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TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Association of High miR-182 Levels with Low-Risk Prostate Cancer

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A subset of men with prostate cancer develops aggressive disease. We sought to determine whether miR-182, an miRNA with reported oncogenic functions in the prostate, is associated with biochemical recurrence and aggressive disease. Prostate epithelial miR-182 expression was quantified via *in situ* hybridization of two prostate tissue microarrays and by laser-capture microdissection of prostate epithelium. miR-182 was significantly higher in cancer epithelium than adjacent benign epithelium ($P < 0.0001$). The ratio of cancer to benign miR-182 expression per patient was inversely associated with recurrence in a multivariate logistic regression model (odds ratio = 0.18; 95% CI, 0.03–0.89; $P = 0.044$). Correlation of miR-182 with mRNA expression in laser-capture microdissected benign prostate epithelium was used to predict prostatic miR-182 targets. Genes that were negatively correlated with miR-182 were enriched for its predicted targets and for genes previously identified as up-regulated in prostate cancer metastases. miR-182 expression was also negatively correlated with genes previously identified as up-regulated in primary prostate tumors from African American patients, who are at an increased risk of developing aggressive prostate cancer. Taken together, these results suggest that although miR-182 is expressed at higher levels in localized prostate cancer, its levels are lower in aggressive cancers, suggesting a biphasic role for this miRNA that may be exploited for prognostic and/or therapeutic purposes to reduce prostate cancer progression. *(Am J Pathol 2019, 189: 911–923; https://doi.org/10.1016/j.ajpath.2018.12.014)*

Prostate cancer (PCa) has the highest incidence of any cancer in men, and although most patients have organ-confined disease, approximately 1 in 10 patients will die of their cancer.[1] Classifying prostate tumors as indolent or aggressive remains a challenge both biologically and clinically. For localized or regional PCa, radical prostatectomy and radiation therapy are potentially curative. However, these treatments often cause morbidities.[2] Tissue-based biomarker panels can be used to complement clinical nomograms to guide treatment decisions[3,4] and have shown utility for predicting disease recurrence after prostatectomy.[5–7] The expression of miRNAs, small non-coding RNAs that regulate mRNA translation,[8] is dysregulated in many cancers, including PCa.[9] Currently, no miRNA biomarker test for PCa is used clinically, although several studies have described promising results that tissue or serum miRNAs have diagnostic and/or prognostic utility for PCa.[10–13] Thus, miRNAs may augment existing PCa prognostic tools and provide insight into cellular pathways that differentiate indolent and aggressive PCa.

miR-182, a member of the miR-183 family, is considered an oncomiR and is consistently present at high levels in PCa.[14–18] miR-182 regulates genes involved in proliferation and the Wnt and PI3K pathways, which are known drivers of PCa.[14,17–22] miR-182 and other miRNAs in the miR-183 family regulate prostate zinc homeostasis through direct translational inhibition of *SLC39A1*, which encodes a key zinc transporter.[14] Zinc depletion has been implicated in prostate carcinogenesis,[23] and patients with biochemical recurrence were found in a retrospective study to have 21% lower levels of zinc in tumor-adjacent benign tissue than

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patients with nonrecurrent disease.[24] In light of these findings with recurrence and the high level of miR-182 in PCa, we hypothesized that miR-182 may associate with biochemical recurrence and that miR-182 in benign tissue may regulate pathways that prime the tissue for aggressive disease.

We examined the expression of miR-182 in relation to clinical markers of aggressive PCa, such as high Gleason grade and biochemical recurrence. miR-182 expression was quantified in tissues from patients with PCa through *in situ* hybridization (ISH) of a prostate tissue microarray (TMA), and quantitative RT-PCR (RT-qPCR) of laser-captured microdissected (LCM) prostate epithelium. Correlation of miR-182 with gene expression in LCM-collected prostate epithelium was used to predict tissue-specific targets. These approaches facilitated investigation of prostate epithelium and avoided analyzing the more prevalent stroma, which can bias results for this epithelial-specific miRNA. Our results suggest that miR-182 is a complex oncomiR that is higher in PCa compared with benign tissues, but within patients with PCa, the levels of the miRNA associated with aggressive tumor characteristics and PCa recurrence are lower.

