Novel lncRNA LINCO0844 Regulates Prostate Cancer Cell Migration and Invasion through AR Signaling

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Abstract

The human genome is mostly transcribed, yielding a rich repository of noncoding transcripts that are involved in a myriad of biological processes including cancer. However, how many noncoding transcripts such as long noncoding RNAs (lncRNA) function in cancer is still unclear. This study identified a novel set of clinically relevant androgen-regulated lncRNAs in prostate cancer. Among this group, LINCO0844 was demonstrated to be a direct androgen-regulated target that is actively transcribed in androgen receptor (AR)–dependent prostate cancer cells. The expression of LINCO0844 is higher in normal prostate compared with malignant and metastatic prostate cancer clinical specimens, and patients with low expression had a poor prognosis and significantly increased biochemical recurrence, suggesting LINCO0844 functions in suppressing tumor progression and metastasis. Indeed, in vitro loss-of-function studies revealed that LINCO0844 prevents prostate cancer cell migration and invasion. Moreover, findings from gene expression profiling analysis indicated that LINCO0844 functions in transcriptional network and the development and progression of prostate cancer.

Implications: This study highlights the function of the lncRNA, LINCO0844, in regulating global AR-regulated genes in prostate cancer by modulating AR binding to chromatin. Mol Cancer Res; 16(12); 1865–78. ©2018 AACR.

Introduction

Prostate cancer is the most common noncutaneous cancer affecting men worldwide (1). Androgen receptor (AR) is a hormone-regulated transcription factor that occupies a central position in both normal prostate development and its carcinogenesis (2). AR mediates its biological effect by controlling the transcription of downstream target genes (3, 4). Therefore, androgens which inhibit the transcriptional activity of AR have become a part of standard care in prostate cancer therapy. Regardless of the favorable initial response, a majority of the patients will relapse into a more lethal and incurable form called metastatic castration-resistant prostate cancer (mCRPC). Extensive research over the years has established that the reactivation or aberrant activation of the AR signaling pathway remains a critical event in the development of mCRPC (5, 6). Hence, it is imperative to elucidate the underlying molecular mechanisms of AR-mediated transcriptional regulation in order to develop better diagnostic markers and improved therapies for the management of advanced prostate cancer.

The discovery that the majority of the human genome is transcribed yielding a rich repository of noncoding RNAs (ncRNA) has generated widespread interest in understanding their functional roles (10). lncRNAs that are greater than 200 bp in length are arbitrarily classified as long noncoding RNA (lncRNA) and have been implicated in a myriad of biological processes including cancer (11, 12). With respect to prostate cancer, a number of lncRNAs have emerged as prostate cancer biomarkers such as Prostate Cancer Antigen 3 (PCA3; refs. 12, 13) and SCLAP1 (14). In gene regulation, SRA (steroid receptor RNA activator) was the first lncRNA that was identified as a transcriptional coregulator for nuclear hormone receptors including AR (15, 16). Since then, additional lncRNAs have been implicated in prostate cancer biology by directly regulating the transcriptional activity of AR. For example, Rosenfeld and colleagues showed two lncRNAs, Prostate Cancer Gene Expression Marker-1 (PCGEM1) and Prostate Cancer Noncoding RNA1 (PRNCR1), which are important for enhancing androgen-dependent transcription by promoting the chromatin looping of AR-bound enhancers to their target gene promoters (17). In addition, CTBP1-AS, an AR-stimulated antisense lncRNA, was found to repress the expression of CTBP1 in a cis-regulatory manner as well as tumor-suppressive genes in trans by recruiting the histone deacetylase corepressor complex via the PTB-associated splicing factor (18). Similarly, HOTAIR was reported to physically interact with AR in order to

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prevent the E3 ubiquitin ligase-mediated degradation of the receptor and thus facilitating an androgen-independent AR transcriptional program and the promotion of mCRPC transformation (19). Although we are beginning to understand the role of a few lncRNAs in prostate cancer, our current knowledge is still limited and a vast repository of lncRNAs have yet to be identified or extensively investigated with respect to AR-mediated transcriptional regulation and prostate cancer biology.

In this study, we have used a combination of bioinformatic analysis and molecular approaches to identify and functionally characterize a novel AR-regulated lncRNA in prostate cancer called Long Intergenic Long Non-Coding RNA 844 (LINC00844). We demonstrate that LINC00844 is clinically significant and potentially plays an important role in metastatic transformation of prostate cancer and provides evidence to show that LINC00844 inhibits migration and invasion of prostate cancer cells without altering the rate of cell proliferation. In addition, we establish that LINC00844 is a critical component of the AR transcriptional network and enhances the global expression of androgen-regulated genes by promoting AR recruitment to chromatin. Finally, our study also reveals that LINC00844 mediates its phenotypic effects in part by regulating the DHT-mediated activation of NDRG1, a known metastatic suppressor gene.

