Synergistic Activity with NOTCH Inhibition and Androgen Ablation in ERG-Positive Prostate Cancer Cells

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Abstract

The oncogenic activation of the ETS-related gene (ERG) due to gene fusions is present in over half of prostate cancers in Western countries. Because of its high incidence and oncogenic role, ERG and components of ERG network have emerged as potential drug targets for prostate cancer. Utilizing gene expression datasets, from matched normal and prostate tumor epithelial cells, an association of NOTCH transcription factors with ERG expression status was identified, confirming that NOTCH factors are direct transcriptional targets of ERG. Inhibition of ERG in TMPRSS2-ERG-positive VCaP cells led to decreased levels of NOTCH1 and 2 proteins and downstream transcriptional targets and partially recapitulated the phenotypes associated with ERG inhibition. Regulation of NOTCH1 and 2 genes by ERG were also noted with ectopic ERG expression in LNCaP (ERG-negative prostate cancer) and RWPE-1 (benign prostate–derived immortalized) cells. Furthermore, inhibition of NOTCH by the small-molecule γ-secretase inhibitor 1, GSI-1, conferred an increased sensitivity to androgen receptor (AR) inhibitors (bicalutamide and enzalutamide) or the androgen biosynthesis inhibitor (abiraterone) in VCaP cells. Combined treatment with bicalutamide and GSI-1 showed strongest inhibition of AR, ERG, NOTCH1, NOTCH2, and PSA protein levels along with decreased cell growth, cell survival, and enhanced apoptosis. Intriguingly, this effect was not observed in ERG-negative prostate cancer cells or immortalized benign/prostate epithelial cells. These data underscore the synergy of AR and NOTCH inhibitors in reducing the growth of ERG-positive prostate cancer cells.

Implications: Combinational targeting of NOTCH and AR signaling has therapeutic potential in advanced ERG-driven prostate cancers. Mol Cancer Res; 1–10. ©2017 AACR.

Introduction

Radical prostatectomy or radiotherapies are effective for treatment of localized organ-confined prostate cancer, inhibition of the androgen receptor (AR), and the androgen biosynthesis remains the major therapeutic strategy for the treatment of metastatic disease (1–3). Androgen deprivation therapy (ADT) is effective initially, but the inevitable transition from ADT-responsive to castration-resistant prostate cancer (CRPC) remains the most significant challenge. Second-generation androgen axis inhibitors, such as abiraterone and enzalutamide, have significantly improved survival of patients with CRPC (4). However, the benefit is short-lived, and resistance to these drugs and treatment side effects usually develop (4, 5). There is an urgent need to continue to develop effective and novel strategies to inhibit AR as well as other prostate cancer drivers that contribute to prostate cancer progression (6, 7).

Frequent activation of the ETS-related gene (ERG) represents one of the most validated oncogenic alterations in prostate cancer (8–11). The androgen-dependent expression of ERG oncogene in more than half of all prostate cancers in Western countries plays a major role in the AR oncogenic network. Accumulating evidence has established that ERG is a key regulator that controls the activity of various signaling pathways recognized as key oncogenic drivers in various malignancies, including prostate cancer (12–14). Given the high incidence and mounting evidence supporting its oncogenic role, ERG and components of ERG network have emerged as promising drug targets for prostate cancer therapies (15–18). However, the prevailing notion is that oncogenic nuclear transcription factors, such as ERG, are challenging therapeutic targets. This scenario prompted us to consider an alternative strategy based on the understanding of the interface between ERG and...
Mechanistic investigations in TMPRSS2-ERG cancer suggested upregulation of NOTCH factors by ERG. Transcriptomes from ERG-positive and ERG-negative prostate and other functionally relevant pathways. Our initial evaluations of transcriptomes from ERG-positive and ERG-negative prostate cancer suggested upregulation of NOTCH factors by ERG. Mechanistic investigations in TMPRSS2-ERG-positive VCaP cells defined NOTCH1 and 2 receptors as direct transcriptional targets of the ERG. The NOTCH signaling pathway controls cell-fate decisions during development, including differentiation, proliferation, stem cell maintenance, and self-renewal of various cell types (19). The NOTCH signaling pathway has complex functions ranging from being tumor suppressor or as an oncogene in a specific cellular context as well as in a signal strength–dependent manner (20). NOTCH pathway has been reported to be involved in drug resistance (21). Pharmacologic inhibition of NOTCH signaling has been shown to increase drug sensitivity to conventional therapies of various types of tumors (22–31). Recently, Cui and colleagues have shown that inhibition of NOTCH signaling by γ-secretase Inhibitor (GSI-1) enhances the antitumor effect of docetaxel in prostate cancer (31). Although NOTCH1 mutations are associated with ERG overexpression in animal models and human T-cell acute lymphoblastic leukemia, direct regulation of NOTCH by ERG noted in this study has not been described before (32–34). This report further describes synergistic effects of NOTCH and androgen axis inhibitors on ERG-positive prostate cancer. The report also revealed unexpected observations of synergy between NOTCH and AR signaling inhibitors currently in clinical use.

