Physical and Functional Interactions between ELL2 and RB in the Suppression of Prostate Cancer Cell Proliferation, Migration, and Invasion

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Abstract

Elongation factor, RNA polymerase II, 2 (ELL2) is expressed and regulated by androgens in the prostate. ELL2 and ELL-associated factor 2 (EAF2) form a stable complex, and their orthologs in Caenorhabditis elegans appear to be functionally similar. In C. elegans, the EAF2 ortholog eaf-1 was reported to interact with the retinoblastoma (RB) pathway to control development and fertility in worms. Because RB loss is frequent in prostate cancer, ELL2 interaction with RB might be important for prostate homeostasis. The present study explored physical and functional interaction of ELL2 with RB in prostate cancer. ELL2 expression in human prostate cancer specimens was detected using quantitative polymerase chain reaction coupled with laser capture microdissection. Co-immunoprecipitation coupled with deletion mutagenesis was used to determine ELL2 association with RB. Functional interaction between ELL2 and RB was tested using siRNA knockdown, BrdU incorporation, Transwell, and/or invasion assays in LNCaP, C4-2, and 22Rv1 prostate cancer cells. ELL2 expression was downregulated in high-Gleason score prostate cancer specimens. ELL2 could be bound and stabilized by RB, and this interaction was mediated through the N-terminus of ELL2 and the C-terminus of RB. Concurrent siRNA knockdown of ELL2 and RB enhanced cell proliferation, migration, and invasion as compared to knockdown of ELL2 or RB alone in prostate cancer cells. ELL2 and RB can interact physically and functionally to suppress prostate cancer progression.

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Introduction

Loss of the retinoblastoma tumor suppressor gene (RB) has been reported in primary prostate tumors [1], and simultaneous gene methylation and allelic deletion of RB are frequent in castration-resistant prostate cancer [2,3]. In one study, deletion of the RB locus was reported in approximately 5% of primary tumors of prostate cancer and was predominantly associated (30%-40%) with castration-resistant prostate cancer [4]. Inactivation of RB has been shown to enhance the expression of androgen receptor, and knockdown of RB in LNCaP cells reduced their sensitivity to androgen ablation [1].

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Conditional deletion of RB in prostate epithelial cells resulted in the induction of focal hyperplasia and murine prostatic intraepithelial neoplasia in mouse models [5,6]. Cumulatively, these studies indicate that loss of function of RB is an important step in prostate carcinogenesis. Although RB disruption is strongly associated with tumorigenesis, the mechanisms by which RB dysfunction promotes prostate cancer progression still remain incompletely understood. Elucidation of interactions of the RB with its binding partners could provide greater insights into its role in suppressing prostate carcinogenesis.

Elongation factor, RNA polymerase II, 2 (ELL2) is a homolog of ELL1, which was initially identified based on its gene fusion with mixed-lineage leukemia in acute myeloid leukemia and named eleven-nineteen lysine-rich leukemia [7–9]. Transcription elongation plays a key role in efficient gene regulation and is frequently deregulated during cancer development and progression [10]. A variety of transcription elongation factors are involved in transcription elongation regulation of various genes [11]. ELL2 is a component of the super elongation complex which is required for rapid transcriptional induction in the presence or absence of paused Pol II [8,10,12]. ELL-associated factor 2 (EAF2) is tightly associated with ELL proteins [8,13]. ELL family proteins enhance transcription elongation, whereas EAF family proteins can stimulate transcription elongation activity of ELL proteins [8,14]. Inactivation of ELL or EAF orthologs in C. elegans resulted in identical phenotypes, suggesting that ELL and EAF family proteins act together to regulate downstream cellular pathways [15]. ELL2 and EAF2 were reported as androgen-response genes in the prostate [16–19]. EAF2 was also shown to act as a tumor suppressor in the murine prostate [17]. More recently, we identified genetic interactions between the EAF2 ortholog in C. elegans and the retinoblastoma protein (RB) signaling pathway [20]. Thus, ELL2 may also suppress prostate cancer and interact with RB signaling. Here we examined the function of ELL2 and its interactions with RB in human prostate cancer cells. Our studies suggest that ELL2 interacts with RB and plays a potential tumor suppressive role in prostate cancer.