Materials and Methods

Reagents and antibodies

Dihydrotestosterone (DHT) was purchased from Tokyo Chemical Industry, and MDV-3100 and Bicalutamide were purchased form TCI Chemicals. The following antibodies were used for chromatin immunoprecipitation (ChIP) and Western blot analyses: anti-AR (sc-815X, sc-H280, and sc-816) from SCBT, anti-NDRG1 (ab-37897) from Abcam, and anti-β-actin and anti-GAPDH from Sigma.

Cell culture

LNCaP, VCaP, and 22Rv1 were purchased from the ATCC. LNCaP and 22Rv1 cells were maintained in RPMI medium 1640 (RPMI; Gibco) supplemented with 10% FBS (Gibco). VCaP cells were grown in DMEM (Gibco) supplemented with 10% FBS, 1 mmol/L sodium pyruvate, and 0.08% sodium bicarbonate. All cells were routinely tested for mycoplasma contamination (once in every 3 months) by MycoAlert (mycoplasma detection kit by Lonza). In addition, VCaP cells were authenticated by DiagCor (Hong Kong). Cells with early passage numbers (<30 for LNCaP and 22Rv1 and <20 for VCaP) were used for all experiments. Prior to Ethanol (ETOH) or DHT treatment, LNCaP and 22Rv1 cells were deprived of hormones for 3 days by growing them in phenol red–free RPMI containing 10% charcoal–dextran-treated FBS (HyClone), whereas VCaP cells were grown for at least a day in phenol red–free DMEM containing 10% charcoal–dextran-treated FBS.

Plasmids and cell transfection

Full-length LINC00844 was synthesized by IDT and cloned into the pcDNA3.1 vector at EcoRI and Apal restriction sites. A PCMV vector expressing full-length human NDRG1 was purchased from Sino Biologicals. LNCaP cells were transiently transfected with plasmids using Lipofectamine 3000 (Invitrogen) according to the manufacturer’s instructions.

siRNA studies

LNCaP and VCaP cells cultured for 24 hours in phenol red–free medium were transfected with 5 nmol/L of Dicer-substrate siRNAs from IDT, using Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer’s protocol. After 24 hours of incubation, the cells were transfected again in a similar manner with 5 nmol/L siRNA. Forty-eight hours after the second round of transfection, cells were treated with ETOH or 10 nmol/L DHT for another 12 hours before harvesting for real-time reverse transcription-qPCR (RT-qPCR), microarray, and Western blot analyses. For every target, 2 separate siRNAs were paired with the control nontargeting siRNA (NC). siRNA and real-time qPCR primer sequences are listed in Supplementary Table S4 in the Supplementary Material. Gene expression profiles for knockdown studies were obtained from at least three independent experiments.

ChIP-qPCR and ChIP-seq

ChIP assays were performed with minor modifications to the protocol described by Carroll and colleagues (20). For knockdown ChIP assays, 3 million LNCaP cells or 5 million VCaP cells were transfected twice with siRNA targeting LINC00844 or control. Forty-eight hours after transfection and 2 hours of 100 nmol/L DHT/ETOH treatment, cells were cross-linked with 1% formaldehyde and harvested. Cells were lysed, nuclei extracted, and DNA was sonicated using a Biorupter (Diagenode) before overnight immunoprecipitation with AR antibody conjugated to protein G magnetic beads (Dynabeads; Invitrogen). The beads were thoroughly washed and reverse cross-linked at 65°C along with 5% input before isolating DNA using a PCR-purification kit (Qiagen). AR enrichment was quantified by real-time qPCR using primer sequences listed in Supplementary Table S3 and represented as percentage of input.

