

# LINC01006 influences cell proliferation, apoptosis, migration and invasion by targeting miR-34a-5p/DAAM1 in prostate cancer

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## Primary research

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# Abstract

**Background:** Large quantities of researches have demonstrated that long noncoding RNAs (lncRNAs) exert crucial function in the development of human cancers by acting as oncogenes or tumor suppressors. LINC01006 was once found to have oncogenic function in pancreatic cancer. However, its role is still unclear in prostate cancer (PCa).

**Methods:** RT-qPCR assay examined LINC01006, miR-34a-5p, DAAM1 expression in PCa cells. RNA pull down and luciferase reporter assay were used to certify the relationship between LINC01006 and its downstream targets.

**Results:** LINC01006 was discovered to be remarkably high in PCa cell lines. Moreover, the functional assay validated that LINC01006 silence hindered proliferation, migration and invasion as well as accelerated apoptosis in PCa cells. The results of nucleus cytoplasm fractionation and FISH assay revealed that LINC01006 had the possibility to modulate downstream targets in ceRNA mechanism. MiR-34a-5p was selected out to be low expressed in PCa cell lines. In addition, miR-34a-5p up-regulation restricted proliferative, migratory and invaded capacities while facilitated apoptosis in PCa cells. DAAM1 was determined to be negatively regulated by miR-34a-5p. More interestingly, DAAM1 knockdown had inhibitory effects on PCa cells. Eventually, data of rescue assays exhibited that overexpression of DAAM1 counteracted the suppressive impacts of LINC01006 down-regulation on PCa progression.

**Conclusions:** LINC01006 was an oncogene in PCa by targeting miR-34a-5p/DAAM1, which offered a novel insight for treating PCa.

## Background

It is widely known that prostate cancer (PCa) is the common disease which brings trouble to elderly males around the world [1]. The high morbidity and mortality of PCa leave it become the third of most common cancer leading to death [2, 3]. However, lack of obvious symptom in early stage makes it harder to diagnose PCa in time. Thus, it is important to have a deep understanding of the underlying molecular mechanism in PCa.

Long noncoding RNAs (lncRNAs) are a type of RNAs whose length was more than 200nts with limited capacities of coding proteins [4]. Recently, abundant of lncRNAs have been identified to have crucial functions in regulating the biological process of multiple cancers, including PCa [5-7]. For example, lncRNA LSAMP-AS1 repressed the process of PCa by binding miR-183-5p and enhancing DCN expression [8]. RHPN1-AS1 was reported to promote the progression of breast cancer by targeting miR-6884-5p/ANXA11 [9]. UCA1 contributed to the proliferation in gastric cancer through modulating PRL-3 expression and sponged miR-495 [10]. Long intergenic non-protein coding RNA 1006 (LINC01006) was studied as an underlying biomarker in gastric cancer [11]. Nevertheless, how LINC01006 participated in the growth of PCa has not been explored.

Increasing studies have explored that lncRNAs acted as sponges of miRNAs to modulate the expression of mRNAs. This mechanism was called competing endogenous RNA (ceRNA) system [12, 13]. LncRNAs bound with miRNAs so that the downstream mRNAs were released from the bond with miRNAs. MAGI2-AS3 modulated the progression of NSCLC via targeting miR-155/SOCS-1 [14]. TTN-AS1 accelerated the course of ovarian cancers through sponging miR-139-5p to elevate ROCK2 expression [15]. In this study, we unveiled the relationship between LINC01006 and its downstream targets.

The main task of our research was to investigate the role of LINC01006 in PCa cells. Moreover, functional assays were carried out to assess the role of LINC01006, miR-34a-5p and dishevelled associated activator of morphogenesis 1 (DAAM1). Rescue assays were performed to certify axis of LINC01006/miR-34a-5p/DAAM1.

## Methods

### Cell lines

Human prostate cancer cells (DU145, PC3, LNCAP, VCaP) and human prostate epithelial cells (RWPE-1) were available from ATCC (Manassas, VA) and grown under 5% CO<sub>2</sub> and 37°C. They were cultured with 10% FBS (Gibco, Rockville, MD) and 1% antibiotics (Gibco) in DMEM culture medium (Gibco).

### Total RNA extraction and qRT-PCR

The extracted total RNAs from cultured cells acquired using TRIzol Reagent (Invitrogen, Carlsbad CA) and converted to cDNA by PrimeScript Reverse Transcriptase Kit (Takara, Shiga, Japan). qPCR assay was conducted with SYBR Green PCR Kit (Takara). Expressions of target genes were calculated by  $2^{-\Delta\Delta Ct}$  method and standardized to U6 or GAPDH.

### Cell transfection

The designed shRNAs for LINC01006 or DAAM1, the pcDNA3.1/DAAM1, as well as their relative control shRNAs and vectors, these were all procured from Genechem (Shanghai, China). The miR-34a-5p mimics/inhibitor and NC mimics/inhibitor were designed at GenePharma (Shanghai, China). DU145 and LNCAP cells were transfected in 24-well plates for 48 h by using Lipofectamine 3000 (Invitrogen).