Material and Methods

**Laser-Capture Microdissection and Quantitative Polymerase Chain Reaction (qPCR)**

Human prostate cancer tissue specimens without any previous chemo-, radio-, or hormone therapy were obtained from the UPCI Tissue and Research Pathology Services. Use of these prostate tissues was approved by the University of Pittsburgh Institutional Review Board. Prostate cancer cells and adjacent normal glandular cells were isolated by laser-capture microdissection using a Leica LMD6000 Microsystems microscope (Wetzlar, Germany) equipped with an HV-D20P Hitachi (Tokyo, Japan) color camera and Leica Laser Microsystems microscope (Wetzlar, Germany). Captured individual tissue specimens were lysed, and RNA isolation, reverse transcription, and real-time qPCR were performed using CellsDirect One-Step qRT-PCR Kit (Invitrogen, Carlsbad, CA). Gene-specific primers and Taqman probes cross exon/exon junctions and were designed previously [21] (Table 1). Probes contained FAM fluorophore and TAMRA quencher. TBP was purchased from Applied Biosystems (Foster City, CA). Primer and probe combinations were optimized and validated previously [21]. Real-time PCR was performed on a Bio-Rad IQ5 (Bio-Rad Laboratories, Hercules, CA) or ABI Step-One Plus (Applied Biosystems, Foster City, CA). Real-time PCR data were analyzed by ∆Cp (crossing point) method as R = 2 [Cp sample – Cp control] [21] to generate the relative expression ratio (R) of each target gene relative to GAPDH.

**Data Analysis Using c-BioPortal**

The c-BioPortal for Cancer Genomics site (http://cibiportal.org) was used to determine the alteration frequency in ELL2 and RB1 in prostate cancer data sets. Data from the Prostate Adenocarcinoma (TCGA, Provisional) case set was used to generate an OncoPrint with individual genes represented as rows and individual patients indicated as columns.

**Cell Culture, Overexpression, and Knockdown**

For cell culture, cell lines LNCaP, 22RV1, HEK 293 (ATCC), and C4-2 (kind gift from Leland K. Chung) were cultured in RPMI-1640 (10-040-CV, Corning cellgro) or Dulbecco’s modified Eagle’s medium (12-604F, Lonza) supplemented with 10% fetal bovine serum (FBS) (S11150, Atlanta Biologicals) and 5% antibiotics.

For overexpression experiments, HEK 293 cells were transiently transfected with indicated expression vector(s) using PolyJet In Vitro Transfection reagent (SL100688, SignaGen Laboratories) according to the manufacturer’s instructions. Cells were harvested and prepared for subsequent experiments 48 hours after transfection. After 48 hours, the transfected cells were directly lysed and analyzed by Western blot. After blocking for 1 hour at room temperature in 5% milk in phosphate-buffered saline/0.1% Tween-20, Western membranes were blotted using appropriate antibodies, including anti-ELL2 antibody (A302-505A, Bethyl) and anti-RB antibody (554136, BD Biosciences).

For knockdown experiments, cells in six-well plates were transfected with control siRNA (sc-37007 Santa Cruz) or siRNAs targeting RB or ELL2 using DharmaFECT siRNA transfection reagent (T-2001-03, Dharmacon). The final concentration of siRNA was 50 nM in each well. The control siRNA was used to complement the amount in single-knockdown groups. Forty-eight hours or at indicated times after transfection, the cells were used for further experiments or harvested. All siRNAs used against RB or ELL2 are listed in Table 2 and ordered from IDT (Integrated DNA Technologies, USA). Two different siRNAs were used for each gene to confirm that the impact of siRNAs was due to knockdown of specific gene(s) and not because of their potential off-target effects.

| Table 2. TaqMan Primers and Probes for qPCR |
|---------------------------------------------|
| **Gene** | **Primer/Probe** | **Species** |
| ELL2 | Forward: TGACTGCATCCACGAAAACAT | Human |
| | Reverse: TGCTTTGTGGACACATGCTGA | |
| | Probe: 6FAMTCCTCAAGGCTTAGGACTTCCATAMRA | |
| GAPDH | Forward: CATGTTGTCATGTTTTTGTA | Human |
| | Reverse: GGCTCAAGCATGTTTGTG | |
| | Probe: 6FAMCCAGCTCAAGATCATCACGAATGCCTTCATAMRA | |
For Western blot analysis of proteins related to invasion, C4-2 cells were transfected with siControl, siELL2-1, and/or siRB-1 for 2 days. The cell lysates were then prepared and resolved on sodium dodecyl sulfite (SDS) polyacrylamide gel electrophoresis, and proteins were transferred to polyvinylidene difluoride membranes. The membranes were incubated overnight at 4°C with antibodies for Snail (3879, Cell Signaling Technology), Slug (9585, Cell Signaling Technology), E-cadherin (3195, Cell Signaling Technology), Vimentin (sc-73258, Santa Cruz Biotechnology), Twist (sc-15393, Santa Cruz Biotechnology), and GAPDH (sc-25778, Santa Cruz Biotechnology). GAPDH was used as loading control. Following secondary antibody incubation, immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Bio-Rad).

