

# Identification and Diagnostic Performance of a Small RNA within the PCA3 and BMCC1 Gene Locus That Potentially Targets mRNA

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## Abstract

**Background:** PCA3 is a long noncoding RNA (lncRNA) with unknown function, upregulated in prostate cancer. lncRNAs may be processed into smaller active species. We hypothesized this for PCA3.

**Methods:** We computed feasible RNA hairpins within the BMCC1 gene (encompassing PCA3) and searched a prostate transcriptome for these. We measured expression using qRT-PCR in three cohorts of prostate cancer tissues ( $n = 60$ ), exfoliated urinary cells ( $n = 484$  with cancer and  $n = 166$  controls), and in cell lines ( $n = 22$ ). We used *in silico* predictions and RNA knockup to identify potential mRNA targets of short transcribed RNAs.

**Results:** We predicted 13 hairpins, of which PCA3-shRNA2 was most abundant within the prostate transcriptome. PCA3-shRNA2 is located within intron 1 of PCA3 and appears regulated by androgens. Expression of PCA3-shRNA2 was upregulated in

malignant prostatic tissues, exfoliated urinary cells from men with prostate cancer (13–273 fold change;  $t$  test  $P < 0.003$ ), and closely correlated to PCA3 expression ( $r = 0.84$ – $0.93$ ;  $P < 0.001$ ). Urinary PCA3-shRNA2 (C-index, 0.75–0.81) and PCA3 (C-index, 0.78) could predict the presence of cancer in most men. PCA3-shRNA2 knockup altered the expression of predicted target mRNAs, including COPS2, SOX11, WDR48, TEAD1, and Noggin. PCA3-shRNA2 expression was negatively correlated with COPS2 in patient samples ( $r = -0.32$ ;  $P < 0.001$ ).

**Conclusion:** We identified a short RNA within PCA3, whose expression is correlated to PCA3, which may target mRNAs implicated in prostate biology.

**Impact:** This short RNA is stable *ex vivo*, suggesting a role as a robust biomarker. We identify cytoplasmic enrichment of this RNA and potential targeting of mRNAs implicated in prostate carcinogenesis. *Cancer Epidemiol Biomarkers Prev*; 24(1); 268–75. ©2014 AACR.

## Introduction

Prostate cancer is the most common cancer in men (1). The diagnosis of prostate cancer is typically based on a combination of digital rectal examination (DRE), serum PSA, and prostate biopsy (2). This approach leads to over diagnosis of indolent cancer and can delay the detection of significant disease. Non-

protein biomarkers have been identified to help this diagnostic approach. To date, Prostate Cancer Associated 3 (PCA3) appears the most promising, and received FDA approval in 2012 (3). The diagnostic assay (PROGENSA) uses quantitative RT-PCR to measure the expression of the PCA3 and PSA mRNA in postprostatic massage urine samples (4). The PCA3 score (PCA3/PSA mRNA ratio  $\times 1,000$ ) is higher in men with prostate cancer than in controls and may be used to guide the need for further prostate biopsy (5, 6).

PCA3 is a long noncoding RNA (lncRNA) that is overexpressed in most prostate cancer specimens. The PCA3 gene is located on chromosome 9q21-22 in an antisense orientation within intron 6 of the BMCC1/PRUNE2 gene (7, 8). PCA3 expression is predominantly restricted to the prostate, under androgen regulation and the gene may produce multiple transcripts through splicing and alternate polyadenylation (8). The adoption of PCA3 assay into clinical practice has been hampered by several factors. First, the assay detects a lncRNA (gene length, 25 kb; PCR target sequence is 380 bases) that is unstable *ex vivo*. Target transcripts require protection from RNases before analysis, making the test expensive and vulnerable to transportation errors. Second, the function of PCA3 is unknown, producing a biologic gap in knowledge. Finally, the test has a poor sensitivity for prostate cancer and is mainly used in conjunction with serum PSA in men with a previous negative biopsy (5).

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**Note:** Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (<http://cebp.aacrjournals.org/>).

