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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b>  MicroRNAs are non-coding small RNAs that exert their silencing functions at the posttranscriptional level; they play a fundamental role in regulation of diverse cellular pathways. In addition to microRNAs, the cell also expresses abundantly other non-coding RNAs such as long non-coding RNAs (>200 bases) (lncRNAs), which could account for 4-9% of transcripts in human genome. In this application we proposed to test whether lncRNAs are dysregulated in prostate cancer. We demonstrated that lncRNA GAS5 is a direct target for miR-21. Furthermore, GAS5 can also suppress miR-21. In addition to GAS5, we identified that PCGEM1 is upregulated in prostate cancer, and it interacted with androgen receptor (AR). More importantly, both AR and PCGEM1 were co-localized in prostate cancer cells as well as in the clinical specimens, especially in high grade prostate tumors, suggesting that their interaction may contribute to aggressiveness of prostate cancer and castration resistance. Together, our study suggests that lncRNAs are important players in prostate cancer biology, and further characterization of these lncRNAs will provide a better understanding of prostate cancer biology. As a result, lncRNAs may serve as biomarkers for prostate cancer.					
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## Introduction

Functional genomics has identified a large number of non-coding genes including microRNAs and long non-coding RNAs (lncRNAs). While microRNAs are well characterized, lncRNAs are poorly characterized. Although little is known about functions of these lncRNAs, evidence suggests that certain lncRNAs can form complementary duplex with microRNAs and has an inverse correlation with corresponding microRNAs. Therefore, the goal of this application was to determine the role of lncRNAs in prostate cancer and to determine whether microRNAs can also target lncRNAs in addition to protein-coding genes. Identification of such lncRNAs would provide a better understanding of microRNA regulation and aid in the development of prostate cancer biomarkers.

## Body

### Task 1. Determine whether lncRNAs negatively correlate with microRNA expression

Results: we found that there was a negative correlation between lncRNA GAS5 and miR-21.

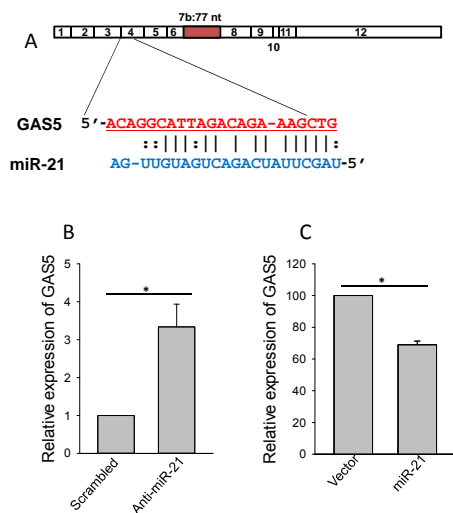
### Task 2. Determine whether alterations of lncRNA levels have any impact on microRNA expression

Results: lncRNA GAS5 can suppress miR-21.

### Task 3. Identify novel lncRNAs in prostate cancer

Results: PCGEM1 is a novel lncRNA that can interact with AR and is upregulated in prostate tumors

## LncRNA GAS5 as a miR-21 target



**Fig. 1 Identification of GAS5 as a miR-21 target.** A, a potential miR-21 binding site in GAS5. B, Anti-miR-21 increases GAS5 expression. C, Ectopic expression of miR-21 suppresses GAS5.

As master gene regulators, microRNAs often have multiple targets of protein-coding genes. However it is not clear whether microRNA can silence long non-coding RNAs (lncRNAs). In this application we chose miR-21 because it plays an important role in prostate cancer. We used a focus group of lncRNAs derived from lncRNA database ([www.lncRNADB.com](http://www.lncRNADB.com)) because there is a commercially available real-time RT-PCR based array (Human Disease Related lncRNA Profiler, #RA920D-1 from SBI) for lncRNA profiling.

Since miR-21 is often upregulated in tumor specimens as well as in cancer cell lines, we knocked down miR-21 in prostate cancer LNCaP cells by anti-miR-21 and then determined the effect of anti-miR-21 on lncRNA

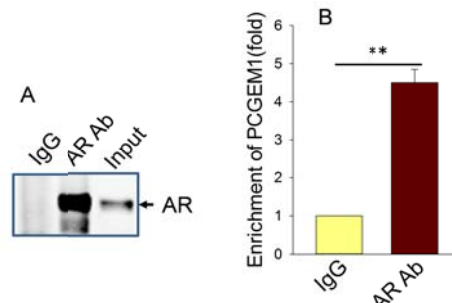
expression. Thus, total RNA extracted from these transfected cells was used for lncRNA profiling. From initial profiling, we found that several lncRNAs were induced by anti-

miR-21 and among them was GAS5. We were particularly interested in GAS5 because in silico analysis with RNA22 program identified a complementary region with miR-21 (Fig. 1A). To further confirm this finding, we designed a different set of primers. While anti-miR-21 increased GAS5 (Fig. 1B), ectopic expression of miR-21 suppressed GAS5 (Fig. 1C).

