

# Overexpression of lncRNA ANRIL promoted the proliferation and migration of prostate cancer cells via regulating let-7a/TGF- $\beta$ 1/Smad signaling pathway

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**Abstract.** Long non-coding RNAs (lncRNAs) were playing critical roles in tumorigenesis. However, in prostate cancer, the roles and mechanisms of lncRNAs especially ANRIL were largely unknown. We investigated the effects of ANRIL on the proliferation and migration of prostate cancer cells using CCK-8 assay and Transwell migration assay. Real-time PCR and western blotting assays were used to analyze the levels of ANRIL, let-7a, TGF- $\beta$ 1, p-Smad2 and p-Smad7. Our results showed that ANRIL was significantly overexpressed in prostate cancer tissues compared with corresponding normal tissues. Knockdown of ANRIL significantly inhibited the proliferation and migration of prostate cancer LNCap, PC3 and DU145 cells. Knockdown of ANRIL significantly decreased the levels of TGF- $\beta$ 1 and p-Smad2, and increased the level of p-Smad7 in prostate cancer LNCap cells. We further found that knockdown of ANRIL significantly enhanced the expression of let-7a, and rescue experiment found that let-7a inhibitor recovered the suppressive effects of ANRIL silencing on the proliferation and migration of prostate cancer LNCap, PC3 and DU145 cells. And let-7a inhibitor recovered the suppressive effects of ANRIL silencing on the activity of TGF- $\beta$ 1/Smad signaling pathway in prostate cancer LNCap cells. Taken together, our findings indicated that overexpression of lncRNA ANRIL promoted the proliferation and migration of prostate cancer cells via regulating let-7a/TGF- $\beta$ 1/Smad signaling pathway.

Keywords: lncRNA ANRIL, prostate cancer, let-7a, TGF- $\beta$ 1/Smad signaling pathway

## 1. Introduction

Prostate cancer is one of the most frequent malignant cancers in the reproductive and urinary system, and the second most common cancer in the world [1,2]. Many studies suggested that long non-coding RNAs (lncRNAs) were playing critical roles in tumorigenesis. However, in prostate cancer, the roles and mecha-

nisms of lncRNAs especially ANRIL were largely unknown.

ANRIL was significantly up-regulated in cervical cancer tumors and cell lines, and high ANRIL expression was associated with advanced the International Federation of Gynecology and Obstetrics (FIGO) stage, lymph node metastasis and poor overall survival than those with low ANRIL expression, and functional study showed that ANRIL could promote the proliferation, migration and invasion of cervical cancer via PI3K/Akt pathways [3]. And Zhang et al. further found that ANRIL knockdown inhibited the proliferation and invasion, and promoted apoptosis of cervical cancer cells by sponging miR-186 [4]. In

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pancreatic cancer, ANRIL was also overexpressed, and its overexpression promoted epithelial-mesenchymal transition by activating the ATM-E2F1 signaling pathway both *in vitro* and *in vivo* study [5]. In osteosarcoma, ANRIL was also upregulated, and HIF-1 $\alpha$  directly bound to putative hypoxia response element in the upstream region of ANRIL and further promoted ANRIL expression [6]. In prostate cancer, the rs4977574, rs1333048 and rs10757278 polymorphisms of lncRNA ANRIL were associated benign prostate hyperplasia and prostate cancer risk [7]. ANRIL was elevated in prostate cancer tissues, and CBX7 within the polycomb repressive complex 1 could bind to ANRIL and then repress the INK4b/ARF/INK4a locus and control senescence [8]. However, the functional roles of ANRIL in prostate carcinogenesis were still unknown.

In the present study, we found that ANRIL was overexpressed in prostate cancer, and knockdown of ANRIL using siRNAs inhibited the proliferation and migration of prostate cancer cells. We further found that knockdown of ANRIL significantly inhibited the TGF- $\beta$ 1/Smad signaling pathway and increased the level of let-7a. Importantly, rescue experiments showed that let-7a inhibitor could reverse the suppression of ANRIL knockdown on the proliferation and migration of prostate cancer cells and the activity of TGF- $\beta$ 1/Smad signaling pathway.