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The importance of lncRNAs in human health and disease is becoming clearer (9, 10). The GENCODE consortium recently annotated 9,277 lncRNA genes, corresponding to 14,880 transcripts (11). In contrast with protein coding genes, lncRNAs typically have few gene exons, can be processed into active short species (12–14), and are not conserved from primitive species (15). As no functional role for PCA3 has been assigned to date, we wondered whether this ncRNA might encode an active shorter species. To test this hypothesis, we searched for possible short ncRNAs derived from sequence within the boundaries of the BMCC1 primary transcript, which spans PCA3, and evaluated their translational role. Here, we report the outcomes of this work.

## Materials and Methods

### Identification of hairpin RNA structures

To identify short potentially transcribed ncRNAs, we searched the BMCC1 gene locus for predicted and energetically feasible RNA hairpins using ProMir II (16) and MiPred (17). These programs compare random sequences within the target hairpin using structure–sequence composition and minimum of free energy of the secondary structure. We then searched a small RNA transcriptome generated from malignant prostatic epithelium using deep sequencing (18) for RNA sequences derived from these predicted hairpins. We measured the expression of any identified short RNA sequences using custom stem loop primers (TaqMan small RNA assays; Applied Biosystems) with real-time qPCR (as described in ref. 19) in cell lines and human tissues samples.

### Cell lines and androgen regulation of RNA

We examined a panel of cell lines representing prostate cancer (DU145, LNCap, LNCap-LN3, LNCaP-pro5, PC2, PC3M, and PC3M-In4) and other common human malignancies [A549 and NCI-H460 (lung), AN3CA (endometrial), EJ/T24, RT112 and RT4 (bladder), HCT-116 (colorectal), HEK293 (human embryonic kidney), HeLa (Vulval), Jurkat (T-Cell lymphoma), MCF-7 and T47D (breast), MRC5 (lung fibroblasts), SKOV-3 (ovarian), and WM793 (melanoma)]. Cell lines were purchased from ATCC, validated by STR profiling, and grown in appropriate media according to standard methods (20). To determine androgen regulation of candidate RNAs, we examined their expression in LNCap cells (chosen for their androgen dependency) growing in androgen-depleted media (phenol red free RPMI-1640 and 10% charcoal stripped serum; Sigma) with no (0 nmol/L), 1 nmol/L, and 10 nmol/L added testosterone (7).

### Expression in prostatic RNA

We measured the expression of candidate short RNAs in prostatic tissues and urinary samples enriched for prostatic cells and secretions. Frozen malignant and histologically benign prostatic tissues were obtained using laser-capture microdissection of fresh radical prostatectomy specimens from the University of Erlangen, Germany. Each sample was marked by a dedicated urothologist (A. Hartmann) and tissue extracted from  $10 \times 10 \mu\text{mol/L}$  sections (>80% tumor purity). RNA from exfoliated prostatic urinary cells and secretions was collected following vigorous prostatic massage in men with prostate cancer and matching controls within separate pilot and validation cohorts from the University of Sheffield, United Kingdom. Controls were matched for age and PSA, and selected if they had undergone two or more prostatic biopsies without finding cancer. Following massage, the first 10 to 20 mL

of urine was collected and centrifuged. The cell pellet was then washed twice in PBS before storage. All samples were frozen at  $-80^\circ\text{C}$  until use. The ethics committee's approval was in place before commencement of this study.

### RNA extraction and quantification

Total RNA was extracted using the mirVana Extraction Kit (Ambion) and measured using a 2100 Bioanalyzer [Agilent; as described elsewhere (19)]. RNA expression was determined using real-time quantitative RT-PCR with primers for PCA3, BMCC1, PSA (4, 7), and two custom stem loop hairpin primers for PCA3-shRNA2 [termed "a" and "b": target sequences (ACTGCACTCCA-GCCTGGGCA) and (CACTGCACTCCAGCCTGGGCA) Ambion: assay IDs, SCSGJ090 and CSHSNF8, respectively] using qRT-PCR (21). Expression of PCA3, BMCC1, and PCA3-shRNA2 was normalized to PSA and fold change calculated using  $\Delta C_t$  values (21). For RNA localization studies, we extracted separate nuclear and cytoplasmic RNA fractions from cells using standardized methodology (methods detailed in ref. 22).