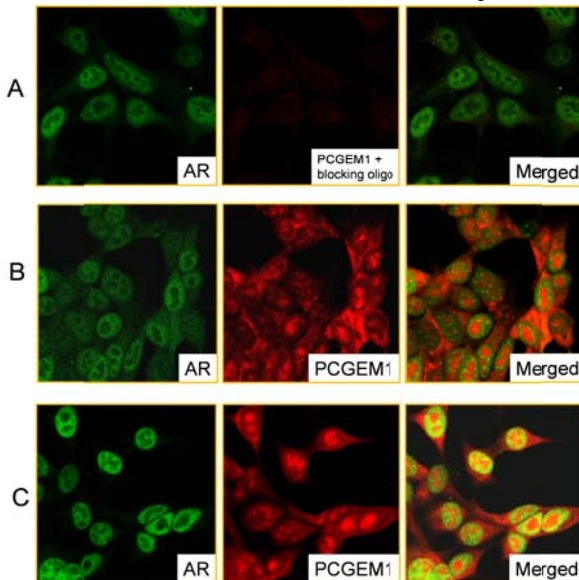
### PCGEM1 is a novel lncRNA that can interact with AR

To search for novel lncRNAs in prostate cancer, we asked what kind of lncRNAs can interact with androgen receptor (AR) because AR is a key regulator of prostate growth and the principal drug target for the treatment of prostate cancer. As a nuclear receptor transcription factor, AR controls expression of genes important to prostate cancer cell growth and progression, and the development of castration-resistant prostate cancer (CRPC). Therefore, we performed RNA immunoprecipitation (RIP) with AR antibody for a focused set of lncRNAs. We used a commercially available real-time

RT-PCR based array (Human Disease-Related lncRNA Profiler from System Biosciences) for this purpose. Although this array carries only 83 lncRNAs, they are curated based on literature evidence ([www.lncRNAdb.com](http://www.lncRNAdb.com)) and many of them are associated with diseases or with known functions. Basically, cellular extract was prepared from LNCaP cells and then was subject to RIP assay with AR antibody. The precipitated RNA was then used for lncRNA



**Fig. 2 Association of AR with PCGEM1, as detected by RNA immunoprecipitation with AR antibody (A) and qRT-PCR (B).** LNCaP extract was used for this experiments. n = 3; \*\*, p < 0.01.



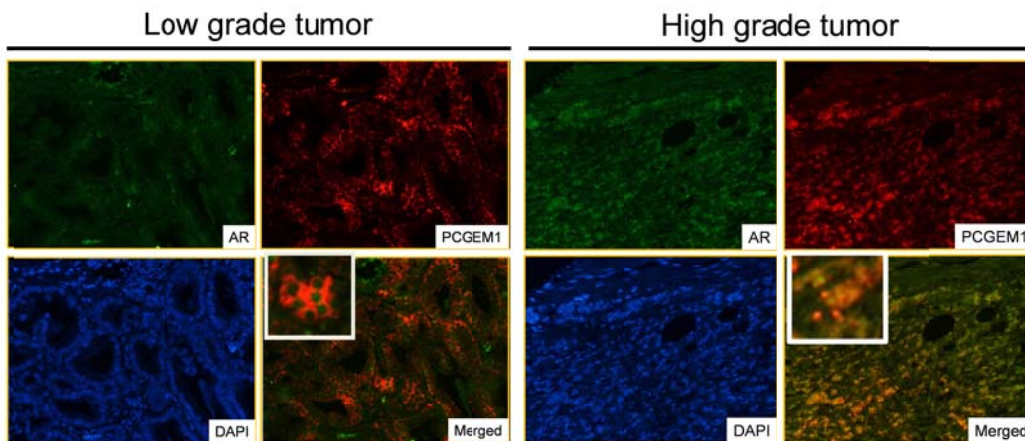
**Fig. 3 Co-localization of AR and PCGEM1 in LNCaP cells.** AR was detected by immunofluorescence (IF) staining with AR antibody; PCGEM1 by fluorescence in situ hybridization (FISH). A, Cells were grown in androgen free medium for 2 days and then IF and FISH were performed in presence of blocking oligo. B, Same as in A, but in the absence of blocking oligo. C, The same cells as in B were then cultured with 2 nM R1881 for overnight.

### Colocalization of PCGEM1 with AR in prostate cancer cells

To further examine their interaction, we carried out immunofluorescence (IF) staining and fluorescence in situ hybridization (FISH) to detect their co-localization in LNCaP cells. Thus, we simultaneously performed IF with AR antibody and FISH with a biotin labeled PCGEM1 probe which was derived from the