## 2. Materials and methods

### 2.1. Patients

Fresh tissues of prostate cancer and adjacent normal tissues from 8 patients were collected by the Department of Urology, Yunnan Tumor Hospital and the Third Affiliated Hospital of Kunming Medical University (Kunming, China). Primary tumor tissues and the adjacent normal tissues from the same patients were separated and then immediately stored at  $-70^{\circ}\text{C}$ . All prostate cancer patients were treated with radical operation, and none of them received any preoperative treatment. All the samples were residual specimens after diagnostic sampling, and all patients signed separate informed consent forms for sampling and molecular analysis.

### 2.2. Cell culture

The human prostate cancer cell lines including LNCap, PC3 and DU145 were (American Type Cul-

ture Collection, Manassas, VA, USA) cultured in RPMI-1640 medium (Invitrogen corporation, USA) with 10% fetal bovine serum (Hyclone, USA), penicillin (100 U/ml) and streptomycin (100 mg/ml). The cells were maintained at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$ .

### 2.3. Cell transfection

The cells were seeded in six-well plates and transfected with lncRNA ANRIL siRNAs or negative control siRNA or let-7a inhibitor using lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The lncRNA ANRIL siRNA-1 sequence was GGUCAUCUCAU-UGCUCUAU; the lncRNA ANRIL siRNA-2 sequence was GCCCAAUUAUGCUGUGGUA. In addition, commercial anti-let-7a oligonucleotide (inhibitor) was purchased from GenePharma (Shanghai, China). And the lncRNA ANRIL siRNAs and negative control siRNA were also synthesized by GenePharma (Shanghai, China).

### 2.4. Cell proliferation assay

Cellular proliferation was measured by Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) based on manufacturer's instructions. Twenty-four hours after transfection, cells were seeded into 96-well plates at a density of  $8 \times 10^3$  cells per well with 100  $\mu\text{l}$  medium and continued to incubate at  $37^{\circ}\text{C}$ . At the indicated time points, 10  $\mu\text{l}$  CCK-8 solutions were added to each well. After incubation at  $37^{\circ}\text{C}$  for 1 h, the absorbance was measured with a plate reader at 450 nm. The growth curves were shown to reveal the growth rates.

### 2.5. Cell migration assay

Transwell assay was performed to detect the migration ability of transfected prostate cancer cells. The cells were digested and seeded in the upper chamber at a density of  $3 \times 10^5$  cells/mL. The lower chamber was filled with medium containing 20% FBS, and then the set was assembled and incubated at  $37^{\circ}\text{C}$  for 36 hours. After incubation, the membrane was stained using 0.1% crystal violet for 30 minutes. Stained cells were washed in PBS, and counted under an optical microscope.

### 2.6. Total RNA extraction and Real-time PCR assay

Total RNA was isolated from cancer cells using the

Table 1  
Characteristics of patients with prostate cancer in the present study

NO.	Age	pT status	pN status	Gleason score
1	45	T2	N0	6
2	54	T2	N0	7
3	61	T3	N0	9
4	39	T4	N0	8
5	48	T3	N0	8
6	55	T2	N1	9
7	64	T4	N1	7
8	54	T2	N1	9

RNeasy Mini Kit as described by the manufacturer (Qiagen, Hilden, Germany) and used for Real-time PCR assay.

Real-time PCR was performed to detect the relative expression levels of lncRNA ANRIL. The PCR reactions were performed in a total volume of 20  $\mu$ l, including 10  $\mu$ l of 2XPower SYBR<sup>®</sup> Green PCR Master Mix (Applied Biosystems, Warrington, UK), 2  $\mu$ l of cDNA (5 ng/ $\mu$ l) and 1  $\mu$ l of primer mix (10  $\mu$ M each). PCR amplification and detection were performed in a Light-Cycler 480 II (Roche Applied Science) as follows: an initial denaturation at 95°C for 10 min; 40 cycles of 95°C for 15 s and 60°C for 1 min. The relative gene expression was calculated using the comparative CT Method. The gene expression of the target gene were normalized to an endogenous reference (GAPDH), and relative to the calibrator were given by the formula  $2^{-\Delta\Delta C_t}$ .  $\Delta C_t$  was calculated by subtracting the average GAPDH CT from the average CT of the gene of interest. The ratio defines the level of relative expression of the target gene to that of GAPDH. The following primer pairs were used for amplification: ANRIL forward, 5'-GGACTACAGATGCACCACC AT-3', ANRIL reverse, 5'-TGAGCACTGTGTCCATA GCA-3'; GAPDH forward, 5'-AAATCCCATCACCA TCTTCCAG-3', GAPDH reverse, 5'-GAGTCCTCC ACGATACCAAAGTTG-3'.

