ERG Is a Critical Regulator of Wnt/LEF1 Signaling in Prostate Cancer

Longtao Wu, Jonathan C. Zhao, Jung Kim, Hong-Jian Jin, Cun-Yu Wang, and Jindan Yu

Abstract
Chromosomal translocations juxtaposing the androgen-responsive TMPRSS2 promoter with the ETS-family transcription factor ERG result in aberrant ERG upregulation in approximately 50% of prostate cancers. Studies to date have shown important roles of ERG in inducing oncogenic properties of prostate cancer. Its molecular mechanisms of action, however, are yet to be fully understood. Here, we report that ERG activates Wnt/LEF1 signaling cascade through multiple mechanisms. ERG bound to the promoters of various Wnt genes to directly increase ligand expression. Consequently, ERG overexpression increased active β-catenin level in the cells and enhanced TCF/LEF1 luciferase reporter activity, which could be partially blocked by WNT-3A inhibitor IWP-2. Most importantly, our data defined LEF1 as a direct target of ERG and that LEF1 inhibition fully abolished ERG-induced Wnt signaling and target gene expression. Furthermore, functional assays showed that Wnt/LEF1 activation phenocopied that of ERG in inducing cell growth, epithelial-to-mesenchymal transition, and cell invasion, whereas blockade of Wnt signaling attenuated these effects. Concordantly, LEF1 expression is significantly upregulated in ERG-high human prostate cancers. Overall, this study provides an important mechanism of activation of Wnt signaling in prostate cancer and nominates LEF1 as a critical mediator of ERG-induced tumorigenesis. Wnt/LEF1 pathway might provide novel targets for therapeutic management of patients with fusion-positive prostate cancer.

Introduction
Prostate cancer is the most commonly diagnosed nonskin cancer and a leading cause of cancer-related death in men of the industrialized world. Over the past years, genetic aberrations such as chromosomal translocations have been reported in a majority of prostate cancers (1). In particular, approximately 50% of human prostate cancers were found to contain chromosomal rearrangements that juxtapose the androgen-responsive TMPRSS2 promoter to the coding region of the oncogenic ETS family transcription factor ERG, resulting in abnormally high expression of ERG protein (2). Mechanistically, TMPRSS2–ERG gene fusions were found to be caused by double-strand DNA breaks and repair that are induced by androgen and/or genomic stress (3–5). Because of the high specificity of TMPRSS2–ERG gene fusion and its being an early molecular event in prostate cancer (6), it has been shown to provide a valuable tool for prostate cancer diagnosis (7, 8). In addition, numerous studies have investigated the potential of TMPRSS2–ERG gene fusions in predicting prostate cancer aggressiveness in various patient cohorts. Although controversial reports exist (9–11), a majority of such studies have suggested that TMPRSS2–ERG gene fusions are associated with aggressive or fatal types of prostate cancer (12–15). Consequently, studies have also begun to evaluate drug sensitivities of this molecular subtype of prostate cancer with an ultimate goal to design therapeutics that specifically target TMPRSS2–ERG gene fusions (16, 17).

Moreover, there are many studies that have attempted to characterize the roles of ERG in prostate tumorigenesis and to decipher the underlying molecular mechanisms. Studies have shown that ERG plays important roles in epithelial-to-mesenchymal transition (EMT) and in increasing cell invasion (18–20). Besides these most prominent functions, ERG has also been shown to increase prostate cancer cell proliferation in vitro; a function that becomes apparent upon stable or relatively long-term ERG dysregulation (21, 22). In addition, transgenic studies have reported that mouse prostate with TMPRSS2–ERG gene fusion alone develops prostatic intraepithelial neoplasia (mPIN; ref. 18), but when cooperated by other oncogenic pathways, such as PTEN deletion or androgen receptor (AR) overexpression, it leads to the development of prostatic adenocarcinoma (3, 23, 24). Mechanistically, ERG is able to regulate multiple oncogenic pathways such as c-Myc, AR, and EZH2, resulting in the
abrogation of epithelial differentiation and promotion of cancerous dedifferentiation (21, 22, 25). Bioinformatic analyses have also revealed increased expression of Wnt-associated pathways in TMPRSS2–ERG fusion-positive human prostate cancer (26) and the expression of Wnt receptor frizzled-4 (FZD4) was experimentally shown to positively correlate with that of ERG (27). Whether and how ERG directly regulates components of the Wnt pathway to induce prostate tumorigenesis, however, have not been carefully examined.