### Cloning primary transcript using 3'RACE

To determine the sequence (and genomic origin) of the primary RNA transcript producing our short RNA, we performed 3' rapid amplification of cDNA ends (RACE) in PC3 cells using the GeneRacer Kit according to the manufacturer's guidelines (Life technologies). Briefly, total RNA was precipitated, cleaned, polyadenylated, adaptor ligated, and amplified with hot-start PCR using primers to the shRNA and the adaptor. The target sequences were cloned into *E. coli* (Top10; Life Technologies), followed by extraction, purification, and Sanger sequencing. Sequences were aligned (Sequencher 5.1, Gene Codes), and genomic matches were identified using BLAST (NCBI).

### mRNA target analysis and RNA knockup

We identified putative target mRNAs with complementary sequences to our candidate short RNA using TargetScan (v4.2; www.targetscan.org). We determined prostate cancer-specific expression of these mRNAs using publicly available gene expression data (Arrayexpress ID: E-GEOD-8218; ref. 23). We analyzed cellular functions and pathway enrichment for these mRNAs with DAVID Bioinformatics Resource of identified mRNAs (24). We focused upon those with carcinogenic or prostate-specific biologic functions.

For exploratory analysis of targeting, we examined the expression of selected mRNA targets in LNCaP cells following PCA3-shRNA2 knockup. Briefly, we transfected cells with a custom-made hairpin precursor designed to generate PCA3-shRNA2, or with an equal amount of control (scrambled) RNA (both from Ambion) using Lipofectamine RNAiMAX (Life Technologies; methods detailed in ref. 21). We determined the success of transfection using qRT-PCR (as described above). All assays were performed in triplicate. We measured the expression of potential targets using qRT-PCR (primer sequences and reaction conditions given in Supplementary Table S1) in these cell lines and in the exfoliated prostatic urinary cells from cohort 2.

### Statistical analysis

RNA expression was compared between cells and tissues using the Student *t* test or ANOVA, and correlated with other RNAs using Pearson coefficient within SPSS v14.0 (SPSS Inc.). Graphs were plotted using PRISM 6.0 (GraphPad Software Inc.). The ability of



(Fig. 1B). We designed Taqman assays to each (given that the 5' end of short RNA is vital for mRNA targeting) and named these assays PCA3-shRNA2a and PCA3-shRNA2b, respectively. We measured their expression in the 22 cell lines. We detected expression of PCA3-shRNA2 in all 7 prostate and 15 other cancer cell lines (Fig. 1C; Supplementary Fig. S2). PCA3 and PCA3-shRNA2 expression did not vary significantly with organ of origin for these cells. We normalized RNA expression to PSA mRNA, as we were keen to compare with the commercial PCA3 assay (which uses this reference gene). Expression of the target RNA was similar whether using normalized PCA3-shRNA2a or PCA3-shRNA2b primers (data not shown;  $r = 0.98$ ;  $P < 0.001$ ). We identified a correlation between the expression of PCA3 mRNA and PCA3-shRNA2 (PCA3-shRNA2a assay:  $r = 0.92$ ;  $P < 0.001$  and PCA3-shRNA2b assay:  $r = 0.93$ ;  $P < 0.001$ ), which was closer than for BMCC1 ( $r = 0.67$  for PCA3-shRNA2a and  $r = 0.72$  for PCA3-shRNA2b,  $P < 0.001$ ).

These data support our transcriptomic analysis, but do not prove that our short RNA is derived from sequence within the PCA3 intron. To analyze this, we used 3' RACE to clone the primary transcript from the PCA3-shRNA2a primer. Sequenced RACE products from selected colonies aligned to the PCA3 intronic locus and supported our *in silico* prediction of a 98-bp hairpin (red box, Fig. 1D). A BLAST search of this 98-bp sequence revealed strong (97%) homology for only one locus in the genome, i.e., that within PCA3 intron 1 (Supplementary Fig. S1B).

It is known that many RNAs important in prostate carcinogenesis are regulated by the androgen receptor. In LNCap cells (chosen

for their androgen dependency), both PCA3 [ $2.1 \pm 0.31$  fold change (mean  $\pm$  SD)] and PCA3-shRNA2 [ $2.75 \pm 0.23$  fold change (mean  $\pm$  SD)] were upregulated in a dose-dependent manner (Supplementary Fig. S3) by testosterone. The changes were less than seen for PSA [ $219.0 \pm 25.2$  fold upregulation (mean  $\pm$  SD)].