Hairpin-itTM let-7a qRT-PCR Primer Set (GenePharma) was used for the measurement of the relative quantity of let-7a. The mRNA expression of let-7a was normalized to the endogenous expression of U6.

### 2.7. Western blotting assay

Cells from each group were detached with trypsin, centrifuged, and washed 2 times with pre-chilled PBS. Cell lysis buffer was subsequently added and incubated on ice for protein extraction. Protein concentration was determined using the BCA Protein Assay Kit (Beyotime Biotechnology, China). Equal amounts of proteins were separated via 12% SDS-PAGE and then then

transferred to a PVDF membrane (Millipore Corporation, Billerica, MA, USA). The membrane was soaked in 10% skimmed milk (in PBS, PH 7.2, containing 0.1% Tween-20) for 2 h and incubated with an appropriate amount of primary antibody at 4°C overnight. Detection was by peroxidase-conjugated secondary antibodies (KPL, Gaithersburg, MD, USA) and chemiluminescence (Millipore Corporation).

### 2.8. Statistical analyses

All quantitative data are presented as mean  $\pm$  standard deviation (SD). Student's *t* test was performed when only two groups were present using GraphPad Prism version 5.01 (GraphPad Software, La Jolla, CA, USA). The differences were judged as statistically significant when the corresponding two-sided *P* value was  $< 0.05$ .

## 3. Results

### 3.1. lncRNA ANRIL is overexpressed in prostate cancer, and knockdown of lncRNA ANRIL inhibits the proliferation and migration of prostate cancer cells

We detected the expression of lncRNA ANRIL in 8 prostate cancer tissues and corresponding normal tissues using Real-time PCR, and the results showed that lncRNA ANRIL was significantly overexpressed (Fig. 1A). Using siRNAs of lncRNA ANRIL, we significantly reduced the expression of ANRIL in three cell lines of prostate cancer including LNCap, PC3 and DU145 (Fig. 1B). Using Transwell assay, we found that knockdown of ANRIL significantly inhibited the migration of prostate cancer cells LNCap, PC3 and DU145 (Fig. 1C and D). Using CCK-8 assay, we further found that knockdown of ANRIL significantly suppressed the proliferation of prostate cancer cells LNCap, PC3 and DU145 (Fig. 1E).

### 3.2. Knockdown of lncRNA ANRIL inactivates the TGF- $\beta$ 1/Smad signaling pathway

TGF- $\beta$ /Smad signaling pathway plays the important roles in the regulation of proliferation, migration and epithelial-mesenchymal transition (EMT) of prostate cancer [9–11]. Therefore, we investigated whether lncRNA ANRIL could regulate the TGF- $\beta$ /Smad signaling pathway in prostate cancer. Interestingly, we