Wnt signaling plays major roles in embryonic development, organogenesis as well as in human diseases including malignancy such as prostate cancer (28). In the canonical Wnt/β-catenin pathway, the binding of Wnts such as WNT3A to the receptors activates a signaling cascade that prevents phosphorylation of cytoplasmic β-catenin by GSK-3β and results in its cytoplasmic accumulation followed by nuclear translocation. Once in the nucleus, β-catenin is recruited by the TCF/LEF1 transcription factors to activate target genes such as c-Myc, MMPs, AXIN2, and LEF1 itself. Upregulation of WNT1, nuclear β-catenin, and LEF1 are strongly correlated with advanced, hormone-refractory prostate cancer (29, 30). Mutations that constitutively stabilize β-catenin and thus activate Wnt signaling have been reported in approximately 5% of prostate cancer (31, 32). For example, a study has reported stabilizing β-catenin mutation in AR-negative prostate cancer cell line, potentially contributing to bone metastasis (33). Moreover, by using the state-of-the-art exome-sequencing, Kumar and colleagues have recently reported mutations in the Wnt pathway molecules specifically in castration-resistant prostate cancer cells (34).

The function of Wnt/TCF signaling in prostate cancer has been extensively explored in various cell lines and mouse models. Activation of β-catenin in mouse prostate has been shown to result in high-grade PIN and continuous prostatic growth after castration (35). Using probasin promoter-directed gene expression, Yu and colleagues have further shown that while the mouse prostate expressing nuclear β-catenin alone or SV40-large T-antigen alone developed mPIN, the activation of both pathways resulted in invasive prostate carcinoma (36). Similarly, Wnt signaling has also been shown to synergize with K-ras to accelerate prostate tumorigenesis in the mice (37). Interestingly, a recent study has reported that increased Wnt paracrine signaling in the surrounding stroma can also initiate mouse prostate cancer (38). These studies strongly support the prostate tumorigenic role of Wnt/TCF signaling in a physiologic setting.

In this study, we report TMPRSS2–ERG as a critical activator of Wnt/LEF1 pathway through multiple mechanisms. We show that ERG directly induces the expression of various Wnt ligands as well as the LEF1 transcription factor. We nominate LEF1 as the most important ERG-targeted component of the Wnt pathway, as LEF1 knockdown completely abolished ERG-induced Wnt signaling, downstream gene expression, and oncogenic properties. Our study thus characterizes a critical signal transduction pathway downstream of TMPRSS2–ERG gene fusions and offers novel therapeutic opportunities for targeting fusion-positive prostate cancer.

Materials and Methods

Cell lines and treatments

Prostate cancer cell lines LNCaP, VCaP, 22Rv1, BPH1, RWPE-1, DU145, and human embryonic kidney cell line HEK293T cells were obtained from American Type Culture Collection. Immortalized PrEC cells were provided by Dr. William C. Hahn (Dana-Farber Cancer Institute, Boston, MA). Control L cells and WNT3A-producing L cells were provided by Dr. Cara Gottardi (Northwestern University, Chicago, IL). All cell lines were maintained according to recommended conditions.

Plasmids and siRNAs

Human ERG and LEF1 cDNA were amplified by reverse transcription PCR (RT-PCR) from VCaP cells and cloned into pcDNA3.2 vector (Invitrogen). Dominant-negative LEF1 (dnLEF1) was provided by Dr. Marian Waterman (University of California, Irvine, Irvine, CA). For RNA interference assays, control luciferase GL2 Duplex siRNA (D-00110-01-20), LEF1 siRNA (L-015396-00-0005), and ERG siRNA (D-003886-01) were obtained from Dharmacon. Lentivirus-mediated stable ERG knockdown in VCaP cells was conducted with ERG short hairpin RNA (shRNA) construct (Open Biosystems). Lentivirus particles were generated according to the manufacturer’s instruction and stable clones were selected. For ERG stable overexpression, prostate cancer cells were infected with lentivirus particle containing ERG overexpression construct, and selected with puromycin for 7 days after viral infection.