Materials and Methods

RWPE1 Spheroid Culture

RWPE1 cells were acquired from ATCC (Manassas, VA) in 2014, used at passage <20, and were maintained in RPMI 1640 medium and 10% fetal bovine serum. Cells were transduced with lentivirus that contained full miR-183 family cluster sequence or a control vector and sorted with fluorescence-activated cell sorting for green fluorescent protein expression.[19] These cells were grown in a 50% Matrigel (Corning, Corning, NY) suspension for 8 days, dissociated with Dispase (Stemcell Technologies, Vancouver, Canada), suspended in Histogel (Thermo Fisher, Waltham, MA), formalin fixed, and paraffin embedded before ISH.

TMA and Prostate Tissue Specimens

The Outcome TMA was constructed by the National Cancer Institute—sponsored Cooperative Prostate Cancer Tissue Resource.[25,26] This TMA was designed as a case-control study for biochemical recurrence after prostatectomy. The specimens were collected between 1988 and 2002. All patients with biochemical nonrecurrence were followed up for a minimum of 5 years and five serum prostate-specific antigen (PSA) measurements. Recurrence was defined as a postsurgical PSA value ≥0.4 ng/mL or two consecutive values ≥0.2 ng/mL. The original TMA contained 404 patients with four tumor cores per patient; however, many cores have been depleted. Data were collected from 133 patients, 56 of whom had both cancer and benign epithelium present. Cores with a diameter of 0.6 mm were taken from tumor regions of tissue. The number of cores analyzed per patient ranged 1 to 4 (mean, 2.4 cores). The TMA is publicly available and completely deidentified through the Cooperative Prostate Cancer Tissue Resource.

The Murphy TMA was constructed based on patients undergoing radical prostatectomy at the Jesse Brown Veterans Affairs Medical Center for clinically localized PCa. Collaborating pathologists performed centralized pathologic review and assembled the TMA from the formalin-fixed, paraffin-embedded prostatectomy specimen with pathologic and clinical data. Cores were selected from the highest Gleason grade region of the prostatectomy specimen with care to punch cores from areas of >75% tumor epithelium and from the contralateral normal benign epithelium. The prostatectomy tissues were collected between 2013 and 2017. Cores with a 1-mm diameter were taken from tumor and benign regions of tissue. The TMA contains cores from 66 patients with three tumor cores and two benign cores per patient. Fifty-five patients were analyzed, and the number of cores analyzed per patient ranged 2 to 4 (mean, 3.7 cores). Patients consented to the use of their tissues for PCa research. Specimens are deidentified. The tissue collection was approved by the Jesse Brown Veterans Affairs Institutional Review Board.

Additional deidentified prostatectomy tissues analyzed were part of a cohort of University of Illinois at Chicago (UIC) patients and the Cooperative Human Tissue Network approved by the UIC Office for the Protection of Research Subjects under UIC Institutional Review Board 2013-0341 as previously described.[27]

Immunofluorescence and Staining

A 5-μm tissue section adjacent to the section used for ISH was probed for rabbit polyclonal cytokeratin 5 (KRT5, clone Poly19055, BioLegend, San Diego, CA) and mouse monoclonal pan-cytokeratin AE1/AE3 (ab27988, Abcam, Cambridge, UK) antibodies diluted to 1:200. Antigens were retrieved using sodium citrate buffer, pH 6, 100°C for 5 minutes at 5 psi. Alexafluor 555— and 488—labeled secondaries (Invitrogen, Carlsbad, CA) were used at 1:200, followed by DAPI nuclear counterstain. Slides were imaged on the Vectra Automated Multispectral Imaging System (PerkinElmer, Waltham, MA) at the Research Histology and Tissue Imaging Core at UIC. The other adjacent section was hematoxylin and eosin (H&E) stained and scanned with Aperio AT2 (Leica, Wetzlar, Germany) at the Research Histology and Tissue Imaging Core.