antisense of PCGEM1 sequence. In addition, this probe was modified with locked nucleic acid (LNA), which was purchased from Exiqon, to increase the stability of probe/target complex. FISH signal was revealed by detection kit with Alexa Fluor 568 (Invitrogen). To determine the specificity of PCGEM1 signal, we introduced 10 x amount of blocking oligo which was complementary to the probe in the hybridization buffer. Thus the blocking oligo served as a competitor to PCGEM1 probe. As shown in Fig. 3A, little PCGEM1 signal was detected in the blocking oligo treated cells. Of interest, while AR was predominantly in the nucleus, PCGEM1 was mainly localized to cytoplasm or nucleoli if it was in the nucleus in the absence of androgen (Fig. 4B). There was a relatively small amount of co-localization of PCGEM1 and AR, especially in “nuclear speckle-like structures” (Fig. 3B). However, in the presence of androgen, more AR moved into the nucleus and the nuclear AR signal became stronger; at the same time, more PCGEM1 was also detected in the nucleus (Fig. 3C). More interestingly, the co-localization signal became much stronger, especially in the nucleoplasm (Fig. 3C), suggesting that androgen may promote both nuclear localization of AR and PCGEM1, and their interaction. This possibility will be further tested by RIP assay with AR antibody.

Next, we examined the expression of AR and PCGEM1, and their subcellular localization in clinical specimens. Although FISH for paraffin-embedded specimens is challenging because they often carry a high background of autofluorescence, we were able to manage to reduce the background and obtain specific signals for AR and PCGEM1 (Fig. 4). For example, in the low grade prostate tumor, the distribution patterns of AR and PCGEM1 were similar to what we observed in LNCaP cells in the absence of androgen. While AR was seen mainly in the nucleus, PCGEM1 was predominantly in the cytoplasm. There as little co-localization. However, for high grade prostate tumors, we observed a relatively strong co-localization signal of PCGEM1 and AR. Since the epitope

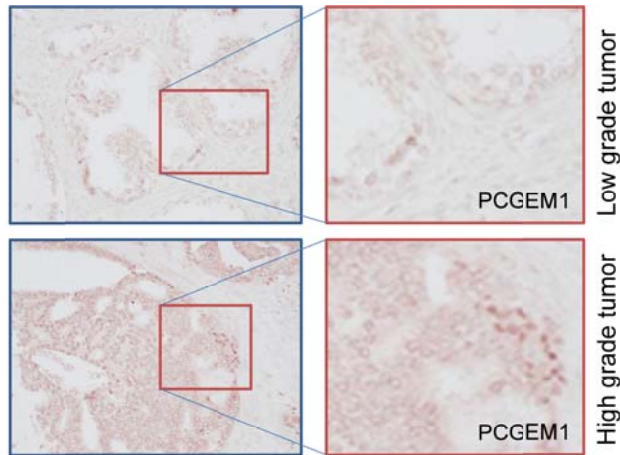


**Fig. 4 Co-localization of AR and PCGEM1 in high grade prostate tumors.** Inserts in the merged pictures were taken from the same field and enlarged to show AR and PCGEM 1 distribution.

of the AR antibody used here was derived from the N-terminus (Cell Signaling, Cat#5153), it is expected to also recognize AR variants lacking ligand binding domain

(LBD) such as AR3-V7, in addition to full length AR. Therefore, it would be of interest to determine whether this increase in co-localization is due to upregulation of alternative AR splicing variants which have been shown to be associated with aggressiveness of prostate cancer and castration resistance. These results suggest that co-localization of AR and PCGEM1 could be important to the AR-regulated gene expression and may have a clinical significance.

### **LncRNA PCGEM1 is upregulated in prostate cancer**



**Fig. 5 Upregulation of PCGEM1 in prostate tumors as detected by in situ hybridization.**

Furthermore, we used a regular in situ hybridization method to detect PCGEM1 expression in prostate tumors because this method would allow us to examine their expression in archived paraffin-embedded specimens. As shown in Fig. 5, PCGEM1 was expressed in both low grade and high grade prostate tumors, however, the level of PCGEM1 was much higher in high grade prostate tumors than in low grade tumors, suggesting that PCGEM1 may play an oncogenic role in prostate cancer.

### **Key research accomplishments**

- By lncRNA profiling, we identify that GAS5 is negatively regulated by miR-21.
- GAS5 can also negatively regulate miR-21, forming a feedback loop
- LncRNA PCGEM1 is upregulated in prostate tumors, especially those high grade tumors
- PCGEM1 interacts with AR; this interaction may contribute to aggressiveness of prostate cancer and castration resistance

### **Reportable Outcomes**

A manuscript regarding PCGEM1 is in preparation.

### **Conclusions**

We have identified lncRNA GAS5 as a miR-21 target. GAS5 carries a putative miR-21 binding site. Thus the results suggest that miR-21 not only target protein-coding gene, but also lncRNAs such as GAS5. Moreover, we demonstrate that PCGEM1 is a novel

lncRNA that is upregulated in prostate cancer, and its expression is correlated with aggressiveness of prostate cancer. Of interest, PCGEM1 can interact with AR and activate AR-regulated gene expression. In clinical specimens, PCGEM1 is co-localized with AR in high grade tumors, suggesting that this interaction may play a role in aggressiveness of prostate cancer and castration resistance. Future work will be to determine whether PCGEM1 can serve as a biomarker to distinguish aggressive prostate cancer from indolent diseases.

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