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

Ahmed A. Mohamed[1], **Shyh-Han Tan**[1], **Charles P. Xavier**[1], **Shilpa Katta**, **Wei Huang**[1], **Lakshmi Ravindranath**[1], **Muhammad Jamal**[1], **Hua Li**[1], **Meera Srivastava**[2], **Eri Srivatsan**[3], **Taduru Sreenath**[1], **David G. McLeod**[1], **Alagarsamy Srinivasan**[1], **Gyorgy Petrovics**[1], **Albert Dobi**[1,*], and **Shiv Srivastava**[1,*]

[1]Center for Prostate Disease Research, Department of Surgery, Uniformed Services University of the Health Sciences and Walter Reed National Military Medical Center, Bethesda, MD

[2]Department of Anatomy, Physiology and Genetics, Uniformed University of Health Sciences, Bethesda, MD

[3]Division of General Surgery, Department of Surgery, VAGLAHS/David Geffen School of Medicine at University of California at Los Angeles, Los Angeles, California, USAJonsson Comprehensive Cancer Center, University of California at Los Angeles, Los Angeles, CA

Abstract

The oncogenic activation of the ETS related gene (*ERG*) due to gene fusions is present in over half of prostate cancer (CaP) in Western countries. Due to its high incidence and oncogenic role, ERG and components of ERG network have emerged as potential drug targets for CaP. 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 NOTCH-1 and -2 proteins and downstream transcriptional targets and partially recapitulated the phenotypes associated with ERG inhibition. Regulation of *NOTCH-1* and *-2* genes by ERG were also noted with ectopic ERG expression in LNCaP (ERG-negative CaP) and RWPE-1 (benign prostate derived immortalized) cells. Furthermore, inhibition

of NOTCH by the small molecule gamma-Secretase inhibitor 1, GSI-1, conferred an increased sensitivity to androgen receptor (AR) inhibitors (Bicalutamide, 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/normal prostate epithelial cells. These data underscore the synergy of AR and NOTCH inhibitors in reducing the growth of ERG-positive CaP cells.

Implications—Combinational targeting of NOTCH and AR signaling has therapeutic potential in advanced ERG-driven prostate cancers.

Keywords

ERG; Oncogene; NOTCH; small molecule; inhibitor; prostate cancer

Introduction

Radical prostatectomy or radiation therapies are effective for treatment of localized organ-confined prostate cancer (CaP), 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 (CRCP) 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 develops (4,5). There is an urgent need to continue to develop effective and novel strategies to inhibit AR as well as other CaP drivers which contribute to CaP progression (6,7).

Frequent activation of the *ETS* Related Gene (ERG) represents one of the most validated oncogenic alterations in CaP (8–11). The androgen dependent expression of ERG oncogene in more than half of all CaPs 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 CaP (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 CaP 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 other functionally relevant pathways. Our initial evaluations of transcriptomes from ERG positive and ERG negative CaP suggested up regulation of NOTCH factors by ERG. Mechanistic investigations in *TMPRSS2-ERG* positive VCaP cells defined NOTCH 1 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). Pharmacological inhibition of NOTCH signaling has been shown to increase drug sensitivity to conventional therapies of various types of tumors (22–31). Recently Cui *et al* have shown that inhibition of NOTCH signaling by Gamma-Secretase Inhibitor (GSI-1) enhances the antitumor effects of Docetaxel in CaP (31).

Although NOTCH 1 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 CaP. The report also revealed unexpected observations of synergy between NOTCH and AR signaling inhibitors currently in clinical use.

Materials and Methods

Reagents

Gamma-Secretase inhibitor I (cat.# 565750) was purchased from Calbiochem/EMD Millipore (Billerica, MA). ERG monoclonal antibody developed by our laboratory (ERG-MAb, 9FY) was obtained from Biocare Medical (Concord, CA). Antibodies against AR (cat.# sc-816), GAPDH (cat.# sc-25778), and α-Tubulin (cat.# sc-5286) were purchased from Santa Cruz (Santa Cruz, CA). Anti-PSA (cat.#A056201-2) antibody was obtained from DAKO cytomation (Carpinteria, CA). 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 Technologies (Danvers, MA). Sheep anti-mouse IgG-HRP (cat #NXA931) and donkey-anti rabbit IgG-HRP (cat #NA934) were from GE Healthcare (Fairfield, CT). Bicalutamide (cat #S1190), Enzalutamide (cat # S1250) and Abiraterone (cat # S1123) were purchased from Selleckchem (Houston, TX).

Cell Lines

VCaP, LNCaP, PC-3, DU145, and RWPE-1 cells were purchased from the American Tissue Culture Collection (ATCC, Manassas, VA) 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) and Dr. Simon Hayward (Vanderbilt University Medical Center), respectively. Cell lines obtained from ATCC have been authenticated and tested for Mycoplasma contamination by the vendor using Short Tandem Repeat (STR) Profiling kit (cat.# 135-XV) and Universal Mycoplasma Detection Kit (cat.#30-1012K). Each cell line was passaged for fewer than six 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 (TRE) upstream of a minimal CMV-multiple cloning site

(MCS) cassette were obtained from Clontech (Clontech, San Diego, CA). *TMPRSS2-ERG3* cDNA(35) was inserted into the pLVX-Tight-puro MCS, 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 RPMI 1640 medium with 10% Tet-system approved FBS, and KSFM medium, respectively.

Inhibition of target genes by small interfering RNA

Two or more different siRNA 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 si-2: cat.# J-007771-12), *NOTCH2* (NOTCH2 si-1: cat.#J-012235-05, and NOTCH2 si-2: cat.# J-012235-06) and non-targeting (NT) siRNA duplexes (cat.# D-001206-13-20). All siRNAs were purchased from Thermo Scientific/Dharmacon (Lafayette, CO). Cells were cultured in their respective medium supplemented with 10% of fetal bovine serum for 48 hours followed by transfection with optimized dosage (50 nM) of target siRNA or NT siRNA, using Lipofectamine 2000 (Life Technologies, Carlsbad, CA). 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 (M-PER) (Thermo, Rockford, IL) containing a protease inhibitor cocktail and phosphatase inhibitor cocktails I & III (Sigma, St Louis, MO). Cell lysates equivalent to 50 μg of total protein were separated on 4%–12% bis-tris gel (Life Technologies, Carlsbad, CA) and transferred to PVDF membrane (Life Technologies, Carlsbad, CA). Membranes were incubated with the primary antibodies at 4°C for 12 hours, followed by three 5-minute washes with wash buffer (1xPBST or 1xTBST) before treatment with secondary antibodies at room temperature for one hour. Finally, membranes were washed three times with wash buffer and developed with ECL Western blot detection reagent (GE Healthcare, Fairfield, CT).

