Novel IncRNA LINC00844 Regulates Prostate Cancer Cell Migration and Invasion through AR Signaling

Shreyas Lingadahalli1, Sudhir Jadhao1, Ying Ying Sung2, Mi Chen1, Lingling Hu1, Xin Chen1, and Edwin Cheung1

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, LINC00844 was demonstrated to be a direct androgen-regulated target that is actively transcribed in androgen receptor (AR)-dependent prostate cancer cells. The expression of LINC00844 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 LINC00844 functions in suppressing tumor progression and metastasis. Indeed, in vitro loss-of-function studies revealed that LINC00844 prevents prostate cancer cell migration and invasion. Moreover, findings from gene expression profiling analysis indicated that LINC00844 functions in trans, affecting global androgen-regulated gene transcription. Mechanistic evidence reveals that LINC00844 is important in facilitating AR binding to the chromatin. Finally, LINC00844 mediates its phenotypic effects in part by activating the expression of NDRG1, a crucial cancer metastasis suppressor. Collectively, LINC00844 is a novel coregulator of AR that plays a central role in the androgen transcriptional network and the development and progression of prostate cancer.

Implications: This study highlights the function of the lncRNA, LINC00844, 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, antiandrogens (2). AR mediates its biological effect by controlling the transcrip-

1Cancer Center, Faculty of Health Sciences, University of Macau, Taipa, Macau.
2Genome Institute of Singapore, Singapore, Singapore.

Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

Current address for X. Chen: School of Automation, Guangdong University of Technology, Guangzhou, China.

Corresponding Author: Edwin Cheung, University of Macau, 4014 E12, Avenida da Universidade, Macau 999078, Macau. Phone: 853-8822-4992; Fax: 853-8822-2314; E-mail: echeung@umac.mo

do: 10.1158/1541-7786.MCR-18-0087
©2018 American Association for Cancer Research.
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 IncRNAs in prostate cancer, our current knowledge is still limited and a vast repository of IncRNAs 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 IncRNA 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 grown in DMEM (Gibco) supplemented with 10% FBS (Gibco). VCaP and LNCaP cells were grown in phenol red–free DMEM containing 10% charcoal–dextran-treated FBS. LNCaP and VCaP cells were transfected with 5 nmol/L of Dicer-substrate siRNA (Invitrogen) according to the manufacturer’s protocol. After 48 hours of incubation, 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 bed-graph files were converted to bigwig format using the bedGraphToBigWig tool and loaded into the IGV browser to obtain screenshots. 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 chemotaxtractant. After 48 hours, cells...
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 using the Spectramax with 560 EX nm/590 EM 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.arrayanalysis.org (25). All the microarray data have been deposited at the GEO database with the accession number, GSE1099336.

Results
LINC00844 is a novel androgen-upregulated prostate cancer–associated lncRNA
To identify and characterize novel clinically relevant lncRNAs 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 we chose metaRNAseq p < 0.01, we identified 370 clinically relevant lncRNAs (Supplementary Table S1). Because our lab is interested in androgen-mediated transcription in prostate cancer cells, we decided to focus on the androgen-regulated lncRNAs within this group. To do this, we first defined a set of androgen-regulated lncRNAs 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 lncRNAs with the differentially regulated list of lncRNAs from the prostate cancer studies. From this, we found 38 lncRNAs that are both clinically relevant and under androgen transcriptional regulation (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 lncRNAs 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 lncRNAs and have yet to be functionally characterized in prostate cancer. We selected several of the more abundantly expressed lncRNAs 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.bcb.pku.edu.cn/; ref. 32). A score of −1.04818 was obtained for LINC00844 which suggests that the open reading frame of this lncRNA lacks the potential to code for a protein. Similarly, when we examined the coding potential of HOTAIR, a well-known lncRNA, 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 lncRNA (ENSG00000237949, Entrez:100507008) transcribed from an intergenic region on chromosome 10q21.1. The basic Gencode (www.gencodegenes.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 lncRNA 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
(ARE) motif (Fig. 2F), suggesting that AR binds directly to this site. Taken together, our results suggest that LINC00844 is a direct AR-regulated lncRNA.

