

Original Article

Long non-coding RNA LOC283070 mediates the transition of LNCaP cells into androgen-independent cells possibly via CAMK1D

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Abstract: Aims: The present study is to investigate the role of long non-coding RNAs (lncRNAs) in the development of androgen independence in prostate cancer and its underlying mechanism. Methods: We established an androgen-independent prostate carcinoma (AIPC) cell line LNCaP-AI from androgen-dependent prostate carcinoma (ADPC) cell line LNCaP. Different expression profiles of lncRNAs and mRNAs between LNCaP and LNCaP-AI cells were investigated using microarray analysis. The expression of RNAs was determined using quantitative real-time polymerase chain reaction. Protein levels were measured using Western blotting. MTT assay was used to test cell viability. Tumor formation assay was performed in nude mice to detect tumor growth in vivo. Flow cytometry was performed to detect cell cycles. Transwell assay was employed to test cell migration and invasion. Results: According to bioinformatics prediction, lncRNA LOC283070 could possibly play an important role in the transition of LNCaP cells into LNCaP-AI cells. LOC283070 was up-regulated in LNCaP-AI cells and frequently up-regulated in AIPC cell lines. Overexpression of LOC283070 in LNCaP cells accelerated cell proliferation and migration, even under androgen-independent circumstances. Knockdown of LOC283070 inhibited LNCaP-AI cell proliferation and migration. Moreover, overexpression of LOC283070 promoted tumor growth in vivo in both normal mice and castrated mice. CAMK1D overexpression had similar effect with LOC283070, and CAMK1D knockdown fully abrogated the effect of LOC283070 overexpression on the transition of LNCaP cells into androgen-independent cells. Conclusions: The present study shows that overexpression of LOC283070 mediates the transition of LNCaP cells into androgen-independent LNCaP-AI cells possibly via CAMK1D.

Keywords: Androgen-independent prostate cancer, androgen-dependent prostate cancer, long non-coding RNA, microarray analysis, gene ontology

Introduction

Prostate cancer is the second commonest cancer and the sixth leading cause of cancer-related mortality all over the world [1, 2]. Incidence of prostate cancer is increasing annually in China [1, 2]. At the early stage, the proliferation of prostate cancer is dependent on serum androgen, and thus prostate cancer can be effectively treated by androgen deprivation using either surgical or medical castration [3]. However, hormone ablation therapy only leads to temporary suppression of pros-

tate tumors. As a result, some tumor cells resume growing, and finally differentiate into androgen-independent cells [4, 5].

Despite extensive researches performed in the past, the mechanisms leading to androgen independence are not fully understood. Long non-coding RNAs (lncRNAs) are RNA molecules longer than 200 nt that do not encode proteins [6]. Recently, they have emerged as major players in governing fundamental biological processes. Abnormal expression of lncRNAs is associated with cancers including pro-

state cancer [7]. Prostate cancer is also found to be closely associated with a variety of lncRNAs, some of which have significant tissue specificity. For example, prostate cancer non-coding RNA 1 (PRNCR1), prostate cancer gene expression marker 1 (PCGEM1), differential display code 3 (DD3)/prostate cancer antigen 3 (PCA3) and prostate cancer-associated ncRNA transcripts 1 (PCAT-1) exhibit significantly increased expression levels only in prostate cancer tissues/cells. PRNCR1 trans-activates the expression of androgen receptor (AR), which is the key factor for the progression of prostate cancer [8]. Both of PCGEM1 and PCAT-1 promote the proliferation and tumorigenesis of prostate cancer cells [9, 10]. DD3/PCA3 exhibits higher tissue specificity than prostate-specific antigen (PSA), although the biological function of PCA3 in prostate cancer is unknown. More importantly, as a prostate cancer-specific lncRNA, DD3/PCA3 can be detected in urine from patients with prostate cancer, appearing to be a non-invasive marker for the early diagnosis of prostate cancer [11, 12]. Therefore, the detection and functional studies of prostate cancer-specific lncRNAs help provide new biomarkers and targets for the diagnosis and treatment of prostate cancer. In recent years, identification of prostate cancer-related lncRNAs and studies on their biological functions were carried out, but it is never reported whether lncRNAs play a role in the development of androgen independence in prostate cancer or the underlying mechanism of action. In the present study, we establish an androgen-independent prostate carcinoma (AIPC) cell line LNCaP-AI (defined as LNCaP cell line that is capable of growing in charcoal-stripped serum) from androgen-dependent prostate carcinoma (ADPC) cell line LNCaP, and investigate the different expression profiles of lncRNAs and mRNAs between LNCaP cells (androgen-dependent, AD) and LNCaP-AI cells (androgen-independent, AI).

Materials and methods

Cells

Androgen-dependent human prostate adenocarcinoma cell line LNCaP and androgen-independent cell lines PC-3 and DU145 were obtained from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). LNCaP, PC-3 and Du-145 cells were cultured in RPMI-1640 (Life Tech-

nologies, Inc., Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT, USA) at 37°C in 5% CO₂. LNCaP-AI cells [11, 12], which were constructed in our lab, were cultured in RPMI-1640 supplemented with 10% charcoal-treated (stripped) fetal bovine serum.

Animals

Male nude mice (4-6 weeks) were obtained from Vital River Laboratories Co., Ltd., Beijing, China. After one week of adaptation, the mice were randomly assigned to two groups (6 mice/group): normal group and castrated group. To assess the effect of LOC283070 expression on the transition of LNCaP cells into androgen-independent cells in vivo, 1×10⁶ stable LNCaP cells transfectants (pcDNA3.1-LOC283070 or parental pcDNA3.1 vector) were suspended in 100 µl serum-free medium and subcutaneously injected into both axilla flanks of the mice. In castrated group, cells (1×10⁶) were injected subcutaneously into both axilla flanks of mice that had been castrated via scrotal approach for 3 days. Before the termination of the experiment, the mice were euthanized by CO₂, and tumors were removed and weighed, the volume of each tumor was calculated (length × width² × 0.5).

LncRNA microarray

Arraystar Human LncRNA Microarray V2.0 (Arraystar Inc., Rockville, MD, USA) was composed of lncRNAs and protein-coding mRNAs from human genome. It contained a total of 33,045 lncRNAs and 30,215 coding transcripts. LncRNAs were carefully selected from the most authoritative databases such as RefSeq, UCSC Knowngenes, and Ensembl as well as many related literatures. Each transcript was represented by a specific exon or splice junction probe that can accurately identify individual transcripts. Positive probes for house-keeping genes and negative probes were also printed onto the array for hybridization quality control. The microarray hybridization and bioinformatics analysis were performed by Kang-Chen Bio-tech, Shanghai, China.

Agilent Feature Extraction software (version 11.0.1.1; Agilent Technologies, Santa Clara, CA, USA) was used to analyze the acquired array images. Quantile normalization and subsequent data processing were performed us-

Table 1. Primer sequences

lncRNAs	Primer sequences for qRT-PCR (5' to 3')
ENST00000444155	S: CGAGCTGTGAGCAGTGGTAGT AS: AGTTCAGTGAGTGGGAGGGAG
NR_028336	S: CAAGATGACCACACTCCAGCC AS: TCAGAGAAGAGCCGTGAAAACA
ENST00000419440	S: GTTAGAGACGCAGCAACGCAT AS: CAGGGCTTTGTCACTGACTTCC
AA827066	S: CAACATTAGGCGAATCGTGAAA AS: AGGAGTGAGGGAAGAGAAAGAAGAC
ENST00000438147	S: AGAGAAGGCTTTGCTCTGTTGC AS: TGGGTCCCTAAGTCGTATGTGG
ENST00000428426	S: GGAGGAGAAATGTGGAATTGAGTG AS: TGGTATGGGAAGCTGAGGTGG
BX953928	S: TGGATACAGATGAGCACCTACCT AS: AGCAAGCTGGTTTACCTGAGAC
BM565532	S: CCTAATAGCCAGCCACTTATTTG AS: CTTGCACATCTCGAAGAAGTACA
ENST00000503819	S: CCCAACAGGGACCAGGAAGAT AS: AGTGTGATGAGATGAATGCTGAGAA
ENST00000419196	S: ATCAATGTAAGAGAGAAGTCTGGGC AS: GGTTTGCTTTTGCTGGTCGT
ENST00000434301	S: GCCCCTGACTCTCCTGACCT AS: GACTCTTCGGCTTGACGCTGG
AK091731	S: TATCTGGGGATTGGATTGGGG AS: AGAACTAATGTGGGCTGGCGG
NR_027716	S: AAGAGCAAATGACCCTGACGC AS: CTCAGCTCGATCCGACGGTAT
AF085831	S: GCTGCTCCTTATTCTCTCTAG AS: CCCAAAGTTCAGGCTCTATCC
LOC283070	S: AGGCGGTCTGAGGAAGATAAGG AS: TCCCACTGACTCTGGAGGCAT

Note: S, Sense; AS, Anti-sense.