miR-182 ISH

The protocol from the miRCURY LNA miRNA ISH optimization kit (Exiqon, Vedbaek, Denmark) was followed with modifications. Formalin-fixed, paraffin-embedded TMA sections (5 μm) were placed onto hydrophilic slides, baked overnight at 60°C, deparaffinized, and incubated for 20 minutes at 37°C with 15 μg/mL of proteinase K for protein digestion. Digoxigenin-labeled miR-182 LNA probe (80 nmol/L),

digoxigenin-labeled U6 LNA probe (10 nmol/L) (positive control), or no probe (negative control) was incubated at 48°C for 60 minutes followed by stepwise 5-minute washes in saline-sodium citrate buffer at 42°C (×1, ×−0.5, ×−0.2, and then ×0.2) at room temperature. Slides were blocked and incubated for 60 minutes with alkaline phosphatase−conjugated anti-digoxigenin antibody (Sigma-Aldrich, St. Louis, MO) at 1:200. Alkaline phosphatase was visualized with Vector Red alkaline phosphatase substrate (Vector Laboratories, Burlingame, CA) for 90 minutes and stopped with KTBT buffer. Slides were counterstained with DAPI.

Imaging and Segmentation

miR-182 ISH was imaged on the Vectra Automated Multispectral Imaging System (PerkinElmer) at the Research Histology and Tissue Imaging Core at UIC. Cores were imaged at ×20 magnification and tiled. Images were unmixed using a spectral library for Vector Red signal created using Nuance software version 3.0.2 (PerkinElmer) with inForm Advanced Image Analysis software version 2.4.1 (PerkinElmer). For the Outcome TMA, cores that could be unambiguously identified and that contained ISH signal were segmented by a pathologist (A.M.A.) using an adjacent H&E reference slide. Epithelium of adenocarcinoma, benign glands, and high-grade prostatic intraepithelial neoplasia (PIN) were manually selected in each core for quantitation of Vector Red fluorescence. Representative areas were circled, and, in cores with more than a single type of epithelium of interest (eg, adenocarcinoma and benign prostate glands), equivalent amounts of each cell type were selected. Adenocarcinoma was identified according to architectural and cytologic parameters: increased nuclear size, decreased nucleocytoplasmic ratio or prominent nucleoli, crowded acini, fused glands, cribriform masses, solid sheets, and/or single infiltrating cells. High-grade PIN was identified by nuclear enlargement, prominent nucleoli, and epithelial cell crowding with visible basal cells and absence of diagnostic criteria for intraductal carcinoma of the prostate.[28] Glands with simple atrophy without inflammation were selected as benign epithelium. All other benign lesions, including histologic variants of atrophy, basal cell hyperplasia, prostatitis, and atypical adenomatous hyperplasia, were excluded. For the Murphy TMA, an adjacent H&E slide was used to select representative benign epithelium, excluding atrophy and PIN from benign cores, and representative adenocarcinoma from tumor cores. Benign epithelium cores were reviewed and approved by a pathologist after segmentation.

Laser-Capture Microdissection and miR-182 Quantification by RT-qPCR

LCM of benign prostate epithelium from frozen prostatectomy tissue and RNA isolation was performed previously.[27] Briefly, cDNA was synthesized from 60 ng of LCM-collected RNA with Universal RT miRNA reagents (Exiqon). qPCR was run in triplicate for miR-182 using the miRCURY LNA miRNA PCR System (Exiqon) and three housekeeping genes (*RNU44*, *RNU48*, and *RNU66*) using SYBR green (BioRad, Hercules, CA) and QuantStudio 6 (Thermo Fisher) with the following settings: 95°C for 10 minutes (×1), 95°C for 15 seconds (×50), and 60°C for 1 minute (×50). For quality control, patients with low housekeeper correlation were excluded from subsequent analysis (Supplemental Figure S1A). miR-182 relative quantity was calculated by the −ΔΔCt method, using the mean Ct of the housekeeping genes for normalization.

Correlation of miR-182 Expression with Gene Expression in LCM Benign Epithelium

RNA from 13 African American and 13 patients of European ancestry were analyzed using GeneChip 1.0 Human Gene ST arrays (Affymetrix, Santa Clara, CA) and previously reported in Richards et al[27] (Gene Expression Omnibus; *https://www.ncbi.nlm.nih.gov/geo*; accession number GSE91037). Expression data were normalized using Robust Multiarray Average method in the R package Affy.[29] Data quality was evaluated using affyPLM,[30] and three arrays (GSM2420027, GSM2420029, and GSM2420033) were excluded from this study based on normalized unscaled SEM plots (Supplemental Figure S1B). Spearman's ρ and *P* values were calculated using the relative quantity of miR-182 expression and the $\log_2$ (Robust Multiarray Average) expression values of each probe set for 21 patients using the cor.test function, specifying Spearman correlation, in the stats package of R version 3.3.3[31] (Supplemental Table S1).