**Co-Immunoprecipitation**

For co-immunoprecipitation, HEK 293 cells were transfected with flag-tagged wild-type ELL2 and myc-tagged wild-type RB, and proteins were co-immunoprecipitated. HEK 293 cells were transfected with flag-tagged wild-type ELL2 and myc-tagged RB deletion mutants [amino acids (aa) 1-380, 373-787, 768-928]. HEK 293 cells were transfected with myc-tagged wild-type RB and flag-tagged ELL2 deletion mutants (aa 1-292, 293-531, 293-640). The reactions were immunoblotted with Flag and Myc antibodies as indicated. Each blot is representative of three repeated experiments.
BrdU Assay

Cells seeded in 24-well plates were transfected with 50 nM of siRNA as indicated for 48 hours. The cells were subsequently cultured in the presence of 10 μM BrdU (B5002-250MG, Sigma) for 2 hours (C4-2, 22RV1) or 16 hours (LNCaP) and then fixed by Carnoy’s fixative (3:1 methanol:glacial acetic acid) for 20 minutes at −20°C. After treatment with 2 M HCl, cells were washed with 0.1 M boric acid, then incubated with 3% hydrogen peroxide for 20 minutes at 37°C, followed by blocking with 10% goat serum for 1 hour at 37°C. Cells were then incubated with anti-BrdU antibody (B2521, Sigma) overnight at 4°C and CY3 labeled goat anti-mouse secondary antibody (A 10521, Life technologies) for 1 hour at 37°C. Nuclei were stained with 1 μM SYTOX Green (S7020, Life technologies) for 15 minutes at room temperature.

Images were acquired using a fluorescence microscope (Nikon TE2000-U). C4-2 and LNCaP cells were counted using Photoshop CS5 counting tool (Adobe, San Jose, CA), whereas 22RV1 cells were measured using ImageJ software (Wayne Rasband, National Institutes of Health, USA). The percentage of BrdU-positive cells was calculated using (BrdU-positive cell number/total cell number)*100% for C4-2 and LNCaP cells or (BrdU-positive fluorescence area/SYTOX Green–positive area)*100% for 22RV1 cells.

Invasion Assay

Prostate cancer cells were transfected with siRNA at 50 nM each as indicated in a 6-well plate for 48 hours, then collected by trypsin treatment, and then embedded into Matrigel (354234, Matrigel matrix basement membrane, Corning) at a density of 125 cells/μl as 15-μl pellets on the bottom of 12-well plates with 1 ml of RPMI-1640 with 10% FBS added. After incubation at 37°C for 48 hours (C4-2) or 72 hours (LNCaP), images were acquired using a Nikon Eclipse TS100 microscope using a 10× objective. The cells were counted with the Photoshop CS5 counting tool (Adobe, San Jose, CA), and the percentage of invadopodia-positive cells was...
calculated using (invadopodia-positive cell number/total cell number) * 100%.

Migration Assay

Cell migration assay was performed in Transwell chambers (24-well, 8-MM pore size, Corning). C4-2 cells were transfected with siRNA at 50 nM each as indicated for 48 hours, collected by trypsin treatment, and then placed in the top chamber with 200 μl of RPMI without FBS (110,000 cells each chamber). As chemoattractant, 600 μl of RPMI-1640 containing 20% FBS was placed in the bottom chamber. After 48 hours, nonmigrated cells on the top surface of the chamber membrane were removed by gentle scraping with cotton swabs. The migrated cells at the lower surface of chamber membranes were stained with 0.1% crystal violet (S25275B, Fisher Science Education). The number of migrated cells in five random high-power fields (10 × 10) per membrane was counted under a microscope.
Statistical Analysis

Data are presented as mean ± SEM. Statistical analyses were performed with Student’s t test. *, **, and *** denote $P < .05$, $P < .01$, and $P < .001$, respectively.