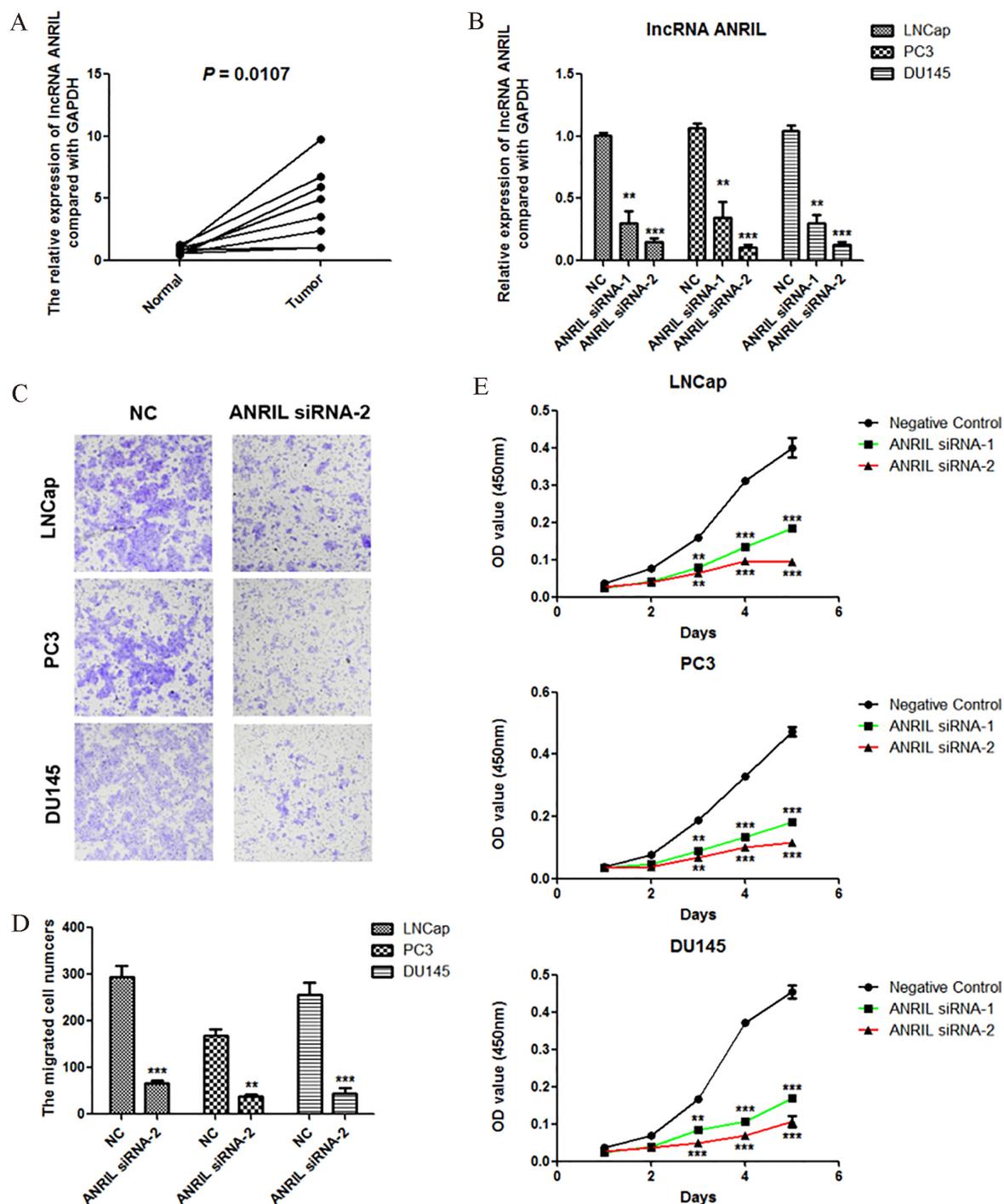


Fig. 1. lncRNA ANRIL is overexpressed in prostate cancer, and knockdown of lncRNA ANRIL inhibits the proliferation and migration of prostate cancer cells. A. lncRNA ANRIL was overexpressed in prostate cancer detected by Real-time PCR assay ( $n = 8$ ). B. Knockdown of ANRIL in LNCap, PC3 and DU145 cells was detected by Real-time PCR. C and D. Effect of ANRIL siRNA on the migration of LNCap, PC3 and DU145 cells. The migration of cells was measured by Transwell migration assay, and statistical analysis was done using Student's test. E. Effect of ANRIL siRNAs on the proliferation of LNCap, PC3 and DU145 cells. The viability of cells was measured by Cell Counting Kit-8, and statistical analysis was done using Student's test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