Western blotting and antibodies

Western blotting was conducted using a standard protocol as previously described (22). Antibodies used in this study are listed in Supplementary Table S1.

Luciferase reporter assay

WNT pathway luciferase reporter assay was conducted as previously described (39). Briefly, cells were seeded in a 24-well plate and cotransfected with the SuperTOPFlash reporter, the Renilla expression plasmid pRL-TK, the ERG expression plasmid pcDNA3-ERG, and pcDNA3–β-catenin or stimulated with WNT3A conditional medium. To confirm the role of LEF1 in the activation of WNT signaling, dnLEF1 was cotransfected with the plasmids as indicated. X-tremeGENE HP (Roche Diagnostics) was used to increase transfection efficiency in LNCaP cells, whereas Lipofectamine 2000 (Invitrogen) was used to transfect other cell lines. Luciferase activities were determined 48 hours posttransfection and normalized against Renilla internal control values.

ChIP, ChIP–qPCR, and qRT-PCR assays

Chromatin immunoprecipitation (ChIP) was conducted as previously described (22, 40). Quantitative PCR (qPCR) was conducted with GoTaq qPCR Master Mix 2× (Promega) using an Applied Biosystems StepOne Plus Real-Time PCR System. All primers were designed using Primer 3 and synthesized by Integrated DNA Technologies and are listed in Supplementary Table S2.
Gene expression microarray and bioinformatics analysis

Microarray profiling was conducted using HumanHT-12 v4.0 Expression BeadChip (Illumina) as previously described (41). The bead-level data were preprocessed using GenomeStudio (Illumina), and the expression data were analyzed and normalized using the beadarray package in Bioconductor. Differential and coordinated expression of a gene set between microarray experimental conditions was conducted using gene set enrichment analysis (GSEA) tools.

Cell proliferation and invasion assay

Cell proliferation assay was conducted using the WST-1 Kit according to the manufacturer's instruction (Clontech Laboratories). For IWP2 treatment, LNCaP cells were treated with 5 nmol/L of IWP2 or DMSO as control for 24 to 72 hours before WST-1 incubation and measurement. Cell invasion was examined as previously described using Boyden Chamber Assay (42).

Accession numbers

The microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database under the accession number GSE47423.

Results

ERG regulates WNT pathway genes in prostate cancer

To evaluate the link between ERG and Wnt signaling, we examined ERG occupancy on a set of 168 Wnt pathway genes defined by Kyoto Encyclopedia of Genes and Genomes (KEGG) functional annotation. ERG ChIP-Seq data obtained from the TMPRSS2–ERG fusion-positive VCaP cells revealed at least one ERG-binding event at the regulatory elements of 72 of 168 KEGG WNT pathway genes (Fig. 1A). As we have previously shown that AR is a critical mediator of ERG function (22), we also examined AR ChIP-Seq data around these regions. Our results showed that AR did not co-occupy these regions, suggesting Wnt signaling as a potentially novel, AR-independent, pathway downstream of ERG. Out of these genes, many are Wnt ligands (Fig. 1B). To confirm these observations, we conducted ERG and immunoglobulin G (IgG) ChIP-qPCR in VCaP cells using primers flanking the binding sites near a number of Wnt ligand genes. Our data confirmed significant ERG enrichment at the promoters of known target genes such as PSA and TMPRSS2 as well as at the binding sites of various Wnt ligands (Fig. 1C). Next, we asked whether these binding events result in ERG-mediated regulation of Wnt pathway gene expression. We carried out RNA interference experiments to

![Figure 1](https://example.com/figure1.png)