RNA obtained by Trizol extraction was purified by processing with RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quantification and quality check were performed with Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and standard denaturing agarose gel electrophoresis. RNA from each sample (1 µg) was reversely transcribed to cDNA using random hexamer primer with RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Thermo Fisher Scientific, Waltham, MA, USA). Primers for each lncRNA (**Table 1**) were designed according to Primer 5 and checked with Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) to ensure unique amplification product. qRT-PCR was performed on an Eppendorf Mastercycler® ep realplex (Eppendorf, Hamburg, Germany) using the SYBR green method according to the manufacturer's instructions. PCR reaction conditions were: denaturation at 95°C for 10 min, followed by 40 PCR cycles at 95°C for 15 s and one cycle at 60°C for 1 min. Relative gene expression levels were quantified based on the cycle threshold (Ct) values and normalized to the internal control gene β-actin. The $2^{-\Delta\Delta Ct}$ method was used to comparatively quantify the levels of lncRNAs.

ing the GeneSpring GX v11.5.1 software package (Agilent Technologies, Santa Clara, CA, USA). After quantile normalization of the raw data, lncRNAs and mRNAs that had flags in Present or Marginal ("All Targets Value") were chosen for further data analysis. Differentially expressed lncRNAs and mRNAs between two samples were identified through "Fold Change" filtering. Hierarchical clustering was performed using the Agilent GeneSpring GX software (version 11.5.1; Agilent Technologies, Santa Clara, CA, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

To isolate total RNA, cells were lysed with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total

Western blotting

Total protein from cells was lysed using radio-immunoprecipitation assay buffer (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA). Equal amounts of protein were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane. After being blocked with Tris-buffered saline with Tween 20 containing 5% skimmed milk at room temperature for 1 h, the membranes were incubated with rabbit anti-human CAMK1D primary antibody (1:2000; Cell Signaling Technology, Danvers, MA, USA) dissolved in Tris-buffered saline with Tween 20

containing 5% skimmed milk overnight at 4°C. Mouse anti-β-actin antibody (1:5000; Santa Cruz, Dallas, TX, USA) was used as control. Immunoblots were detected using an electrochemiluminescence kit (Santa Cruz, Dallas, TX, USA) and visualized after X-ray film exposure. Protein levels were quantified using ImageJ software (<http://wiki.imagej.net/>).

Transfection

For identified dys-regulated lncRNAs, we performed preliminary screening and selected LOC283070 as the main subject for the present study. Molecular assays demonstrated that LOC283070 indeed plays a role in the development of androgen independence in prostate cancer. The cDNA encoding lncRNA-LOC283070 was amplified by the Prime STAR HS DNA Polymerase (Takara, Tokyo, Japan), cloned into pcDNA 3.1 vector (Invitrogen, Carlsbad, CA, USA), and named as pcDNA3.1-LOC283070. The primers used were 5'-CGG-ATCCTGGGCTCATAGGAAATATCTGTAGTAGGATG3' (sense) and 5'-CGGATCCTGAAGGTGATTAATTGTCTTTATTGGAGGAAAACAG-3' (antisense). Then, the amplified fragments were sequenced, and conformed to have no errors in nucleotides. The pcDNA3.1 empty vector was used as negative control (NC). CAMK1D overexpression vector GV219-CAMK1D was purchased from Genechen (Shanghai Genechen Co., Ltd, Shanghai, China).

LOC283070 siRNA sequences (GenePharma, Shanghai, China) were as follows: si-LOC-1: 5'-GACAGCUUAACCGAUUAATT-3' (sense). 5'-UUAUACAGGUUAAGCUGCCTT-3' (antisense). si-LOC-2: 5'-GUGCACGAAACCUUUGCAUTT-3' (sense). 5'-AUGCAAAGGUUUCGUGCACTT-3' (antisense). si-LOC-3: 5'-GAACUUCUCAUUCGAUUAUTT-3' (sense). 5'-AUAAUCGAAUGAGAAGUUCTT-3' (antisense).

The negative control sequences were: 5'-UUCUCCGAACGUGUCACGUTT-3' (sense) and 5'-ACGUGACACGUUCGGAGAATT-3' (antisense) (GenePharma, Shanghai, China). CAMK1D siRNA sequences were: 5'-CCCAGAAACCUUACAGCAATT-3' (sense) and 5'-UUGCUGUAAGGUUUCUGGGTT-3' (antisense) (GenePharma, Shanghai, China). Transfection was performed at about 30-50% confluence of LNCaP-AI or LNCaP cells using Lipofectamine 2000 reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. Express-

ion of LOC283070 was validated by qRT-PCR, while expression of CAMK1D was validated by Western blotting.

LOC283070 (pcDNA3.1-LOC283070) or vector (pcDNA3.1) packaged with Lentivirus were purchased from Genechen (Shanghai Genechen Co., Ltd., Shanghai, China). Then, lentiviral particles were transfected into LNCaP cells.

MTT assay

For all cell viability studies, cells were plated in 96-well plates in both regular medium and charcoal-treated medium. After being cultured for 24 h, cells were transfected with pcDNA3.1-LOC283070 or pcDNA3.1 vector. Tetrazolium salt (MTT, 10 μl, 5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) cell proliferation assay was then carried out on various days following the manufacturer's instructions. Assays were performed in triplicate.

Cell growth curve

Cells were inoculated in triplicate in 2 mL media in 6-well plates 24 h after transfection at a density of 1×10^5 /well. Cell numbers were counted every day for one week using a hemocytometer. A cell growth curve was drawn with cell number on the y-axis and time on the x-axis to assess the cell proliferation ability. Assays were performed in triplicate.

Flow cytometry

For the cell cycle assay, LNCaP cells were transfected with pcDNA3.1-LOC283070 or pcDNA3.1 vector for 24 h. Then, the cells were cultured in either regular medium or charcoal-treated medium for 3 days, collected and fixed in 75% ethanol at 4°C overnight. After washing twice with phosphate-buffered saline, the cells were incubated with 30 μg/mL propidium iodide (PI), 0.2 mg/mL RNase A, and 0.2% Triton X-100 (all from Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 30 minutes. The cells were then analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's manual. Assays were performed in triplicate.