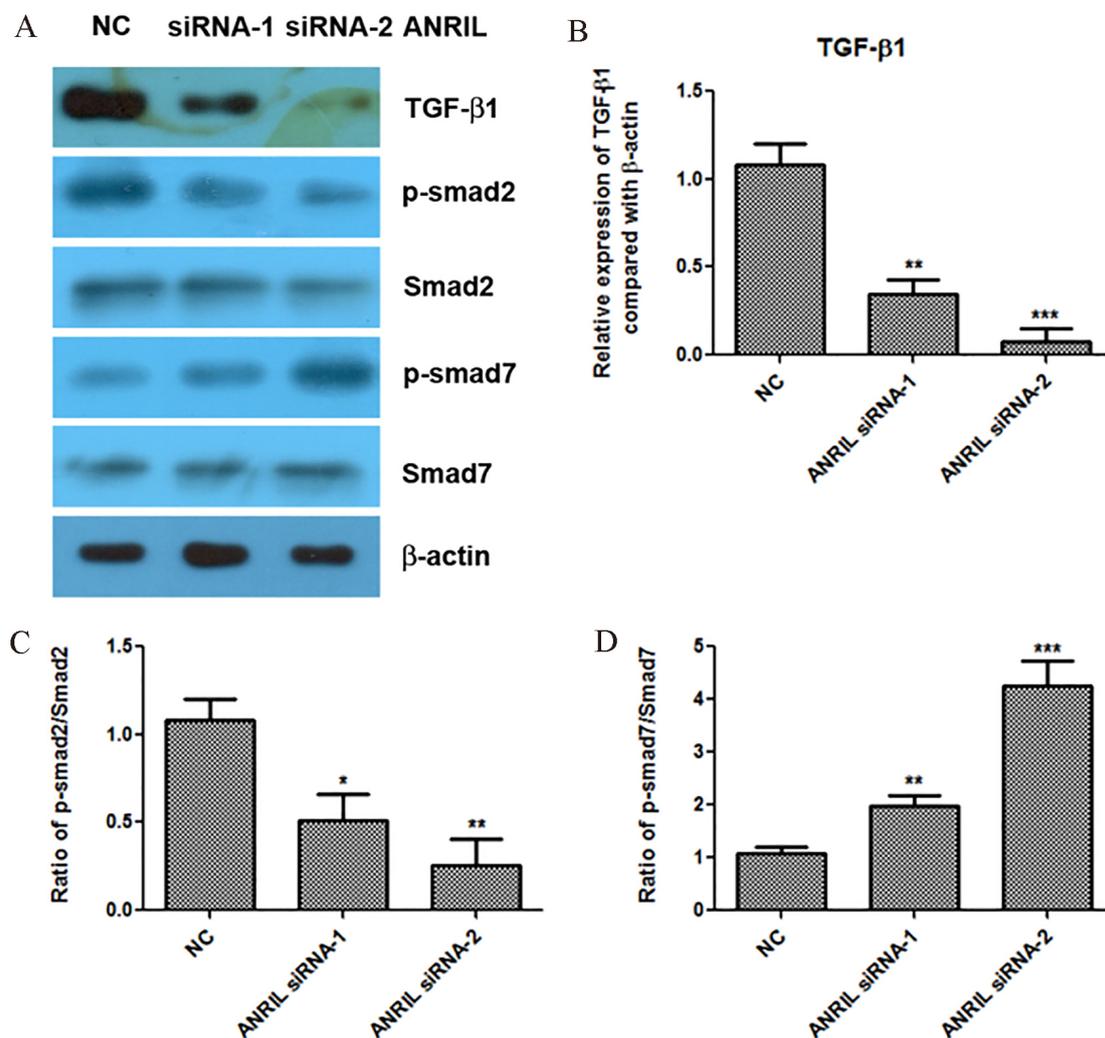


Fig. 2. Knockdown of lncRNA ANRIL inactivates the TGF- $\beta$ 1/Smad signaling pathway. A to D. Effect of ANRIL siRNAs on the expression levels of TGF- $\beta$ 1, p-smad2, smad2, p-smad7 and smad7 detected by western blotting assay, and statistical analysis was done using Student's test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

found that knockdown of ANRIL significantly reduced the protein expression of TGF- $\beta$ 1, and decreased the level of p-smad2 and increased the level of p-smad7, but had no effects on the protein levels of smad2 and smad7 in LNCap prostate cancer cells (Fig. 2A–D). Our results suggested that silence of lncRNA ANRIL inactivated the TGF- $\beta$ 1/Smad signaling pathway.

### 3.3. Knockdown of lncRNA ANRIL increases the expression of let-7a, and let-7a inhibitor reactivates the TGF- $\beta$ 1/Smad signaling pathway at the condition of ANRIL silencing

We further found that knockdown of lncRNA ANRIL significantly increased the expression of let-7a

in the prostate cancer cells LNCap, PC3 and DU145 (Fig. 3A). In order to confirm that let-7a is involved in the regulation of TGF- $\beta$ 1/Smad signaling pathway by lncRNA ANRIL, we reduced the expression of let-7a at the condition of ANRIL silencing, and then analyzed the activity of TGF- $\beta$ 1/Smad signaling pathway in LNCap cells. Importantly, we found that knockdown of let-7a at the condition of ANRIL silencing could increase the protein expression of TGF- $\beta$ 1, and promote the phosphorylation of smad2 and inhibit the phosphorylation of smad7 compared with ANRIL silencing group (Fig. 3B–F). These results indicated that knockdown of lncRNA ANRIL inactivated the TGF- $\beta$ 1/Smad signaling pathway through upregulation of let-7a.

