs. We will now generate gene profiles for each mutant and wt AR in RWPE cells using microarray. We will also validate changes in AR target gene expression in VCaP with wt and mutant ARs once established.
Task 4: Identify small molecules that alter AR activity in a promoter specific manner.

Generate vectors for high-throughput screening (HTS). The multiplexed promoter screen utilizes four promoters: multimerized cAREs driving a minimal thymidine kinase (tk) promoter, multimerized sAREs driving tk, a PSA enhancer/promoter (containing both cAREs and sAREs, and binding sites for other transcription factors), and the SV40 promoter (as a control lacking hormonal response). A similar screen successfully identified small molecules that selectively regulate glucocorticoid receptor activity (10). Our promoters were cloned upstream of fluorescent protein (FP) reporter genes for mCherry, citrine, mOrange2 or cerulean, obtained from Roger Tsien (UCSD) or Joel Swanson (UMichigan). This was accomplished by excising luciferase from pGL3-basic ARE reporters by digesting with NcoI and FseI, and replacing with FPs that had been amplified by PCR with primers containing NcoI or FseI restriction sites. For flexibility in determining the optimal ARE-FP combinations for screening, each FP was cloned downstream of each hormone-responsive promoter (cARE, sARE, PSA), resulting in 12 unique reporters (see Fig. 5A, B). To control for off-target and nonspecific effects, LacZ in the SV40-βgal vector was replaced with mCherry. Vector accuracy was verified by DNA sequencing.

HeLa cells containing a high level of endogenous AR were obtained from Elizabeth Wilson (9) and serve as the host cell line for ARE-FP transfection. The level of AR expression in HeLa is similar to VCaP, the cell line originally proposed for these studies (Fig. 5C). However, VCaP cells are not hearty enough to withstand the various manipulations involved in HTS. Another advantage of the HeLa-AR cells is that they contain a stably integrated PSA-luciferase, allowing us to use one less ARE-FP vector. To verify activation, several ARE-FP vectors were plated in charcoal stripped serum, treated with hormone and analyzed for activity using a flourimeter. As shown in Fig. 5D, cARE and sARE responded to hormone while SV40-driven mCherry levels were unaffected.
Fig 1. Reporters and host cells for screening. A) The androgen response elements (sAREs, cAREs, PSA) are upstream of luciferase in pGL3-basic vectors. Luc was removed using NcoI and FseI restriction sites (left). Each fluorescent protein (FP) coding region was amplified by PCR with primers (P1, P2) containing NcoI and FseI sites (middle). Each FP was inserted downstream of the AREs in pGL3 (right), generating 12 unique vectors (YFP – Citrine, OFP – mOrange, CFP – Cerulean) (B). A constitutive SV40-mCherry reporter (SV40-RFP) was generated similarly. C) Relative AR expression in VCaP and two HeLa lines with stably transfected AR. Whole cell lysates were resolved by SDS-PAGE and membranes from transferred gels probed with anti-AR (AR-N20) and rabbit secondary antibody. Note amounts of lysate loaded differ. D) HeLa-AR cells (high) were transiently transfected with cARE-eGFP, sARE-OFP or SV40-RFP, and treated with 100 nM R1881 or no hormone for 24 hrs. Lysates were read at appropriate excitation/emission wavelengths on a fluorimeter. Data is in relative fluorescent units.

Optimizing HTS. The fluorescent protein reporter assay was translated from 12-well to 384-well high-throughput screening format (HTS) at the University of Michigan Center for Chemical Genomics (CCG). Mass handling of cells was optimized for transfection efficiency and induction ratio, and several different fluorescent plate readers were tested for most sensitive signal detection and least overlap between wavelengths. These parameters were tested first for single reporters and subsequently for simultaneously transfected reporters in order to develop a multiplex assay.

The following protocol was used for all HTS experiments:
Day 1 – plate HeLA-AR cells in 10 cm dishes to adhere overnight.
Day 2 – transfect cells with reporters using xTREMEGENE HP transfection reagent.
Day 3 – replate cells into 384-well plates with a multidrop dispenser; treat with 100 nM R1881, 100 nM R1881 plus 100 µM bicalutamide, or no additional treatment
Day 4 – read fluorescent reporter expression or luminescence (for endogenous PSA-luc).

HeLa-AR cells did not survive in charcoal stripped serum and subsequent experiments were performed in full serum (FBS). This does not affect the results because the assay is carried out in saturating ligand, and we aim to identify ligands that suppress AR activity. Bicalutamide was used as a control for suppression.
The CCG has several instruments for reading fluorescent assays, including several plate readers and an image analysis system. After testing several machines, the EnSpire multimode monochromter (fluorescence) and the PHERAstar (luciferase) produced the best results. We also ruled out using ARE-driven mOrange or cerulean vectors due to poor induction of the two FPs. With a focus on citrine and EGFP, Fig. 6A shows cAREs and sAREs upstream of citrine (YFP) were strongly induced by hormone, an effect that was completely antagonized by bicalutamide. EGFP read more poorly, with less activation and suppression compared to basal expression in FBS. For the endogenous PSA-luciferase in the HeLa-AR cells, the PHERAstar detected high baseline luminescence due to plating in FBS, an effect completely suppressed by bicalutamide (Fig. 6B).