### Colony formation

Transfected DU145 and LNCAP cells were added in 6-well plates, with 500 cells in each well. Following culturing for 14 days, resulting colonies were fixed in 4% PFA for 30 min, then stained in 0.5% crystal violet solution for 5 min. Cloned were counted manually.

### EdU assay

EdU assay was undertaken in DU145 and LNCAP cells after transfection by use of BeyoClick™ EdU Cell Proliferation Kit (Beyotime, Shanghai, China) with Alexa Fluor 594. Samples were counterstained with EdU medium and DAPI solution, finally visualized under inverted microscope (Olympus, Tokyo, Japan).

### **TUNEL assay**

Following transfection, the collected DU145 and LNCAP cells were washed using PBS and treated with 4% PFA. TUNEL reagent was purchased from Merck KGaA (Darmstadt, Germany) for staining the apoptotic cell samples. After DAPI staining, samples were analyzed with optical microscopy (Olympus).

### **Flow cytometry**

Apoptosis of transfected DU145 and LNCAP cells was estimated with application of double Annexin V/PI staining kit (Invitrogen). Cell samples were double-stained in 6-well plates with Binding Buffer for 15 min, then subjected to flow cytometer (BD Biosciences, Franklin Lakes, NJ).

### **Transwell assay**

Transfected cells ( $1 \times 10^5$ ) were placed to the upper chambers of transwell inserts coating with Matrigel (BD Biosciences) for assessing cell invasion. The 100% complete culture medium was added into the lower chambers. Cell migration was assessed without Matrigel. After 24 h, the inserts were fixed and cells invading or migrating through the membrane were stained by crystal violet for counting.

### **Subcellular fractionation**

The nuclear and cytoplasmic RNAs were isolated from DU145 and LNCAP cells by subcellular fractionation assay, using PARIS™ Kit (Invitrogen) as guided. GAPDH was examined as the indicator of cell cytoplasm for quantification, and U6 was examined as the indicator of cell nucleus.

### **FISH**

The LINC01006-specific FISH probe in this study was designed by Ribobio (Guangzhou, China). Cells of DU145 and LNCAP were hybridized with probe in buffer, then counterstained in Hoechst solution and visualized using confocal laser microscope (Olympus).

### **Luciferase reporter assay**

LINC01006 or DAAM1 fragments covering wild-type and mutant miR-34a-5p binding sites were acquired and inserted into the pmirGLO dual-luciferase vector (Promega, Madison, WI). They were co-transfected with miR-34a-5p mimics and NC mimics in DU145 and LNCAP cells for luciferase assay, then assayed by dual-luciferase reporter assay system (Promega).

### **RNA pull down**

The miR-34a-5p sequence covering LINC01006 or DAAM1 wild-type or mutant binding sites were acquired and biotinylated. Then, the biotin-labelled probes were mixed with cell proteins and magnetic beads (Invitrogen). The final RNA-protein binding mixture was assayed by qRT-PCR.

### **RNA immunoprecipitation (RIP)**

RIP assay was undertaken in DU145 and LNCAP cells with application of Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit, following the protocol (Millipore, Bedford, MA). The cell lysates were treated in RIP buffer with magnetic beads and control IgG antibody or human Ago2 antibody, followed by qRT-PCR analysis.

### **Statistical analyses**

Data of three individually performed experiments were presented as the mean  $\pm$  SD. Statistical analyses of this study were undertaken with Student's t-test or one-way ANOVA by use of GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA). The experimental results were recorded when  $p < 0.05$ .

## **Results**

### **LINC01006 had high expression and facilitated the progression of PCa cells**

As shown in GEPIA database, LINC01006 was high expressed in PCa tissues compared with the adjacent normal tissues (Fig. 1A). Without doubt, the expression of LINC01006 was conspicuously overexpressed in PCa cell lines (DU145, PC3, LNCAP and VCaP) in comparison with normal human prostate epithelial cells (RWPE-1) (Fig. 1B). To figure out the function of LINC01006 in the development of PCa cells, sh-LINC01006#1/2 was transfected into DU145 and LNCAP cells to reduce the expression of LINC01006 (Fig. 1 C). The results of colony formation and EdU assays manifested that proliferation was hindered by LINC01006 silence (Fig. 1D-E). In contrast, apoptosis rate was promoted by down-regulation of LINC01006 in TUNEL and flow cytometry analysis (Fig. 1F-G). At the same time, data of transwell assays exhibited that migration and invasion were decreased by LINC01006 knockdown (Fig. 1H). In summary, LINC01006 was up-regulated and boosted the process of PCa cells.