Figure 1. ERG regulates Wnt pathway genes in prostate cancer. A, heatmap of ERG-binding sites around Wnt pathway gene promoters. ERG and AR ChIP-Seq was conducted in VCaP cells as previously described (22). Heatmap shows ChIP-Seq read intensity around the transcription start site of Wnt pathway genes. Genes were ranked by the height of ChIP-Seq peaks. B, ERG occupancy near the promoters of representative Wnt pathway genes. ERG ChIP-Seq was conducted in VCaP cells as in A. Chromosomal positions are shown at the top and the gene structure at the bottom. Black arrow indicates transcription start site and direction. C, ERG directly binds Wnt ligand gene promoters. ERG and IgG ChIP was conducted in VCaP cells. ChIP-qPCR was conducted using gene-specific primers. PSA and TMPRSS2 are known target genes of ERG. Error bars indicate triplicate experiments, mean ± SEM. D, Wnt ligand expression is reduced following ERG knockdown. VCaP cells were infected with ERG shRNA lentivirus and selected for clones with stable knockdown. Gene expression was assayed using qRT-PCR and normalized to GAPDH. E, Western blot analysis confirmed ERG knockdown by siRNA. VCaP cells were transfected with siRNA duplex targeting control luciferase or ERG gene. F, Wnt pathway genes are enriched for downregulation upon ERG knockdown. VCaP control (siCtrl) and ERG knockdown (siERG) cells as confirmed in D were analyzed by expression microarray. GSEA was used to determine whether the expression of Wnt pathway genes was significantly different between siCtrl and siERG cells.
knockdown ERG expression in VCaP cells. Quantitative reverse transcriptase PCR (qRT-PCR) analysis showed that stable ERG knockdown led to significant inhibition of WNT2, WNT3A, and WNT11 gene expression, whereas WNT1 expression was not detectable (Fig. 1D). To obtain a global view of gene expression changes, expression microarrays were then conducted in the control and ERG-knockdown VCaP cells (Fig. 1E). Using GSEA (43), we found that Wnt pathway genes were significantly (P = 0.012) enriched for downregulation by ERG knockdown, suggesting that ERG may induce Wnt signaling in prostate cancer (Fig. 1F). We next sought to confirm this in another independent prostate cancer cell line and further examine how ERG regulates Wnt signaling in prostate cancer.

**ERG directly induces Wnt ligand expression in prostate cancer cells**

To validate ERG occupancy on the promoter regions of Wnt ligands in an independent prostate cell line, we carried out ectopic ERG overexpression in the TMPRSS2–ERG fusion-negative LNCaP cells to generate stable cell lines. Western blot analysis showed that ectopic ERG expression in LNCaP cells was at a level comparable with endogenous ERG in VCaP cells resulted from gene fusion (Supplementary Fig. S1A). ERG ChIP was then conducted in the control and ERG-expressing LNCaP cells. ChIP-qPCR analysis confirmed highly significant ERG enrichment at the promoters of various Wnt ligands such as WNT1, WNT2, WNT3A, and WNT11 following ERG overexpression (Fig. 2A). Next, we asked whether these binding events result in the regulation of gene expression. Using qRT-PCR, we compared Wnt gene expression between the control and ERG-expressing LNCaP cells (Fig. 2B). Through analysis of previously reported ERG targets, we first validated that ERG indeed induced PLAT and PLAU and inhibited PSA and TMPRSS2 gene expression (18, 22). Interestingly, our data showed that ERG drastically induced Wnt ligand gene expression. Similar patterns of ERG-mediated regulation of target genes were also observed in another independent prostate cancer cell line, 22Rv1 (Fig. 2C). We next decided to focus on WNT3A, one of the previously reported ERG targets, we further and examined how ERG regulates other key components of the Wnt pathway.

**ERG increases active β-catenin and stimulates Wnt signaling in prostate cancer cells**

As Wnt ligand binding to receptors turns on a series of biochemical reactions, ultimately leading to accumulation of unphosphorylated, active β-catenin, which subsequently associates with TCF/LEF1 family transcriptional factors to induce Wnt downstream genes, we conducted Western blot analysis of β-catenin. Our analysis of control and ERG-expressing 293T cells revealed significantly increased active β-catenin level by ectopic ERG overexpression (Fig. 3A). This is especially prominent in cells that were cotransfected with ectopic wild-type β-catenin, and thus there were much more total β-catenin available. In contrast, ERG did not alter the level of total β-catenin. On the contrary, Western blot analysis showed that ERG knockdown in VCaP cells resulted in dramatic decrease of active β-catenin level (Fig. 3B).