Quantitative Reverse Transcription- Polymerase Chain Reaction (QRT-PCR)

Total RNA prepared from ERG siRNA or NT siRNA treated cells was reverse transcribed into cDNA by OmniScript® RT Kit (QIAGEN, Germantown, MD). Primers used are shown in Supplementary Table 1. 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 post-treatment and viable cells were counted by hemocytometer by using trypan blue exclusion method (Cat. # 15250-061, Life Technologies, Carlsbad, CA).

Clonogenic cell survival assay

VCaP cells (50K cell/10cm 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 1xPBS and replaced with regular growth medium. Cells were then allowed to recover for 14 days and evaluated for their survivability. The colonies which consisted of at least 50 cells were selected for assessment. Colonies were fixed with 4% Paraformaldehyde (PF), stained with crystal violet (0.5% w/v), and counted under inverted microscope.

Chromatin Immuno-precipitation (ChIP) assay

VCaP cells transfected with 50 nM of *ERG* siRNA or 50 nM of NT siRNA were processed for ChIP assay as previously described (37). Amplification reactions were carried out on T-Gradient Thermoblock (Biometra, Göttingen, Germany) by using 95°C, 5 s; ,95°C, 15 s; 54°C 30 s; 72°C 60 s program setting. For detecting genomic input DNA and specific ChIP products 35 and 40 PCR amplification cycles were used, respectively. ETS binding sites within the target regions were identified (Supplementary Table 2) by matrix match analysis using the MatInspector software (Genomatix GmbH, Munich, Germany). 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) were used to identify ERG associated relevant oncogenes that could be utilized as surrogates of ERG targeted therapy in CaP (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 10μM), 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, Houston, TX) were also evaluated for the synergistic effect with the AR inhibitors but only GSI-1 show significant synergy with AR inhibitors and selected for further evaluation.

Statistical analysis

Two-tailed Student's t-test was used to compare between specific groups within a data set. $P < 0.05$ was considered statistically significant difference. Data are presented as the mean ± standard error of the mean.

Results

NOTCH2 expression in prostate cancer correlates with *TMPRSS2-ERG* status

To identify functionally and therapeutically relevant targets of *ERG* oncogene for CaP, 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 (WD) and 20 poorly-differentiated (PD) CaP tumors (37–39). The results revealed that the expression of *NOTCH2* correlated with the expression of *ERG* expression while the expression of *NOTCH1, NOTCH3* and *NOTCH4* showed no significant correlation with *ERG* expression (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 eight 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&E). To gain further insights into regulation of NOTCH transcription factors by ERG, we performed Chromatin Immuno-precipitation (ChIP) analysis examining the promoter upstream regions of the *NOTCH1* and 2 genes. Using MatInspector software (Genomatix GmbH, Munich, Germany), a matrix match survey of −1,500bp 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 2 for locations of the ETS binding sites). The recruitment of ERG to these sites was significantly reduced in response to ERG siRNA- knock-down (Fig. F and G). The recruitment of ERG suggests involvement of ERG oncoprotein on 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 CaP 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&C2. Knock-down of *NOTCH1* or *NOTCH2* alone in VCaP cells by siRNA did not show significant cell growth inhibition (Fig. 2C1&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 non-malignant epithelial shape in VCaP cells (Fig. 2C1). These findings are consistent with reports on the modulation of 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 were tested with a combination of GSI-1 and AR axis inhibitors (Abiraterone, Bicalutamide, or Enzalutamide). In contrast to the observations 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 to mock treated control cells (Fig. 5A&B).

Discussion

The dysregulation and high frequency of *ERG* expression and function in CaP and other malignancies have demonstrated ERG protein and its functional net-work 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 oncogenic activation influences cancer biology; e.g, activation of cell invasion and other pro-cancer 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 *NOTCH*1, 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 non-responsive to *ERG* overexpression or knockdown.

The NOTCH signaling pathway is critical in controlling cell-fate decisions during development (19,40). The ligands driven NOTCH signaling is activated when the NOTCH receptors of a signal-sending cell physically interacts 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 (tumor necrosis factor-α-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* knock-out mouse model it was shown that Notch1- expressing cells are indispensable for prostate branching morphogenesis, growth, differentiation and re-growth, 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 activation by ERG. The knock-down of either *NOTCH1* or *NOTCH2* or both in *TMPRSS2-ERG* positive CaP cells recapitulated the phenotypes associated with *ERG* knock-down. 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 GSI-1 inhibited ERG, AR and AR targets only in ERG positive CaP 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 GSI-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 GSI-1. These findings imply that pharmacological inhibition of both NOTCH and AR could be used effectively to treat ERG positive CaP 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 (MET) and inhibition of Epithelial-Mesenchymal Transition (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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

AR	Androgen Receptor
ERG	EST Related Gene
PSA	Prostate Specific Antigen

GSI-1	Gamma-Secretase Inhibitor 1
CaP	Prostate Cancer
ADT	Androgen Ablation Therapy
CRPC	Castration Resistant Prostate Cancer
EMT	Epithelial-Mesenchymal Transition
MET	Mesenchymal-Epithelial Transition
FBS	Fetal Bovine Serum

References

1. Rodrigues DN, Boysen G, Sumanasuriya S, Seed G, Marzo AM, de Bono J. The molecular underpinnings of prostate cancer: impacts on management and pathology practice. J Pathol. 2016; doi: 10.1002/path.4826.

2. Yap TA, Smith AD, Ferraldeschi R, Al-Lazikani B, Workman P, de Bono JS. Drug discovery in advanced prostate cancer: translating biology into therapy. Nat Rev Drug Discov. 2016; 15(10):699–718. DOI: 10.1038/nrd.2016.120 [PubMed: 27444228]

3. Sharp A, Welti J, Blagg J, de Bono JS. Targeting Androgen Receptor Aberrations in Castration-Resistant Prostate Cancer. Clin Cancer Res. 2016; 22(17):4280–2. DOI: 10.1158/1078-0432.CCR-16-1137 [PubMed: 27330057]

4. Francini E, Taplin ME. Prostate cancer: Developing novel approaches to castration-sensitive disease. Cancer. 2016; doi: 10.1002/cncr.30329.