### **MiR-34a-5p inhibited the progression of PCa**

After exploring the function of LINC01006, we intended to investigate the function of its downstream targets. The data of nucleus cytoplasm fractionation and FISH assay displayed that LINC01006 was accumulated in cytoplasm rather than in nucleus (Fig. 2A-B). StarBase (<http://starbase.sysu.edu.cn/>) exhibited that miR-148b-3p, miR-34a-5p and miR-6783-3p were predicted to bind to LINC01006 (CLIP-Data  $\geq 1$  Degradome-Data  $\geq 0$  pan-Cancer  $\geq 6$ ). RT-qPCR data exhibited that only miR-34a-5p was low expressed in PCa cells (Fig. 2C). The binding sites between LINC01006 and miR-34a-5p were showed in Fig. 2D. To verify the binding sites, RNA pull down assay was performed. Data disclosed that biotinylated miR-34a-5p could pull down LINC01006 in PCa cells (Fig. 2E). MiR-34a-5p mimics were transfected into cells to increase miR-34a-5p expression (Fig. 2F). Luciferase reporter assays data showed that

overexpression of miR-34a-5p decreased the activity of LINC01006-WT successfully but failed to work on the activity of LINC01006-Mut (Fig. 2G). Then, we analyzed the effects of miR-34a-5p on the progression of PCa. The proliferation was reduced by overexpression of miR-34a-5p in colony formation and EdU assays (Fig. 2H-I). Conversely, miR-34a-5p up-regulation increased apoptosis rate in TUNEL assay and flow cytometry analysis (Fig. 2J-K). Migratory and invaded capacities were diminished by up-regulation of miR-34a-5p in transwell assay (Fig. 2L). In a word, miR-34a-5p was low expressed and suppressed the growth of PCa.

### **DAAM1 expression was negatively regulated by miR-34a-5p**

Then, we explored the downstream of miR-34a-5p. StarBase predicted SYT1, LMAN2L and DAAM1 had binding sites for miR-34a-5p. RT-qPCR data revealed that only DAAM1 expression was decreased by up-regulation of miR-34a-5p in PCa cells (Fig. 3A). Besides, DAAM1 was detected to be up-regulated in PCa cells (Fig. 3B). Then, we carried out RIP assays. Data exhibited that LINC01006, miR-34a-5p and DAAM1 were all enriched in Ago2 antibody but not in IgG antibody (Fig. 3C). The binding sites between miR-34a-5p and DAAM1 were predicted by bioinformatics in Fig. 3D. RNA pull down assays data verified that miR-34a-5p could bind to DAAM1 (Fig. 3E). Luciferase reporter assay displayed that DAAM1-WT activity was diminished by up-regulation of miR-34a-5p but DAAM1-Mut activity showed no apparent changes (Fig. 3F). MiR-34a-5p inhibitor was transfected into cells to cut down the expression of miR-34a-5p (Fig. 3G). Furthermore, DAAM1 expression was lessened by LINC01006 silence but then recovered by miR-34a-5p depletion (Fig. 3H). In brief, DAAM1 was high expressed and negatively modulated by miR-34a-5p in PCa cells.

### **DAAM1 accelerated the progression of PCa**

Additionally, the influence of DAAM1 was examined by functional assays. To begin with, DAAM1 was targeted by sh-DAAM1#1/2 to lessen its expression (Fig. 4A). The outcomes of colony formation and EdU assays disclosed that proliferation was decreased by DAAM1 knockdown (Fig. 4B-C). The apoptosis rate was enhanced by DAAM1 down-regulation in TUNEL assays and flow cytometry analysis (Fig. 4D-E). In transwell assays, migration and invasion were cut down by down-regulated DAAM1 (Fig. 4F). To sum up, DAAM1 boosted the process of PCa cells.

### **LINC01006 promoted the progression of PCa by sponging miR-34a-5p to increase DAAM1**

To corroborate LINC01006 regulated DAAM1 to promote the growth of PCa cells, rescue assays were constructed. At the beginning, DAAM1 expression was elevated by pcDNA3.1/DAAM1 (Fig. 5A). The descending tendency of proliferation induced by LINC01006 knockdown was restored by overexpression of DAAM1 in colony formation and EdU assay (Fig. 5B-C). On the contrary, the rising apoptosis rate imposed by down-regulation of LINC01006 was reversed by up-regulated DAAM1 in TUNEL and flow cytometry analysis (Fig. 5D-E). Meanwhile, the falling trend of migration and invasion caused by LINC01006 silence was countervailed by up-regulation of DAAM1 (Fig. 5F). Taken together, LINC01006 facilitated the progression of PCa by enhancing DAAM1 expression.

## Discussion

LncRNAs were discovered to exert vital functions in the progression of various cancers [16, 17]. For instance, lncRNA PCGEM1 contributed to the process of renal carcinoma by targeting miR-433-3p/FGF2 [18]. PVT1 facilitated the proliferation of NSCLC through sponging miR-526b to regulate EZH2 [19]. OIP5-AS1 boosted the process of gastric cancer via targeting miR-367-3p to enhance HMGA2 [20]. LINC01006 was described as an oncogene in pancreatic cancer by binding with miR-2682-5p to elevate HOXB8 [21]. In the current study, LINC01006 was detected to be high expressed in PCa cells. Besides, down-regulation of LINC01006 restricted the course of PCa cells. These findings manifested that LINC01006 had oncogenic feature in PCa cells.

