ERG Oncoprotein Inhibits ANXA2 Expression and Function in Prostate Cancer

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Abstract

Overexpression of ERG in the prostate epithelium, due to chromosomal translocations, contributes to prostate tumorigenesis. Here, genomic analysis of ERG siRNA-treated prostate cells harboring the endogenous TMPRSS2–ERG fusion revealed an inverse relationship between ERG and Annexin A2 (ANXA2) expression at both the RNA and protein level. ANXA2, a Ca2+-dependent and phospholipid-binding protein, is involved in various cellular functions, including maintenance of epithelial cell polarity. Mechanistic studies defined the prostate-specific transcription start site of ANXA2 and showed that the recruitment of ERG to the ANXA2 promoter is required for transcriptional repression by ERG. Knockdown of ERG enhanced the apical localization of ANXA2, the bundling of actin filaments at cell–cell junctions and formation of a polarized epithelial phenotype. ERG overexpression disrupted ANXA2-mediated cell polarity and promoted epithelial–mesenchymal transition (EMT) by inhibiting CDC42 and RHOA, and by activating coflin. Immunohistochemistry demonstrated a reciprocal relationship of ANXA2 and ERG expression in a large fraction of primary prostate cancer clinical specimens. ANXA2 was absent or markedly reduced in ERG(+) tumors, which were mostly well differentiated. ERG(–) tumors, meanwhile, expressed moderate to high levels of ANXA2, and were either poorly differentiated or displayed subsets of poorly differentiated cells. Taken together, the transcriptional repression of ANXA2 by ERG in prostate epithelial cells plays a critical role in abrogating differentiation, promoting EMT, and in the reciprocal correlation of ERG and ANXA2 expression observed in human prostate cancer.

Implications: ANXA2 is a new component of the ERG network with potential to enhance biologic stratification and therapeutic targeting of ERG-stratified prostate cancers. Mol Cancer Res; 13(2): 368–79. ©2014 AACR.

Introduction

Chromosomal translocations in prostate cancer largely consist of gene fusions involving ETS transcription factors and promoters of androgen-driven genes. Among the established translocations, the TMPRSS2–ERG fusion, which places ERG under the regulation of androgen receptor (AR), constitutes about 50% of prostate cancers in the Western countries (1–3). ERG overexpression leads to the induction of C-MYC expression, inhibition of prostate luminal epithelial differentiation markers (KLK3/PSA and SLC45A3/Prostein), and subsequent reversal of differentiation (6, 7). In an effort to continue to refine our understanding of the oncogenic functions of ERG and ERG network in prostate cancer (3, 4), this study reports a novel reciprocal relationship of ERG and Annexin A2 (ANXA2) that may, in part, contribute to the ERG-induced mesenchymal phenotype.

We have noted earlier the inverse correlation between ERG and ANXA2 gene expression in human prostate cancer specimens and in in vitro cell culture models (2, 6). ANXA2 is a calcium-dependent phospholipid-binding protein involved in a number of cell biologic processes, including endosome trafficking, cell adhesion, and cytoskeleton organization (8, 9). It is also involved in the plasminogen activation system in initiating wound healing, tissue remodeling, and angiogenesis by activation of plasminogen (10). At the cellular level, ANXA2 exists as a monomer in the cytoplasm, but it forms a heterotrimer with S100-A10, which results in its association with the plasma membrane (11–13). It has also been reported to be translocated into the nucleus (14, 15), expressed on the cell surface (10), and secreted into the extracellular milieu (16).

ANXA2 protein is present in the luminal and basal epithelium of glands in the normal prostate or benign prostatic hyperplasia (BPH). However, ANXA2 expression is absent or focally detected in the epithelium of prostatic intraepithelial neoplasia (PIN) and moderately differentiated adenocarcinomas. Surprisingly, ANXA2 expression reemerges in a subset of poorly differentiated (PD) high-grade adenocarcinomas (13, 17–19). The mechanism, by which ANXA2 expression is modulated in prostate cancer progression, remains to be understood.