Previous studies have shown that WNT3A is able to induce phosphorylation of AKT and the downstream GSK-3β, which in turn leads to accumulation of active β-catenin (46–48). In addition, ERG overexpression has been shown to cooperate with AKT upregulation in producing adenocarcinoma in mice (23). We thus investigated the levels of these proteins in prostate cancer cells following ERG overexpression. Interestingly, Western blot analysis revealed that AKT and GSK-3β phosphorylation were indeed drastically increased by ERG overexpression, being consistent with the accumulation of active β-catenin (Fig. 3C).

Active β-catenin is known to translocate to the nucleus where it interacts with TCF/LEF1 proteins for transcriptional activation of downstream genes. We thus examined the effect of β-catenin and/or ERG overexpression on SuperTOPFlash reporter activity in 293T cells. Our results confirmed that either ERG or ectopic β-catenin overexpression indeed dramatically
increased SuperTOPFlash activity (Fig. 3D). Similar effects were also observed in prostate cancer cell lines such as 22Rv1 and LNCaP (Fig. 3E and Supplementary Fig. S2A). In addition, there is a strong synergistic effect between β-catenin and ERG overexpression, being consistent with the level of active β-catenin shown in Fig. 3A. It is also plausible that ERG may be able to induce additional components of the Wnt pathway that are downstream of β-catenin. To further test the role of ERG in activating Wnt signaling, we examined the expression of AXIN2, a direct target and also a negative feedback regulator of the Wnt/TCF pathway (49). ERG was overexpressed in a panel of prostate cell lines that do not harbor TMPRSS2–ERG gene fusions (Supplementary Fig. S2B). qRT-PCR analysis showed significant AXIN2 upregulation following ectopic ERG overexpression in prostate cell lines such as BPH1, RWPE, and 22Rv1 (Fig. 3F). Being consistent with this, ERG knockdown in VCaP cells, on the other hand, resulted in drastic decrease of AXIN2 expression (Fig. 3G).
LEF1 is a critical mediator of ERG-induced Wnt signaling

LEF1 is a member of the TCF family transcription factors that interact with active β-catenin to turn on downstream Wnt pathway genes. LEF1 has been implicated in promoting prostate cancer progression (50). Intriguingly, analysis of VCaP ERG ChIP-Seq data revealed several strong ERG-binding events near the LEF1 promoter, suggesting that LEF1 may be a direct target of ERG in prostate cancer (Fig. 4A). To confirm this, LNCaP cells were infected with a series dilution of ERG-expressing adenovirus and subjected to ERG ChIP. ChIP-qPCR analysis revealed significant ERG occupancy on the LEF1 promoter in ERG-overexpressing LNCaP cells (Fig. 4B). Being concordant with this, LEF1 protein level was drastically induced following increasing amount of ERG overexpression, whereas that of AR, an ERG-inhibited gene, was gradually repressed (Fig. 4C). The ability of ERG to dramatically increase LEF1 expression was also confirmed in independent prostate cancer cell lines such as 22Rv1 and DU145 (Fig. 4D). In contrast, other members of TCF family transcription factors such as TCF1, TCF3, and TCF4 were not significantly altered by ERG (Supplementary Fig. S3A). Moreover, Western blot analysis revealed that ERG knockdown in VCaP cells drastically reduced LEF1 expression (Fig. 4E). Taken together, our data suggest that LEF1 may be the most important TCF family
transcription factor that conveys the effects of ERG on Wnt signaling in prostate cancer.