Transwell assay

Cell migration and invasion were evaluated using Transwell migration chambers (8 μm pore size; Corning, New York, NY, USA). Cells (5×10^4)

Aberrant lncRNA levels in LNCaP-AI cells

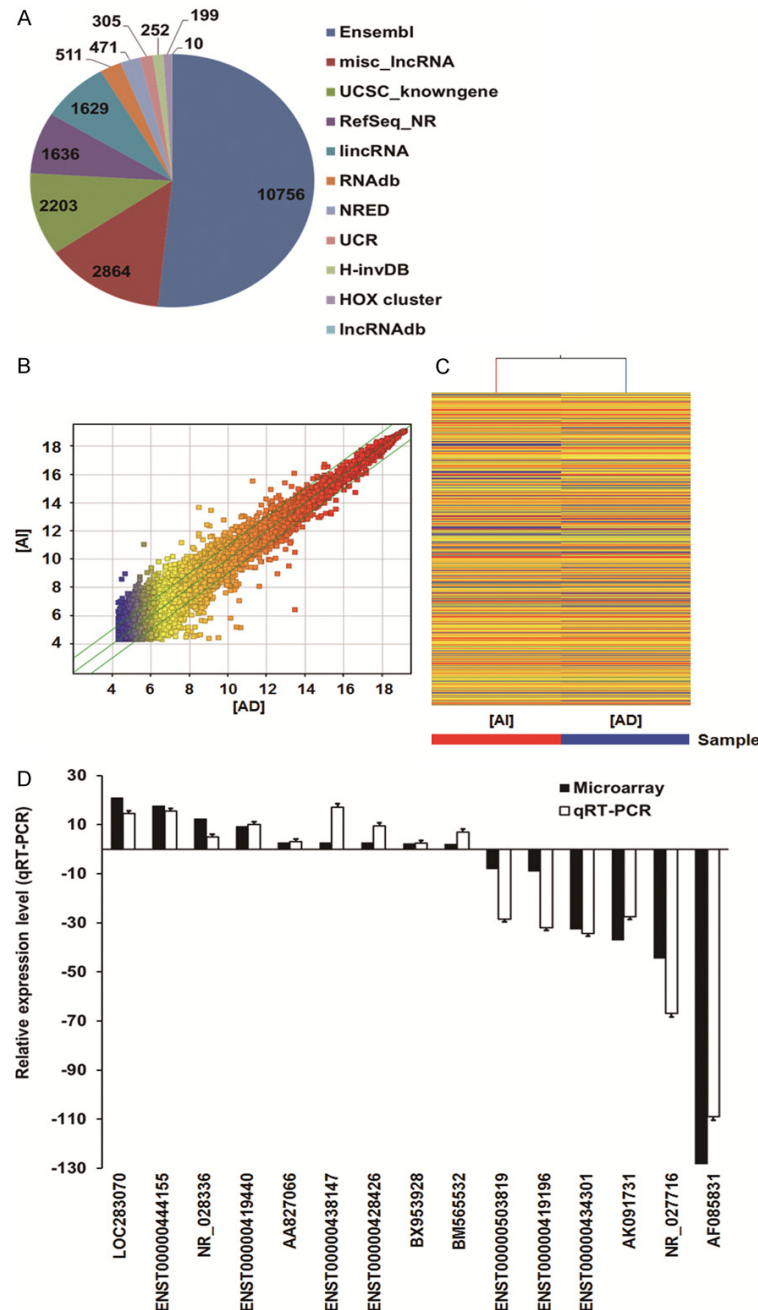


Figure 1. Differentially expressed lncRNAs in LNCaP (androgen-dependent, AD) and LNCaP-AI cells (androgen-independent, AI). A: Pie chart showing the relative number of lncRNAs from the most authoritative databases. B: Scatter plot showing lncRNAs expression variation between LNCaP-AI and LNCaP cells. The lncRNAs above the top green line and below the bottom green line indicated ≥ 2.0 -fold change of lncRNAs in LNCaP-AI cells compared with that in LNCaP cells. C: Hierarchical clustering showing lncRNA expression profiles in LNCaP-AI and LNCaP cells. "Red" indicates high relative expression, and "blue" indicates low relative expression. D: Comparison of lncRNA expression levels between microarray and qRT-PCR. Fifteen differentially expressed lncRNAs were validated by qRT-PCR. The Y-axis represents the log-transformed median fold changes (AI/AD) in expression levels of 15 samples ($P < 0.05$).

in 100 μ L of serum-free medium were added to the upper chamber of the transwell after transfection, whereas 600 μ L of medium containing 10% fetal bovine serum was added to the lower chamber. After 48 hours of incubation at 37°C, non-invaded cells on the top of the membrane were scraped and removed by cotton swabs, and the invaded cells were fixed, stained with 0.1% crystal violet, and counted under a light microscope. The migration assay was repeated three times with duplicate wells.

Statistical analysis

All data were analyzed using SPSS 17.0 software package (SPSS, Chicago, IL, USA). All values were expressed as means \pm standard deviation (SD) from at least three independent experiments, and statistical significance was defined as $P < 0.05$.

Results

The expression of lncRNAs in LNCaP-AI cells is distinct from that in LNCaP cells

To determine lncRNA expression variation and patterns in both LNCaP and LNCaP-AI cell lines, we performed second-generation lncRNA microarray based on the lncRNA expression profile data from the microarray analysis, as well as scatter plot analysis and hierarchical clustering. A total of 20836 lncRNAs were detected (Figure 1A). Based on these data, lncRNA expression levels between androgen-independent LNCaP-AI cells and androgen-dependent LNCaP cells were com-

Aberrant lncRNA levels in LNCaP-AI cells

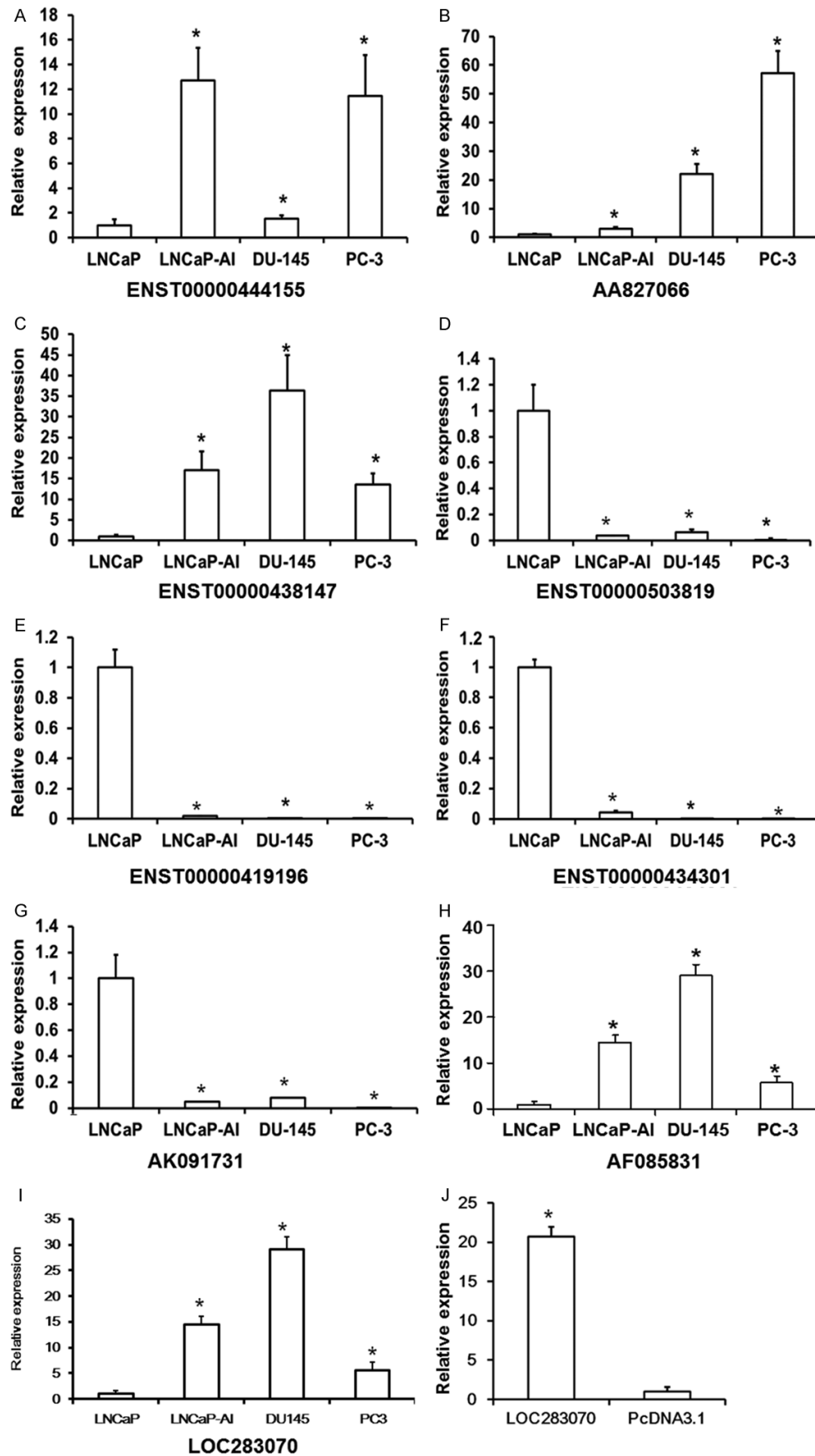


Figure 2. Relative expression of lncRNAs (A) ENST00000444155, (B) AA827066, (C) ENST00000438147, (D) ENST00000503819, (E) ENST00000419196, (F) ENST00000434301, (G) AK091731, (H) AF085831 and (I) LOC283070 in prostate cancer cell lines LNCaP, LNCaP-AI, DU-145 and PC-3 determined using qRT-PCR. Data are means \pm SD. *, $P < 0.05$ compared with LNCaP. (J) Overexpression of LOC283070 in LNCaP cells confirmed by qRT-PCR.

pared using scatter plot (**Figure 1B**) and hierarchical clustering (**Figure 1C**). We identified 1277 up-regulated and 495 down-regulated lncRNAs in LNCaP-AI cells compared with LNCaP cells by setting a filter of fold change ≥ 2.0 . These results suggest that the expression of lncRNAs in LNCaP-AI cells is distinct from that in LNCaP cells.