Mounting essays have illustrated that ceRNA mechanism was the main system when it comes to the analysis of lncRNA function in diseases [22]. BCAR4 facilitated the progression of bladder cancer through sponging miR-370-3p and activating Wnt signaling pathway [23]. LINC00152 accelerated the process of hepatocellular carcinoma via targeting miR-139/PIK3CA and PI3k/Akt signaling pathway [24]. In this study, we first identified the ceRNA feature of LINC01006 by using FISH assay and nucleus cytoplasm fractionation. Then, the candidate miRNAs were detected by RT-qPCR and miR-34a-5p was selected to be low expressed in PCa cells. In the previous study, miR-34a-5p was reported to suppress migration and invasion in human epidermal keratinocytes [25]. Our study illustrated that miR-34a-5p up-regulation could hinder the course of PCa cells. The above data showed miR-34a-5p was a tumor suppressor.

A plentiful of articles highlighted that mRNAs were modulated by miRNAs [26, 27]. In this study, we discovered that DAAM1 was negatively by miR-34a-5p. Besides, DAAM1 had high expression in PCa cells. The former research displayed that overexpression of DAAM1 was related to proliferation and poor prognosis in breast cancer. We also conducted the functional assays of DAAM1 and found DAAM1 down-regulation inhibited the progression of PCa cells. The former study verified that DAAM1 contributed to migration and invasion in ovarian cancer cells, which implied DAAM1 was an oncogene [28]. Finally, rescue assays certified that DAAM1 up-regulation could counterbalanced the inhibitory effects of LINC01006 silence on the process of PCa cells.

## Conclusion

In short, all these data in our study revealed that LINC01006 promoted the progression of PCa via targeting miR-34a-5p/DAAM1. LINC01006 could be used as an underlying target for PCa treatment in the future.

## Abbreviations

Long intergenic non-protein coding RNA 1006 (LINC01006)

Dishevelled associated activator of morphogenesis 1 (DAAM1)

Long non-coding RNAs (lncRNAs)

Competing endogenous RNAs (ceRNAs)

Prostate cancer (PCa)

microRNAs (miRNAs)

messenger RNA (mRNA)

American type culture collection (ATCC)

Dulbecco's modified Eagle's medium (DMEM)

fetal bovine serum (FBS)

radioimmunoprecipitation assay (RIPA)

sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

polyvinylidene fluoride (PVDF)

RNA extraction and quantitative real-time polymerase chain reaction (RT-qPCR)

horseradish peroxidase (HRP)

Fluorescence in situ hybridization (FISH)

WT (wild-type)

Mut (mutant)

standard deviation (SD)

analysis of variance (ANOVA)

## **Declarations**

### **Ethics approval and consent to participate**

Not applicable.

### **Consent for publication**

Not applicable.

### **Availability of data and material**

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

## Funding

None

## Authors' contributions

ME designed this study. ME and WQ performed experiments. LJ and ZX conducted functional assays. GZ and YX made statistical analysis. ME reviewed manuscript. All authors approved final manuscript.