This study evaluated the potential role of ERG-mediated repression of ANXA2 in epithelial–mesenchymal transition (EMT). EMT is a cellular process important during tumor development and metastasis, whereby epithelial cells lose characteristic features of a...
differentiated phenotype ([cell–cell adhesion, planar and apical–basal polarity, and lack of motility]) to gain mesenchymal features, which include motility, invasiveness, and an increased resistance to apoptosis (20). Previous studies have shown that ERG promotes EMT through facilitators such as ZEB1 (21) and Frizzled-4 (FZD4; ref. 22). In contrast, ANXA2 binds to both phosphorylated phosphatidylinositol (4,5)P2 (PIP2) and CDC42, and establishes the localization of E-cadherin to cell junctions, all of which are key components of adherens junctions (AJ) formation, required for a differentiated epithelial phenotype (23, 24). The formation of intercellular adhesive junctional complexes represented by AJ, tight junctions (TJ), desmosomes, and gap junctions contribute to the lateral and apical–basal polarity of epithelial cells (25).

During EMT, components of junctional complexes (E-cadherin, β-catenin, and claudins or occludins from TJs, including ZO-1) are downregulated by transcriptional repression, delocalization, or proteolytic degradation (26).

ANXA2 is a key orchestrator of cell–cell adhesion through its control of actin remodeling (9, 27). The association of actin filament bundles with AJ complexes at intercellular contact sites, a crucial step in establishing intercellular adhesion and epithelial polarity (28) is accomplished by the activation Rho-GTPases, CDC42, RHOA, or RAC1 (29, 30). These Rho-GTPases are also critical regulators of microtubule dynamics and intracellular membrane trafficking pathways (20).

Here, we demonstrate that a negative regulation of ANXA2 transcription by ERG contributes to the abrogation of prostate epithelial differentiation and promotion of the EMT. Transcriptional repression of ANXA2 disrupts the maintenance of a polar epithelial phenotype through the inhibition of CDC42 and, to a lesser extent, RHOA, and activation of coflin. We validated the reciprocal correlation between ERG and ANXA2 expression in human prostate tumor specimens. We noted that while ERG(+)/ANXA2(--), tumors tend to be PD or contain secondary or tertiary patterns that are PD. Although the functional impact of the dichotomy of ERG/ANXA2 expression status between moderate and aggressive cancer needs to be better understood, we observed an association of ERG(--)/ANXA2(+) expression status with aggressive cancers. More importantly, the mechanistic insights developed from this study highlighted the critical biologic role of ERG in repressing ANXA2 and this expression phenotype was prevalent in a majority of WD tumors, a common feature of early detected prostate cancer.

**Materials and Methods**

**Prostate tissue specimen**

Under an Institutional Review Board (IRB)–approved protocol (Protocol No. 20405–28), 40 cases were selected from over 300 patients with prostate cancer treated with radical prostatectomy at Walter Reed National Medical Center (WRNMMC, Bethesda, MD). Patients, matched for age (42–74 years) and race (Caucasians), were segregated on the basis of differentiation status of the tumors from which mRNA was derived: 20 cases each of WD tumors and PD tumors (2). WD tumors had Gleason score of 6 to 7, no seminal vesicle invasion, and WD cells, whereas the PD tumors had Gleason score of 8 to 9, seminal vesicle invasion, and PD cells. Total mRNA extracted from WD and PD tumors and from their matched normal tissues by laser capture microdissection (LCM) were analyzed for gene expression by using Affymetrix Human Genome U133 Plus 2.0 arrays (GSE32446).

**Immunohistochemistry**

Specimens for ERG and ANXA2 immunohistochemistry (IHC) were cut, stained, and prepared according to previously described methods (31, 32). Adjacent whole-mounted sections were analyzed for malignant and benign cells by hematoxylin and eosin (H&E) staining and for ERG and ANXA2 expression status by IHC. The status of ERG and ANXA2 expression of index tumors were correlated with differentiation status and Gleason grade.