We next attempted to determine whether LEF1 inactivation is able to attenuate ERG-induced Wnt signaling using SuperTOPFlash reporter assays. A dnLEF1 was used to block LEF1 activity as previously reported (51). Importantly, luciferase reporter assays showed that while ERG substantially increased SuperTOPFlash activities in addition to that mediated by β-catenin overexpression, dnLEF1 fully blocked ERG-induced Wnt signaling. This result was observed in both 22Rv1 (Fig. 4F) and HEK293T cells (Supplementary Fig. S3B). In addition, we used siRNA to target LEF1 in ERG-expressing cells (Supplementary Fig. S3C) and examined the effect on SuperTOPFlash activity. Our results confirmed that siLEF1 significantly reduced Wnt signaling mediated by β-catenin and fully blocked ERG-induced SuperTOPFlash activities, suggesting LEF1 as a predominant mediator of ERG regulation of Wnt signaling (Supplementary Fig. S3D). We thus asked whether LEF1 knockdown is able to reverse ERG regulation of downstream gene expression.

To address this, we conducted microarray profiling of LNCaP cells treated with control, ERG overexpression, or ERG overexpression with concomitant LEF1 knockdown (Supplementary Fig. S3C). Analysis of the microarray data revealed 359 and 449 genes that were respectively induced and repressed by ERG with a 2-fold cutoff. GSEA analysis showed that ERG-induced genes were significantly enriched for repression upon LEF1 knockdown (Fig. 4G). In contrast, ERG-repressed gene expression was restored following LEF1 depletion (Supplementary Fig. S3E). Together, these data strongly suggest that LEF1 is a critical modulator of ERG regulation of downstream gene expression.
gene expression. This prompted us to examine whether Wnt/LEF1 signaling may also be critical in mediating ERG oncogenic function.

Wnt/LEF1 signaling mediates the oncogenic properties of ERG

ERG has been previously shown to regulate cell proliferation (21, 22). We first confirmed this using WST-1 cell growth assay in LNCaP cells treated with control or ERG overexpression (Fig. 5A). Next, we treated these cells with IWP-2 to block Wnt ligands production. Interestingly, WST-1 assays showed that IWP-2 significantly reduced the proliferation of control and ERG-expressing LNCaP cells, likely due to its suppression of both endogenous and ERG-stimulated Wnt ligands secretion. We also noted that ERG continued to significantly increase cell growth even in the presence of IWP-2, albeit at a much lower level. This is likely due to its upregulation of other downstream components of Wnt pathway such as LEF1. To test this, we conducted LEF1 knockdown in control and ERG-expressing LNCaP cells. WST-1 cell proliferation assays showed that LEF1 knockdown not only significantly reduced the growth of control cells, but also fully blocked ERG-mediated LNCaP prostate cancer cell growth (Fig. 5B).

We next examined how Wnt/LEF1 pathway regulates the effects of ERG on cell motility. We have earlier shown that ERG further induces SuperTOPFlash reporter activities in addition to β-catenin (Fig. 3D and E), showing an additive/synergistic effect in enhancing WNT/LEF1 signaling. In concordance with this, Boyden Chamber Assay illustrated that while ectopic β-catenin dramatically induced LNCaP cell invasion, ERG overexpression significantly increased the invasion capability of both control and β-catenin–overexpressing cells (Fig. 5C and D). To determine whether LEF1 is the key mediator of ERG-induced cell invasion as was in the case of SuperTOPFlash assay described in Fig. 4E, cell invasion assays were conducted in an independent cell line DU145 with stable LEF1, ERG, or ERG and dnLEF1 overexpression (Fig. 5E and F). We confirmed that ERG overexpression increased cell invasion and that LEF1 overexpression led to a comparable level of induction of cell invasion. Moreover, inactivation of LEF1 using dnLEF1 in the ERG-expression cells suppressed invasion of ERG-overexpressing cells. These results, along with the cell proliferation data shown in Fig. 5A and B, strongly highlight LEF1 as a critical modulator of tumorigenic roles of ERG in prostate cancer. As increased cell invasion is often causally associated with EMT and ERG overexpression has been previously associated with
EMT (19, 20, 27), we decided to reexamine this in the context of Wnt/LEF1 signaling.