Materials and Methods
Reagents
γ-Secretase inhibitor I (cat.# 565750) was purchased from Calbiochem/EMD Millipore. ERG mAb developed by our laboratory (ERG-MAb, 9FY) was obtained from Biocare Medical. Antibodies against AR (cat.# sc-816), GAPDH (cat.# sc-25778), and α-tubulin (cat.# sc-5286) were purchased from Santa Cruz Biotechnology. Anti-PSA (cat.# A056201-2) antibody was obtained from Dako cytometry. Antibodies against NOTCH1 (cat.# 3268), NOTCH2 (cat.# 4530), EMT Antibody Sampler Kit (cat.# 9782), and Apoptosis Antibody Sampler Kit (cat.# 9915) were purchased from Cell Signaling Technology. Sheep anti-mouse IgG-HRP (cat.# NXA931) and donkey-anti rabbit IgG-HRP (cat.# NA934) were from GE Healthcare. Bicalutamide (cat.# S1190), enzalutamide (cat.# S1250), and abiraterone (cat.# S1123) were purchased from Selleckchem.

Cell lines
VCaP, LNCaP, PC-3, DU145, and RWPE-1 cells were purchased from ATCC and were grown as recommended by the supplier. LAPC-4 and BPH-1 were generous gifts from Dr. Charles Sawyers (then at University of California at Los Angeles, Los Angeles, CA) and Dr. Simon Hayward (Vanderbilt University Medical Center, Nashville, TN), respectively. Cell lines obtained from ATCC have been authenticated and tested for mycoplasma contamination by the vendor using Short Tandem Repeat Profiling Kit (cat.# 135-XV) and Universal Mycoplasma Detection Kit (cat.# 30-1012K). Each cell line was passaged for fewer than 6 months after resuscitation. Reference data were not available for authentication of LAPC-4 and BPH-1 cell lines; hence, these cell lines were not authenticated.

Construction of lentiviral ERG expression vectors
LVX tet-on advanced vector system that includes the pLVX-tet-on plasmid with the tet inducible transactivator element (rtTR-advanced) and pLVX-Tight-puro plasmid harboring the tetracycline response element upstream of a minimal CaMV-multiple cloning site (MCS) cassette were obtained from Clontech Laboratories. TMPRSS2-ERG cDNA (35) was inserted into the pLVX-Tight-puro MCS, and the two plasmids were packaged; high-titer lentiviral particles containing the plasmids were generated and transfected into the HeLa cells. The cells were selected using G418 (800 μg/mL) and puromycin (2 μg/mL) for the retention of pLVX-tet-on and pLVX-Tight-puro plasmids (36). Stable transfectants of LNCaP (lentiviral TMPRSS2-ERG, LNCaP-LTE3) and RWPE1 (lentiviral TMPRSS2-ERG, RWPE1-LTE3) cells were maintained in RPMI1640 medium with 10% Tet-system approved FBS, and Iscove medium, respectively.