5. Watson PA, Arora VK, Sawyers CL. Emerging mechanisms of resistance to androgen receptor inhibitors in prostate cancer. Nat Rev Cancer. 2015; 15(12):701–11. DOI: 10.1038/nrc4016 [PubMed: 26563462]

6. Barbieri CE, Chinnaiyan AM, Lerner SP, Swanton C, Rubin MA. The Emergence of Precision Urologic Oncology: A Collaborative Review on Biomarker-driven Therapeutics. Eur Urol. 2016; doi: 10.1016/j.eururo.2016.08.024.

7. Roychowdhury S, Chinnaiyan AM. Translating genomics for precision cancer medicine. Annual review of genomics and human genetics. 2014; 15:395–415. DOI: 10.1146/annurev-genom-090413-025552

8. Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW, et al. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science. 2005; 310(5748):644–8. DOI: 10.1126/science.1117679 [PubMed: 16254181]

9. Petrovics G, Liu A, Shaheduzzaman S, Furusato B, Sun C, Chen Y, et al. Frequent overexpression of ETS-related gene-1 (ERG1) in prostate cancer transcriptome. Oncogene. 2005; 24(23):3847–52. DOI: 10.1038/sj.onc.1208518 [PubMed: 15750627]

10. Barbieri CE, Rubin MA. Genomic rearrangements in prostate cancer. Curr Opin Urol. 2015; 25(1):71–6. DOI: 10.1097/MOU.0000000000000129 [PubMed: 25393273]

11. Sreenath TL, Dobi A, Petrovics G, Srivastava S. Oncogenic activation of ERG: A predominant mechanism in prostate cancer. J Carcinog. 2011; 10:37.doi: 10.4103/1477-3163.91122 [PubMed: 22279422]

12. Adamo P, Ladomery MR. The oncogene ERG: a key factor in prostate cancer. Oncogene. 2016; 35(4):403–14. DOI: 10.1038/onc.2015.109 [PubMed: 25915839]

13. Abou-Ouf H, Zhao L, Bismar TA. ERG expression in prostate cancer: biological relevance and clinical implication. J Cancer Res Clin Oncol. 2016; 142(8):1781–93. DOI: 10.1007/s00432-015-2096-x [PubMed: 26711283]

14. Huang Y, Thoms JA, Tursky ML, Knezevic K, Beck D, Chandrakanthan V, et al. MAPK/ERK2 phosphorylates ERG at serine 283 in leukemic cells and promotes stem cell signatures and cell proliferation. Leukemia. 2016; 30(7):1552–61. DOI: 10.1038/leu.2016.55 [PubMed: 27055868]

15. Rahim S, Minas T, Hong SH, Justvig S, Celik H, Kont YS, et al. A small molecule inhibitor of ETV1, YK-4-279, prevents prostate cancer growth and metastasis in a mouse xenograft model. PLoS One. 2014; 9(12):e114260.doi: 10.1371/journal.pone.0114260 [PubMed: 25479232]

16. Brenner JC, Ateeq B, Li Y, Yocum AK, Cao Q, Asangani IA, et al. Mechanistic rationale for inhibition of poly(ADP-ribose) polymerase in ETS gene fusion-positive prostate cancer. Cancer cell. 2011; 19(5):664–78. DOI: 10.1016/j.ccr.2011.04.010 [PubMed: 21575865]

17. Wang S, Kollipara RK, Humphries CG, Ma SH, Hutchinson R, Li R, et al. The ubiquitin ligase TRIM25 targets ERG for degradation in prostate cancer. Oncotarget. 2016; 7(40):64921–31. DOI: 10.18632/oncotarget.11915 [PubMed: 27626314]

18. Wang X, Qiao Y, Asangani IA, Ateeq B, Poliakov A, Cieslik M, et al. Development of Peptidomimetic Inhibitors of the ERG Gene Fusion Product in Prostate Cancer. Cancer cell. 2017; 31(4):532–48. e7. DOI: 10.1016/j.ccell.2017.02.017 [PubMed: 28344039]

19. LaFoya B, Munroe JA, Mia MM, Detweiler MA, Crow JJ, Wood T, et al. Notch: A multi-functional integrating system of microenvironmental signals. Developmental biology. 2016; 418(2):227–41. DOI: 10.1016/j.ydbio.2016.08.023 [PubMed: 27565024]

20. Guruharsha KG, Kankel MW, Artavanis-Tsakonas S. The Notch signalling system: recent insights into the complexity of a conserved pathway. Nat Rev Genet. 2012; 13(9):654–66. DOI: 10.1038/nrg3272 [PubMed: 22868267]

21. Takebe N, Miele L, Harris PJ, Jeong W, Bando H, Kahn M, et al. Targeting Notch, Hedgehog, and Wnt pathways in cancer stem cells: clinical update. Nat Rev Clin Oncol. 2015; 12(8):445–64. DOI: 10.1038/nrclinonc.2015.61 [PubMed: 25850553]

22. Majidinia M, Alizadeh E, Yousefi B, Akbarzadeh M, Zarghami N. Downregulation of Notch Signaling Pathway as an Effective Chemosensitizer for Cancer Treatment. Drug Res (Stuttg). 2016; 66(11):571–9. DOI: 10.1055/s-0042-111821 [PubMed: 27701712]

23. Yahyanejad S, Theys J, Vooijs M. Targeting Notch to overcome radiation resistance. Oncotarget. 2016; 7(7):7610–28. DOI: 10.18632/oncotarget.6714 [PubMed: 26713603]

24. Su F, Zhu S, Ruan J, Muftuoglu Y, Zhang L, Yuan Q. Combination therapy of RY10-4 with the gamma-secretase inhibitor DAPT shows promise in treating HER2-amplified breast cancer. Oncotarget. 2016; 7(4):4142–54. DOI: 10.18632/oncotarget.6769 [PubMed: 26716652]

25. Lee HW, Kim SJ, Choi IJ, Song J, Chun KH. Targeting Notch signaling by gamma-secretase inhibitor I enhances the cytotoxic effect of 5-FU in gastric cancer. Clin Exp Metastasis. 2015; 32(6):593–603. DOI: 10.1007/s10585-015-9730-5 [PubMed: 26134677]

26. Vinson KE, George DC, Fender AW, Bertrand FE, Sigounas G. The Notch pathway in colorectal cancer. Int J Cancer. 2016; 138(8):1835–42. DOI: 10.1002/ijc.29800 [PubMed: 26264352]

27. Ikezawa Y, Sakakibara-Konishi J, Mizugaki H, Oizumi S, Nishimura M. Inhibition of Notch and HIF enhances the antitumor effect of radiation in Notch expressing lung cancer. Int J Clin Oncol. 2016; doi: 10.1007/s10147-016-1031-8.