The microarray data are further confirmed by qRT-PCR analysis

To test and verify the microarray data, 9 up-regulated lncRNAs (LOC283070, ENST00000444155, NR_028336, ENST00000419440, AA827066, ENST00000438147, ENST00000428426, BX953928, and BM565532) and 6 down-regulated lncRNAs (ENST00000503819, ENST00000419196, ENST00000434301, AK091731, NR_027716, and AF085831) with bioinformatics analysis, were selected for qRT-PCR analysis. qRT-PCR data showed that 9 lncRNAs were up-regulated, and 6 lncRNAs were down-regulated in LNCaP-AI cells compared with LNCaP cells, being consistent with the microarray data (**Figure 1D**). Furthermore, 4 up-regulated lncRNAs and 5 down-regulated lncRNAs were randomly selected from the 15 validated lncRNAs to detect the expression level in different prostate cancer cell lines, including LNCaP, LNCaP-AI, DU-145 and PC-3. The qRT-PCR results showed constant consistency in the expression of lncRNAs between AIPC cells and ADPC cells (**Figure 2A-I**). These results indicate that the microarray results are consistent with qRT-PCR results.

Overexpression of LOC283070 promotes LNCaP cell proliferation

To further identify the role of LOC283070 in prostate cancer cells, we performed functional assays on LNCaP cells with LOC283070 overexpression (pcDNA3.1-LOC283070). qRT-PCR data showed that pcDNA3.1-LOC283070 was over-expressed compared with pcDNA3.1 vector ($P < 0.05$) (**Figure 2J**). MTT assay showed that proliferative ability of LNCaP cells was remarkably increased in the presence of LOC283070 overexpression compared with those

transfected with pcDNA3.1 vectors ($P < 0.05$) (**Figure 3A**). When treated with charcoal-treated medium that suppresses the proliferative ability of LNCaP cells, ectopic expression of LOC283070 still promoted cell proliferative ability ($P < 0.05$) (**Figure 3B**). Furthermore, cell growth curve analysis indicated that overexpression of LOC283070 increased the number of LNCaP cells (**Figure 3C**). Of note, treatment with charcoal-treated medium further accelerated the proliferation LNCaP cells (**Figure 3D**). These results suggest that overexpression of LOC283070 promotes LNCaP cell proliferation.

Overexpression of LOC283070 accelerates cell cycle of LNCaP cells treated with charcoal-treated medium

To analyze cell cycle progression of LNCaP cells in the presence of LOC283070 overexpression, the cells were cultured in both regular medium and charcoal-treated medium. When treated with regular medium, no distinct changes were observed in the distribution of cells in each cell cycle phase (**Figure 3E**). After being treated with charcoal-treated medium, the percentage of S-phase cells was dramatically increased at 48 h after LOC283070 overexpression compared with control (**Figure 3E**). These results indicate that overexpression of LOC283070 accelerates cell cycle of LNCaP cells treated with charcoal-treated medium.

Overexpression of LOC283070 promotes LNCaP cell migration

To investigate whether lncRNA LOC283070 participates in cell migration, transwell assay was performed. LNCaP cells with LOC283070 overexpression showed a significantly higher migration potential than cells transfected with pcDNA3.1 vector in regular medium, and LOC283070 induced $> 50\%$ increase of migrated tumor cells compared with vector control ($P < 0.05$, **Figure 3F**). In addition, in charcoal-treated medium, the migration ability of LNCaP cells was promoted by 66% compared with vector control ($P < 0.05$) (**Figure 3F**). The result suggests that overexpression of

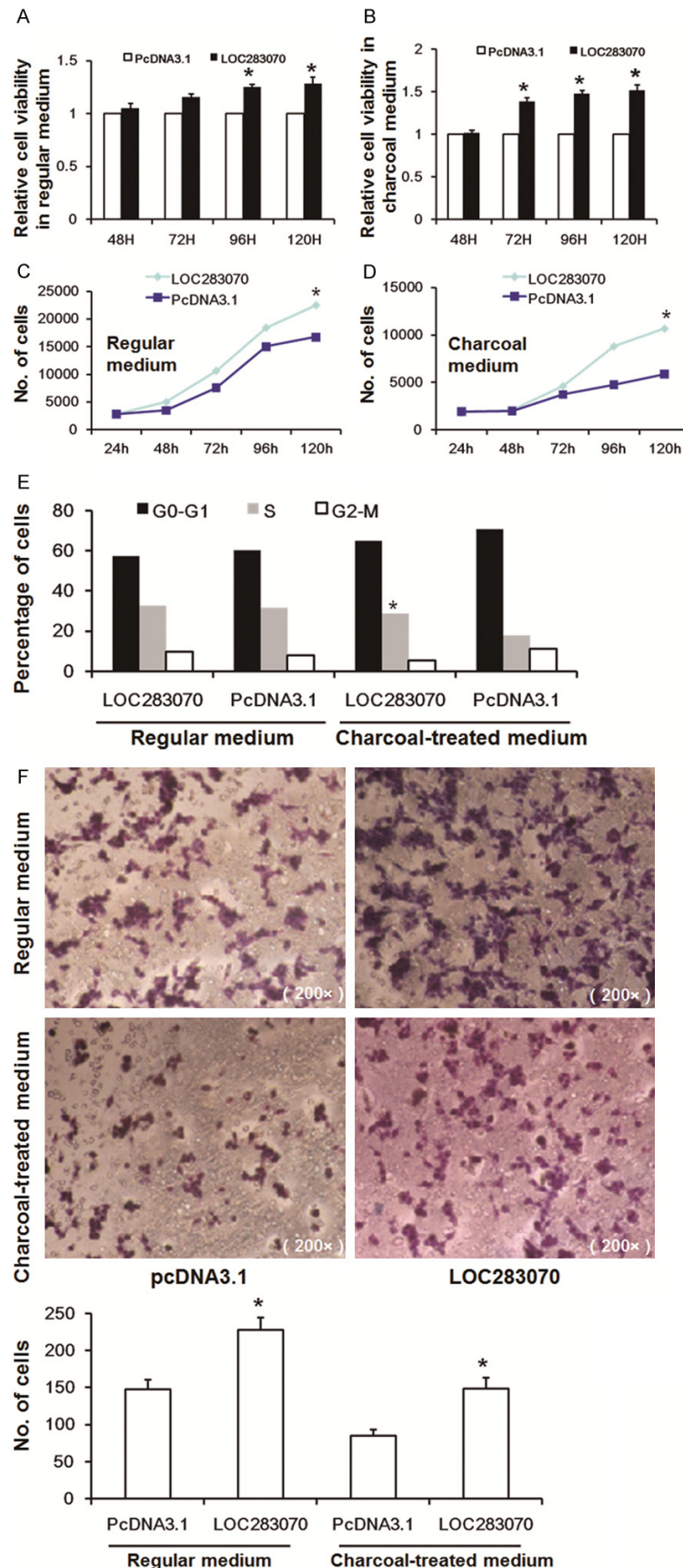


Figure 3. Effect of LOC283070 overexpression on cell proliferation and migration. (A, B) The cell viability of LNCaP cells with LOC283070 overexpression cultured in (A) regular medium and (B) charcoal-treated medium. Cell viability was determined by MTT assay. (C, D) Cell growth curve analysis showing the proliferative ability of LNCaP cells with the overexpression of LOC283070 cultured in (C) regular medium and (D) charcoal-treated medium. (E) Cell cycle progression in cells with LOC283070 overexpression cultured in regular medium and charcoal-treated medium. (F) Migration potential of LNCaP cells with the overexpression of LOC283070 or pcDNA3.1 vector alone cultured in regular medium and charcoal-treated medium (magnification 200 \times).