#### Expression of PCA3-shRNA2 in prostate tissue

Having identified the existence and origin of this short RNA, we investigated its expression in malignant and benign prostatic tissues from 60 radical prostatectomy specimens (Table 1, Fig. 1E). We identified expression of PCA3 and PCA3-shRNA2 were correlated ( $r = 0.88$ ;  $P < 0.001$ ), and there was upregulation of each RNA in malignant tissues when compared with benign samples. The extent of this difference was largest for PSA [ $8.6 \pm 1.2$  fold change (mean  $\pm$  SD)] and least for PCA3-shRNA2 ( $1.4 \pm 1$ ; data not shown). When normalized to PSA, we found significant differences in expression for PCA3-shRNA2, PCA3, and BMCC1 between malignant and benign tissues ( $t$  test  $P < 0.01$ , and Supplementary Fig. S4). Once again, there was close correlation in the detection of PCA3-shRNA2 using PCA3-shRNA2a or PCA3-shRNA2b primers ( $r = 0.99$ ;  $P < 0.001$ ). There was less correlation between PCA3-shRNA2 and BMCC1 expression ( $r = 0.51$ ;  $P < 0.001$ ).

#### Analysis of PCA3-shRNA2 in urinary samples

The clinical utility for PCA3 is a test for prostate cancer using exfoliated prostatic urinary cells. To explore this role for PCA3-shRNA2, we examined expression in 179 post-DRE urinary

**Table 1.** Patient samples analyzed in this report

Material	Radical prostatectomy specimens	Urinary pilot cohort	Urinary validation cohort
	Tissue	Disaggregated urinary cells	
Total	60	179	471
Benign tissue/controls			
Total	29	50	116
Age			
Median	67.0	68.3	66.2
SD	5.5	8.9	7.2
PSA			
Median	7.1	7.8	6.2
SD	4.9	5.9	6.2
Prostate cancer			
Total	31	129	355
Stage			
pT1-2	7	34	279
pT3	24	30	61
Metastatic	0	49	15
Missing	0	7	0
Gleason sum			
5	0	3	0
6	11	54	191
7	6	29	125
8-10	14	21	31
Missing	0	14	8
Age, years			
Median	67		65.1
SD	5.5		7.0
PSA			
Median	7.1	7.5	7.4
SD	4.9	175.1	418.9
0-10	17	79	248
11-20	12	27	64
>20	2	16	37
Missing	0	0	9