**Cell culture and siRNA knockdown**

Human prostate tumor cell lines, VCaP and LNCaP, were purchased from the American Type Culture Collection (ATCC) and maintained as recommended. VCaP cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) or with 10% charcoal-stripped serum (CSS) for androgen-deprived conditions (Gemini Bio-Products). In siRNA knockdown experiments, cells were deprived of androgen for 72 hours before transfection with siRNA oligonucleotides (oligos) against non-targeting (NT) control, ERG (5′-CGACGAGGACUCCUCUGCUCAAU1-3′ and 5′-UGAUGUUGAAAGGCCGUAUUA-3′), and ANXA2 (5′-CGACGAGGACUCCUCUGCUCAAU1-3′, 5′-AUCCAUGUUGCUCGUAUUA-3′, 5′-GAAAAAGAAGUCCUCGACUUA-3′, and 5′-AAACGACUCCUGGAAUUA-3′) by using Lipofectamine 2000 (Invitrogen). Cells were treated with 0.1 nmol/L of R1881 (PerkinElmer) 24 hours after transfection, and incubated for another 24, 48, or 72 hours before they were analyzed for gene or protein expression. The analysis of total RNA from control and ERG siRNA-treated VCaP cells using GeneChip HGU133 Plus 2.0 arrays was described by Sun and colleagues (6).
siRNA transfection. Cells were induced with 0.1 nmol/L R1881 24 hours after transfection and cultured for another 48 hours. Cells were fixed with 4% paraformaldehyde buffered in PBS, permeabilized with 0.1% Triton X-100 in PBS, and blocked with 1% normal horse serum (Vector Laboratories) before incubating with appropriate primary antibodies. Species-specific secondary antibodies (Alexa Fluor-594 goat anti-mouse and Alexa Fluor-488 goat anti-rabbit; Invitrogen) were subsequently applied together with DAPI (4′,6-diamidino-2-phenylindole). F-actin was stained with Alexa Fluor-594 phalloidin (Invitrogen). Images were captured using a 40×/0.65 N-Plan objective on a Leica DMIRE2 upright microscope with a QImaging Retiga-EX CCD camera controlled by OpenLab software (PerkinElmer), converted into color, and merged by using Adobe Photoshop.

Confocal microscopy images were acquired on a LSM 510 Meta confocal system equipped with an Axio Observer Z1 inverted microscope (Carl Zeiss) and a Hamamatsu Photonics EM-CCD Digital Camera (Hamamatsu Corp.) using a 40× objective. Total internal reflection fluorescence (TIRF) microscopy imaging was conducted on a Zeiss Axio Observer Z1 inverted microscope equipped with oil immersion alpha Plan-Apochromat 63x/1.46 objective and motorized Laser TIRF 3 system (Carl Zeiss). Excitation was achieved by using 530-nm laser. Images were acquired on a Hamamatsu Photonics EM-CCD digital camera and processed using HC Image acquisition software (Hamamatsu Corp.). Approximately 15 cells were imaged from knockdown slides, from which the signal intensity of ANXA2 at the apical plasma membrane was averaged.

**Immunoblot and immunofluorescence assays**

Cells were lysed in Mammalian Protein Extraction Reagent (M-PER, Thermo Scientific) containing protease inhibitor and phosphatase inhibitor cocktails I and II (Sigma-Aldrich). Cell lysates equivalent to 30 μg of protein were separated on 4% to 12% Bis-Tris gels (Invitrogen) and transferred onto polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with blocking buffer (Li-COR Biosciences) and incubated with the appropriate primary antibodies. After washing, membranes were incubated with horseradish peroxidase (HRP)-linked (GE Healthcare) or anti–phospho-linked secondary antibodies (Li-COR Biosciences). Membranes were developed with ECL Western blot detection reagent (GE Healthcare) and exposed to X-ray film for detection or scanned with Odyssey infrared scanner (Li-COR Biosciences). Antibodies used for immunoblot, immunofluorescence, and IHC assays against specific proteins were obtained from the following sources: ERG (9Fy) from Biocare Medical; GAPDH (sc-25778) and β-catenin (H102; # sc-7199) from Santa Cruz Biotechnology; ANXA2 (610068) from BD Biosciences; cofilin (#3312) and ZO-1 (#40–2200) from Invitrogen; and phospho-cofilin (Ser3, 7G2; #3313) and E-cadherin (clone 24E10; #3195) from Cell Signaling Technology.

**5′ Rapid Amplification of cDNA Ends**

A total of 5 μg of mRNA isolated from VCaP cells was subjected to 5′ oligo-capping procedure by using the FirstChoice RLM-RACE Kit (Invitrogen). The 5′ end of ANXA2 promoter was amplified by 5′ RNA Ligase Mediated Rapid Amplification of cDNA Ends (5′RLM-RACE) by pairing the 5′ RACE outer primer, 5′-CTCATGGCGATGATGACACTG-3′ with the inner primer, 5′-GGTTATCCAGAGATGTTCCAGAC-3′. Amplicons were gel-purified, cloned into pCR-BluntII-TOPO vector, and subjected to DNA sequencing.