LOC283070 promotes LNCaP cell migration.

Knockdown of LOC283070 inhibits LNCaP-AI cell proliferation and migration

To study the effect of LOC283070 knockdown on cell proliferation and migration, LOC283070 siRNA was transfected into LNCaP-AI cells. After co-transfection with siRNA, a markedly decreased expression of LOC283070 was detected with qRT-PCR (Figure 4A). Meanwhile, LOC283070 knockdown significantly decreased cell proliferation rate, as measured by MTT assay (Figure 4B) and cell growth curve analysis (Figure 4C). Furthermore, analysis of cell cycle progression in LNCaP-AI cells with LOC283070 knockdown showed that compared with negative control, a decrease in the percentage of S-phase cells was observed in LNCaP-AI cells at 48 h after transfection with LOC283070 siRNA (Figure 4D). Subsequently, evaluation of cell

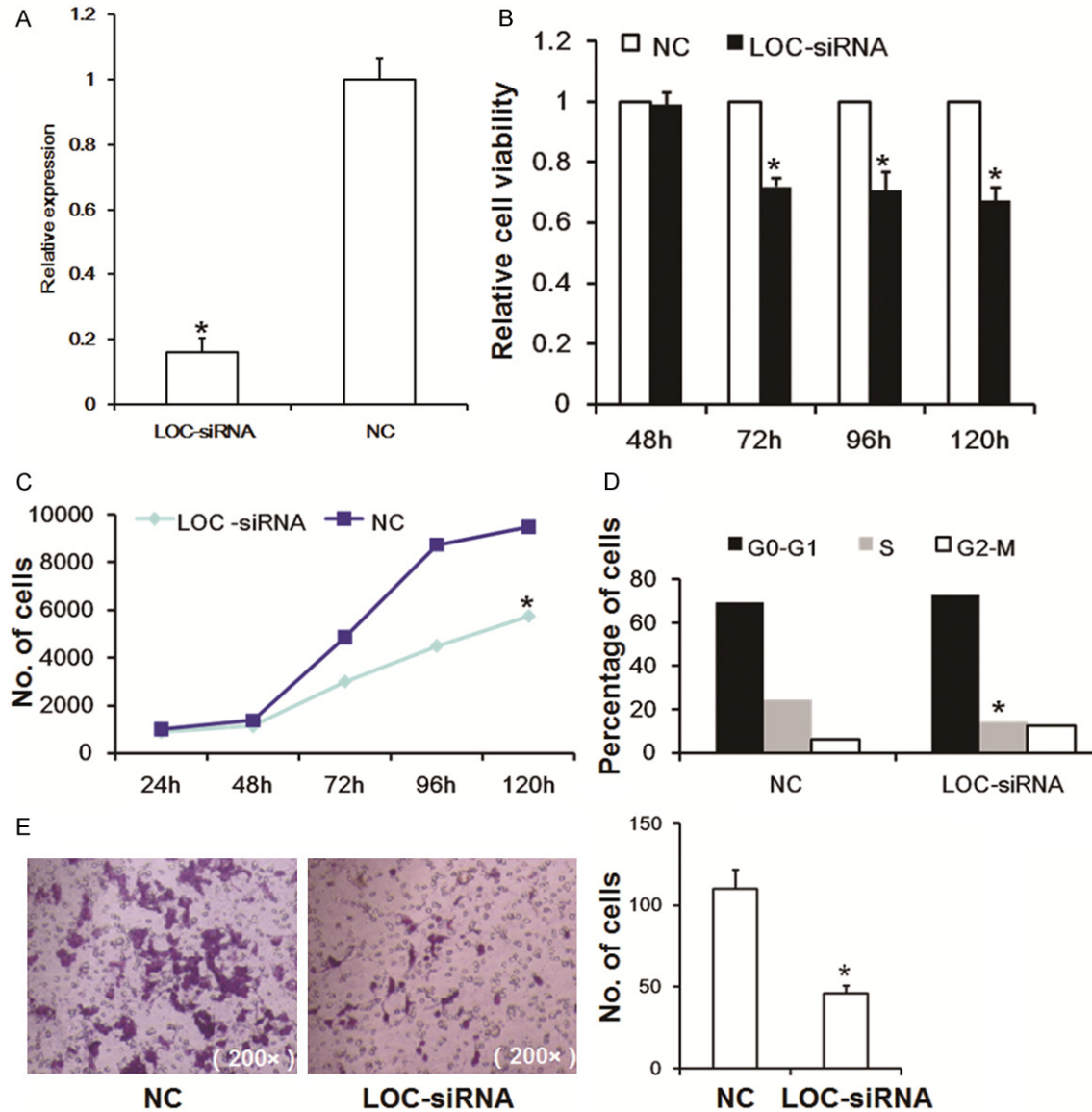


Figure 4. Effect of LOC283070 knockdown on cell proliferation and migration. A: Knockdown of LOC283070 in LNCaP-AI cells confirmed by qRT-PCR. B: LNCaP-AI cell viability determined by MTT assay. C: Cell growth curve analysis of LNCaP-AI cells with the knockdown of LOC283070. D: Cell cycle assay with the knockdown of LOC283070. E: Migration ability of LNCaP-AI cells with the knockdown of LOC283070 at 48 h.

migration ability showed that compared with negative control, the migration ability of LNCaP-AI cells was decreased by 60% at 48 h after the knockdown of LOC283070 ($P < 0.05$) (Figure 4E). These results indicate that knockdown of LOC283070 inhibits LNCaP-AI cell proliferation and migration.

Overexpression of LOC283070 promotes tumor growth in vivo

To test the effects of lncRNA LOC283070 on tumor growth in vivo, LNCaP cells with stable

overexpression of LOC283070 or control vector were injected subcutaneously into the opposite axilla flanks of nude mice. qRT-PCR analysis showed the overexpression of LOC283070 in LNCaP cells (Figure 5A). As shown in Figure 5B and 5C, the growth of tumors derived from LOC283070-overexpressed LNCaP cells was significantly increased compared with those derived from pcDNA3.1-transfected cells. The average tumor weight and volume in LOC283070 group of normal mice was significantly higher than that in pcDNA3.1 group (Figure 5D, 5E). Moreover, tumor weight and volume in LOC-