28. Sakakibara-Konishi J, Ikezawa Y, Oizumi S, Kikuchi J, Kikuchi E, Mizugaki H, et al. Combined antitumor effect of gamma-secretase inhibitor and ABT-737 in Notch-expressing non-small cell lung cancer. Int J Clin Oncol. 2016; doi: 10.1007/s10147-016-1060-3.

29. Yu P, Petrus MN, Ju W, Zhang M, Conlon KC, Nakagawa M, et al. Augmented efficacy with the combination of blockade of the Notch-1 pathway, bortezomib and romidepsin in a murine MT-1 adult T-cell leukemia model. Leukemia. 2015; 29(3):556–66. DOI: 10.1038/leu.2014.241 [PubMed: 25118879]

30. Zhang T, Armstrong AJ. Docetaxel Resistance in Prostate Cancer: Taking It Up a Notch. Clin Cancer Res. 2015; 21(20):4505–7. DOI: 10.1158/1078-0432.CCR-15-1613 [PubMed: 26307134]

31. Cui D, Dai J, Keller JM, Mizokami A, Xia S, Keller ET. Notch Pathway Inhibition Using PF-03084014, a gamma-Secretase Inhibitor (GSI), Enhances the Antitumor Effect of Docetaxel in Prostate Cancer. Clin Cancer Res. 2015; 21(20):4619–29. DOI: 10.1158/1078-0432.CCR-15-0242 [PubMed: 26202948]

32. Baldus CD, Thibaut J, Goekbuget N, Stroux A, Schlee C, Mossner M, et al. Prognostic implications of NOTCH1 and FBXW7 mutations in adult acute T-lymphoblastic leukemia. Haematologica. 2009; 94(10):1383–90. DOI: 10.3324/haematol.2008.005272 [PubMed: 19794083]

33. Ben Abdelali R, Asnafi V, Leguay T, Boissel N, Buzyn A, Chevallier P, et al. Pediatric-inspired intensified therapy of adult T-ALL reveals the favorable outcome of NOTCH1/FBXW7 mutations, but not of low ERG/BAALC expression: a GRAALL study. Blood. 2011; 118(19):5099–107. DOI: 10.1182/blood-2011-02-334219 [PubMed: 21835957]

34. Stankiewicz MJ, Crispino JD. AKT collaborates with ERG and Gata1s to dysregulate megakaryopoiesis and promote AMKL. Leukemia. 2013; 27(6):1339–47. DOI: 10.1038/leu. 2013.33 [PubMed: 23380710]

35. Hu Y, Dobi A, Sreenath T, Cook C, Tadase AY, Ravindranath L, et al. Delineation of TMPRSS2-ERG splice variants in prostate cancer. Clin Cancer Res. 2008; 14(15):4719–25. DOI: 10.1158/1078-0432.CCR-08-0531 [PubMed: 18676740]

36. Soh H, Venkatesan N, Veena MS, Ravichandran S, Zinabadi A, Basak SK, et al. Cystatin E/M Suppresses Tumor Cell Growth through Cytoplasmic Retention of NF-kappaB. Molecular and cellular biology. 2016; 36(12):1776–92. DOI: 10.1128/MCB.00878-15 [PubMed: 27090639]

37. Sun C, Dobi A, Mohamed A, Li H, Thangapazham RL, Furusato B, et al. TMPRSS2-ERG fusion, a common genomic alteration in prostate cancer activates C-MYC and abrogates prostate epithelial differentiation. Oncogene. 2008; 27(40):5348–53. DOI: 10.1038/onc.2008.183 [PubMed: 18542058]

38. Dubovenko A, Serebryiskaya T, Nikolsky Y, Nikolskaya T, Perlina A, JeBailey L, et al. Reconstitution of the ERG Gene Expression Network Reveals New Biomarkers and Therapeutic Targets in ERG Positive Prostate Tumors. J Cancer. 2015; 6(6):490–501. DOI: 10.7150/jca.8213 [PubMed: 26000039]

39. Dobi A, Furusato B, Shaheduzzaman S, Chen Y, Vahey M, Nydam T, et al. ERG Expression Levels in Prostate Tumors Reflect Functional Status ofthe Androgen Receptor (AR) as a Consequence of Fusion of ERG with ARRegulated Gene Promoters. The Open Cancer Journal. 2010; 3:101–8.

40. Dontu G, Jackson KW, McNicholas E, Kawamura MJ, Abdallah WM, Wicha MS. Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells. Breast cancer research : BCR. 2004; 6(6):R605–15. DOI: 10.1186/bcr920 [PubMed: 15535842]

41. Hori K, Sen A, Artavanis-Tsakonas S. Notch signaling at a glance. J Cell Sci. 2013; 126(Pt 10): 2135–40. DOI: 10.1242/jcs.127308 [PubMed: 23729744]

42. Deng G, Ma L, Meng Q, Ju X, Jiang K, Jiang P, et al. Notch signaling in the prostate: critical roles during development and in the hallmarks of prostate cancer biology. J Cancer Res Clin Oncol. 2016; 142(3):531–47. DOI: 10.1007/s00432-015-1946-x [PubMed: 25736982]

43. Wang XD, Leow CC, Zha J, Tang Z, Modrusan Z, Radtke F, et al. Notch signaling is required for normal prostatic epithelial cell proliferation and differentiation. Developmental biology. 2006; 290(1):66–80. DOI: 10.1016/j.ydbio.2005.11.009 [PubMed: 16360140]