## Acknowledgements

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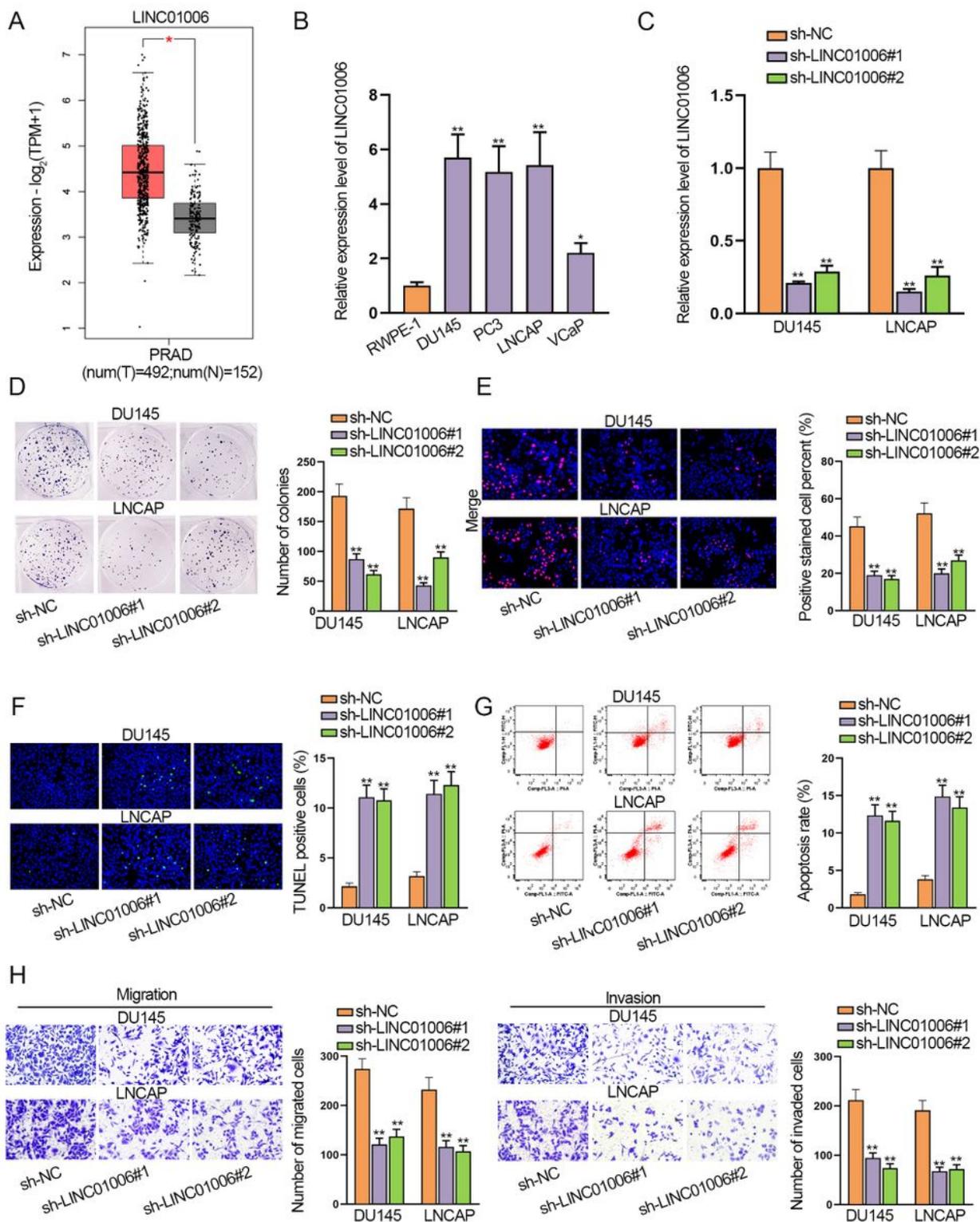
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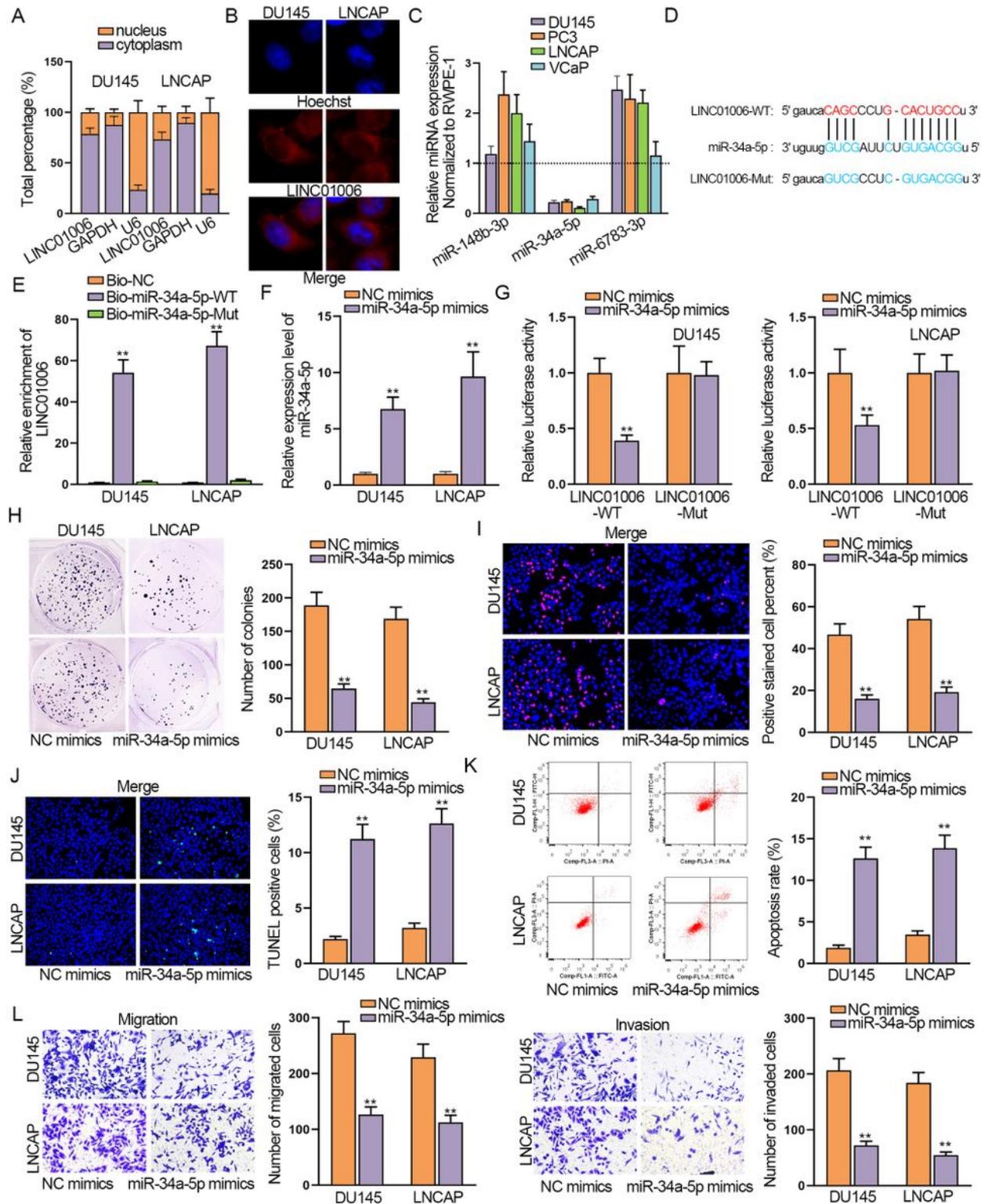
## Figures