Validation of Predicted miR-182 Targets

Primary prostate epithelial cells were isolated from deidentified radical prostatectomy tissue specimens as previously described under UIC Office for the Protection of Research Subjects−approved Institutional Review Board 2011-1138.[14] Cells were grown in PrEGM media (Lonza, Basel, Switzerland) and transfected with miR-182 pre-miRNAs (Thermo Fisher) or mock transfected using siPORT NeoFX (Life Technologies, Carlsbad, CA). After 24 hours, RNA was Trizol (Invitrogen) extracted. cDNAs were made using High-Capacity cDNA RT kit (Invitrogen). Primers are listed in Table 1. qPCR was run using SYBR green and QuantStudio 6 (Thermo Fisher). PCR settings were as follows: 95°C for 10 minutes (×1), 95°C for 15 seconds, 58°C for 30 seconds, and 72°C for 30 seconds (×40). Relative quantity was calculated by the −ΔΔCt method, using β_2-microglobulin for normalization.

Gene-Set Enrichment Analysis

Gene Set Enrichment Analysis version 3.0 software is from the Broad Institute.[32] The correlation coefficients for

Table 1 Primers for Quantitative RT-PCR Validation of Predicted miR-182 Gene Targets

Gene	Primers	
CD164	FWD	5′-TTAGCTTTCTCCCGAACGCC-3′
	RVS	5′-GCAGCTGTTTCGACCTTCAC-3′
ELL2	FWD	5′-ATGTGAAGCTCACCGAGACG-3′
	RVS	5′-TTTGACAAGCCCGTGGAGTC-3′
PRKAR1A	FWD	5′-ACACCCAGAGAGGGACAGAGAA-3′
	RVS	5′-GAGCTCACATTCTCGAAGGCT-3′
RNF152	FWD	5′-AGACTCGGTGACAGATACAGAAAT-3′
	RVS	5′-TGCAGGTAATGGCAAGCTCA-3′
NR3C1	FWD	5′-GTTGTTTATCTCGGCTGCGG-3′
	RVS	5′-TCAGTGAATATCAACTCTGGCA-3′
B2M	FWD	5′-CCTGAATTGCTATGTGTCTGGG-3′
	RVS	5′-TGATGCTGCTTACATGTCTCGA-3′

FWD, forward; RVS, reverse.

genes with multiple probe sets were averaged together. To mimic expression data input, the negative correlation coefficients were multiplied by −1 and input in triplicate as negative correlation samples and the positive correlation coefficients in triplicate as positive correlation samples. Because of the small sample size, gene set permutation was used to calculate statistics. The miR-182-5p targets were predicted from TargetScan version 7.1 with a cumulative-weighted context score <-0.5,[33] miRDB with a target score >0.85,[34] and TarBase version 8 with a prediction score >0.85.[35] Other gene sets were downloaded from MSigDB version 6.1, including Kyoto Encyclopedia of Genes and Genomes version 6.1,[36] CHANDRAN_METASTASIS_TOP50_UP (ID M18970),[37] and WALLACE_PROSTATE_-CANCER_RACE_UP (ID M10319).[38]

The Cancer Genome Atlas Analysis

The results shown are in part based on data generated by The Cancer Genome Atlas (TCGA) Research Network (*http://cancergenome.nih.gov*; last accessed May 2, 2018). TCGA prostate adenocarcinoma miRNA sequencing counts data and RNA sequencing counts data (level 3 publicly available data) were downloaded from the National Cancer Institute Genomic Data Commons (*https://portal.gdc.cancer.gov*; last accessed August 2017) and matched by case identifiation. For small RNA sequencing, counts per million miRNA counts were used as reported, and for RNA sequencing, gene counts were divided by the total number of unique reads per patient $\times$1,000,000 for counts per million. There were 499 tumor cases with both data sets.