**ERG and LEF1 induces EMT and LEF1 is selectively upregulated in TMPRSS2–ERG fusion-positive prostate cancer**

We examined the morphology of DU145 cells with stable overexpression of vector control or ERG. Importantly, we observed that while the control cells tend to grow in clusters or patches that are characteristic of epithelial cells, ERG overexpression led to a phenotypical switch of the cells to a more spindle-like shape and the ERG-expressing cells grow in isolated form with much less cell-to-cell contact (Fig. 6A). Consistent with this morphologic change, qRT-PCR analysis showed that epithelial markers such as E-cadherin and Claudin-1 were indeed dramatically downregulated by ERG and that LEF1 overexpression showed a similar effect (Fig. 6B). Immunoblot analysis confirmed the loss of E-cadherin and Claudin-1 protein in ERG-expressing cells and further showed that this effect was phenocopied by LEF1 overexpression (Fig. 6C). With this understanding of a primary role of LEF1 in ERG-mediated oncogenic properties, we analyzed LEF1 expression in human prostate cancer to see if their expression levels are correlated in vivo.

We reanalyzed a previously published microarray dataset that profiled global gene expression of 472 primary prostate cancer tissues (52). We rank-ordered all samples by the level of ERG transcript and arbitrarily separated them into TMPRSS2–ERG fusion-negative and -positive groups based on the expression profile (Fig. 6D). Statistical analysis confirmed that ERG level is substantially ($P = 7.8E-164$) different between these two groups and found that LEF1 is indeed significantly ($P = 0.0036$) overexpressed in the ERG-high prostate cancers (Fig. 6E).

**Discussion**

Over the past years, ERG has been shown to regulate various oncogenic pathways in prostate cancer. The first link to the Wnt pathway was suggested by bioinformatics analysis, which revealed that Wnt pathway genes are upregulated in TMPRSS2–ERG fusion-positive prostate cancer (26). Later studies have shown that the Wnt receptor FZD4 was positively regulated by ERG and mediated its effect on regulating E-cadherin and β-integrin expression (27). Another study further showed that integrin-linked kinase is a target of ERG and is important in ERG-induced EMT and invasive characteristics.

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**Figure 6.** ERG and LEF1 induces EMT and are dysregulated in prostate cancer. A, ERG overexpression induces EMT of prostate epithelial cells. Phase-contrast microscopy images of stable control and ERG-expressing DU145 cells are shown. ERG-expressing cells lost cell-to-cell contact and showed a spindle-like shape. B, the expression of epithelial markers is decreased by ERG and LEF1. qRT-PCR analysis of E-cadherin and Claudin-1 was conducted in control and DU145 cells with ERG and LEF1 overexpression. C, ERG and LEF1 inhibit the expression of epithelial markers. Stable control, ERG-expressing, and LEF1-expressing DU145 cells were analyzed by Western blot analysis. D, categorization of ERG$^-$ and ERG$^+$ prostate cancer specimens. A total of 472 primary prostate cancer tissues were separated into ERG$^-$ and ERG$^+$ groups based on the expression level of ERG gene (52). E, LEF1 is significantly upregulated in the ERG$^+$ prostate cancers. Box plot analysis shows the expression level of ERG and LEF1 in the ERG$^-$ and ERG$^+$ prostate cancer specimens.
cancer cell lines, especially LNCaP cells, we carried out several SuperTOPFlash assays. Usually, SuperTOPFlash assays do not work very well in prostate Wnt signaling pathway. Reduced or abolished ERG-induced luciferase activity. Moreover, we have observed that 22Rv1 prostate cancer cell line is much easier to transfect, which was used as a prostate cancer model, in addition to the conventional HEK293 cell line for SuperTOPFlash assays. Therefore, this study is the first to comprehensively analyze how ERG regulates Wnt signal transduction and how this process contributes to prostate tumorigenesis.