**Chromatin immunoprecipitation**

VCaP cells transfected with 50 nmol/L of NT siRNA or ERG siRNA for 48 hours were processed for chromatin immunoprecipitation (ChIP) as previously described (6, 33) using 25 μg of chromatin and 2 μg of ERG MAb (9Fy). Enrichment of regions at the ANXA2 promoter compared with input was determined by 40 PCR amplification cycles on T-Gradient Thermoblock (Biometa). Amplified DNA was visualized by separation on agarose gel and measured by using Quantity One (Bio-Rad). The enrichment was normalized against input DNA. The location of ETS sites (VSETSF1 to #8) within the ANXA2 (Gene ID:302) core promoter identified by MatInspector software (Genomatix) is shown in Fig. 2C. Primer sequences and PCR conditions used are listed in Supplementary Table S1. As positive control, regions from the C-MYC promoter upstream and HPGD core promoter were amplified (6, 33). A region lacking ETS motifs, approximately 15 kb upstream of the ANXA2 transcription initiation site, was amplified as internal negative control.

**RHOA, CDC42, and RAC1 G-LISA assays**

VCaP cell transfected with NT siRNA, ERG siRNA, or ANXA2 siRNA were analyzed for activated forms of RHOA, CDC42, and RAC1 72-hours after transfection. Cells were stimulated with 50 ng/mL of epidermal growth factor (EGF; Sigma-Aldrich) for CDC42 and RAC1 activation and with 10 μmol/L of nocodazole (Sigma-Aldrich) for RHOA activation in G-LISA colorimetric or luciferase-based ELISA assays (Cytoskeleton). Active GTPases were captured in wells of a 96-well plate and after the removal of inactive forms, GTPase activities were detected by using HRP-conjugated antibodies against the specific GTPase. After developing with HRP detection reagents, absorbance was measured at 490 nm and luminescence was measured on a microplate luminometer. The mean relative Rho-GTPase activity from three replicates was normalized relative to NT siRNA treated cells after subtracting background signal.

**Results**

ANXA2 and ERG expression are inversely correlated in prostate tumors and in VCaP cells

Our previous GeneChip-based evaluations of prostate adenocarcinomas from 40 patients revealed a reciprocal correlation between ERG and ANXA2 (6). The mean differences of gene expression for ANXA2 between ERG(+) and ERG(−) tumors showed a sharp decrease compared with other members of the Annexin family (Fig. 1A, middle row). An inverse correlation between ERG and ANXA2 expression is observed in a majority of the 40 cases analyzed (Fig. 1B). This correlation was further examined by the siRNA knockdown of ERG in VCaP cells, which carry the TPMRSS2–ERG fusion and overexpress ERG protein. An approximately 2.5-fold increase of ANXA2 expression was noted in ERG siRNA versus control NT siRNA-treated VCaP cells (Fig. 1C). Consistent with the ANXA2 mRNA data, a 4-fold increase in ANXA2 protein expression was noted in response to ERG knockdown in VCaP cells (Fig. 1D).

**Mapping and characterization of prostate-specific ANXA2 promoter**

To examine whether the reciprocal correlation between ERG and ANXA2 was the result of ERG-mediated transcriptional repression of ANXA2, we analyzed the transcription regulation
of the ANXA2 promoter. We first identified the prostate-specific ANXA2 promoter by RACE from cDNA of VCaP cells. Transcripts amplified using distinct primer pairs were found to be identical by sequence analyses (Fig. 2A). These data suggested for transcription initiation site within the GXP269017 promoter sequence (ElDorado 12–2012 annotation, NCBI build 37; ref. 34). This ANXA2 transcript is composed of a noncoding exon 1 spliced to exon 2, which are 12 kb apart in the genome (Fig. 2B). Analysis of the ANXA2 promoter for potential ERG-binding sites using the MatInspector identified at least eight sites represented by the V$ETSF matrix. ChIP assays using chromatin from VCaP cells confirmed the recruitment of ERG to binding sites #1, #2, #3, #6, and #7, which were significantly reduced upon siRNA treatment (Fig. 2C). Transcription regulation by ERG were analyzed in prostate cancer cell lines by using luciferase reporter constructs containing ANXA2 promoter upstream sequences that span V$ETSF #1 to #8. Overexpression of ERG by using doxycycline-inducible ERG lentivirus in LNCaP-LTE3 cell line followed by luciferase assay showed repression of transcription from reporter constructs incorporating 959 and 1146 bp upstream sequences from the start site (Fig. 2D, top), while the knockdown of ERG in VCaP cells alleviated transcription repression in reporter construct containing 959 bp upstream sequences from the start site (Fig. 2D, bottom).