44. Upadhyay P, Nair S, Kaur E, Aich J, Dani P, Sethunath V, et al. Notch pathway activation is essential for maintenance of stem-like cells in early tongue cancer. Oncotarget. 2016; 7(31): 50437–49. DOI: 10.18632/oncotarget.10419 [PubMed: 27391340]

45. Zhou J, Jain S, Azad AK, Xu X, Yu HC, Xu Z, et al. Notch and TGFbeta form a positive regulatory loop and regulate EMT in epithelial ovarian cancer cells. Cellular signalling. 2016; 28(8):838–49. DOI: 10.1016/j.cellsig.2016.03.016 [PubMed: 27075926]

46. Shao S, Zhao X, Zhang X, Luo M, Zuo X, Huang S, et al. Notch1 signaling regulates the epithelial-mesenchymal transition and invasion of breast cancer in a Slug-dependent manner. Molecular cancer. 2015; 14:28.doi: 10.1186/s12943-015-0295-3 [PubMed: 25645291]

47. Zhang X, Zhao X, Shao S, Zuo X, Ning Q, Luo M, et al. Notch1 induces epithelial-mesenchymal transition and the cancer stem cell phenotype in breast cancer cells and STAT3 plays a key role. International journal of oncology. 2015; 46(3):1141–8. DOI: 10.3892/ijo.2014.2809 [PubMed: 25544568]

48. McMullin RP, Dobi A, Mutton LN, Orosz A, Maheshwari S, Shashikant CS, et al. A FOXA1-binding enhancer regulates Hoxb13 expression in the prostate gland. Proceedings of the National

Academy of Sciences of the United States of America. 2010; 107(1):98–103. DOI: 10.1073/pnas. 0902001107 [PubMed: 20018680]
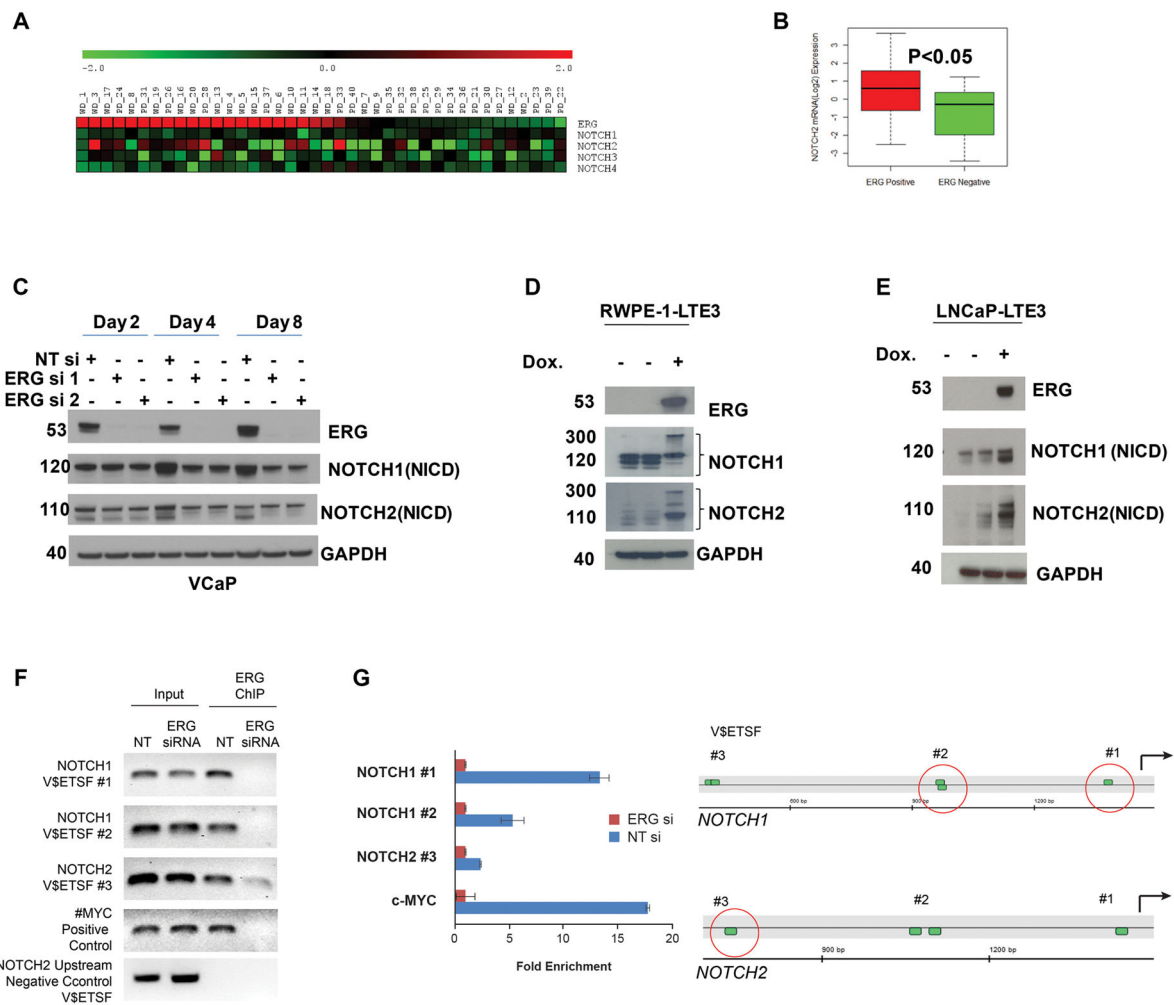
Figure 1.