LEF1 is the transcriptional effector of Wnt signaling in that it turns on the downstream transcriptional program downstream upon binding by nuclear β-catenin. Interestingly, LEF1 itself is also a Wnt target gene that is induced by Wnt activation. In this study, we observed strong ERG protein-binding events around the LEF1 promoter, suggesting its being a direct target of ERG. But being a Wnt target, LEF1 can also be induced indirectly by ERG through Wnt signaling. Using immuno blot analysis, we have repeatedly observed drastic upregulation of LEF1 protein following ERG overexpression. It is plausible that ERG may also be able to stabilize LEF1 protein, which may be interesting lines for future studies. Nevertheless, our results illustrated that ERG exerts tight control of LEF1 protein and thus its transcriptional activity through multiple mechanisms. In addition, LEF1 knockdown is able to sufficiently abolish the effect of ERG in regulating target gene expression and inducing oncogenic properties. These results establish LEF1 as a critical target and mediator of ERG function in prostate cancer. Wnt/LEF1 signaling thus contribute to the Wnt/TCF pathway genes as important contributors to castration resistance. In addition, using transgenic mice several groups have shown that Wnt/TCF pathway activation promotes prostate cancer initiation and progression.

Although both Wnt/LEF1 pathway and ERG gene have been shown to promote prostate cancer in vivo using xenografts and transgenic mice, it will be extremely important in future studies to block Wnt/LEF1 signaling in ERG-increasing mouse models to further show the physiologic importance of the ERG–Wnt–LEF1 axis in prostate cancer.

Exomes of prostate cancer tissues and have revealed mutations to the Wnt/TCF pathway genes as important contributors to castration resistance. In addition, using transgenic mice several groups have shown that Wnt/TCF pathway activation promotes prostate cancer initiation and progression. Therefore, evidence are emerging that strongly support the physiologic importance of the Wnt/TCF pathway in prostate cancer. However, it is important to note that, as AR can compete with TCF/LEF1 factors for elevated β-catenin as previously documented, increase of some components of the Wnt/TCF pathway may affect AR signaling rather than downstream Wnt target genes. Nonetheless, the strong upregulation of LEF1 protein by ERG shall directly lead to the elevation of Wnt/LEF1 pathway, which would be highly significant, as LEF1 has been previously shown upregulated and physiologically important in castration-resistant prostate cancer. Although both Wnt/LEF1 pathway and ERG gene have been shown to promote prostate cancer in vivo using xenografts and transgenic mice, it will be extremely important in future studies to block Wnt/LEF1 signaling in ERG-increasing mouse models to further show the physiologic importance of the ERG–Wnt–LEF1 axis in prostate cancer.

ERG Gene Fusions Activate Wnt/LEF1 Signaling

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and a vast majority of its target genes such as PSA and TMPRSS2. We proposed that ERG suppression of AR signaling inhibits epithelial differentiation, thus contributing to prostate cancer progression. In contrast, in this study ERG activation of the Wnt/LEF1 pathway results in transition of the cells to more mesenchymal (thus poorly differentiated) cell types. Although the primary role of ERG in prostate cancer is in regulating cell motility, studies have shown that ERG can also induce cell growth, especially upon stable or long-term overexpression (21, 22). In this study, we also found that Wnt/LEF1 pathway can also mediate this effect of ERG on cell growth. Moreover, LEF1 has been previously shown to upregulate AR expression and transcriptional activity (50). These results strongly suggest a model wherein ERG inhibits prostatic differentiation by disrupting AR transcriptional regulation and induces mesenchymal maldifferentiation through activation of Wnt/LEF1 signaling. In addition, LEF1 upregulation may compensate the antiproliferative effect of ERG resulted from its inhibition of AR, thus leading to a net gain of EMT and cell invasion. Taken together, Wnt/LEF1 signaling is a critical downstream pathway that is important for ERG-mediated tumorigenesis.

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

References

4. Lin C, Yang L, Tanasa B, Hutt K, Ju BG, Ohgi K, et al. Nuclear receptor-RBP-Jk transcriptional activity (50). These results strongly suggest a model wherein ERG inhibits prostatic differentiation by disrupting AR transcriptional regulation and induces mesenchymal maldifferentiation through activation of Wnt/LEF1 signaling. In addition, LEF1 upregulation may compensate the antiproliferative effect of ERG resulted from its inhibition of AR, thus leading to a net gain of EMT and cell invasion. Taken together, Wnt/LEF1 signaling is a critical downstream pathway that is important for ERG-mediated tumorigenesis.

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

References


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