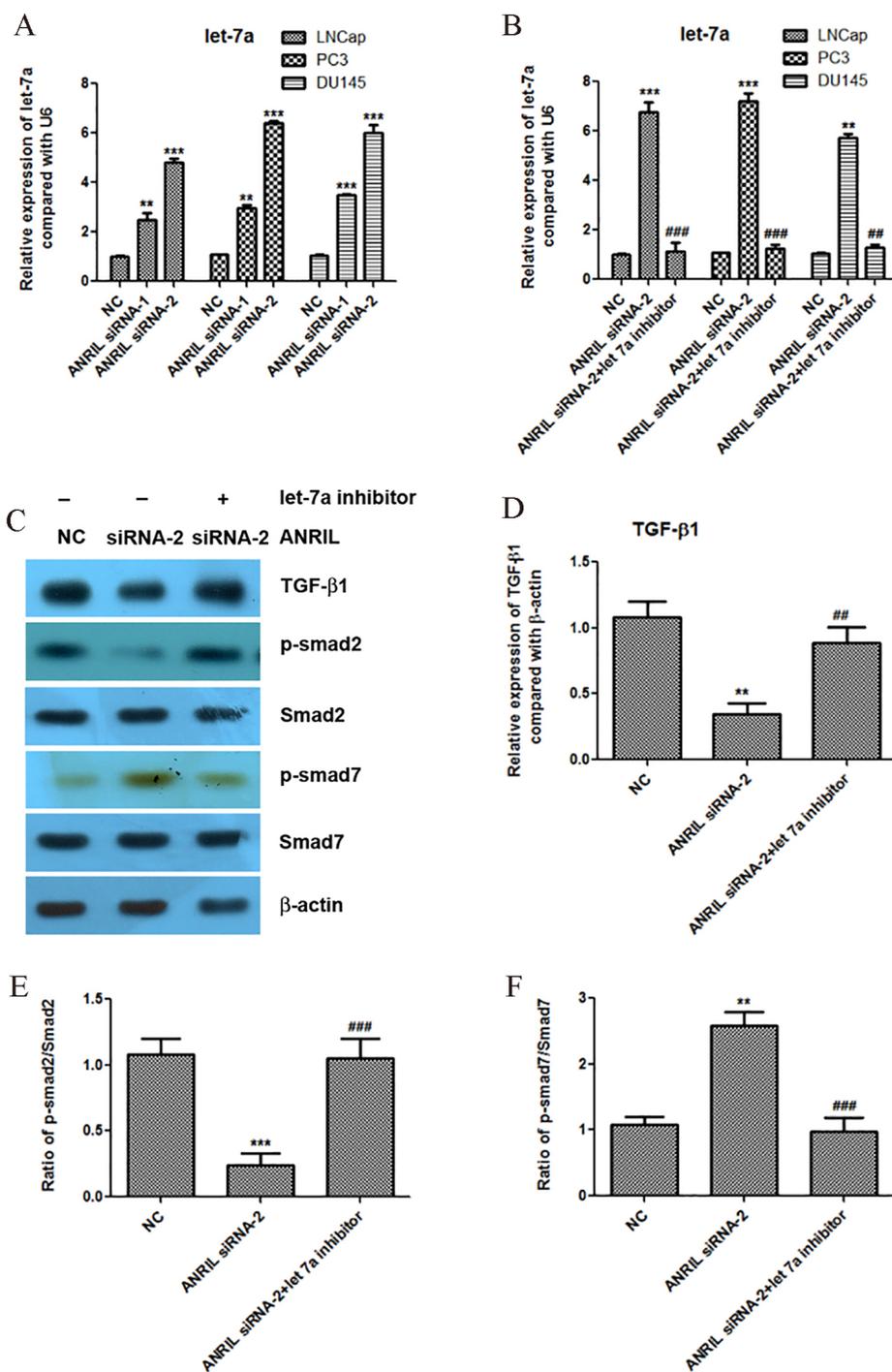


Fig. 3. Knockdown of lncRNA ANRIL increases the expression of let-7a, and let-7a inhibitor reactivates the TGF- $\beta$ 1/Smad signaling pathway at the condition of ANRIL silencing. A. Knockdown of lncRNA ANRIL increases the expression of let-7a detected by Real-time PCR. B. Knockdown of let-7a inhibitor in LNCap, PC3 and DU145 cells was detected by Real-time PCR. C to F. Effect of let-7a inhibitor at condition of ANRIL silencing on the expression levels of TGF- $\beta$ 1, p-smad2, smad2, p-smad7 and smad7 detected by western blotting assay, and statistical analysis was done using Student's test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