Inhibition of target genes by siRNA
Two or more different siRNAs were used for each gene transcript knockdown. ERG-specific siRNA sequences and conditions were previously reported by us (37). NOTCH1 (NOTCH1-si1: cat.# J-007771-10, and NOTCH1-si2: cat.# J-007771-12), NOTCH2 (NOTCH2-si1: cat.# J-012235-05, and NOTCH2-si2: cat.# J-012235-06), and nontargeting (NT) siRNA duplexes (cat.# D-001206-13-20) were purchased from Thermo Scientific/Dharmacon. Cells were cultured in their respective medium supplemented with 10% of fetal bovine serum for 48 hours followed by transfection with optimized dosage (50 nmol/L) of target siRNA or NT siRNA, using Lipofectamine 2000 (Life Technologies). Cells were harvested at desired time points post treatment and processed for Western blot analysis.

Immunoblot assays
Cells were lysed in Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) containing a protease inhibitor cocktail and phosphatase inhibitor cocktails I and III (Sigma). Cell lysates equivalent to 50 μg of total protein were separated on 4% to 12% bis-tris gel (Life Technologies) and transferred onto nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS) for 1 hour, followed by three 5-minute washes with wash buffer (1× PBST or 1× TBST) before treatment with secondary antibodies at room temperature for one hour. Finally, membranes were washed three times with wash buffer and developed with ECL detection reagent (GE Healthcare).

qRT-PCR
Total RNA prepared from ERG siRNA or NT siRNA-treated cells was reverse transcribed into cDNA by Omniscript RT Kit (Qiagen). Primers used are shown in Supplementary Table S1. Primers and probes were designed using the Primer3 online software (Martinsried, Germany).

Cell proliferation assay
Cells were grown in their respective media containing 10% FBS for 48 hours and then treated with indicated concentrations of drugs, either alone or in combination at the indicated time points. Cells were harvested by trypsinization at the indicated time points posttreatment, and viable cells were...
**Clonogenic cell survival assay**

VCaP cells (5 × 10^4 cells/10-cm dish) were plated using the recommended medium for 48 hours. Cells were then treated with the indicated concentrations of AR and NOTCH inhibitors as a single agent or in combination for 48 hours with replenishment of inhibitors every 24 hours. After 48 hours, the medium was removed, washed with 1× PBS, and replaced with regular growth medium. Cells were then allowed to recover for 14 days and evaluated for their survivability. The colonies that consisted of at least 50 cells were selected for assessment. Colonies were fixed with 4% paraformaldehyde, stained with crystal violet (0.5% w/v), and counted under inverted microscope.

**Chromatin immunoprecipitation assay**

VCaP cells transfected with 50 nmol/L of ERG siRNA or 50 nmol/L of NT siRNA were processed for chromatin immunoprecipitation (ChIP) assay as described previously (37). Amplification reactions were carried out on T-Gradient Thermoblock (Biomera) by using 95°C, 5 seconds; 95°C, 15 seconds; 54°C, 30 seconds; 72°C, 60 seconds program setting. For detecting genomic input DNA and specific ChIP products, 35 and 40 PCR amplification cycles were used, respectively.

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**Figure 1.**

**NOTCH1 and NOTCH2 are transcriptionally regulated by ERG.**

A, Association of NOTCH factors with ERG expression status. Association of ERG and NOTCH expression were evaluated in a cohort of well (WD) and poorly differentiated (PD) prostate cancer samples. B, Among the four NOTCH transcription factors, NOTCH2 showed significant association with ERG status. C, Decreases protein levels NOTCH1 and 2 (NICD: NOTCH intracellular domain) in response to knockdown of ERG in VCaP cells. D and E, Heterologous expression of TMPRSS2:ERG3 (ERG) under the control of stable integrant doxycycline (Dox)-inducible promoter resulted in increased protein levels of NOTCH1 and 2 in LNCaP (LNCaP-LTE3) cell line or in benign prostate-derived immortalized prostate epithelial cells (RWPE-1). F, ERG is recruited to predicted ERG-binding sites of NOTCH1 (NOTCH1_V$ETSF#1, NOTCH1_V$ETSF#2) and NOTCH2 (NOTCH2_V$ETSF#3) gene promoter upstream sequences, which was diminished in response to ERG siRNA treatment. The recruitment of ERG to the C-MYC promoter (37) was used as a positive and a distal upstream sequence within 15 kb of the NOTCH2 transcription initiation site lacking ETS motifs was as negative control. Input DNA was used as technical control. In quantitative ChIP PCR, the fold enrichment was calculated as the ratio of input normalized fold changes between NTsi (2^ΔΔCt) and ERGsi (2^ΔΔCt) values (48). G, Position of predicted ERG-binding sites (V$ETSF) relative to the 5' end of evaluated NOTCH1 and NOTCH2 gene promoter upstream sequences. Red circles, position of detected ERG recruitment.
ETS-binding sites within the target regions were identified (Supplementary Table S2) by matrix match analysis using the MatInspector software (Genomatix GmbH). Fold enrichment values were calculated by first normalizing the average fold changes of NT or ERGsi to the average of corresponding input values of target sequence amplicons (NOTCH1/V$ETS#1, NOTCH1/V$ETS#2, NOTCH2/V$ETS#3, and C-MYC (37)). Then the ratio of normalized fold changes between NT and ERGsi were calculated.