To determine crosstalk in reading multiple fluorescent reporters, sARE-citrine, cARE-EGFP and SV40-mCherry were individually transfected into HeLa-AR cells and fluorescence at each setting was read sequentially on the EnSpire (YFP, eGFP) and PHERAstar (mCherry) plate readers (Fig. 6C). Reporter fluorescence was not detected at nonoptimal settings (i.e., eGFP and mCherry do not read in the citrine channel), confirming absence of crosstalk among these FPs. Unfortunately, when sARE-YFP and cARE-EGFP were cotransfected and read sequentially, suppression by bicalutamide was greatly diminished for both reporters. Z scores, calculated for each reporter to define assay quality, suggest co-transfection of cAREs and sAREs would not sufficiently distinguish the effect of a compound from background noise (Z’ < 0.5).
**Pilot Screen.** To achieve the best separation between positive (R1881) and negative (bicalutamide) controls, thus increasing ability to identify hit compounds, we chose to screen cARE and sARE separately, each multiplexed with PSA-luciferase. I first ran a pilot screen to ensure that the signal window between controls was maintained in the presence of 0.5% DMSO, the vehicle used for the compound library. Cells transfected with cARE- or sARE-YFP were treated with R1881 and 0.5% DMSO or bicalutamide. Reporters were repressed by bicalutamide (Fig. 7), and Z’ scores were 0.66 for cARE-citrine, 0.53 for sARE-citrine and 0.62 for PSA-luciferase, acceptable for HTS.

![Fig. 7](image)

**HTS.** I performed a HTS screen of the Spectrum FDA-approved compound library, a collection of over 2000 small molecules, and the NIH library of 450 compounds. Each 384-well plate in the assay contained two columns of positive controls (suppression by bicalutamide) and 2 columns of negative controls (stimulation by R1881); each well in between received a test compound at 25 µM concentration.

![Fig. 8](image)

I used 75% suppression of ARE-driven citrine fluorescence, and less than 300 counts of PSA-luciferase, as values that signify AR antagonists. As shown in Fig. 8, of the 200 compounds suppressed AREs more than 75%, the majority suppressed cAREs and sAREs similarly. When PSA suppression is included in analysis, more evidence for differential effects of compounds appears. For selective suppression, we distinguished compounds that suppressed one ARE by more than 75% but the other by less than 25%. Interestingly, 22 compounds strongly suppressed cAREs but had little effect on sAREs. These compounds may potentially suppress cell proliferation but not differentiation. Eight compounds had the opposite effect and suppressed sAREs but not cAREs.
**Dose Response:** I performed dose response assays on 120 compounds that included: 1) compounds that suppressed on cAREs but not sAREs, 2) natural compounds, 3) compounds that suppressed on cAREs and PSA, 4) compounds that suppressed only on cAREs and 5) various chemotherapeutic agents, androgens and anti-androgens. Compounds were tested for suppression on cAREs because this is the reporter we aim to inhibit. The dose response contained two columns of positive controls (bicalutamide) and two columns of negative controls (R1881). The remainder of the plate contained each of the 120 compounds in eight duplicate doses. Of the 120 compounds, eight suppressed in a dose-dependent manner on cAREs, 24 on PSA and 46 on both reporters, summing to 78 compounds. Notably, of the 22 compounds that suppressed cAREs but not sAREs in the primary screen, four showed a dose response. Those four compounds included a chemotherapeutic agent, an interferon inducer, a natural compound and a MOA inhibitor. These drugs are being tested in our lab for their ability to selectively suppress cAREs, but not sAREs, and their effects on endogenous genes and proliferation.

**Key Research Accomplishments**

- I generated an RWPE cell line harboring wild-type and mutant ARs that can be used for future high-throughput gene expression analysis and xenograft models.
- I created vectors for a multiplexed reporter assay to identify compounds that selectively target AR activity in a high-throughput screen (HTS).
- I ran a primary HTS of 2000 compounds and identified 120 compounds of interest.
- I ran a dose response assay on the 120 compounds and selected several for follow-up screening in our lab.

**Reportable Outcomes**

- Compound information will be made publicly available once reported via publication.
- Screen data is currently available to other users of the Center for Chemical Genomics at the University of Michigan

**Conclusions**

There is therapeutic interest in understanding the tumor-promoting versus tumor-differentiating effects of AR in prostate cancer. Mutant ARs provide a way to study the unique and common pathways that AR uses to promote tumorigenesis. I have generated a cell line that stably expresses wild-type and mutant ARs in order to probe the biological effects and pathways characteristic of tumor progression to identify genes that should be targeted for therapy. This presents an opportunity to seek selective androgen receptor modulators (SARMs) that affect AR in a promoter-specific manner. To this end, I have optimized a compound screen that measures AR activity at multiple promoters simultaneously. I ran a primary screen of 2000 compounds, of which 22 suppressed cAREs but not sAREs. Dose response assays identified four compounds of interest that will be assessed for effects on endogenous gene expression and cell proliferation.
References