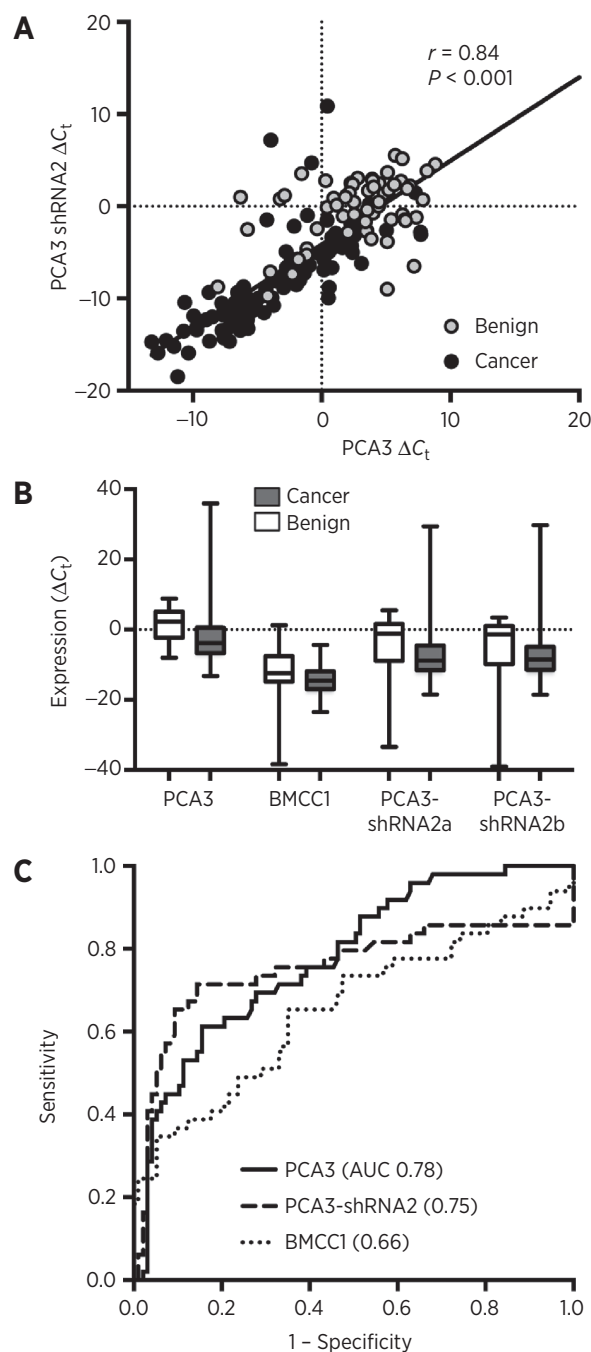
samples (Table 1) from men with ( $n = 129$ ) and without prostate cancer ( $n = 50$ ). Once again, we identified close correlation between PCA3 and PCA3-shRNA2 expression (Fig. 2A;  $r = 0.84$ ;  $P < 0.001$ ), between the two PCA3-shRNA2 assays (i.e., PCA3-shRNA2a vs. PCA3-shRNA2b;  $r = 0.95$ ;  $P < 0.001$ ), and less close correlation with BMCC1 expression ( $r = 0.30$ ;  $P < 0.001$ ). Overall, there was upregulation of PCA3 [ $86.2 \pm 53.1$  fold change (mean  $\pm$  SD)], BMCC1 ( $2.7 \pm 0.1$ ) and PCA3-shRNA2 ( $273 \pm 0.1$ ) in specimens from men with cancer, when compared with controls (all  $t$  test  $P < 0.003$ , Fig. 2B). This allowed the identification of malignancy in most men, [Fig. 2C: concordance indices suggest that PCA3 (C-index 0.78) and PCA3-shRNA2 (C-index 0.75) had similar accuracy for cancer, and were superior to BMCC1 (C-index 0.66)]. Scatterplots comparing PCA3 with PCA3-shRNA2 expression (Figs. 1E and 2A) suggested less variation in malignant samples than benign samples. Thus, we plotted RNA expression in the frozen tissues and urinary samples according to cancer presence (Supplementary Fig. S5) and saw less variation for malignant ( $r = 0.90$ ;  $P < 0.001$ ) than for benign ( $r = 0.58$ ;  $P < 0.001$ ) samples.

To explore the robustness of these findings, we examined a separate larger validation cohort of 471 urinary samples (Fig. 3). Samples were collected and processed in a similar manner to the pilot cohort. Quantitative analysis revealed that PCA3-shRNA2 expression was higher in samples from men with prostate cancer than controls [ $13.0 \pm 2.8$  fold upregulation (mean  $\pm$  SD) in malignant samples;  $t$  test  $P < 0.001$ ; Supplementary Fig. S6]. Expression of PCA3-shRNA2 did not vary with tumor stage (Fig. 3B), as reported for PCA3 (4), but could correctly identify prostate cancer (C-index 0.81, Fig. 3C), supporting our pilot exploration outcomes.

#### The functional role of PCA3-shRNA2

To date, little is known about the function of PCA3. To explore a functional role for PCA3-shRNA2, we investigated its cellular localization. qRT-PCR of total and nuclear fractions revealed a cytoplasmic enrichment (nuclear:cytoplasmic ratio = 0.6) for PCA3-shRNA2, close to that seen for established microRNAs (Supplementary Fig. S7), and very different from PCA3 (with its mostly nuclear localization). As this suggests a potential mRNA-targeting capacity, we searched the genome for complementary sequences. Using TargetScan, we identified 178 mRNAs with complementary seed sequences (Supplementary Table S3). Gene enrichment analysis revealed significant associations (Bonferroni adjusted  $P < 0.05$ ) with pathways important for cell regulation (such as cell adhesion and growth, cell signaling) and prostate biology (such as response to steroids, TGF $\beta$  signaling, and urogenital development). We annotated these mRNAs with their expression in human prostate cancer samples (23), and preferentially selected those known to be downregulated in cancer (reflecting our hypothesized targeting by upregulated PCA3-shRNA2: defined as fold change  $< 1.0$  and  $t$  test  $P < 0.05$ ) or implicated in prostate cancer biology, and having high predicted binding affinity (e.g., 8-mer seed). The resultant panel (Table 2) included interesting potential targets; such as ETS variant genes 1 and 5 (ETV1 and ETV5), mitogen-activated protein kinase kinase 1 (MAPK31), noggin, N-cadherin, and TEA domain family member 1 (SV40 transcriptional enhancer factor).