**ANXA2 induced by ERG knockdown is localized to the apical surface of the VCaP cells**

In VCaP cells, the localization of ANXA2 to peripheral cell junctions is increased upon ERG knockdown, as observed by immunofluorescence staining (Fig. 3A). Further evaluation by scanning the XZ- and YZ-sections of cells using confocal microscopy (Fig. 3B) showed the accumulation of ANXA2 not only to the cellular junctions but also to the apical surface of cells. We also showed that the upregulated ANXA2 is localized to the apical plasma membrane of immunofluorescence-labeled cells by using trans-TIRF microscopy, which selectively detects protein molecules nearest to or associated with the plasma membrane (35). Quantitative evaluation of the distribution of ANXA2 within the TIRF field of excitation in ERG-depleted cells compared with control NT-transfected VCaP cells showed a 3.7-fold increase in signal intensity at the cell plasma membrane (Fig. 3C).

**ERG represses CDC42 activity via inhibition of ANXA2**

Because the inhibition of differentiation by ERG is accompanied by the development of a more pronounced mesenchymal phenotype (21, 22), we reasoned that the knockdown of ERG would reverse the mesenchymal phenotype and regain the features of differentiated epithelial cells, characterized by apical-
Figure 2.
Mapping of prostate-specific ANXA2 transcript, analysis of ERG binding, and transcription regulation ANXA2 promoter. 5’ RACE analysis identified a prostate-specific transcript (A). The 5’ end of the transcript is spliced together from exons 1 and exon 2 (red triangles) of ANXA2 mRNA, variant 1 (NM_001002858.2), and variant 3 (NM_004039.2), and is translated starting from exon 2 (B). ChIP of chromatin from NT and ERG siRNA treated VCaP cells using ERG MAb showed relatively strong recruitment of ERG to V$ETSF$ motifs #1, #2, #3, #6, and #7. The ratio of immunoprecipitated chromatin to input chromatin is shown adjacent to each amplified region. The recruitment of ERG to the C-MYC and HPGD promoter regions were evaluated as positive controls and a region upstream of ANXA2 promoter was tested as a negative control (C). Analysis of luciferase reporter constructs containing ANXA2 promoter upstream sequences transfected in doxycycline-inducible ERG-expressing LNCaP-LTE3 cell line (D, top; †, P < 0.05; ‡‡, P < 0.05) and in NT and ERG siRNA-treated VCaP cells (D, bottom; †, P < 0.05). Mean relative luciferase activities were calculated from triplicate transfections.
basal polarity. This was confirmed by an increased accumulation of AJ components, E-cadherin and β-catenin, and ZO-1, a major constituent of TJ, at intercellular junctions following the knockdown of ERG (Fig. 4A). Because the formation of intercellular adhesion and epithelial polarity depend on the association of actin filament bundles with AJs, we monitored the extent of actin polymerization by using fluorescent conjugated phalloidin. We observed that the depletion of ERG enhanced the accumulation of f-actin along intercellular junctions (Fig. 4B).

To assess further whether the ERG-mediated inhibition of differentiation and promotion of EMT is dependent or independent of ANXA2, we examined the effect of ERG knockdown on downstream targets of ANXA2. Because members of the Rho family of GTPases are involved in cell–cell adhesion, cell polarity, and actin cytoskeletal rearrangement (9, 30, 36), we examined whether the effects of ERG siRNA-induced ANXA2 expression may be mediated through CDC42, RHOA, and RAC1 GTPases. Activated forms of CDC42, RHOA, and RAC1 GTPases in VCaP cells were measured using G-LISA activation assays following the siRNA knockdown of ERG or ANXA2. Cells were added with either EGF or nocodazole briefly to stimulate GTPase activity. We observed an increase in the activated forms of CDC42 following ERG siRNA knockdown (Fig. 4C, (i)), suggesting that CDC42 was repressed by the ERG-mediated repression of ANXA2. In contrast, the siRNA knockdown of ANXA2 decreased the active form of CDC42 and GTPases. Compared with CDC42, RHOA showed a similar but weaker response that was enhanced by EGF stimulation (Fig. 4C, (ii)). RAC1, however, was not responsive to the...
knockdown of ERG and ANXA2. To demonstrate further that the bundling of f-actin is enhanced upon ANXA2 overexpression, we monitored the activity of coflin. Coflin is an actin-severing protein downstream of the CDC42 and RHOA pathway that becomes inactivated following phosphorylation. ERG knockdown resulted in increased levels of phosphorylated and inactive form of coflin. Consistent with these data, ANXA2 knockdown resulted in decreased levels of phosphorylated coflin (Fig. 4D).