For the high-throughput sequencing of the ChIP DNA (ChIP-seq), DNA libraries were constructed using the NEBNext Ultra DNA Library Prep Kit for Illumina (NEB; catalogue # E7370L) according to the manufacturer’s protocol. The libraries were amplified for 12 cycles by PCR and purified using AMPure XP beads (Beckman Coulter; catalogue # A63880). The barcoded libraries were mixed in equimolar ratio and sequenced using the Illumina HiSeq 2000. Sequencing reads were mapped with BWA (21) to the indexed reference genome UCSC hg19. The obtained BAM files (as replicates) were subjected for peak calling using MAC2 (22) using default parameters. Specific and overlapping peaks between different test conditions were identified using the HOMER package (23). Heatmaps of the read density and the average binding profiles around the center of peaks (± 1 kb) were produced using the R heatmap package. Genome coverage bedgraph files were converted to bigwig format using the bedGraphToBigWig tool and loaded onto the IGV browser to obtain screen-shots. The raw sequencing data can be found at the GEO with the accession number GSE108704.

Migration and invasion assay

Migration and invasion assays were carried out using LNCaP cells transfected with siRNA and/or plasmids and seeded in Boyden’s Transwell Chamber inserts (uncoated for migration and 1:20 diluted Matrigel coating for invasion) in RPMI media containing 5 nmol/L of siRNA in 100 μL of RPMI with 1% FBS. Note that 600 μL of RPMI with 10% FBS containing ETOH or DHT in the lower chamber acted as chemoattractant. After 48 hours, cells were deprived of hormones for 3 days by growing them in phenol red–free RPMI containing 10% charcoal–dextran-treated FBS (HyClone), whereas VCaP cells were grown for at least a day in phenol red–free DMEM containing 10% charcoal–dextran-treated FBS.
were fixed with 4% formalin and stained with crystal violet, and cells migrated to the other side of the insert were counted using an inverted bright-field microscope at 20X magnification.

Alamar blue cell proliferation assay
LNCaP cells transfected with siRNA for 24 hours were seeded in multiple 96-well plates (as triplicates) at a concentration of 10,000 cells/well followed by a second round of siRNA transfection. To assess for proliferation, cells were incubated with ALAMAR blue reagent for 3 hours, and the fluorescence was measured by the Spectramax with 560EX nm/590EM nm filter settings. The results from three independent experiments are presented as mean ± SEM.

Microarray expression profiling
Microarray analysis was performed as described previously (24). Briefly, purified total RNAs from three independent biological replicates were converted to cRNA using the TargetAmp-Nano Labeling Kit for Illumina Expression BeadChip (Epibio) according to the manufacturer's instructions. cRNA was hybridized onto HumanHT-12 v4 Expression BeadChips (Illumina). The BeadChips were scanned with the iScanner (Illumina), and the image data were processed using GenomeStudio. The gene expression data were analyzed using Lumi-based tools from www.arrayana lysis.org (25). All the microarray data have been deposited at the GEO database with the accession number, GSE109336.

Results
LINC00844 is a novel androgen-upregulated prostate cancer-associated IncRNA
To identify and characterize novel clinically relevant IncRNAs that are associated with prostate carcinogenesis, we processed and analyzed RNA sequencing (RNA-seq) expression data from two independent prostate cancer studies (Fig. 1A; ref. 26, 27). We first used DESeq2 (28) to obtain a list of differentially expressed transcripts (malignant vs. benign or normal tissue) from each cohort and then used metaRNAseq (29) to perform meta-analysis on the two lists. The combined P value from multiple testing was further corrected by the Benjamini and Hochberg method, and using an FDR < 0.01, we identified 370 clinically relevant IncRNAs (Supplementary Table S1). Because our lab is interested in androgen-mediated transcription in prostate cancer cells, we decided to focus on the androgen-regulated IncRNAs within this group. To do this, we first defined a set of androgen-regulated IncRNAs with a minimum of 1.5-fold expression change from the RNA-seq data of LNCaP and VCaP cells that have been treated with or without DHT (30). Next, we overlapped this list of androgen-regulated IncRNAs with the differentially regulated list of IncRNAs from the prostate cancer studies. From this, we found 38 IncRNAs that are both clinically relevant and under androgen transcriptional reg ulation (Supplementary Table S2). We further ranked this list based on their abundance in LNCaP and VCaP cells and validated the top 15 candidates by qPCR (Fig. 1B). Among the top 15 candidates, we identified PCAT1 (26) and RP11-279F6.2 (DRAIC; ref. 31), two IncRNAs that have been previously shown to play an important role in the androgen signaling pathway. As for the other candidates, they are novel androgen-regulated IncRNAs and have yet to be functionally characterized in prostate cancer. We selected several of the more abundantly expressed IncRNAs in LNCaP and VCaP cells for further investigation. In this study, we present our findings for LINC00844.