Results

Down-Regulation of ELL2 Gene Expression in Human Prostate Cancer Tissue Specimens

The expression of ELL2 was examined in human prostate tissue specimens by laser capture microdissection coupled with qPCR. ELL2 expression was not significantly different in tumors with Gleason score ≤ 8 compared to matched normal adjacent tissues. However, in tumors with Gleason score ≥ 9, ELL2 expression levels were significantly decreased ($P = .02$) (Figure 1A). In a study of prostate tumor specimens by Tomlins et al. [22], ELL2 expression was significantly decreased in metastases compared to primary prostate tumors, PIN lesions, and normal adjacent epithelial cells (Figure 1B). ELL2 loss was also examined in several large-scale genomics data sets available through the cBioPortal for Cancer Genomics [23,24]. Of the 11 prostate cancer genomics studies available at the time of query, 9 data sets included ELL2 (Figure 1C). Interestingly, ELL2 alteration was most frequent in the Prostate Adenocarcinoma, Metastatic (Michigan, Nature 2012) data set (Figure 1C). This data set included 50 lethal heavily pretreated castration-resistant prostate cancers and 11 treatment-naive, high-grade localized prostate cancers [3]. These findings suggest that ELL2 loss is associated with advanced prostate cancer.

Co-Immunoprecipitation of ELL2 with RB

Co-immunoprecipitation was used to determine the possibility of a physical interaction between ELL2 and RB in transiently transfected HEK 293 cells. MYC-RB was co-precipitated with FLAG-ELL2, suggesting that ELL2 and RB can be present in the same protein complex (Figure 1C). Attempts to co-immunoprecipitate endogenous ELL2 and RB proteins were unsuccessful, perhaps due to low expression levels of endogenous ELL2 and lack of high-quality anti-ELL2 antibodies suitable for co-immunoprecipitation. Subsequently, we used RB deletion mutants to explore which segment of RB was required for the interaction with ELL2. FLAG-ELL2 was co-precipitated by MYC-RB (aa768-928) or MYC-RB (full length)
using anti-MYC antibody-conjugated agarose beads (Figure 2B). However, when MYC-RB (aa1-380) or MYC-RB (aa373-787) was used, no FLAG-ELL2 was co-precipitated (Figure 2B). These results suggested that the C-terminal domain of RB (aa768-928) was necessary for physical interaction of RB and ELL2. Truncated ELL2 mutants were used to identify which domain of ELL2 was required for interaction with RB. Myc-RB could be co-precipitated by Flag-ELL2 (aa1-292) and FLAG-ELL2 (full length) using anti-FLAG antibody conjugated agarose beads. In contrast, when FLAG-ELL2 (aa293-531) or FLAG-ELL2 (aa293-640) was used, there was no MYC-RB co-precipitated (Figure 2C). Thus, the N-terminal domain of ELL2 (aa1-292) was required for physical interaction of RB and ELL2.

The Effect of RB on ELL2 Protein Level

Because RB and ELL2 can interact with each other, we tested if RB could affect the protein level of ELL2. We transfected equal amount of FLAG-ELL2 plasmids with increasing amount of MYC-RB plasmids into HEK 293 cells, complemented with MYC empty vector. Western blot analysis showed increased protein levels of FLAG-ELL2 corresponding to increased levels of MYC-RB protein (Figure 3A), which suggested that RB could stabilize ELL2.

Because the C-terminal domain of RB (aa768-928) mediates physical interaction with ELL2, we tested whether it could stabilize ELL2. We transfected HEK 293 cells with increasing amount of MYC-RB (aa768-928) plasmids, complemented with MYC empty vector. FLAG-ELL2 protein level increased when increasing amount of MYC-RB (aa768-928) was co-transfected (Figure 3B), indicating that the C-terminal of RB was critical for the stabilization of ELL2. To test whether other domains of RB could stabilize ELL2 protein, we transfected HEK 293 cells with equal amount of FLAG-ELL2 plasmid together with MYC-RB (full length), MYC-RB (aa1-380), MYC-RB (aa373-787), MYC-RB (aa768-928), or the empty MYC empty vector. FLAG-ELL2 protein level was enhanced when MYC-RB (aa768-928) or full-length MYC-RB vector was co-transfected but not when MYC-RB (aa1-380) or MYC-RB (aa373-787) was co-transfected. These observations suggested that the C-terminal of RB was necessary and sufficient for ELL2 stabilization (Figure 3C). We also tested if FLAG-ELL2 could stabilize MYC-RB in co-transfection experiments. However, the effect of FLAG-ELL2 co-transfection on MYC-RB protein level was marginal (data not shown).

As a control, we transfected HEK 293 cells with equal amount of GFP vector with different amounts of full-length MYC-RB complemented with MYC empty vector. No increase in GFP protein level was observed when it was co-transfected with different amounts of MYC-RB, which indicated that MYC-RB stabilization of FLAG-ELL2 was specific (Figure 3D).