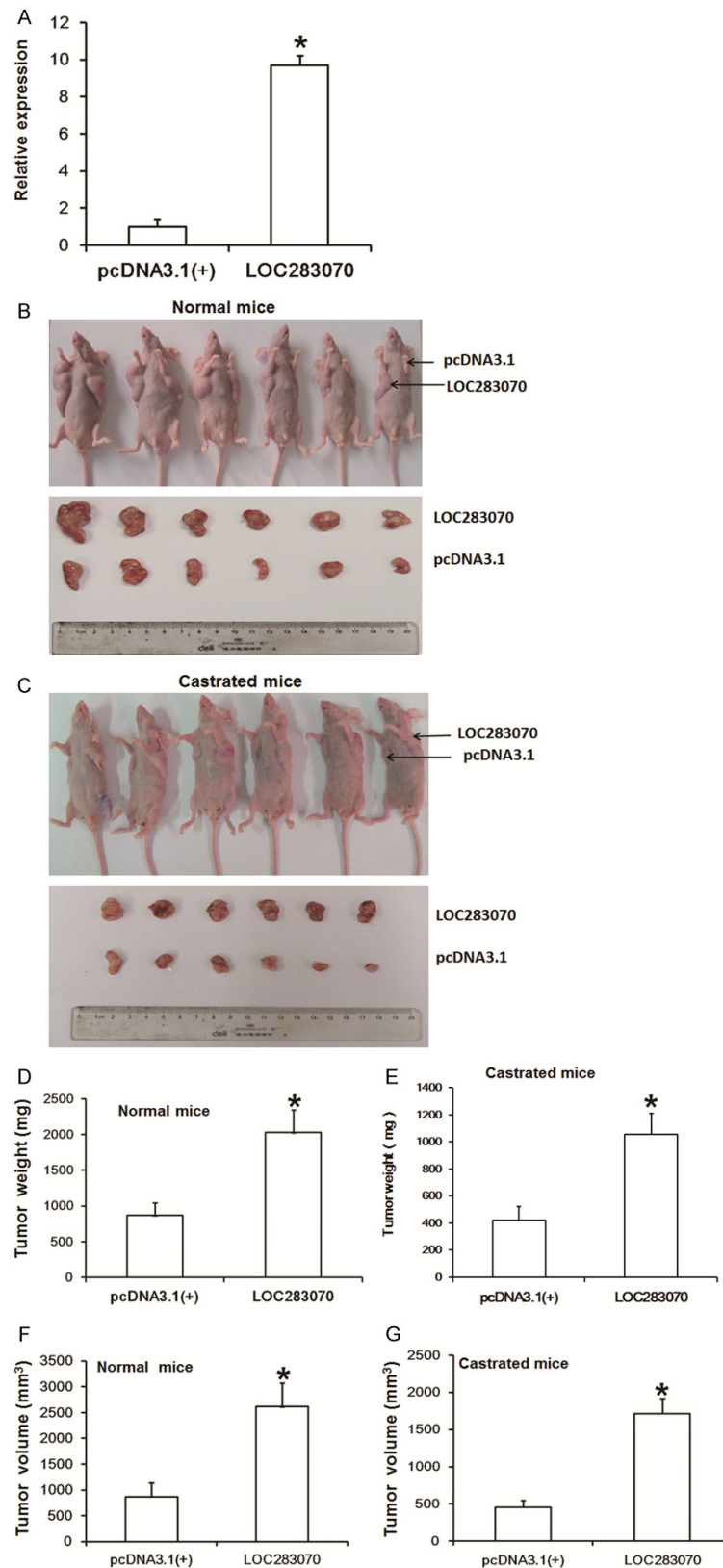


Figure 5. Effect of overexpression of LOC283070 on the growth of xenograft tumors. A: Expression of LOC283070 in LNCaP cells with stable overexpres-

sion of LOC283070 measured by qRT-PCR. B: In normal group, the stable LOC283070-overexpressed LNCaP cells were injected subcutaneously into the right axilla flanks of nude mice and the control were injected subcutaneously into the left axilla flanks of nude mice. Mice were sacrificed at 21 days post-injection. C: In castrated group, the stable LOC283070-overexpressed LNCaP cells were injected into the left axilla flanks and the control into the right axilla flanks of nude mice. D, F: Xenografted tumors were separated and their weights were measured. E, G: Xenografted tumors were separated and their sizes were measured. Data were expressed as means \pm standard errors of the mean. *, $P < 0.05$ compared with control.

283070 group of castrated mice was significantly higher than that in pcDNA3.1 group (Figure 5F, 5G). These results suggest that overexpression of LOC283070 promotes tumor growth in both normal mice and castrated mice.

CAMK1D is related to LOC283070

To test whether CAMK1D is related to LOC283070, Western blotting was performed. The data showed that the protein level of CAMK1D in LNCaP-AI cells was much higher than in LNCaP cells, and overexpression of LOC283070 significantly increased the protein level of CAMK1D in LNCaP cells. In addition, knockdown of LOC283070 significantly decreased the protein level of CAMK1D in LNCaP-AI cells. Of note, CAMK1D siRNA evidently reduced the protein level of CAMK1D. Compared with negative control, LNCaP cells co-transfected with LOC283070 and CAMK1D siRNA

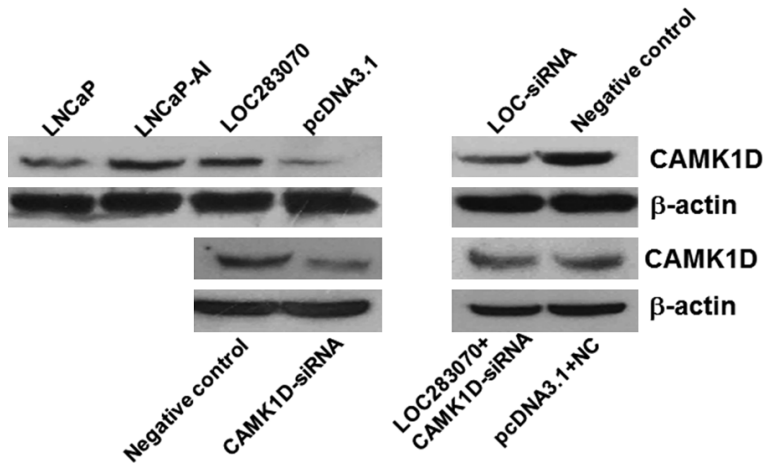


Figure 6. CAMK1D protein expression measured by Western blotting.

had similar CAMK1D protein level (**Figure 6**). The results suggest that CAMK1D is related to LOC283070.

CAMK1D overexpression has similar effect with LOC283070 overexpression on the transition of LNCaP cells into androgen-independent cells

In order to test whether CAMK1D up-regulation plays a role in LOC283070-mediated pathogenesis, we overexpressed CAMK1D in LNCaP cells. The data showed that ectopic expression of CAMK1D had similar promotional role of LOC283070 on LNCaP cell proliferation in both regular medium and charcoal-treated medium (**Figure 7A** and **7B**). At the same time, cell growth curve assay revealed similar tendency, showing significant acceleration in proliferation ratio of treated LNCaP cells, compared with negative control (**Figure 7C** and **7D**). Furthermore, when treated with charcoal-treated medium, the percentage of S-phase cells was also dramatically increased at 48 h after CAMK1D overexpression compared with control (**Figure 7E**). Subsequently, evaluation of cell migration ability showed that compared with control, the migration ability of LNCaP cells with CAMK1D overexpression was greatly increased in both regular medium and charcoal-treated medium ($P < 0.05$) (**Figure 7F**). These results indicate that CAMK1D overexpression has similar effect with LOC283070 overexpression on the transition of LNCaP cells into androgen-independent cells.

CAMK1D knockdown fully abrogates the effect of LOC283070 overexpression on the transition of LNCaP cells into androgen-independent cells

To further test whether CAMK1D up-regulation plays a role in LOC283070-mediated pathogenesis, then we used siRNA of CAMK1D in LNCaP cells with the overexpression of LOC283070. MTT assay showed that the promotional role of LOC283070 on LNCaP cell proliferation was evidently weakened with co-transfection of the siRNA of CAMK1D

in both regular medium and charcoal-treated medium (**Figure 8A** and **8B**). On the other hand, cell growth curve assay revealed similar tendency, showing no significant difference in proliferation ratio of treated LNCaP cells, compared with negative control (**Figure 8C**). Furthermore, after treatment with charcoal-treated medium, increased cell-cycle progression was attenuated by CAMK1D siRNA (**Figure 8D**). At last, cell migration showed no difference between co-transfected cells and cells with negative control vector in both regular medium and charcoal-treated medium (**Figure 8E**). These results indicate that CAMK1D knockdown fully abrogates the effect of LOC283070 overexpression on the transition of LNCaP cells into androgen-independent cells.