We began our characterization of LINC00844 by determining its coding potential to confirm whether it is indeed a noncoding transcript. We did this by examining the DNA sequence of LINC00844 using the Coding Potential Calculator (http://cpc. cbio.pku.edu.cn/; ref. 32). A score of −1.04818 was obtained for LINC00844 which suggests that the open reading frame of this IncRNA lacks the potential to code for a protein. Similarly, when we examined the coding potential of HOTAIR, a well-known IncRNA, we obtained a score of −1.19011. In contrast, we obtained a score of 12.246 for GAPDH.

Next, we examined the basic features of the LINC00844 transcript. LINC00844 is an annotated IncRNA (ENSG00000237949, Entrez:100507008) transcribed from an intergenic region on chromosome 10q21.1. The basic Gencode (www.gencode genes.org) annotation maps it as a 477 bp transcript consisting of two exons transcribed from a region of 1.98 kb. To characterize LINC00844 further, we interrogated several publicly available datasets including the recently published Cap Analysis of Gene Expression (CAGE; ref. 33) on prostate tissues, H3K27 acetylation (H3K27ac) ChIP-seq in LNCaP cells (34), and the RNA-seq expression data from LNCaP and VCaP cells (35), as well as prostate cancer patient samples (27). As shown in Fig. 1C, there is a strong H3K27ac peak at the TSS of LINC00844 (marked by the CAGE peak) which increases upon DHT stimulation. Moreover, there is a concomitant increase in the transcript level of LINC00844 in both LNCaP and VCaP cells. In patient samples, LINC00844 is expressed in normal and prostate cancer. Taken together, our results suggest that LINC00844 is a novel prostate cancer–associated IncRNA that is expressed in prostate tissues and is regulated by androgen in prostate cancer cells.

LINC00844 is a direct AR target
Next, we assessed the expression and regulation of LINC00844 in different prostate cancer cell lines. LINC00844 transcript level is highest in the AR-dependent cell lines, LNCaP and VCaP, whereas it is minimally expressed in the AR-sensitive cell line, 22Rv1, and undetectable in the AR-independent cell line, PC3 (Fig. 2A). With respect to its regulation, LINC00844 was stimulated by DHT in a time- and concentration-dependent manner similar to KLK3/PSA, the model AR-regulated gene (Fig. 2B and C; Supplementary Fig. S1A and S1B). In contrast, DHT did not significantly affect the expression of LINC00844 in 22Rv1 cells (Supplementary Fig. S1C). To further validate the AR-mediated regulation of LINC00844, we treated LNCaP and VCaP cells prior to DHT stimulation with either antiandrogens (Bicalutamide or MDV-3100) or siRNA against AR. In both conditions, we found DHT-mediated stimulation of LINC00844 was greatly reduced (Fig. 2D and E).

To determine if AR directly regulates the expression of LINC00844 in prostate cancer by binding in the upstream or downstream regulatory region of the transcript, we interrogated AR ChIP-Seq datasets from cell lines (36, 37) and patient samples (38). As shown in Fig. 2F, there is an ARBS located at the TSS of LINC00844 in LNCaP and VCaP cells as well as in patient samples. In addition, DHT enhanced the recruitment of AR to this site similar to other ARBS associated with known AR-regulated genes (Supplementary Fig. S1D). We verified the binding of AR to the TSS of LINC00844 by ChIP-qPCR and showed that recruitment was abolished by antiandrogens in both LNCaP and VCaP cells (Fig. 2G). Upon examination of the DNA sequence of the ARBS, we identified the presence of a canonical AR response element

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(ARE) motif (Fig. 2F), suggesting that AR binds directly to this site. Taken together, our results suggest that LINC00844 is a direct AR-regulated IncRNA.

LINC00844 is suppressed in malignant and metastatic prostate cancer

To begin understanding the clinical significance of LINC00844 in prostate cancer, we examined the expression profile of the transcript in patient samples. The expression level of LINC00844 is significantly lower in malignant tumor samples compared with their matched controls or benign samples in four prostate cancer cohorts (26, 27, 39), including the two cohorts that we had utilized for our initial characterization of IncRNAs (Fig. 3A–D). This result suggests a potentially important tumor-suppressive functional role for LINC00844. Notably, two out of the four cohorts which also contain the expression information for metastatic prostate cancer samples showed the levels of LINC00844 transcript in these metastatic samples are further suppressed (Fig. 3B and D). Taken together, our results show LINC00844 is the first AR-regulated IncRNA to demonstrate profound association with both malignant and metastatic prostate cancer.