We transfected DU145 cells (chosen as they have low endogenous PCA3-shRNA2 expression) with the PCA3-shRNA2 plasmid and a scrambled RNA sequence, and measured RNA

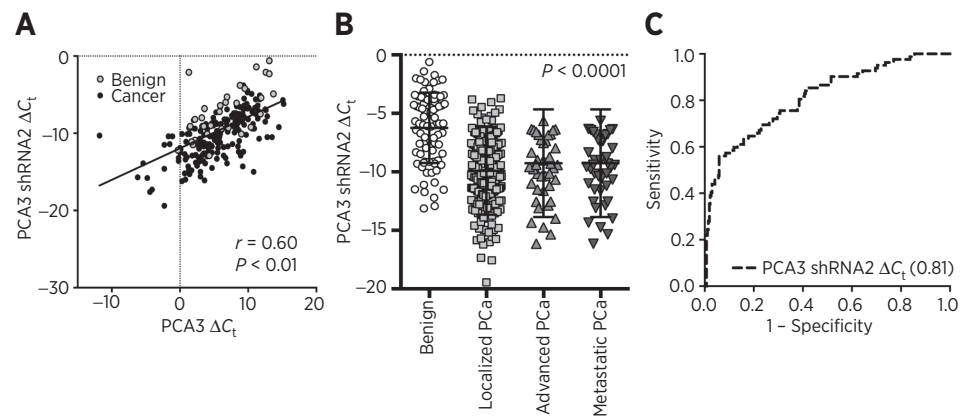


**Figure 2.** Expression of PCA3, BMCC1, and PCA3-shRNA2 in the urinary RNA from men with and without prostate cancer. A, expression of PCA3-shRNA was closely correlated with PCA3 expression ( $r = 0.84$ ). B, expression was higher in urinary pellets from men with prostate cancer than in benign controls for each RNA. C, expression of each RNA could identify the presence of the disease in most men. In comparison, PC3 and PCA3-shRNA2 expression were more reliable (C-indices 0.78, 0.75, respectively) than BMCC1 (C-index 0.66).

expression of these 12 predicted targets (Supplementary Fig. S8A). We identified reciprocal knockdown of COPS2 (COP9 signalosome subunit 2), SOX11 (sex determining region Y - box 11), WDR48, TEAD1, and Noggin, suggestive of targeting

**Figure 3.**

Detection of prostate cancer using PCA3-shRNA2. A, expression of PCA3-shRNA was correlated with PCA3 expression. B, expression was higher in urinary pellets from men with prostate cancer (PCa) than in benign controls, but did not vary with cancer stage (ANOVA  $P = 0.46$  between stages). C, expression could identify the presence of the disease in most men (C-index 0.81).



(Table 2). We measured the mRNA expression of the two strongest candidates (COPS2 and SOX11) in the largest urinary sample cohort to look for biologic associations *in vivo*. We identified reduced expression of COPS2 in the prostate cancer samples (fold change,  $0.29 \pm 0.5$ ;  $t$  test  $P < 0.001$ ) when compared with controls, and a significant inverse correlation between the expression of COPS2 and PCA3-shRNA2 ( $r = -0.32$ ;  $P < 0.001$ ; Supplementary Fig. S8B and Supplementary Fig. S8C). Nonsignificant lower expression for SOX11 was also seen in malignant samples (fold change,  $0.74 \pm 1.5$ ;  $P = 0.08$ ) when compared with controls and this mRNA was not significantly correlated with PCA3-shRNA2 expression ( $r = -0.1$ ;  $P = 0.48$ ).