Discussion

Recent researches are focused on the molecular mechanisms by which prostate cancer cells become androgen-independent in the treatment of prostate cancer, with some progresses being made in understanding the molecular basis of the development of AIPC. This process involves multiple mechanisms, including: i) androgen receptor (AR) gene mutation/amplification that leads to increased sensitivity or identification of AR-specific changes and hence, causing abnormal activation of AR signaling pathway; ii) androgen receptor pathway, such as phosphatidyl inositol 3-kinase (PI3K)/protein kinase B (Akt) pathway activation, neuroendocrine-like differentiation, and apoptosis gene

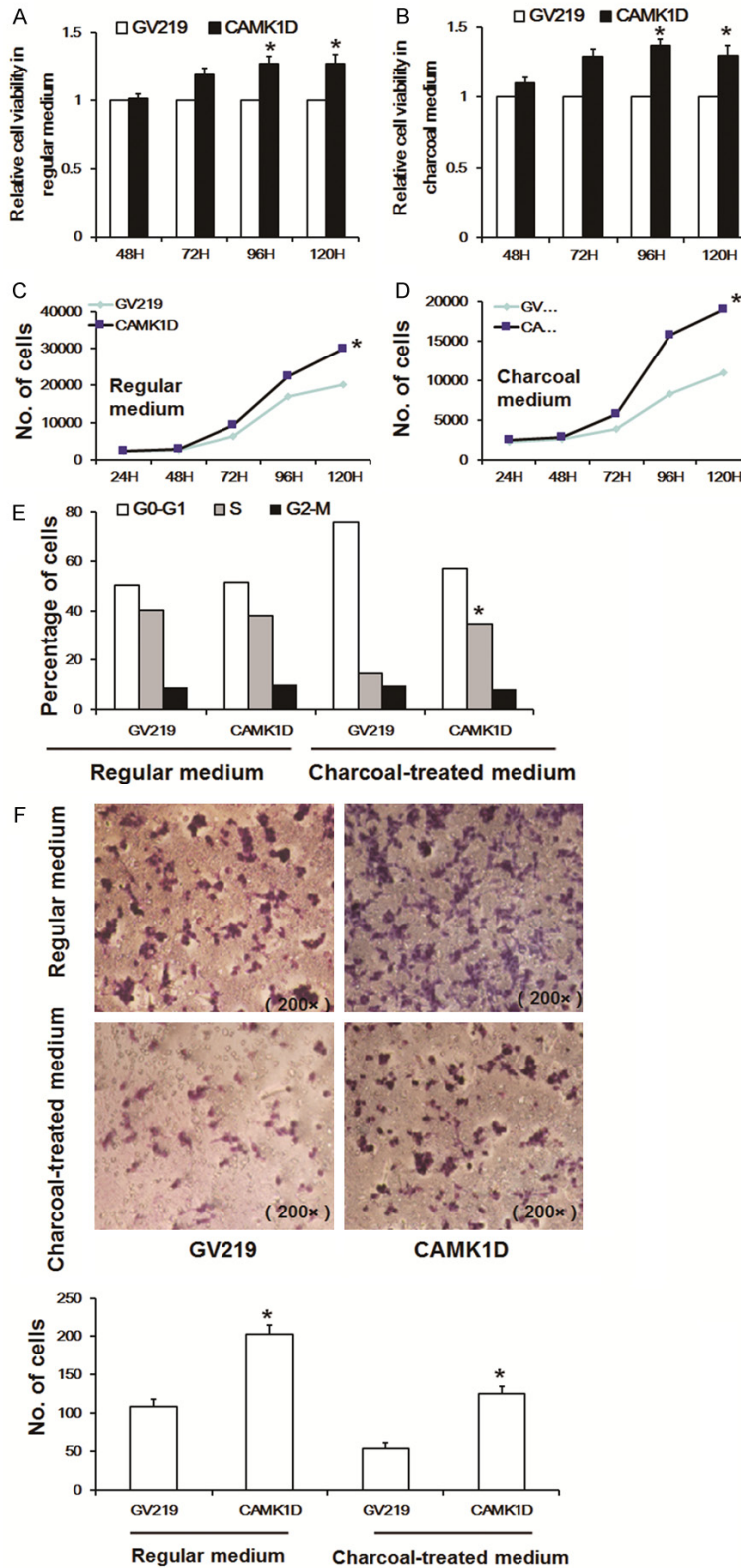


Figure 7. Effect of CAMK1D overexpression on cell proliferation and migration. (A, B) The cell viability of LNCaP cells with CAMK1D overexpression cultured in (A) regular medium and (B) charcoal-treated medium. Cell viability was determined by MTT assay. (C, D) Cell growth curve analysis showing the

proliferative ability of LNCaP cells with the overexpression of CAMK1D cultured in (C) regular medium and (D) charcoal-treated medium. (E) Cell cycle progression in cells with CAMK1D overexpression cultured in regular medium and charcoal-treated medium. (F) Migration potential of LNCaP cells with the overexpression of CAMK1D or GV219 vector alone cultured in regular medium and charcoal-treated medium (magnification 200×).

abnormalities; iii) overexpression of erythroblast transformation-specific family-related gene (ERG) caused by the fusion of prostate-specific androgen-dependent gene TMPRSS2 and ERG gene; iv) hidden cell replacement. Different mechanisms may take effect at the same time [13].

Development of androgen-independent growth of prostatic carcinoma cells, a major obstacle for the treatment of human prostate cancer, is dependent on multiple factors. Recently, next generation transcriptome profiling and accumulating functional evidence shows that a number of differentially expressed lncRNAs are associated with cancers [14]. For example, a number of lncRNAs are detected to express differentially in prostate cancer tissues compared with normal prostate tissues by microarray or transcriptome sequencing (RNA-Seq) [10]. Subsequent functional studies discover several better characterized lncRNAs that are associated with prostate cancer biology, including PRNCR1, PCGEM1, DD3 (PCA3) and PCAT-1.