We further examined the association of LINC00844 expression level with additional clinical parameters such as Gleason score, pathologic tumor–node–metastasis (TNM) stage, spread of cancer to lymph nodes, and biochemical recurrence. LINC00844 expression level exhibits a minor but significant negative correlation with Gleason score (i.e., tumors with higher Gleason score which denotes more aggressive form of the cancer show the least
expression for LINC00844) and pathologic TNM stage of the tumor (Fig. 3E and F). Similarly, tumors with lower expression of LINC00844 not only have higher rate of biochemical recurrence but also have increased spread of the tumors to the neighboring or distant lymph nodes (Fig. 3G and H). Finally, we examined the expression level of LINC00844 with respect to prostate cancer patient recurrence-free survival. As expected, patients with higher expression of LINC00844 had a significantly lower recurrence-free survival (log-rank \( P < 0.02 \); Fig. 3I; Supplementary Fig. S2A and S2B). Taken together, the clinical expression data of LINC00844 suggest it may be involved in not only prostate carcinogenesis but also metastatic transformation.

LINC00844 inhibits cell migration and invasion

Because the above clinical observations indicate that LINC00844 may play an important role in metastatic transformation of malignant prostate cancer, we decided to test this out by examining the effect of LINC00844 knockdown and overexpression on LNCaP cell migration and invasion using the Boyden’s chamber assay. In LNCaP cells that were cultured in normal growth media or after androgen stimulation, knockdown of LINC00844 (Fig. 4A, left) significantly increased both migration (Fig. 4B; Supplementary Fig. S3) and invasion (Fig. 4C; Supplementary Fig. S4) of the cells, whereas ectopic expression (Fig. 4A, right) had the opposite effect on both processes (Fig. 4D).
and E; Supplementary Fig. S5A and S5B). Interestingly, LINC00844 also had a significant effect on basal migration and invasion levels, indicating that LINC00844 potentially may also act through AR-independent pathways. Next, we determined if the observed changes in cell migration and invasion were due to an increase in cell proliferation. As shown in Fig. 4F, we did not observe any significant increase in cell proliferation after LINC00844 depletion. Although many reported lncRNAs in prostate cancer are known to affect cell proliferation and migration (35, 40), our results show LINC00844 affects exclusively prostate cancer cell migration and invasion, without affecting cell proliferation. Collectively, our results suggest that LINC00844 (i) is critical for preventing a metastatic phenotype in prostate cancer cells and (ii) mediates its effects in part through the AR signaling pathway.

LINC00844 is an integral component of the AR transcriptional network

To explore whether LINC00844 has a role in regulating AR signaling in prostate cancer, we performed microarray analysis on siNC- or siLINC00844-treated LNCaP cells before and after DHT stimulation. Overall, we detected 917 DHT-responsive genes (fold change ≥ 1.5 and P < 0.01) with 529 up- and 388 downregulated genes (Fig. 5A). Among the 917 DHT-regulated genes, 520 genes (327 up and 193 down) were affected by LINC00844 knockdown (fold change ≥ 1.2). With respect to
the DHT-upregulated genes that were affected, 316 genes were repressed, whereas 11 genes were increased. Moreover, this group of DHT-upregulated genes included well-known model genes such as KLK2, KLK3, FKBP5, and GREB1. As for the DHT-downregulated genes, 177 were upregulated, whereas 16 were further suppressed.

We examined the expression of several model AR-regulated genes by qPCR and observed similar trends in both LNCaP and VCaP cells (Fig. 5C and D). In general, the depletion of LINC00844 suppressed the activation of androgen-regulated genes in both cell lines, although the effects were more profound in LNCaP cells. Thus, our findings suggest that LINC00844 is important regulator of androgen-dependent gene transcription.

We next performed Gene Ontology (GO) analysis using DAVID Functional Annotation Bioinformatics Microarray Analysis (https://david.ncifcrf.gov; ref. 41) on the above androgen-regulated genes that we identified as dependent on LINC00844. In support of our functional studies, we found that androgen-regulated genes that are dependent on LINC00844 for expression are enriched in processes associated with cytoskeletal organization, extracellular matrix organization, microtubule cytoskeleton organization, cell adhesion, structural component of cytoskeleton, and anatomical structural morphogenesis, all of which are GO terms linked to cell migration or metastasis (Fig. 5B). Taken together, our results indicate that LINC00844 is an integral component of the AR transcriptional network and a decrease or loss of LINC00844 expression may promote malignant transformation of prostate cancer by suppressing the DHT-mediated activation of genes.