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) CaP 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&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^

dCT) and ERGsi (2^ dCT) 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 indicate position of detected ERG recruitment.
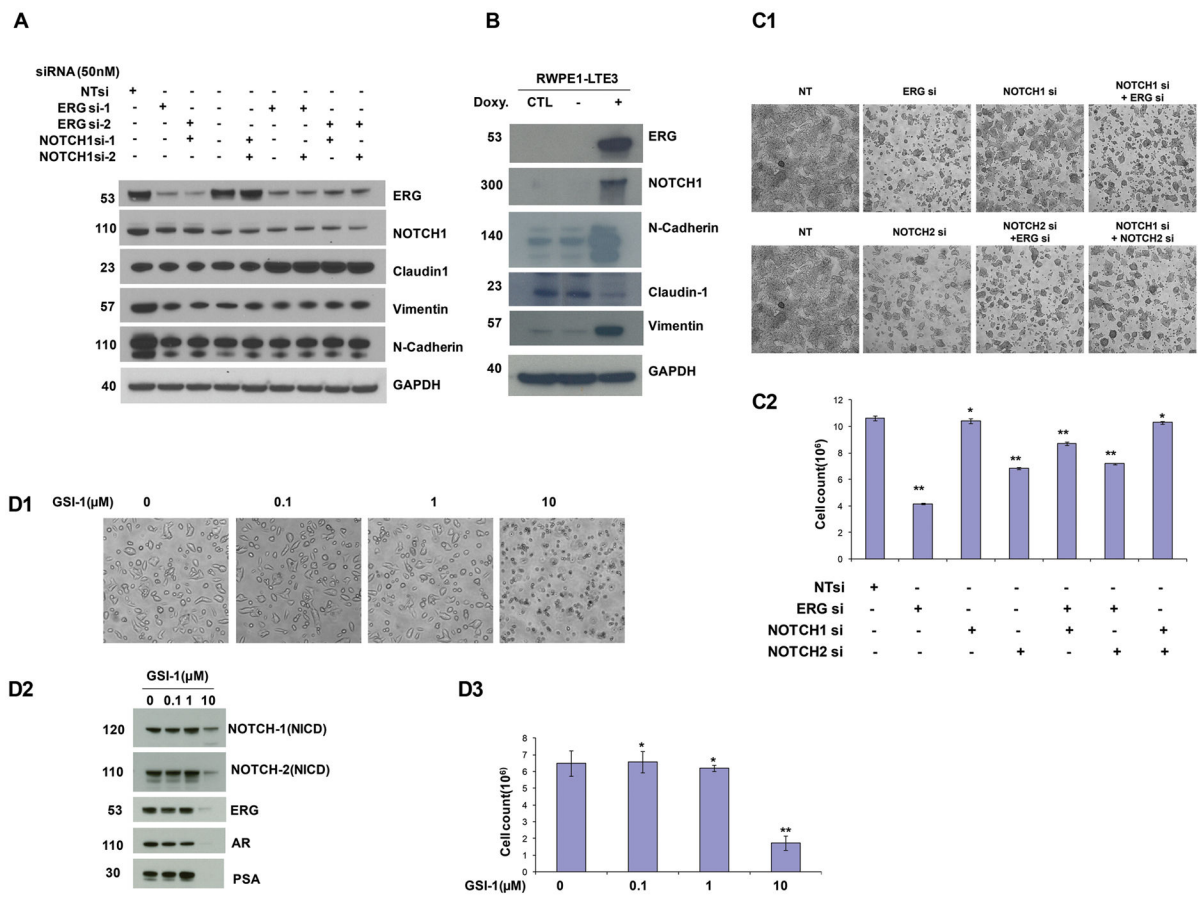
Figure 2.
ERG affects EMT in prostate cancer through modulation of NOTCH transcription factors. **A**, Inhibition of ERG in the *TMPRSS2:ERG* positive VCaP cells resulted in the inhibition of NOTCH1 and the mesenchymal markers (N-Cadherin and Vimentin) while it induced the epithelial marker (Claudin-1). **B**, Overexpression of *TMPRSS2:ERG3* in normal immortalized prostate epithelial cells resulted in an increased expression of NOTCH1, N-Cadherin, and Vimentin, while inhibiting Claudin-1 protein. **C1&C2**, The knock-down of NOTCH1 and NOTCH2 recapitulates the effect of ERG knock-down on prostate epithelial differentiation. Experiments were performed in triplicates and results are shown as mean ±SD of three independent experiments. Student's t test was used to