Correlation of ERG and NOTCH expression in human prostate cancer

Gene expression dataset from matched normal epithelial cells and prostate tumor epithelial cells with known ERG gene expression status previously developed in our laboratory (NCBI, GSE32448) was used to identify ERG-associated relevant onco-genes that could be utilized as surrogates of ERG-targeted therapy in prostate cancer (9, 38, 39). The transcriptomes were derived from tumor cells with well-to-moderately differentiated (n = 20) and poorly differentiated morphology (n = 20) representing two groups of prostate tumor types.

Treatment of cells with NOTCH and AR inhibitors

To evaluate the combined effect of the GSI-1 and the AR inhibitors, cells were grown in recommended media containing 10% FBS for 48 hours. After 48 hours at confluency of 50%, cells were then treated with the indicated concentrations of the drugs (0, 1, 5, or 10 μmol/L), either alone or in combination at the indicated time points. Initially other γ-secretase inhibitors, including DAPT, MK-0752, semagacestat, and R04929097 (all from Selleckchem), were also evaluated for the synergistic effect with the AR inhibitors, but only GSI-1 showed significant synergy with AR inhibitors and was selected for further evaluation.
Statistical analysis
Two-tailed Student’s t test was used to compare between specific groups within a dataset. \( P < 0.05 \) was considered statistically significant difference. Data are presented as the mean ± SEM.

Results

**NOTCH2 expression in prostate cancer correlates with TMPRSS2-ERG status**
To identify functionally and therapeutically relevant targets of ERG oncogene for prostate cancer, an association study was performed in a gene expression dataset from laser capture microdissected matched prostate tumor and normal epithelial cells. We evaluated the correlation between tumor/benign normalized NOTCH1, NOTCH2, and ERG expression status in the gene expression dataset analysis of 20 well-to-moderately differentiated and 20 poorly differentiated prostate cancer tumors (37–39). The results revealed that the expression of NOTCH2 correlated with ERG expression, whereas the expression of NOTCH1, NOTCH3, and NOTCH4 showed no significant correlation with ERG (Fig. 1A). Fifty-seven percent of ERG-positive samples expressed NOTCH2 (15/26) versus 35% of the ERG-negative samples (5/16; Fig. 1B).

**NOTCH transcription factors are direct targets of ERG**
To further understand the regulation of NOTCH transcription factors by ERG, we performed Western blot analyses on ERG siRNA-treated VCaP cells. Both NOTCH1 and NOTCH2 protein expression levels were reduced in response to inhibition of ERG by the siRNA (Fig. 1C). This effect was even more pronounced in sustained inhibition of ERG expression over 8 days. The functional connection between ERG and NOTCH1 and NOTCH2 was further examined in stable transfectants of LNCaP or RWPE-1 cells harboring doxycycline-inducible TMPRSS2-ERG3 lentiviral expression vector (LNCaP-LTE3 and RWPE1-LTE3). NOTCH1 and NOTCH2 protein levels were increased in response to doxycycline-induced ERG expression (Fig. 1D and E). To gain further insights into regulation of NOTCH transcription factors by ERG, we performed ChIP analysis examining the promoter upstream regions of the NOTCH1 and 2 genes. Using MatInspector software (Genomatix GmbH), a matrix match survey of ~1,500-bp promoter upstream sequences of the NOTCH1 and NOTCH2 genes identified ETS-matrix matches, which are potential binding sites for ERG. ChIP assay employing VCaP cells confirmed the specific recruitment of ERG oncoprotein to multiple distinct clusters of ETS sites upstream of the NOTCH1 and NOTCH2 promoters (see Supplementary Table S2 for locations of the ETS-binding sites).