LINC00844 regulates AR binding to chromatin

Next, we focused on understanding the underlying mechanism on how LINC00844 might control the expression of androgen-regulated genes. Previously, our lab and others have demonstrated that AR collaborative factors such as NKX3-1 (36) and lncRNAs like PlncRNA (42, 43) act through a feed-forward mechanism to regulate AR mRNA and protein levels to increase the expression
Figure 5.
LINCO0844 regulates a subset of AR target genes. A, Gene-expression profiling was performed in LNCaP cells transfected with NC or siLINCO0844 with DHT or ETOH stimulation for 12 hours. Heatmap represents all the AR-regulated genes (NC-ETOH vs. NC-DHT, fold change ≥ 1.5, \( P < 0.01 \)) and their corresponding expression in siLINCO0844 condition. The fold change in expression is mentioned below. The AR-upregulated genes that were suppressed by ≥1.2-fold after LINCO0844 knockdown are represented by orange asterisks. B, GO terms associated with biological processes enriched in LINCO0844-dependent genes. The dotted line represents \( P < 0.05 \). The GO terms associated with cell migration and invasion are highlighted in green. The mRNA expression of several genes selected from the microarray list and identified to be regulated by LINCO0844 was quantified by qPCR in (C) LNCaP and (D) VCaP cells. Results are shown as the mean fold change normalized to NC-ETOH ± SEM from at least three independent experiments.
Figure 6.
LINC00844 promotes AR association to the chromatin. LNCaP and VCaP cells treated with siLINC00844 or NC and 10 nmol/L of DHT or ETOH for 12 hours. A, The mRNA levels were quantified by qPCR, and the bar graph represents AR expression as fold change normalized to NC-ETOH ± SEM from at least three independent experiments. B, AR protein levels were analyzed by Western blot (left) and then quantified by Imagelab (Biorad). Bar graph (right plots) represents the mean normalized (to β-actin) band quantity/SEM from at least three independent experiments. C and D, AR ChIP-qPCR was performed in LNCaP and VCaP cells transfected with siLINC00844 or NC and treated with 100 nmol/L of DHT for 2 hours. Graph represents AR enrichment at the ARBS within ±50 kb from the TSS of the specified gene. All results represent the average of three individual experiments/SEM. E, Venn diagram representing the common ARBS identified from 2 independent AR ChIP-seq experiments in LNCaP cells treated with siLINC00844 or NC and 100 nmol/L of DHT for 2 hours. F, A heatmap representing the sorted AR ChIP-seq signals corresponding to NC-DHT-specific regions in all the tested conditions. Signals are plotted in reference to the center of AR ChIP-seq cluster peak (−/+1 kb). G, A graph comparing the average ChIP-seq tag density around ±1 kb from the AR peak center in NC-DHT compared with siLINC00844-DHT (solid lines) and NC-ETOH compared with siLINC00844-ETOH (dotted lines). H, Snapshots showing the AR ChIP-seq peak at the enhancer region of 4 model AR-regulated genes (KLK3, FKBP5, KLK2, and GREB1). Black arrow represents the ARBS with marked reduction in AR binding after LINC00844 knockdown. I, A table listing the GO terms associated with cell migration that are enriched in the ARBS specific to NC-DHT was calculated by the GREAT tool.
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![Graphs and diagrams showing molecular cancer research data](image-url)
of downstream AR target genes. Thus, we examined if LINC00844 could also function in a similar fashion. As shown in Fig. 6A and B, knockdown of LINC00844 did not significantly alter the mRNA or protein level of AR in both LNCaP and VCaP cells, indicating that LINC00844 likely functions by directly or indirectly influencing the activity of AR, such as through chromatin binding.

Hence, we next determined whether LINC00844 is important for the recruitment of AR to ARBS by performing ChIP assays. For this, we began by examining ARBS associated with AR-regulated genes including KLK2, KLK3, FKBP5, and GREB1, whose expression we have shown is dependent on LINC00844. We tested the recruitment of AR to these binding sites in LNCaP and VCaP cells that were treated with siLINC00844 or siNC. Interestingly, AR recruitment at these regions was markedly reduced by LINC00844 depletion (Fig. 6C and D). These results suggest that LINC00844 influences the expression of the AR-regulated transcription in part by facilitating the recruitment of AR to the chromatin.