These observations suggested that the destabilization of actin polymerization is controlled through the downregulation of ANXA2 by the ERG oncoprotein in prostate cancer cells. Taken together, the reestablishment of polarized epithelial features, organized bundling of actin filaments at cell–cell junctions, activation of CDC42 and RHOA GTPases, and coflin phosphorylation suggest that ERG regulates differentiation and EMT activities through the repression of ANXA2 and the activity of Rho-GTPases (Fig. 4E).

Inverse relationship of ERG and ANXA2 expression in prostate cancer

The reciprocal relationship between ERG and ANXA2 gene expression established by the GeneChip-based evaluations of prostate tumors (Fig. 1A and B) and the induction of ANXA2 by ERG inhibition in the TMPRSS2–ERG–positive prostate cancer cell culture model, prompted us to further examine whether this correlation held for ERG and ANXA2 protein status in prostate tumors. We evaluated ERG and ANXA2 protein expression status in relation to the Gleason score and differentiation phenotype of the index tumors of representative whole-mounted prostate sections from the same cohort of patients whose specimens were analyzed by GenChip (Table 1). Positive ERG expression was confirmed in the index tumors of 26 out of the 40 cases examined (37). Our evaluation of ANXA2 expression by IHC (Fig. 5) validated previous reports showing that ANXA2 is localized to the membrane and cytoplasm of basal and epithelial secretory cells of normal/benign prostatic glands (13, 38). Although the presence or absence of ERG in tumors was more uniform, ANXA2 expression was variable and tumors were scored as positive if more than 10% of cells stained positive for ANXA2. Tumors were referred to as WD, if the primary Gleason pattern was 3 or less and as PD if the primary Gleason pattern was 4 or 5. On this basis, we classified 28 tumors as WD and 12 as PD. Among the 28 WD tumors, a majority (67.8% or 19/28) were ERG(+) and ANXA2(–), while half of the 12 PD tumors were ERG(–) and ANXA2(+) (Table 1; Fig. 5). Most of the ERG(+) tumors (22 WD and 4 PD) exhibited absent or markedly reduced ANXA2 expression (Table 1 and Fig. 5A–C). In contrast, ERG(–) tumors (11 of 14), regardless of differentiation status, exhibited moderate to high levels of ANXA2 expression (Table 1 and Fig. 5D and E). Figure 5E shows a papillary variant of prostatic ductal adenocarcinoma, a rare histologic subtype, characterized by stratified columnar epithelium. The tumor shown here is ERG(–) but is populated by both ANXA2(–) and ANXA2(+) cells. A very small number of tumors (3 of 40: 1 WD and 2 PD) were negative for both ERG and ANXA2 expression (Fig. 5F and G).

Discussion

The comparison of ANXA2 RNA expression to other members of the Annexin gene family from the CPDR 40-patient GeneChip dataset (GSE32448) showed a notable decrease in ANXA2 expression in ERG(+) versus ERG(–) tumors, suggesting a transcriptional regulation of ANXA2 by ERG. This reciprocal correlation of ERG and ANXA2 gene expression observed in prostate tumors was further validated by RNA and protein expression analysis in VCaP cells (Fig. 1C and D). The identification of a prostate-specific ANXA2 promoter allowed us to map putative ERG-binding motifs matching the VSETISF matrix and demonstrate significant recruitment of ERG to five of the eight ERG-binding sites by ChIP assay. Deletion analysis of the ANXA2 promoter in LNCaP cells with inducible ERG expression revealed marked repression in response to ERG overexpression in constructs pGL4.11-ANXA2–1146 and pGL4.11-ANXA2–959, which contain ERG-binding sites #6 and #7 (Fig. 2D, top). However, the analysis of the reporter constructs in VCaP cells showed relieve of transcriptional repression upon ERG siRNA knockdown, only with pGL4.11-ANXA2–959 (Fig. 2D, top and bottom). The smaller increase in transcription activity upon ERG siRNA knockdown in VCaP cells compared with that observed in LNCaP cells when ERG overexpression is induced may reflect the interaction of ERG with other cell-specific transcription factors. Furthermore, the recruitment of ERG to multiple ERG-binding sites of the ANXA2 promoter in the context of the chromatin, as shown by ChIP assay, supports the notion that the regulation of ANXA2 expression may involve other cofactors. Our results support the hypothesis of transcriptional repression of ANXA2 expression by the ERG oncogenic activation in TMPRSS2–ERG–positive prostate cancer cells.