compare treated cells with control (0μM), * p> 0.05, ** p< 0.05. **D1–D3**, Treatment of the NOTCH inhibitor GSI-1 significantly reduced expression of AR, ERG and PSA as well as NOTCH1 and NOTCH2 proteins and inhibited cell growth at higher concentrations (10μM). Experiments were performed in triplicates and results are shown as mean ±SD, * p> 0.05, ** p< 0.05, (t test, n=3).
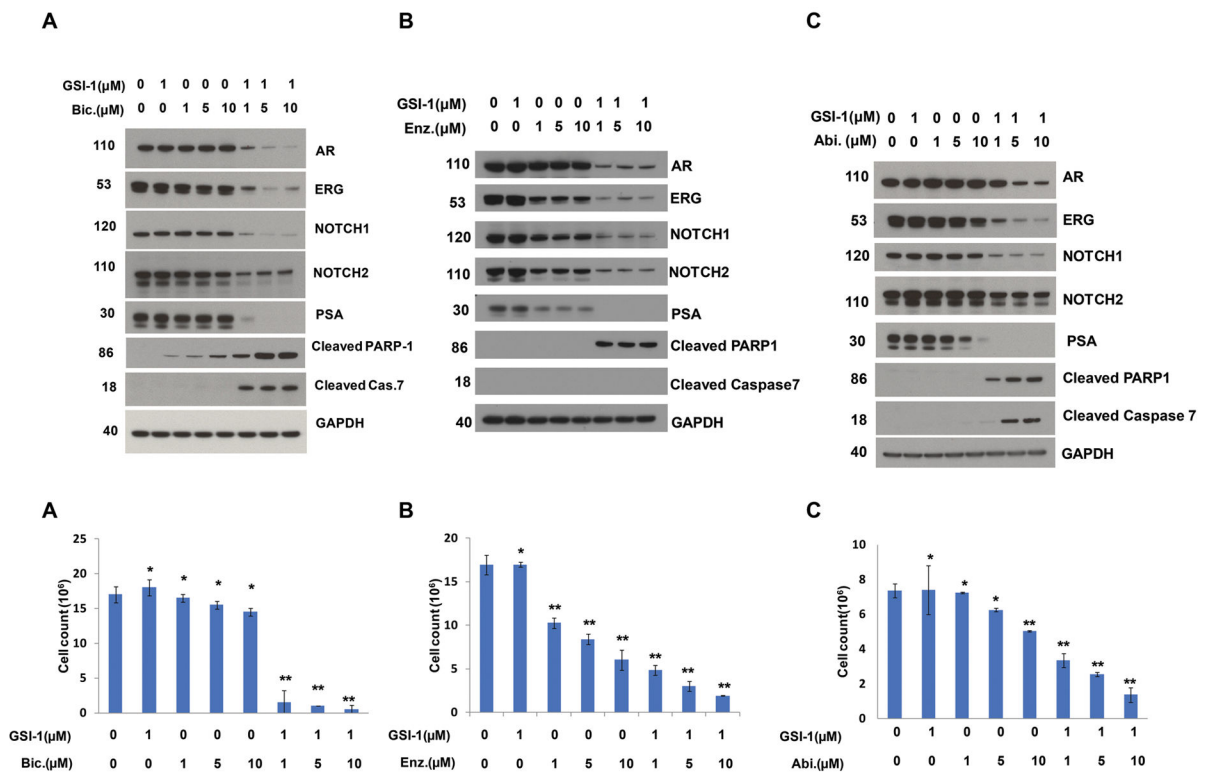
Figure 3.
Increased sensitivity of ERG positive tumor cells to AR inhibitors in combination with NOTCH inhibitor. **A, B & C** (upper panels), Treatment of VCaP cells with NOTCH inhibitor, GSI-1, in combination with either Abiraterone (Abi), Bicalutamide (Bic), or Enzalutamide (Enz) augmented their effects on the inhibition of AR, ERG, PSA, NOTCH1 and NOTCH2 expression and induced cleavage of PARP1 and Caspase 7. **A, B & C** (lower panels), GSI-1 increases sensitivity of ERG positive CaP 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 were performed three times and results are shown as mean ±SD, * p> 0.05, ** p< 0.05.
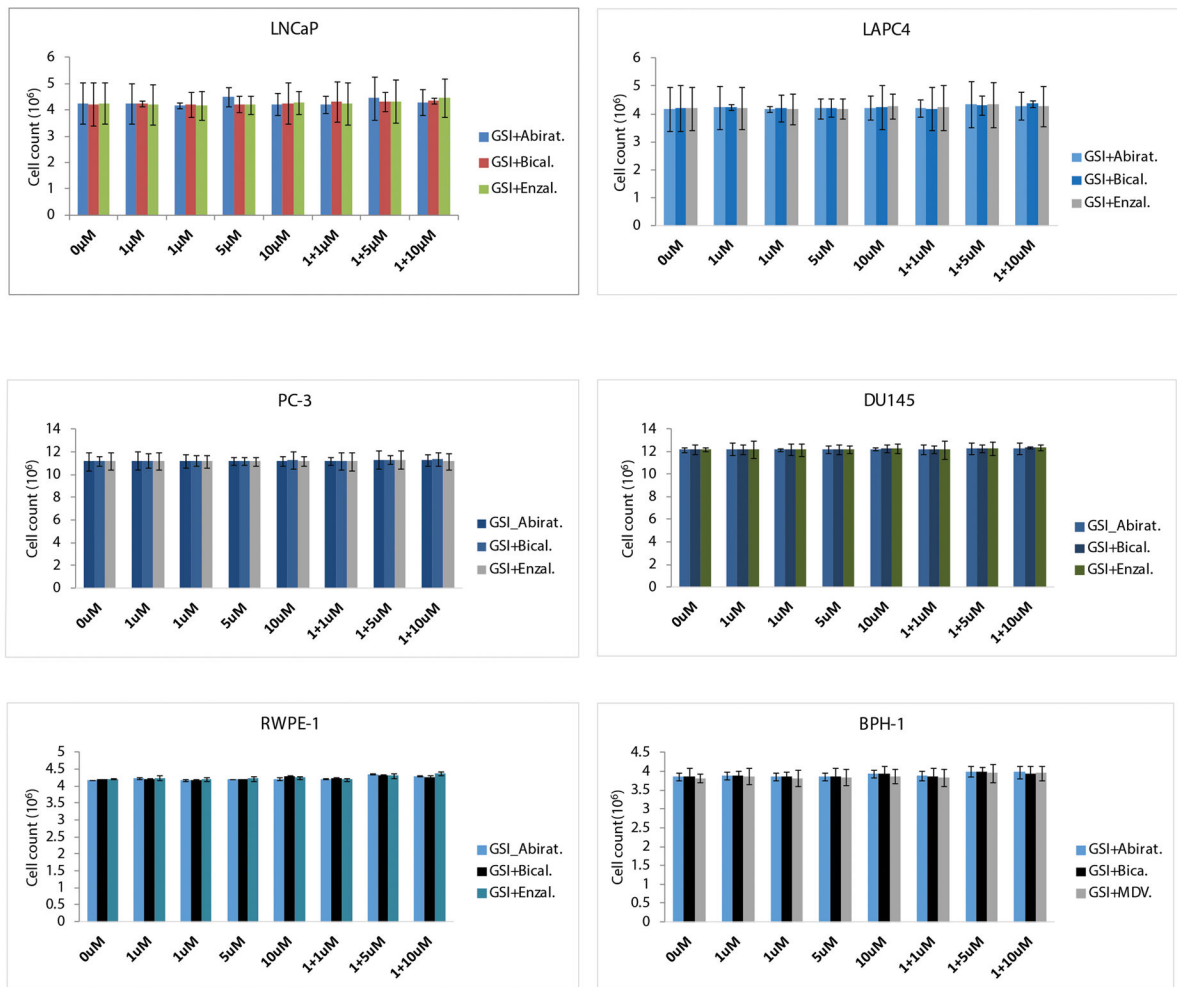
Figure 4.