To examine whether LINC00844 has a global effect on AR binding, we performed AR ChIP-seq on LNCaP cells that have been treated with or without siLINC00844. The experiment was performed in duplicates for increased confidence, and we considered only the common ARBS for downstream analysis. Overall, the depletion of LINC00844 resulted in a genome-wide reduction of a large number of ARBS (Fig. 6E; Supplementary Table S3). Specifically, 4,227 ARBS (~30%) in the NC-DHT condition were lost after LINC00844 knockdown. Moreover, the ChIP-seq tag intensity of these 4,227 ARBS was markedly reduced after LINC00844 depletion (Fig. 6F–H; Supplementary Fig. S6A–S6F). Interestingly, in the LNCaP cells containing the LINC00844 promoter was also reduced after LINC00844 knockdown, suggesting a possible feed-forward regulation (Supplementary Fig. S6G). GREAT analysis of the ARBS that were affected by LINC00844 depletion showed many terms enriched for cell migration which is consistent with our initial finding that LINC00844 regulates the expression of genes associated with prostate cancer cell migration and invasion (Fig. 6I). Taken together, our results suggest that LINC00844 regulates global androgen-dependent transcription in trans by modulating the binding of AR to chromatin.

**LINC00844 inhibits prostate cancer cell migration and invasion by upregulating the expression of NDRG1**

Next, to identify the potential target gene(s) responsible for mediating the LINC00844-associated phenotype, we performed in-depth analysis of the androgen-regulated genes that requires LINC00844 for expression. We specifically concentrated on genes constituting the GO terms enriched for migration and invasion processes (Fig. 5B) and have demonstrated to have tumor and/or metastasis-suppressing properties. Based on these criteria, we identified 44 LINC00844-dependent genes. After extensive literature review, we narrowed the list further to known metastasis suppressors that are suppressed after LINC00844 knockdown. We ended up with the following potential candidate genes: TUBA3C, SNAI2, TPM1, ITGA7, NKK3-1, and NDRG1. Currently, we are examining the function of several of these genes. In this work, we focus our attention on N-Myc Downstream Regulated 1 (NDRG1) as a DHT- and LINC00844-dependent gene. Our results show that knockdown of LINC00844 markedly reduced the DHT-mediated activation of NDRG1 transcript level in LNCaP and VCaP cells without any effects on its basal levels. Although NDRG1 mRNA levels were suppressed in both cell lines, protein reduction was observed only in LNCaP cells (Fig. 7A and B). In addition, we show that knockdown of LINC00844 reduced the binding of AR to an ARBS located approximately 30 kb upstream from the TSS of NDRG1 (Fig. 7C; Supplementary Fig. S7A).

NDRG1 is associated with a number of different types of cancers including breast, colon, pancreas, and prostate cancer (44–48). In addition, NDRG1 expression is negatively correlated with the cancer status, and overexpression studies with NDRG1 in prostate cancer cells markedly reduced in vivo metastasis (47). NDRG1 expression is also significantly reduced in prostate tumors with lymph node or bone metastasis compared with localized prostate cancer (47, 49, 50). Interrogation of data from the study by Presnner and colleagues (26) showed a reduction in NDRG1 expression in malignant and metastatic samples as compared with normal tissue (Fig. 7D). Similarly, NDRG1 expression was also negatively correlated with Gleason score (Fig. 7E), and low expressing patients showed significantly low recurrence-free survival in the MSKCC dataset (ref. 51; Fig. 7F).

From the above results, we hypothesized that LINC00844 may control the invasiveness of prostate cancer cells in part by regulating the expression of NDRG1. To test this possibility, we transfected LNCaP cells with siNDRG1 and assayed for their migration and invasion ability. If the activation of NDRG1 is essential for inhibiting cell migration and invasion, then we should observe an increase in migration and invasion upon the knockdown of NDRG1. Indeed, depletion of NDRG1 (Supplementary Fig. S7B) significantly increased both the migration (Fig. 7G; Supplementary Fig. S8A) and invasion (Fig. 7H; Supplementary Fig. S8B) activity of LNCaP cells. To provide further supporting evidence that NDRG1 is a direct downstream target of LINC00844, we carried out a set of rescue experiments. First, we...
overexpressed LINC00844 and demonstrated that knockdown of NDRG1 abrogated LINC00844-mediated migration and invasion (Fig. 7J and Supplementary Fig. S9A and S9B). Next, we depleted LINC00844 and showed that overexpression of NDRG1 (Supplementary Fig. S10A) overcame the knockdown effect of LINC00844 on migration and invasion (Fig. 7K and L; Supplementary Fig. S10B and S10C), suggesting that NDRG1 is a downstream target of LINC00844. Collectively, our results suggest that AR and LINC00844 control prostate cancer metastasis by directly regulating the transcription of NDRG1.