**Figure 3.**
Increased sensitivity of ERG-positive tumor cells to AR inhibitors in combination with NOTCH inhibitor. **A–C,** Top, treatment of VCaP cells with NOTCH inhibitor, GSI-1, in combination with either abiraterone (Abi), bicalutamide (Bic), or enzalutamide (Enz) augmented their effects on the inhibition of AR, ERG, PSA, NOTCH1, and NOTCH2 expression and induced cleavage of PARP1 and caspase-7. **A–C,** Bottom, GSI-1 increases sensitivity of ERG-positive prostate cancer cells to AR inhibitors. Concomitant treatment of GSI-1 with abiraterone, bicalutamide, or enzalutamide significantly enhanced the inhibition of cell growth in VCaP cells. Each experiment was performed three times, and results are shown as mean ± SD; 
\( \ast \), \( P > 0.05 \); 
\( \ast \ast \), \( P < 0.05 \).
The recruitment of ERG to these sites was significantly reduced in response to ERG siRNA knockdown (Fig. 1F and G). The recruitment of ERG suggests involvement of ERG oncoprotein in the regulation of NOTCH1 and NOTCH2 in prostate tumor cells.

ERG modulates EMT through NOTCH signaling

To determine the role of NOTCH transcription factors in ERG-positive prostate cancer cell lines, inhibition of ERG, NOTCH1, and NOTCH2 alone, or in combination was examined on cell growth and cell morphology. We have reported previously that suppressing ERG expression induced the differentiation markers of prostate epithelial cells (37). Inhibition of ERG and NOTCH1, either alone or in combination, resulted in the inhibition of the mesenchymal markers N-cadherin and vimentin. Simultaneous knockdown of ERG and NOTCH1 resulted in a marked increase of the epithelial marker Claudin-1 (Fig. 2A). Overexpression of ERG protein in the immortalized benign prostate epithelial cells, RWPE-1, resulted in the concomitant overexpression of mesenchymal markers N-cadherin and vimentin, and inhibition of the epithelial marker, Claudin-1 (Fig. 2B).

Inhibition of ERG and NOTCH abrogate tumor cell growth

To assess the effect of ERG and NOTCH1 and NOTCH2 on cell proliferation, we treated VCaP cells with ERG, NOTCH1, NOTCH2-specific siRNAs or NT siRNA, either alone or in combination as shown in Fig. 2C1 and C2. Knockdown of NOTCH1 or NOTCH2 alone in VCaP cells by siRNA did not show significant cell growth inhibition (Fig. 2C1 and C2). However, inhibition of NOTCH1 or NOTCH2 resulted in a characteristic change in cell morphology similar to the morphology observed in response to ERG inhibition, resembling reversal of the mesenchymal and nonmalignant epithelial shape in VCaP cells (Fig. 2C1). These findings are consistent with reports on the modulation of epithelial–mesenchymal

Figure 4.
GSI-1 does not augment the inhibition of cell growth by AR inhibitors in benign or transformed prostate epithelial cells, or in ERG-negative prostate cancer cell lines, irrespective of their AR expression status. Simultaneous treatment of GSI-1 with AR inhibitors (either abiraterone, bicalutamide, or enzalutamide) did not show any synergistic effect in benign prostate epithelial cells, RWPE-1, transformed prostate epithelial cells, BPH-1-1, and a panel of ERG-negative prostate cancer cell lines, regardless whether they are AR positive, LNCaP, LAPC-4, or AR negative, DU145 and PC-3.

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transition (EMT) by NOTCH transcription factors in several cancer types.