**Figure 1**

LINC01006 had high expression and facilitated the progression of PCa cells (A) GEPIA data displayed that LINC01006 was up-regulated in PCa tissues. (B) RT-qPCR detected the expression of LINC01006 in PCa cell lines (DU145, PC3, LNCAP and VCaP) and normal human prostate epithelial cells (RWPE-1). (C) The efficiency of sh-LINC01006#1/2 was examined by RT-qPCR. (D-E) Colony formation and EdU assays were performed to appraise proliferation in DU145 and LNCAP. (F-G) Apoptosis rate was examined in TUNEL

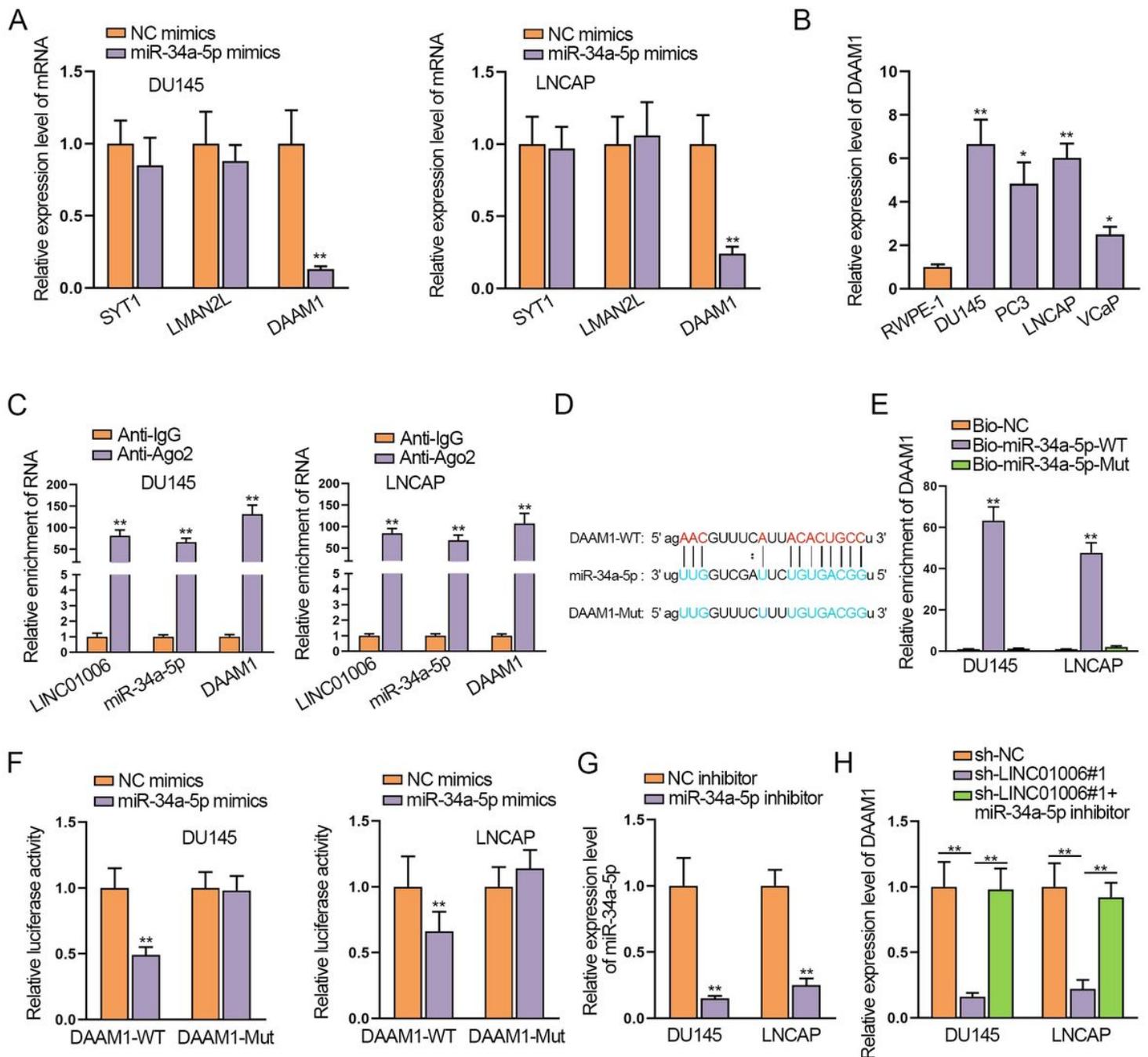
assay and flow cytometry analysis in different groups. (H) Transwell assays were built to assess migration and invasion. \*P < 0.05; \*\*P < 0.01.