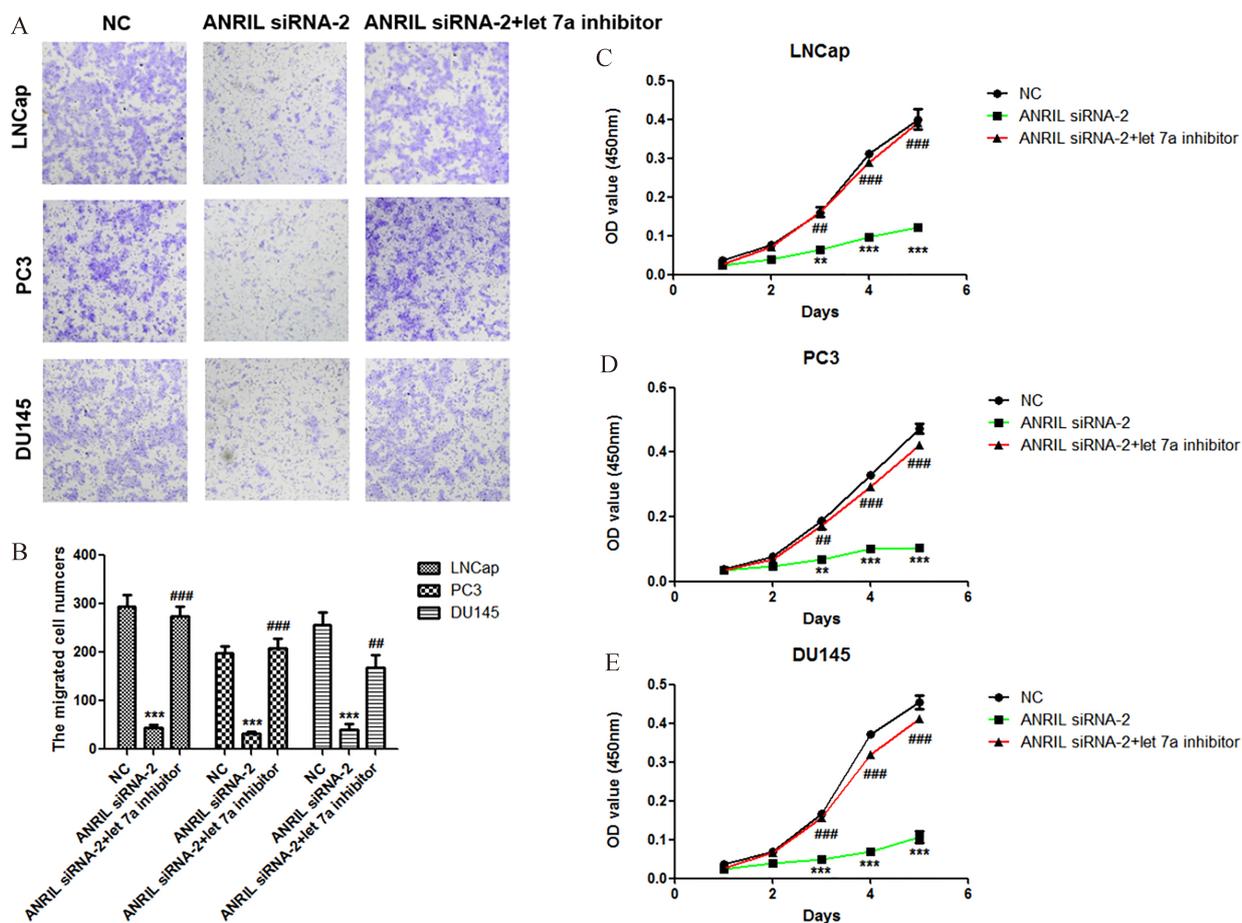


Fig. 4. Knockdown of let-7a recovers the abilities of prostate cancer cells proliferation and migration at the condition of ANRIL silencing. A. Effect of let-7a inhibitor at condition of ANRIL silencing on the migration of LNCap, PC3 and DU145 cells. The migration of cells was measured by Transwell migration assay, and statistical analysis was done using Student's test. E. Effect of let-7a inhibitor at condition of ANRIL silencing on the proliferation of LNCap, PC3 and DU145 cells. The viability of cells was measured by Cell Counting Kit-8, and statistical analysis was done using Student's test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

### 3.4. Knockdown of let-7a recovers the abilities of prostate cancer cells proliferation and migration at the condition of ANRIL silencing

In order to confirm that let-7a is involved in the regulation of prostate cancer cell proliferation and migration by lncRNA ANRIL, we reduced the expression of let-7a at the condition of ANRIL silencing, and then analyzed the activities of cell proliferation and migration. Importantly, we found that knockdown of let-7a at the condition of ANRIL silencing significantly promoted the migration and proliferation of prostate cancer cells LNCap, PC3 and DU145 compared with ANRIL silencing group (Fig. 4A–E). These results indicated that knockdown of lncRNA ANRIL inhibited the prostate cancer cell proliferation and migration through upregulation of let-7a.

## 4. Discussion

The present study found that lncRNA ANRIL was overexpressed in prostate cancer, and knockdown of ANRIL inhibited the proliferation and migration of prostate cancer cells. We further found that knockdown of lncRNA ANRIL inactivated the TGF- $\beta$ 1/Smad signaling pathway via decreasing the TGF- $\beta$ 1 expression and inhibiting the phosphorylation of Smad2 and promoting the phosphorylation of Smad7. Up to now the understanding of the correlations between ANRIL and TGF- $\beta$  signaling pathway is limited. Zhao et al. found that silencing ANRIL could up-regulate the expressions of TGF- $\beta$ 1 and p-Smad2/3 [12]. Different from the suppressive role of ANRIL on the TGF- $\beta$  signaling pathway, overexpression of ANRIL activated the TGF- $\beta$  signaling pathway in prostate cancer. Previ-