We showed that ANXA2 is localized to the intercellular junction of cells (Fig. 3A), the apical cellular surface (Fig. 3B), and the plasma membrane (Fig. 3C) upon ERG knockdown. These results are consistent with observations that ERG overexpression abrogates cell differentiation (6, 7) and with the function of ANXA2 in establishing a polar and differentiated epithelial phenotype (23, 24). ERG knockdown not only enhanced the localization of E-cadherin and β-catenin, and ZO-1 at intercellular junctions (Fig 4A), but also increased the accumulation of actin filament bundles, a critical step in establishing intercellular adhesion and epithelial polarity (Fig 4B). In contrast, decreased expression of ANXA2 led to an apparent destabilization of the actin cytoskeletal network. These structural changes that resulted from ERG siRNA

Figure 4. A, the knockdown of ERG promotes the reversal of EMT and reestablishes the polarized epithelial phenotype of the VCaP cells, demonstrated by the overexpression of determinants of the polarized epithelial cells, and components of the AJ (E-Cadherin and β-catenin) and TJ (ZO-1). B, compared with control NT-siRNA, knockdown of ERG increases organized f-actin polymerization, while knockdown of ANXA2 results in disorganized f-actin network. Cells were fixed and stained with Alexa Fluor-594 phalloidin 72 hours after transfection. C, ERG inhibits the CDC42 and RHOA activity through repression of ANXA2. The relative activity of activated GTP-bound forms of (i) CDC42, (ii) RHOA, and (iii) RAC1 in were measured by G-LISA assays 72 hours after siRNA transfection (C; two-tailed Student t test, **P < 0.01; ***P < 0.001). For control, see Table 1. C1, C2, C3, and C4 indicate cells treated with control, NT-siRNA, ERG-siRNA, or ANXA2-siRNA, respectively. D, by combined ERG and ANXA2 knockdown (lanes 7 and 8; D), ERG regulates the transition between the polar-differentiated epithelial phenotype and the mesenchymal phenotype by inhibiting CDC42 and RHOA-mediated actin-polymerization through the repression of ANXA2 (E).
knockdown highlight the reversal from a mesenchymal to a differentiated epithelial phenotype (25, 26) and support the function of ANXA2 in establishing and maintaining the epithelial phenotype of prostate glands.

ANXA2 organizes actin remodeling and cell–cell adhesion through Rho-GTPases, which are critical regulators of cell polarity that stabilize the polymerization of actin and its interaction with AJ complexes (9, 30, 36). The increase of activated CDC42 and RHOA upon ERG depletion, and decrease upon ANXA2 knockdown, suggests that the inhibition of differentiation by ERG appears to be achieved, in part, by the activation of CDC42 and, to a lesser extent, RHOA through ANXA2 (Fig. 3C). In addition, the enhanced formation of actin bundles along intercellular junctions upon ERG knockdown coincides with increased phosphorylation and inactivation of cofillin, whereas ANXA2 knockdown decreased cofillin phosphorylation (Fig. 3D). Cofilin phosphorylation is regulated by Rho-GTPases through pathways involving ROCK (RHO-associated protein kinase), PAK1 (p21-activated kinase), and LIMK1 (LIM-kinase 1; refs. 39, 40). Inactivation of cofillin releases ATP*-G-actin monomers, which promotes the elongation of actin filaments. In contrast, the activation of cofillin increases severing of actin filaments and generates free barbed ends that increase branching. These results supports the proposal that ERG inhibits differentiation and promotes EMT, which involve the dissolution of polarized epithelium and actin bundles at cell–cell junctions through the repression of ANXA2, CDC42, and RHOA (Fig. 5).

In a majority of cancers, such as breast (41), gastric (42), pancreatic (43), colorectal (44), and hepatocellular carcinomas (45), the upregulation of ANXA2 during oncogenic transformation is indicative of aggressive disease or poor clinical outcome. In some other malignancies, such as laryngeal and squamous cell carcinoma (46), head and neck dysplasia (47), and osteosarcoma (48), ANXA2 is downregulated and its absence is correlated with PD tumors and with poor prognosis. When upregulated, ANXA2 functions as a coreceptor for plasminogen, tPA and pro-cathepsin B and promotes the conversion of plasminogen into plasmin, which is required for invasion, metastasis, and angiogenesis (10, 41). It is hypothesized that in tumors where ANXA2 is overexpressed, its role as a plasminogen receptor prevails and drives the metastatic process, whereas in tumors where it is downregulated, malignancy is achieved by plasminogen-independent mechanisms such as anaplastic transformation or dedifferentiation (49).