**Figure 2**

MiR-34a-5p inhibited the progression of PCa (A-B) Nucleus cytoplasm fractionation and FISH assays were carried out to judge subcellular place of LINC01006. (C) RT-qPCR assays measured 3 miRNAs in PCa cell lines. (D) Binding sites between miR-34a-5p and LINC01006. (E) RNA pull down assays were

contrasted to validate binding sites. (F) RT-qPCR was used to evaluate miR-34a-5p expression in cells with miR-34a-5p mimics. (G) Luciferase reporter assays were carried out to examine binding sites. (H-I) Proliferation was assessed in colony formation and EdU assays in cells with miR-34a-5p mimics. (J-K) Apoptosis rate was appraised in TUNEL and flow cytometry analysis in cells with miR-34a-5p mimics. (L) Transwell assays evaluated migration and invasion. \*\*P < 0.01.



**Figure 3**

DAAM1 expression was negatively regulated by miR-34a-5p (A) RT-qPCR evaluated 3 mRNA expression in cells with miR-34a-5p mimics. (B) DAAM1 expression was detected in PCa cell lines. (C) RIP assay was performed to validate LINC01006, miR-34a-5p and DAAM1 coexisted in RNA induced silencing complexes

(RISCs). (D) Binding sites between miR-34a-5p and DAAM1 were predicted by bioinformatics. (E-F) RNA pull down and luciferase reporter assays were contrasted to certify binding sites. (G) RT-qPCR measured miR-34a-5p expression in cells with miR-34a-5p inhibitor. (H) DAAM1 expression was detected by RT-qPCR in cells with sh-LINC01006#1 and miR-34a-5p mimics. \*P < 0.05; \*\*P < 0.01.

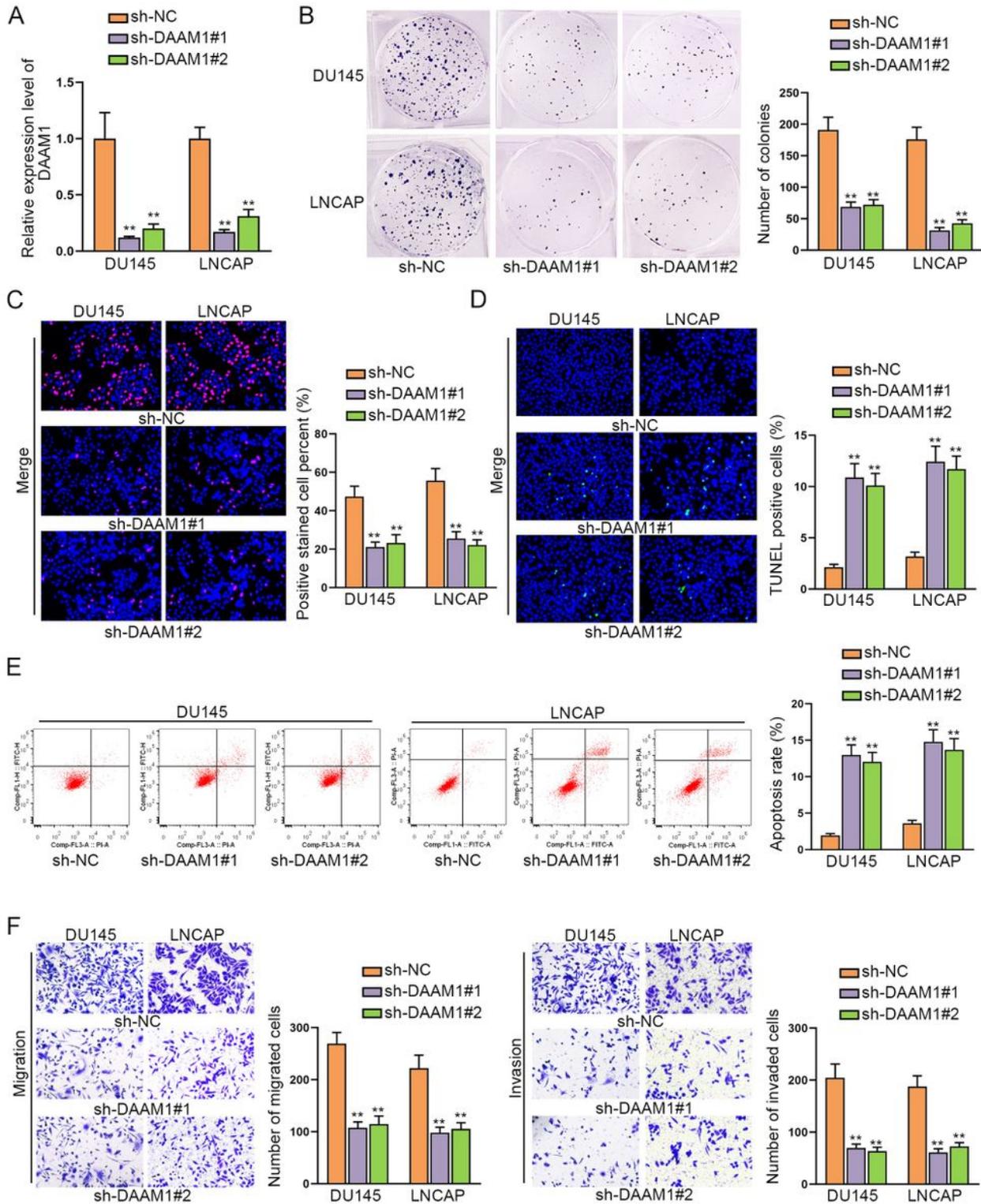


Figure 4

DAAM1 accelerated the progression of PCa (A) RT-qPCR examined sh-DAAM1#1/2 efficiency in PCa cells. (B-C) Proliferation was assessed in colony formation and EdU assays. (D-E) Apoptosis rate was appraised in TUNEL and flow cytometry analysis. (F) Migration and invasion were assessed by transwell assay. \*\*P < 0.01.