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.
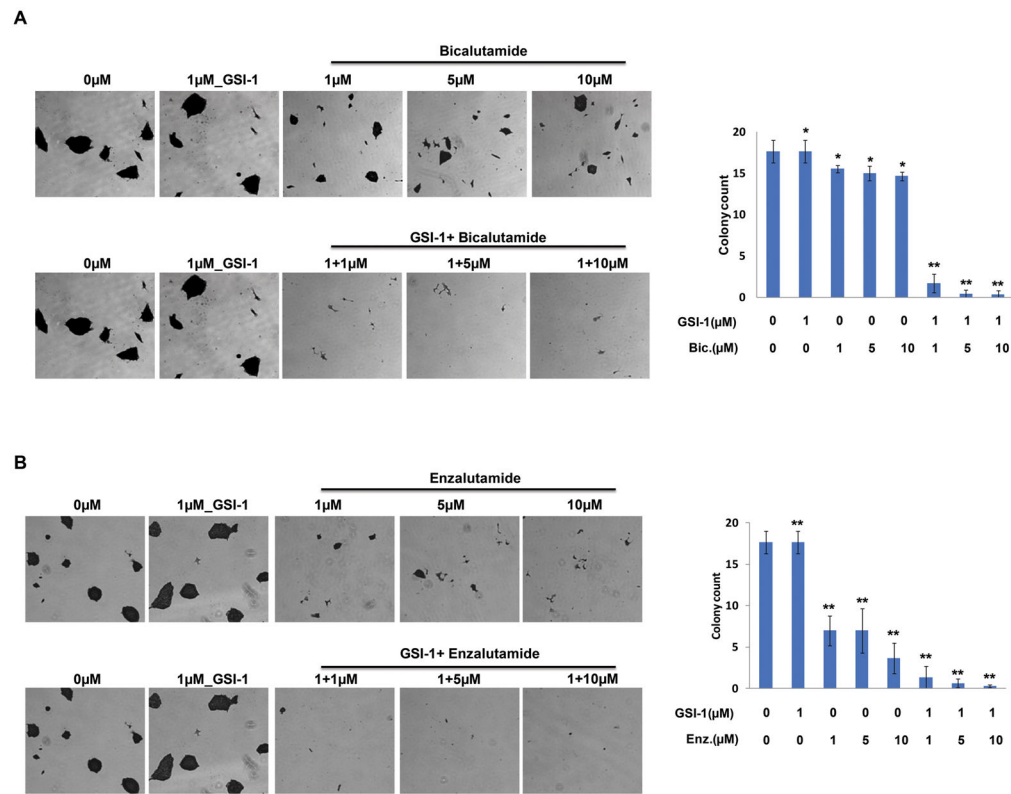
Figure 5.
Combination of GSI-1 and AR inhibitors delays colony survival formation. **A&B**, Treatment of VCaP cells with NOTCH inhibitor, GSI-1, in combination with either Bicalutamide or Enzalutamide significantly inhibited survival colony formation capability of VCaP cells. Colonies with more than 50 cells were counted (right bar diagrams). Results are shown as mean ± SD of triplicate experiments, * p> 0.05, ** p< 0.05.
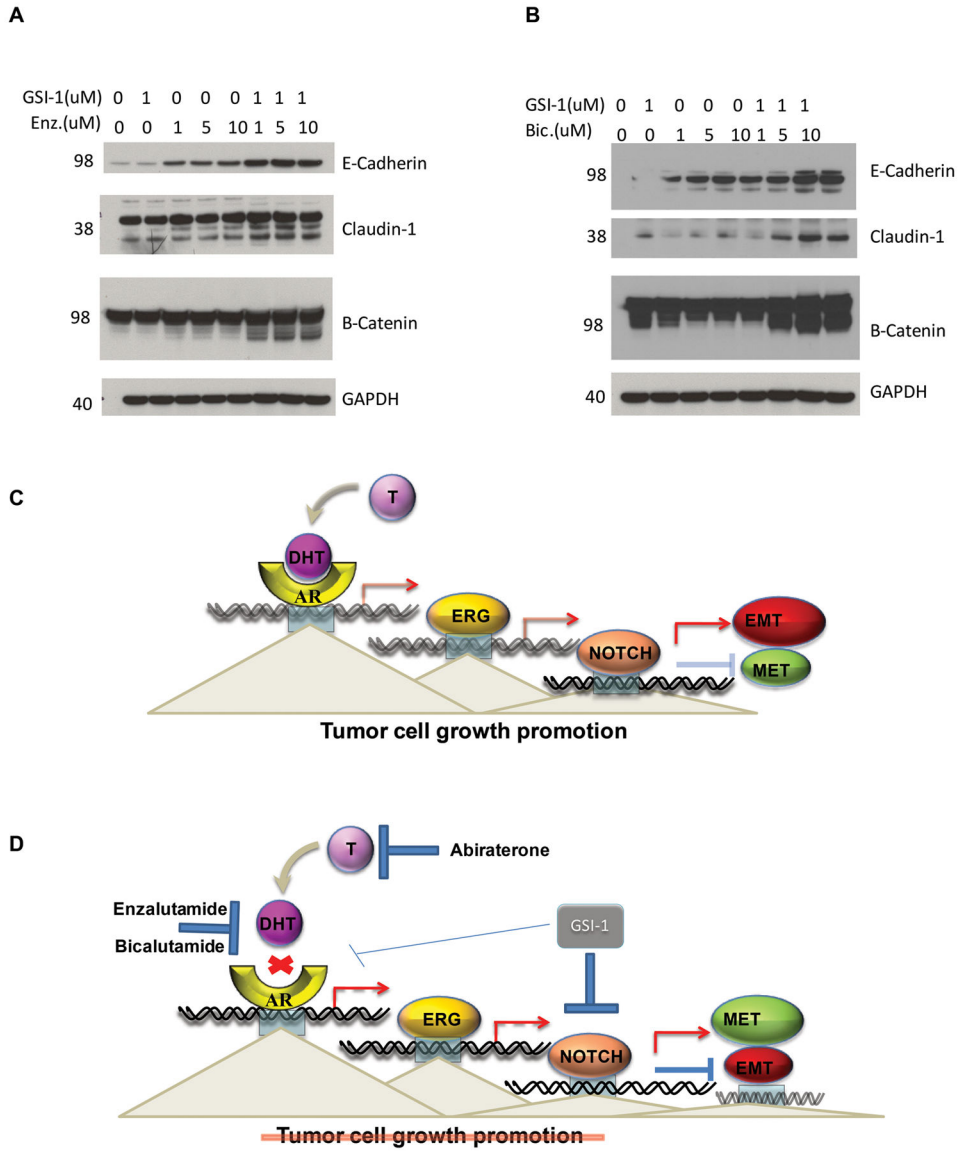
Figure 6.

Synergistic inhibition of NOTCH and ERG signaling reverses EMT. **A&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&D**, The proposed mechanism is that inhibition of AR's tumor promoting function in combination with the pro-tumorigenic functions of NOTCH is more effective therapeutic approach than any of the compounds alone.