Recently, accumulating evidence suggests that lncRNAs

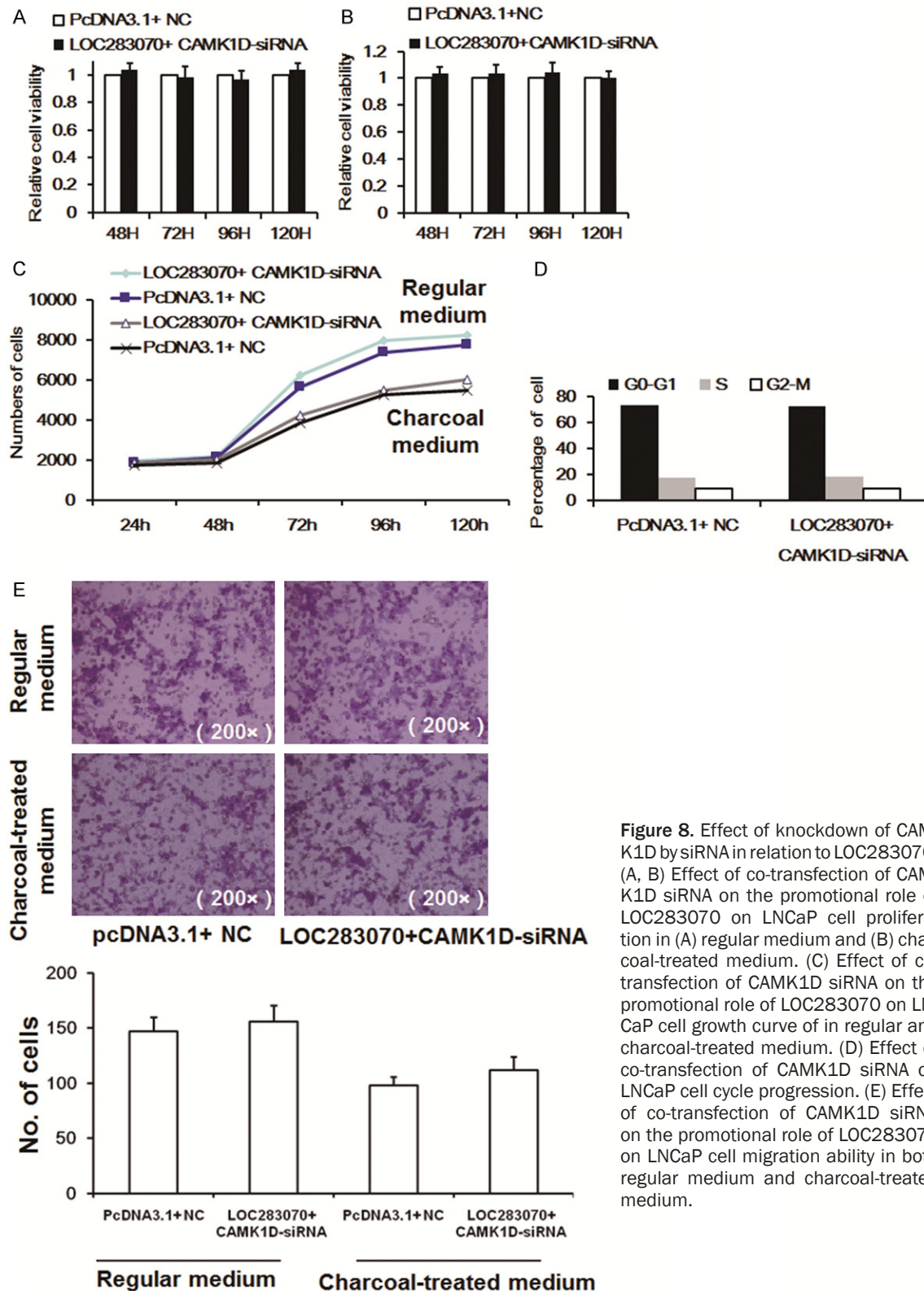


Figure 8. Effect of knockdown of CAMK1D by siRNA in relation to LOC283070. (A, B) Effect of co-transfection of CAMK1D siRNA on the promotional role of LOC283070 on LNCaP cell proliferation in (A) regular medium and (B) charcoal-treated medium. (C) Effect of co-transfection of CAMK1D siRNA on the promotional role of LOC283070 on LNCaP cell growth curve of in regular and charcoal-treated medium. (D) Effect of co-transfection of CAMK1D siRNA on LNCaP cell cycle progression. (E) Effect of co-transfection of CAMK1D siRNA on the promotional role of LOC283070 on LNCaP cell migration ability in both regular medium and charcoal-treated medium.

are involved in numerous biological roles such as imprinting [10], epigenetic regulation [15], translational regulation, splicing, cell develop-

ment and differentiation [16, 17], aging [18], stem cell pluripotency [19], cell growth and apoptosis and cancer metastasis [20, 21].

Naturally, some lncRNAs should be involved in the progression of AIPC. For this reason, we established an androgen-independent cell line LNCaP-AI from ADPC cell line LNCaP by prolonging androgen deprivation culture. Because LNCaP-AI cells are generated under androgen deprivation condition that mimics androgen ablation therapy for human prostate cancer, the comparison between LNCaP-AI cell line and LNCaP cell line should be an excellent model for studying the molecular mechanism of AIPC development. Therefore, we explored the different expression of lncRNAs in LNCaP and LNCaP-AI cells using microarray. The lncRNA expression profiling data show that a number of lncRNAs are expressed differentially in LNCaP-AI cells compared with their expression in LNCaP cells. The microarray expression profiles showed 20836 expressed lncRNAs, in which 1277 were significantly up-regulated and 495 were significantly down-regulated (fold change ≥ 2.0) in LNCaP-AI cells compared with those in LNCaP cells. Subsequently, we randomly selected 15 lncRNAs for validation using qRT-PCR. Furthermore, among these validated lncRNAs, 9 were randomly selected to observe their expression levels in different prostate cell lines, such as LNCaP, LNCaP-AI, DU-145 and PC-3. DU-145 is derived from brain metastasis and PC-3 is derived from bone metastasis. Both of DU-145 and PC-3 have relatively high degrees of malignancy. The qRT-PCR data show that a series of lncRNAs are constantly differentially expressed between AIPC and ADPC cells. With the increase of cell malignancy, the tendency rises, indicating that the dysregulated lncRNA plays a role in the progression of AIPC.

To gain insights into the underlying biology of the differentially expressed transcripts, we performed gain-of-function studies with all of these verified lncRNAs to assess the effect of lncRNAs on malignant behaviors of cancer cells *in vitro*. All 15 verified lncRNAs were cloned into pcDNA3.1 vector, 9 up-regulated lncRNAs were transfected into LNCaP cells and 6 down-regulated lncRNAs were transfected into LNCaP-AI cells for gain-of-function assay. Meanwhile, observation about malignant behaviors of cancer cells were done to find lncRNAs that play a role in the development of androgen independence in prostate cancer. At last, we found LOC283070. We find that the expression of lncRNA LOC283070 in LNCaP-AI cells is signifi-

cantly higher than that in LNCaP cells, and LOC283070 plays a role in the progression of AIPC.

The qRT-PCR assay of a series of prostate cell lines verified elevated expression levels of LOC283070 in AIPC cells, suggesting that LOC283070 might take part in the progression of AIPC. Then, we enhanced the expression of LOC283070 in LNCaP cells to detect its function. In a series of assays on malignant cell behaviors, cancer cells over-expressing LOC283070 showed significantly increased proliferation and migration. In addition, after being treated with androgen deprivation culture, LNCaP cells exhibited reduction in malignant cell behavior and G0/G1 cell cycle arrest, while cells with the overexpression of LOC283070 still exhibited significantly increased growth and migration compared to vector-alone control cells, as well as increased cell-cycle progression (evidenced by increased S phase fraction). In line with these results, inhibition of LOC283070 expression decreases LNCaP-AI cell proliferation, cell-cycle progression, and migration. At last, *in vivo* experiment confirmed that overexpression of LOC283070 promotes tumor growth in both normal mice and castrated mice. All these findings provide evidence for the important role of LOC283070 in the transition of AIPC.

A lot of functions of lncRNAs in human cancer may have connections to their ability to regulate protein expression [22-24]. For example, lncRNA-Dreh can repress the expression of the intermediate filament protein vimentin by binding to it [22]. Next, we attempt to describe a mechanistic basis for the role of LOC283070. Bioinformatics prediction reveals that the sequence of LOC283070 is located on 3'-UTR of the gene CAMK1D (<http://genome.ucsc.edu>). In addition, Anna et al. shows that CAMK1D promotes cell proliferation and increases cell-cycle progression by directly targeting the CREB pathway [7]. Given this close proximity, we hypothesize that LOC283070 regulates CAMK1D to achieve its functions. Indeed, the cis-regulation of neighboring protein-coding genes is a common mechanism for numerous lncRNAs, such as the well-studied lncRNAs H19 and Xist [7, 25]. As CAMK1D is an identified oncogene in basal-like tumors [26-28], many studies clearly identify CAMK1D amplification

and overexpression in a subset of breast cancers, and define a connection to cell proliferation and EMT. Subsequently, much higher CAMK1D protein expression is found in LNCaP-AI cells than in LNCaP cells. Elevated protein expression level of CAMK1D is observed in cells with LOC283070 overexpression and reduced protein expression level of CAMK1D is observed in cells with LOC283070 knockdown. Moreover, CAMK1D overexpression in LNCaP cells has similar effect with LOC283070 overexpression on the transition of LNCaP cells into androgen-independent cells, and LNCaP cells co-transfected with LOC283070 and CAMK1D-siRNA present similar malignant behaviors with negative control group, suggesting that the role of LOC283070 is remarkably diminished when CAMK1D knocked down.

The present study still has a limitation. We demonstrated that the effect of LOC283070 on prostate cancer cells is attributed to the up-regulation of CAMK1D protein expression, but the detailed mechanism underlying this regulation is still not known. Investigation on this issue will be conducted in further studies. In summary, we presented an in vitro model of AIPC in this study. Our study was focused on the molecular mechanisms of AIPC in lncRNA level using AIPC progression model formed by LNCaP and LNCaP-AI cells. Significantly different expression of lncRNAs between LNCaP and LNCaP-AI cells indicates that lncRNAs may play important roles in the progression of AIPC. Further functional studies demonstrate that lncRNA LOC283070 indeed takes part in the progression of AIPC, which can promote the proliferation and migration, partly by increasing CAMK1D protein expression. The present study suggests that lncRNAs may be modulators of biological activities that can become potential biomarkers for the diagnosis or prognosis, or potential treatment targets for AIPC.

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Disclosure of conflict of interest

None.

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