Discussion
Recent discoveries and advancements in transcriptomics have revealed crucial roles for the so-called ‘junk DNA’ or ncRNA in a myriad of biological processes including cancer (11). To further our understanding of ncRNAs in prostate cancer, we have systematically analyzed two well-established prostate cancer cohorts of different ethnicity to identify and characterize a novel lncRNA, LINC00844.

AR is critical for the normal development of prostate tissue and plays an important role in its carcinogenesis (3). AR-mediated transcriptional regulation is central in defining the transcriptomic landscape associated with different stages of prostate cancer like hormone-sensitive and mCRPC. The AR-regulatory complex consists of various pioneer factors, coactivators, and corepressors that are recruited to gene promoters in a coordinated and sequential pattern (4). Recent discoveries have exemplified the critical role of IncRNAs in this complex, such as mediating AR-long-range chromatin interactions and altering the chromatin configuration to regulate AR binding to the chromatin (17). Yet the current list of IncRNAs associated with AR transcriptional complex still remains small. In this study, we demonstrate LINC00844 is a novel AR-regulated and prostate cancer–associated IncRNA that is required for regulating the core AR transcriptional network.

Currently, a limited number of prostate cancer–associated IncRNAs have been identified, and only a fraction of them have been studied in detail to divulge the underlying molecular mechanisms. Interestingly, most of the IncRNAs studied so far are known to be upregulated in malignancy; however, our findings show LINC00844 is suppressed in malignancy which suggests a tumor-suppressive functional role. In this work, we showed LINC00844 is significantly suppressed not only in two test cohorts (26, 27) but also in much larger validation datasets, including TCGA (52) and MiTranscriptome (39). Interestingly, we also found the expression of LINC00844 is further suppressed in metastatic prostate cancer samples which suggest that it be may be crucial for inhibiting the metastatic transformation of malignant prostate cancer. In support of a tumor-suppressive functional role for LINC00844, we observed that prostate tumors with low expression of LINC00844 have worst prognostic outcomes and high biochemical recurrence.

Similarly, by means of loss-of-function studies, we demonstrated that the loss of LINC00844 resulted in increased cell migration and invasion without affecting the rate of proliferation. Interestingly, we also observed that the effect of LINC00844 was more pronounced on DHT-mediated cell migration and invasion compared with cells grown in normal growing media or ETOH-stimulated cells. Hence, we argue that LINC00844 mediates its phenotypic effects on prostate cancer cells by primarily targeting the AR transcriptional network.

Our current work indicates that LINC00844 is a critical component of the AR transcriptional machinery. From our microarray analysis, we showed that a loss of LINC00844 leads to a suppression of a subset of DHT-activated genes that are mainly associated with cytoskeletal organization or proliferation of prostate processes. Mechanistically, LINC00844 appears to be crucial for the binding of AR to the chromatin. To probe deeper into the mechanism through which LINC00844 regulates the binding of AR to the chromatin, we performed AR-RNA immunoprecipitation (RIP) and biotinylated LINC00844-mediated protein pull-down assays to examine if LINC00844 physically interacts with AR. Surprisingly, we did not find evidence of any direct physical interactions between LINC00844 and AR (data not shown).

Hence, at this stage, we can only speculate an indirect regulation of AR by LINC00844. Plausible candidates could be coactivator(s) or collaborative factor(s) of the AR; however, this will need to be further studied.

We also provided evidence to show that LINC00844 could potentially regulate migration and invasion of prostate cancer through NDRG1. A previous study has shown that a loss of NDRG1 expression significantly increases in vivo metastasis of prostate cancer cells (47). Similar to LINC00844, NDRG1 is also significantly suppressed in malignant and metastatic prostate cancer samples, and patients with low expression have significantly increased rates of recurrence. However, significant correlation between LINC00844 and NDRG1 expression was not observed. Herein, we demonstrated in vitro DHT-mediated activation of NDRG1 suppresses prostate cancer cell migration, and invasion and LINC00844 is critical for AR recruitment to its enhancer and its expression.

In summary, our work showed LINC00844 is a novel AR-regulated IncRNA that plays an important role in the regulating prostate cancer cell migration and invasion. In addition, LINC00844 appears to regulate AR recruitment to chromatin and enhances the activation of a large number of canonical target genes.

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

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Lingadahalli, S. Jadhao, Y.Y. Sung, M. Chen, X. Chen, E. Cheung
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