ous study reported that the expression of TGF- $\beta$ 1 was significantly higher in prostate cancer tissues than in normal prostate tissues; and upregulation of lncRNA PlncRNA-1 promoted epithelial-mesenchymal transition (EMT) in prostate cancer cells via inducing the TGF- $\beta$ 1 expression [9]. In prostate cancer, TGF- $\beta$  could promote PI3K/AKT signaling and cell migration through the TRAF6-mediated ubiquitylation of p85 $\alpha$  [11]. TMPRSS2:ERG gene fusion activated the TGF- $\beta$  signaling via increasing VIM, MMP1, CDH2 and SNAI2, and induced the epithelial to mesenchymal transition in human prostate cancer cells [10]. The smad7 was commonly regulated by ubiquitin-mediated degradation via Smurf2 and ITCH, however, how its phosphorylation was regulated was still needed to explore. Our study first reported that lncRNA ANRIL positively regulated the TGF- $\beta$ 1/Smad signaling pathway.

Although Sun et al. reported that downregulation of ANRIL repressed tumorigenicity and enhanced cisplatin-induced cytotoxicity via regulating let-7a in nasopharyngeal carcinoma [13]. However, the relationships among ANRIL, let-7a and TGF- $\beta$  signaling pathway were still unknown. Our study further revealed that knockdown of lncRNA ANRIL increased the expression of let-7a, and let-7a inhibitor reactivated the TGF- $\beta$ 1/Smad signaling pathway, and recovered the abilities of prostate cancer cells proliferation and migration at the condition of ANRIL silencing.

Taken together, our findings revealed that overexpression of lncRNA ANRIL promoted the proliferation and migration of prostate cancer cells via regulating let-7a/TGF- $\beta$ 1/Smad signaling pathway.

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### Conflict of interest

None.

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