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 lncRNAs (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 lncRNA 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 the 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).
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 NXX3-1 (36) and lncRNAs like PlncRNA (42, 43) act through a feed-forward mechanism to regulate AR mRNA and protein levels to influence the expression...
Figure 5.
LINC00844 regulates a subset of AR target genes. A, Gene-expression profiling was performed in LNCaP cells transfected with NC or siLINC00844 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 siLINC00844 condition. The fold change in expression is mentioned below. The AR-upregulated genes that were suppressed by ≥1.2-fold after LINC00844 knockdown are represented by orange asterisks. B, GO terms associated with biological processes enriched in LINC00844-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 LINC00844 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.**

*LINCO0844* promotes AR association to the chromatin. LNCaP and VCaP cells treated with si*LINC00844* 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 si*LINC00844* 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 si*LINC00844* 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 si*LINC00844*-DHT (solid lines) and NC-ETOH compared with si*LINC00844*-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 *LINCO0844* 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.
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 transcriptome 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, we identified 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 require LINC00844 for expression. We specifically concentrated on genes constituting the GO terms enriched for migration and invasion processes (Fig. 5B) and have been 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, ITGA5, NKKX3-1, 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 Prensner 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

Figure 7.

LINC00844 mediates prostate cancer migration and invasion in part by regulating NDRG1 expression. 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 NDRG1 expression as fold change normalized to NC-ETOH ± SEM from at least three independent experiments. B, NDRG1 protein levels were analyzed by Western blot (right) and were quantified by ImageLab (BioRad; left). Bar graphs represent the mean normalized (to GAPDH) band quantity ± SEM from at least three independent experiments. C, AR ChIP-qPCR was performed in LNCaP and VCaP cells treated with siLINC00844 or NC and 10 nmol/L of DHT or ETOH for 2 hours. AR binding to the ARBS at the enhancer region of the NDRG1 gene (30 kb from the TSS) is presented as the mean % of input ± SEM from at least three independent experiments. Box-plot showing the expression of NDRG1 in (D) Prensner and colleagues as log2(FPKM+1) and (E) patients in MSKCC datasets segregated based on their Gleason score and presented as log2 mRNA expression values. F, Kaplan–Meier analysis of prostate cancer outcome. One hundred forty patients from the MSKCC dataset were divided as higher or lower than median NDRG1 expression and analyzed for period of biochemical recurrence-free survival. G–L, LNCaP cells were treated with the indicated siRNA and/or plasmid and seeded in the upper chamber of the Boyden’s transwell support. After 48 hours, cells were fixed and counted. Graphs representing the number of LNCaP cells per high power field (20X objective) migrated across transwell supports with 8 μm pores. Transwell supports were coated with Matrigel in 1/2 dilution for invasion assay or left uncoated for migration assay. G and H, Migration and invasion assay in LNCaP cells with knockdown of NDRG1 and L migration and invasion assay in LNCaP cells with NDRG1 knockdown and LINC00844 rescue. K and L, Migration and invasion assay in overexpression of NDRG1 in LNCaP cells with knockdown of LINC00844. Error bars show the SEM of cells counted in 5 high power fields of view in 3 independent experiments. *,$ P < 0.05; **, $ $ P < 0.01; and $ $$, $ $ $ P < 0.001.
overexpressed LINC00844 and demonstrated that knockdown of NDRG1 abrogated LINC00844-mediated migration and invasion (Fig. 7I and J; Supplementary Fig. S9A and S9B). Next, we depleted LINC00844 and showed that overexpression of NDRG1 (Supplementary Fig. S10A) overcame the knockdown effect of LINC00844 and 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 IncRNA, 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 landscapes 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 worse 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. Hence, 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.

Authors’ Contributions

Conception and design: S. Lingadahalli, Y.Y. Sung, E. Cheung

Development of methodology: S. Lingadahalli, Y.Y. Sung

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y.Y. Sung

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

Writing, review, and/or revision of the manuscript: S. Lingadahalli, E. Cheung

Administrative, technical, or material support (i.e., reporting and organizing data, constructing databases): J. Hu, E. Cheung

Study supervision: E. Cheung

Acknowledgments

This work was supported by a University of Macau Multi-Year Research Grant (MYRG2015-001/36-FHS), a University of Macau Start-up Research Grant (SRC2014-00004-FHS), and the Macau Science and Technology Development Fund (FDCT/023/2014/A1 and FDCT102/2015/A3) to E. Cheung.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 29, 2018; revised June 22, 2018; accepted August 9, 2018; published first August 16, 2018.
Regulation of AR-Mediated Transcription by LINC00844

References

Novel IncRNA *LINC00844* Regulates Prostate Cancer Cell Migration and Invasion through AR Signaling

Shreyas Lingadahalli, Sudhir Jadhao, Ying Ying Sung, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-18-0087

Supplementary Material
Access the most recent supplemental material at:
http://mcr.aacrjournals.org/content/suppl/2018/08/16/1541-7786.MCR-18-0087.DC1

Cited articles
This article cites 52 articles, 15 of which you can access for free at:
http://mcr.aacrjournals.org/content/16/12/1865.full#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://mcr.aacrjournals.org/content/16/12/1865.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://mcr.aacrjournals.org/content/16/12/1865.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.