Inhibition of NOTCH selectively enhances the effect of AR inhibitors in ERG-positive prostate cancer cells

The NOTCH inhibitor, γ-secretase inhibitors-1 (GSI-1) exhibited inhibition of NOTCH1 and NOTCH2 protein levels and VCaP prostate cancer cell growth in a dose-dependent manner (Fig. 2D1–D3). An unexpected observation of this study was the inhibition of AR and ERG by the GSI-1, suggesting for other, as yet unknown, effects of this inhibitor (Fig. 2D2). This prompted us to assess the inhibition of ERG-positive prostate cancer cell growth by combining AR axis inhibitors used for the ADT (abiraterone, or bicalutamide, or enzalutamide) with GSI-1. Combination of low dose of GSI-1 with each of the AR axis inhibitors resulted in enhanced inhibition of the ERG and also AR, PSA, NOTCH1, and NOTCH2 protein levels. In addition to these observations, significant reduction of cell growth and increased apoptosis was noted as shown by the presence of cleaved PARP1 and cleaved caspase-7 (Fig. 3A–C). However, combination of GSI-1 and enzalutamide did not induce cleaved caspase-7, indicating a different mechanism of apoptosis induction by this treatment. Next, a panel of ERG-negative prostate or prostate cancer cell lines that included transformed or benign prostate epithelium derived cells was tested with a combination of GSI-1 and AR axis inhibitors (abiraterone, bicalutamide, or enzalutamide). In contrast to the response in ERG-positive VCaP cells, no appreciable inhibition of cell growth was seen in these cells using the same drug combinations (Fig. 4). These results suggested selectivity of NOTCH and AR inhibitor combinations for ERG-positive prostate cancer cells.

Combination of AR and NOTCH inhibitors delay survival colony formation of VCaP cells

To further evaluate the ability of VCaP cell survival after treatment with combination of AR and NOTCH inhibitors, survival colony formation assay was employed for VCaP cells treated with the AR and NOTCH inhibitors in combination or as a single agent for 48 hours. Combination of low concentrations of GSI-1 and bicalutamide or enzalutamide resulted in significant reduction in cell growth and cell survival when compared with mock treated control cells (Fig. 5A and B).

Discussion

The dysregulation and high frequency of ERG expression and function in prostate cancer and other malignancies have

![Figure 5](image_url)

Combination of GSI-1 and AR inhibitors delays colony survival formation. A and B, Treatment of VCaP cells with NOTCH inhibitor, GSI-1, in combination with either bicalutamide or enzalutamide significantly inhibited survival colony formation capability of VCaP cells. Colonies with more than 50 cells were counted (right bar diagrams). Results are shown as mean ± SD of triplicate experiments; *, P > 0.05; **, P < 0.05.
demonstrated ERG protein and its functional network into promising candidates for ERG-targeted therapeutic intervention. As a transcription factor, ERG biochemical and biological functions are diverse, including maintenance of cellular differentiation and stem cell phenotypes. Within the context of cancer, ERG onco-genic activation influences cancer biology, for example, activation of cell invasion and other procancer signaling pathways, and inhibits cell differentiation (11).

Positive correlation between ERG and NOTCH2 expression was found assessing laser capture microdissected human prostate cancer tumor/normal matched transcriptome datasets. Although correlation with NOTCH1, 3, and 4 was not apparent, we have evaluated all known NOTCH transcription factors in ERG-positive (TMPRSS2-ERG harboring) VCaP cells. Evaluation by ERG knockdown and overexpression approaches suggests that NOTCH1 and NOTCH2 are positively regulated by ERG. In contrast, NOTCH3 and NOTCH4 were nonresponsive to ERG overexpression or knockdown.

The NOTCH signaling pathway is critical in controlling cell-fate decisions during development (19, 40). The ligand-driven NOTCH signaling is activated when the NOTCH receptors of a signal-sending cell physically interact with a signal-receiving cell through receptor–ligand interaction. Ligand binding triggers a succession of proteolytic events, whereby NOTCH receptor is cleaved twice, first by an extracellular matrix metalloprotease (TNFα-converting enzyme, TACE) and then by the transmembrane protease γ-secretase complex. Thus, γ-secretase inhibitor (GSI-1) has been studied for NOTCH targeted treatment of human malignancies (41). NOTCH signaling also plays crucial role in the development of both normal prostate gland and prostate cancer (42, 43). Using Notch1 knockout mouse model, it was shown that Notch1-expressing cells are indispensable for prostate branching morphogenesis, growth, differentiation, and regrowth, suggesting its role in defining progenitor cells in the prostate (43).