The expression of ANXA2 during the neoplastic transformation of the prostate epithelium reveals a biphasic profile: high in normal tissues, is diminished or lost in WD tumors, but reemerges in advanced tumors. This expression pattern encapsulates the critical cellular functions of ANXA2, which include establishing the differentiated and polarized epithelium, protein trafficking, angiogenesis, and metastasis (8, 11, 49). We can infer that ANXA2 plays a dominant role in the formation of intercellular junctions and apical–basal polarity to support a differentiated epithelial phenotype in normal cells. To facilitate the transition to a mesenchymal phenotype, it may be necessary to silence or repress ANXA2 expression. However, as tumor cells acquire invasive properties, ANXA2 is reenlisted to promote metastasis through its interaction with plasminogen on the cell surface.

Our analysis of ERG and ANXA2 expression in whole-mount prostate sections showed moderate or strong reciprocal ERG and ANXA2 expression in 80% (32 of 40) of the cases examined.
ERG Inhibits ANXA2 Expression and Function in Prostate Cancer

Figure 5.
IHC staining of consecutive sections of representative whole-mounted prostate sections with H&E, ERG and ANXA2 antibodies demonstrate the inverse correlation of ERG and ANXA2 in prostate adenocarcinoma. A, a representative whole-mounted prostate section with index tumor of ERG(+)ANXA2(−) phenotype. In the second row, the region indicated by the rectangle is magnified, showing membranous and cytoplasmic staining of ANXA2 in the luminal and basal cells of benign prostate epithelium (blue arrows). Black arrows indicate WD ERG(+)ANXA2(−) cells. B, WD tumors with both ERG(+)ANXA2(−) (black arrows) and ERG(−)/ANXA2(−) (red arrow) phenotype. Blue arrow indicates benign gland. C and D, PD tumors with inversely correlated ERG(+)ANXA2(−) and ERG(−)/ANXA2(+) phenotype, respectively. E, prostatic ductal adenocarcinoma (papillary variant) that is ERG(−) but has both ANXA2(−) and ANXA2(+) cells. WD and PD tumors of ERG(−)/ANXA2(−) phenotype are represented in F and G, respectively. Benign glands (blue arrows) and endothelial cells (green arrowheads) are ANXA2(+).
The association WD tumors with ANXA2(−) expression status observed here have been noted in previous reports describing the absence or focal presence of ANXA2 in low- and WD prostate cancer (Gleason pattern 3 or lower), but its detection in approximately half of high-grade (Gleason score of 8 or greater) lesions [13, 18, 38, 50]. We observed a significant association between ERG(+)/ANXA2(−) status with WD tumors of low Gleason score (19 of 21). In addition, ERG(−)/ANXA2(+), expression status are associated with PD tumors of higher Gleason score (6 of 11) or WD tumors with secondary or tibatary patterns that are PD (5 of 11). These observations suggest that the evaluation of ERG and ANXA2 expression status has potential utility to enhance the diagnosis and prognosis stratification of prostate cancer. In fact, ANXA2 has been tested as an auxiliary marker together with high-molecular weight cytokeratin, p63, and AMACR for prostate cancer, and with other markers in some other malignancies [8, 38, 49]. Further confirmation of whether tumors of ERG(−)/ANXA2(+) phenotype or the less frequent ERG(−)/ANXA2(−) phenotype may contribute to poor prognosis compared with ERG(+) ANXA2(−) would require studies using larger cohorts of patients.

In summary, we have established the inverse correlation of ERG and ANXA2 in cell culture models and prostate cancer specimens. We showed that the inverse correlation is a result of transcriptional repression of ANXA2 by ERG overexpression. The negative regulation of ANXA2 and subsequently its downstream effectors, the Rho-GTPases, CDC42, and RHOA, are critical in the inhibition of differentiation and reversal of EMT. Whether the pleiotropic function of ANXA2 may regulate the stability and biologic function of ERG remains to be explored. However, the differential reciprocal expression of ERG and ANXA2 in low-grade and high-grade prostate tumors may be useful for the prognostic stratification of prostate cancer.

Disclosure of Potential Conflicts of Interest
A. Dobi has ownership interest (including patents) in Biocare Medical. S.-H. Tan has ownership interest (including patents) in ERG Monoclonal Antibody Patent. No potential conflicts of interest were disclosed by the other authors.

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ERG Oncoprotein Inhibits ANXA2 Expression and Function in Prostate Cancer

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