## Discussion

It is known that many transcribed RNAs do not encode proteins. These are termed ncRNAs and are currently best classified according to size and cellular location. Although a fraction of short RNAs, known as microRNAs (around 20–22 bp in size), has been extensively studied (14), little is known about the function of most long ncRNAs (reviewed in refs. 9, 10). Identified roles for longer ncRNAs include direct involvement in chromatin remodeling and androgen receptor regulation (26), and processing into shorter more-active ncRNAs. For example, many microRNAs are clustered together and derived from single primary transcripts (such as miRs-24-2/27a/23a; ref. 27). Recently, Rogler and colleagues reported that RNase MRP (a 268 bp noncoding RNA component of mitochondrial RNA processing endoribonuclease) was the source for two short (around 20 bp) RNAs important in the biology of cartilage-hair hypoplasia (13). As such, we hypothesized that one role for PCA3 could be as a source for short biologically active species.

Here, we present a combination of *in silico* and *in vitro* data suggesting that a short RNA hairpin is produced during processing of the PCA3 primary transcript, and that this may have a biologic activity. This short RNA is located within intron 1 of PCA3, close to a sequence of species conservation, suggesting biologic protection. Our findings have direct and indirect clinical implications. First, expression of the short ncRNA appeared closely correlated with that for PCA3. This was expected, given our data suggesting that the short RNA is derived from the PCA3 transcript. In contrast with long mRNAs, short ncRNAs are stable molecules and do not decay with repeated freeze thawing or prolonged storage at room temperature without RNase inhibition. For example, we recently

reported that short RNAs do not dramatically degrade with prolonged storage at room temperature (in plain clean universal containers without RNase inhibitors) and with freeze thawing (28). As such, PCA3-shRNA2 may be a more stable biomarker for prostate cancer than PCA3. Assays to detect PCA3-shRNA2 would not be so vulnerable to delays in handling or variations in stringency in collection, and so should be more reproducible. In post-DRE urinary cell pellets from two large patient cohorts, we found that PCA3-shRNA2 detected cancer with a similar accuracy to PCA3. Although the PCA3 test is currently normalized to PSA mRNA expression, it is likely that short RNAs (such as prostate-specific microRNAs) could replace the need for this mRNA.

Second, our data suggest a potential functional role for PCA3 and derivative short RNAs. An unbiased genome-wide computational search identified genes and pathways implicated in the biology of prostate cancer. Although many annotated genes in these pathway enrichment datasets are implicated in cell homeostasis and regulation, relatively few are annotated for steroidal regulated pathways, TGF $\beta$  signaling and urogenital development. As such, the identification of genes involved in these pathways is extremely pertinent and adds support for a role of PCA3-shRNA2 in prostate biology. Our preliminary targeting analysis identified changes in COPS2, SOX11, WDR48, TEAD1, and Noggin with PCA3-shRNA2 upregulation. These mRNAs play roles in the regulation of gene transcription, urogenital tract development, and in cell growth and signaling. As such, they appear ideal carcinogenic gene candidates. In a further analysis, we explored the expression of COPS2 and SOX11 in exfoliated urinary cell pellets. We found that COPS2 expression was correlated to PCA3-shRNA2, suggesting biologic validation, and that a trend for SOX11 was also seen. COPS2 is a transcription corepressor that underwent a 4-fold loss of expression in cells with PCA3-shRNA2 knockup. COPS2 is a component of the COP9 signalosome complex that acts to regulate the ubiquitin conjugation pathway during various cellular and developmental processes, including phosphorylation of p53 and c-jun. COPS2 is abundantly expressed in most human tissues, suggesting an important role in cellular homeostasis, and has not been studied in depth with respect to human malignancies. SOX11 is a transcription factor belonging to the SRY-related HMG-box (SOX) family. These regulate multiple biologic processes, such as hematopoiesis, vasculogenesis, and cardiogenesis during embryonic development (29), and some members are negative regulators of the WNT-beta-catenin-TCF pathway (30) which is implicated in



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# BLOOD CANCER DISCOVERY

## Identification and Diagnostic Performance of a Small RNA within the PCA3 and BMCC1 Gene Locus That Potentially Targets mRNA

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