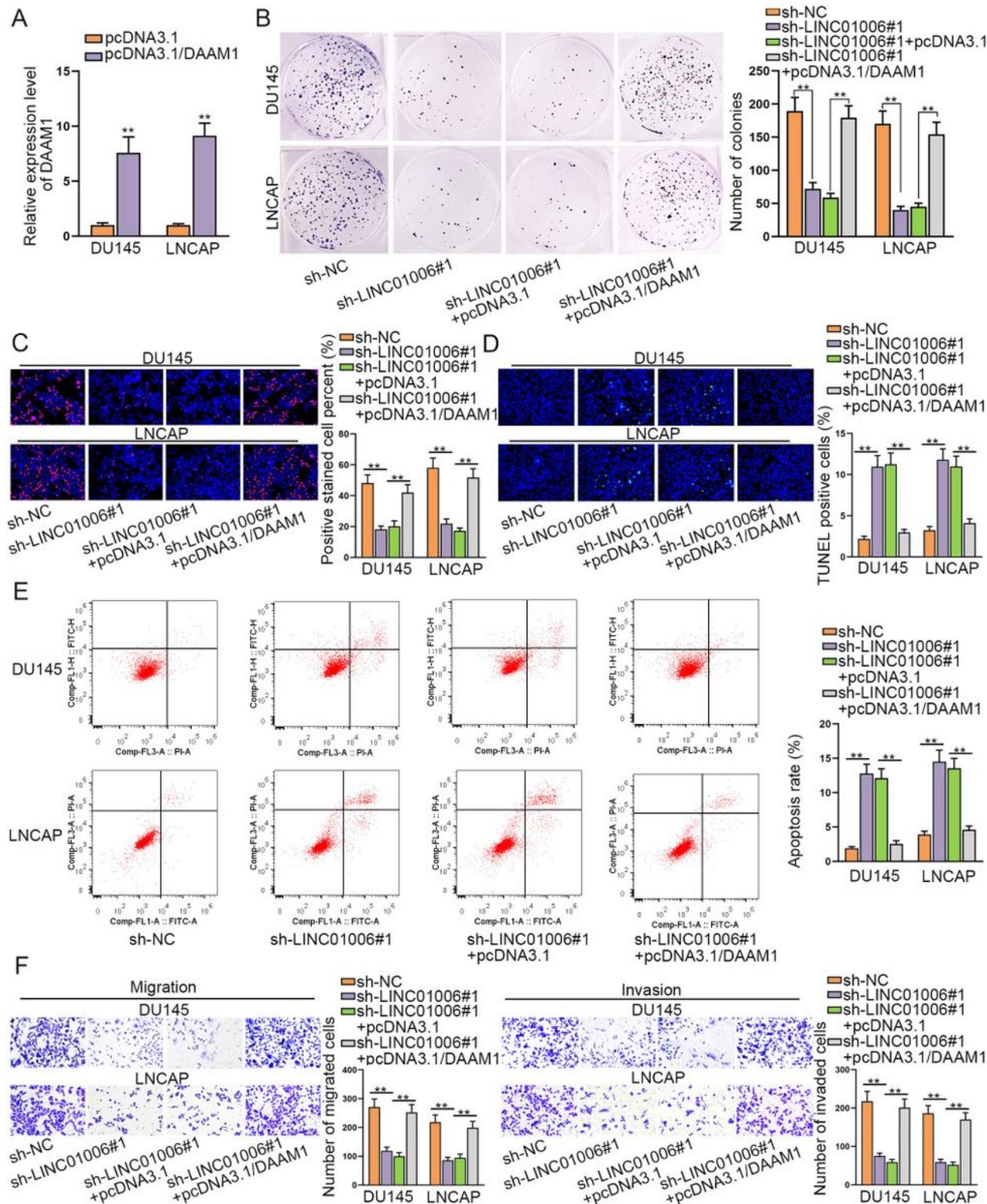


Figure 5

LINC01006 promoted the progression of PCa by sponging miR-34a-5p to increase DAAM1 (A) RT-qPCR measured DAAM1 expression in cells with pcDNA3.1/DAAM1. (B-C) Proliferation was examined in cells with sh-NC, sh-LINC01006#1, pcDNA3.1/DAAM1 among colony formation and EdU assays. (D-E) TUNEL and flow cytometry analysis were built to detect apoptosis rate in different groups. (F) Transwell assays assessed migration and invasion in cells with different groups. \*\*P < 0.01.