In an earlier study, we showed that ERG negatively regulates the expression of a number of prostate differentiation genes and abrogates the prostate epithelial differentiation program (37). In the current study, we further extend these observations by showing that expression of ERG is directly correlated to the expression of NOTCH1 and NOTCH2 factors. Our analysis of the promoter upstream regions of NOTCH1 and NOTCH2 genes showed the specific recruitment of ERG protein to binding sites for ERG, suggesting the likely basis for

Figure 6. Synergistic inhibition of NOTCH and ERG signaling reverses EMT. A and B, The concurrent inhibition of AR pathway and NOTCH signaling by GSI-1 and either bicalutamide (Bic) or enzalutamide (Enz) induces epithelial markers Claudin-1, E-cadherin, and β-catenin, indicating activation of MET and inhibition of EMT. This might be responsible for the observed enhanced synergy between AR inhibitors and GSI-1. C and D, The proposed mechanism is that inhibition of AR’s tumor-promoting function in combination with the protumorigenic functions of NOTCH is a more effective therapeutic approach than any of the compounds alone.
activation by ERG. The knockdown of either NOTCH1 or NOTCH2 or both in TMPRSS2-ERG–positive prostate cancer cells recapitulated the phenotypes associated with ERG knockdown. Moreover, we demonstrate that ERG regulates EMT in part through NOTCH signaling pathway, and increased NOTCH2 is associated with ERG expression in a cohort of prostate cancer patients.

Our investigations revealed an unexpected finding showing that C5I-1 inhibited ERG, AR, and AR targets only in ERG-positive prostate cancer cells. This prompted us to assess potential synergistic effect of a combination of NOTCH and AR inhibitors. When VCaP cells are treated with low doses of the C5I-1 in combination with AR inhibitors, we observed the enhanced inhibition of ERG, AR, PSA, NOTCH1, and NOTCH2 proteins, the induction of apoptosis, and significant cell growth inhibition (Fig. 3). These effects were not observed in a panel of similarly treated ERG-negative prostate cancer or benign or normal transformed prostate epithelial cells regardless of their AR status (Fig. 4). Among the evaluated AR inhibitors, bicalutamide showed most robust synergistic effect with C5I-1. These findings imply that pharmacologic inhibition of both NOTCH and AR could be used effectively to treat ERG-positive prostate cancer patients.

In summary, the results presented in this study show that there is a “functional cross-talk” between AR, ERG, NOTCH signaling pathways. This finding further enabled us to uncover the synergistic effect of a combination of NOTCH and AR inhibitors on ERG-positive prostate cancer cells. The mechanism responsible for the observed synergy deserves more investigation and is the subject of follow-up study. However, we propose that the observed synergy is due to induction of mesenchymal–epithelial transition and inhibition of EMT markers, which in turn sensitizes cells to the drugs (Fig. 6); as has been reported before, activation of NOTCH signaling is essential for the maintenance of EMT and cancer stem cells (44–47). We propose that the therapeutic approach involving a combination of NOTCH and AR inhibitors will be of potential utility in the treatment of advanced prostate cancer with ERG defects.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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The views expressed in this article are those of the authors and do not reflect the official policy of the Department of the Army, Department of Defense, or the U.S. Government.

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Acknowledgments
The authors are grateful to all members of the CPDR, USU, HIF, and especially Stephen Doyle for the artwork and Chantal Falade for administrative support.

Grant Support
This work was supported in part by CPDR-UISSI program HU0001-10-2-0002 (to D.G. McLeod) and NIH grants RO1 DK065977 (to S. Srivastava).

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Received February 1, 2017; revised May 4, 2017; accepted June 6, 2017; published OnlineFirst June 12, 2017.

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Mol Cancer Res  Published OnlineFirst June 12, 2017.

Updated version  Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-17-0058

Supplementary Material  Access the most recent supplemental material at:
http://mcr.aacrjournals.org/content/suppl/2017/06/10/1541-7786.MCR-17-0058.DC1

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