To explore if endogenous RB could stabilize ELL2, we tested whether knockdown of endogenous RB could reduce the level of endogenous ELL2 protein in C4-2 cells. The result showed that RB knockdown could reduce the ELL2 protein level. We also tested the effect of ELL2 knockdown on RB level in C4-2 cells, and the result showed that ELL2 knockdown led to lower RB protein level (Figure 3E). These findings suggest that endogenous RB and ELL2 could stabilize each other.

Figure 6. Effect of ELL2 and/or RB knockdown on C4-2 cell migration. (A) Haptotactic Transwell migration assay in C4-2 cells following siRNA knockdown of ELL2 and RB individually or concurrently after 48 hours with 10% FBS as chemotactant. (B) Quantification of migrated cells. Results are expressed as mean ± SEM and are representative of three individual experiments.
The Effect of RB and/or ELL2 Knockdown on Prostate Cell Proliferation, Invasion, and Migration

Because RB and ELL2 can be present in the same complex (Figure 2) and downregulation of ELL2 and RB expression was observed in prostate cancer specimens (Figure 1), we sought to determine whether RB and ELL2 downregulation individually or concurrently could impact proliferation, migration, and invasion of prostate cancer cells. We used BrdU assay to evaluate cell proliferation level after knockdown of RB and ELL2 separately and in combination in C4-2, LNCaP, and 22RV1 prostate cancer cell lines. Knockdown of RB enhanced BrdU incorporation in C4-2, LNCaP, and 22RV1 cell lines (Figure 4). All cell lines showed a slight increase of BrdU-positive cells in ELL2 knockdown group when compared to controls; however, the increase was not statistically significant (for C4-2, \( P = .0576 \); for LNCaP, \( P = .0883 \); and for 22RV1, \( P = .0724 \)). With depletion of both proteins, proliferation level was significantly enhanced when compared to either RB or ELL2 knockdown group (Figure 4). Knockdown of ELL2 or RB enhanced invasion of C4-2 and LNCaP when compared to the control groups, and combined knockdown of both proteins further enhanced invasion compared to single-knockdown groups (Figure 5). We also tested the migration ability of C4-2 cells after knockdown of RB and/or ELL2 protein. Although knockdown of RB or ELL2 individually could enhance migration, double knockdown significantly promoted cell migration compared to single-knockdown groups (Figure 6). Similar results were achieved in C4-2 cells treated with a second set of siRNA to control for potential off-target effects of siRNA (Supplemental Figure S1 in the online version at http://dx.doi.org/10.1016/j.neo.2017.01.001). Cumulatively, these results suggested that loss of both RB and ELL2 could profoundly stimulate prostate cancer proliferation, invasion, and migration.

Because ELL2 and RB knockdown enhanced invasion and migration of prostate cancer cells, we explored the effect of ELL2 and/or RB silencing on expression of a few genes related to invasion and metastasis, including Snail [25], E-cadherin [26], Vimentin [27], Twist [28], and Slug [29]. Knockdown of ELL2 and/or RB had no significant effect on the expression of these genes (Supplemental Figure S2 in the online version at http://dx.doi.org/10.1016/j.neo.2017.01.001).

Discussion

This study presented evidence for ELL2 as a potential tumor suppressor in the prostate because knockdown of ELL2 increased cell proliferation in multiple prostate cancer cell lines and ELL2 is down-regulated in high–Gleason grade and metastatic prostate cancer cells. These observations suggest that the role of ELL2 in prostate carcinogenesis is similar to EAF2, an ELL-associated factor and a potential tumor suppressor [13,17,18,30]. Our previous studies showed that inactivation of ELL2 and EAF2 orthologs caused similar phenotypes in the C. elegans model, suggesting that these two factors regulate similar signaling pathways [15]. Elucidating the mechanism of ELL2 action could provide new insights into the mechanism of EAF2 action and vice versa because they have similar functions.

Concurrent knockdown of RB and ELL2 enhanced proliferation, invasion, and migration in prostate cancer cell lines much more dramatically than the effect of RB or ELL2 knockdown individually (Figures 4-6). This observation suggests that RB and ELL2 can work together to suppress prostate carcinogenesis, and their combined loss should synergistically promote prostate cancer progression. The tumor suppressive activity of RB is stimulated by ELL2, and reciprocity, tumor suppression by ELL2 can be enhanced by RB. Thus, prostate tumor cells with concurrent loss of RB and ELL2 are likely more aggressive. Future studies will be needed to define the signaling pathways mediating synergistic interactions between ELL2 and RB to evaluate whether concurrent loss of ELL2 and RB can be used to identify patients with high-risk prostate cancer.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neo.2017.01.001.

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