Statistical Analysis

Statistical analyses were performed in GraphPad Prism software version 5.04 (GraphPad Software Inc., San Diego, CA) for *U*-test, Kruskal-Wallis test, and *t*-test. Statistics were performed in R version 3.3.3[31] for Spearman correlation of microarray gene expression with miR-182 and logistic regression analysis. Interclass correlation coefficients

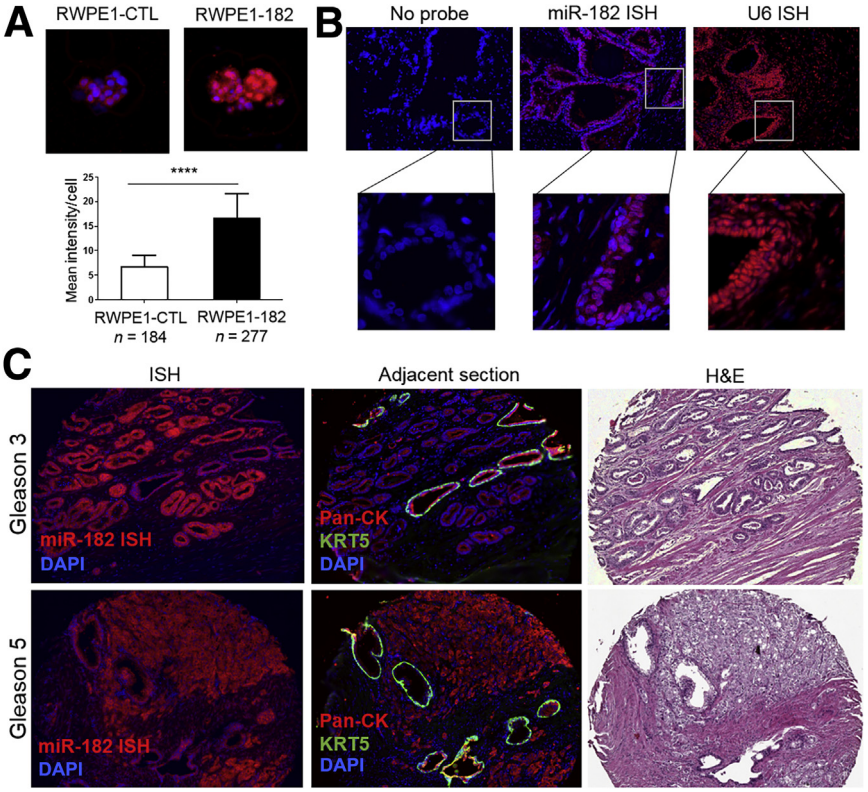

Figure 1 miR-182 is expressed exclusively in prostate epithelium and higher in areas of prostate cancer. **A:** Specificity of miR-182 LNA *in situ* hybridization (ISH) probes (red) with nuclear counterstain DAPI (blue) assessed in RWPE1 benign prostate cells transfected with control (RWPE1-CTL) or miR-183 cluster—expressing lentivirus (RWPE1-182). Bar graph showing quantification of miR-182 ISH intensity per cell (two-tailed unpaired *t*-test). **B:** Representative of no-probe secondary antibody-only control, miR-182 ISH, and U6-positive control developed with Vector Red in benign prostate tissue. DAPI (blue) as nuclear stain. **Boxed areas** in **top row** of **B** are shown in higher magnification in the **bottom row**. **C:** miR-182 ISH (red) on representative tissue cores of primary Gleason grade 3 and Gleason grade 5 tumor tissue. Adjacent tissue sections stained for basal epithelium (KRT5, green), pan-epithelial (pan-CK, red) markers, and hematoxylin and eosin (H&E) are included for comparison. Loss of green KRT5 was used to confirm areas of cancer. Cores were magnified and tiled. $^{****}P < 0.0001$. Original magnification, $\times$20.

were calculated using SAS version 9.4 (SAS Institute Inc., Cary, NC). $P<0.05$ was considered significant.

Results

miR-182 Is Expressed Exclusively in Prostate Epithelium and Is Higher in Areas of Cancer as Determined by ISH

The spatial expression pattern of miR-182 within the prostate was examined by ISH. Specificity of the miR-182 ISH probe was evaluated using RWPE1 cells over-expressing miR-182 and control cells[19] grown as spheroids and formalin-fixed, paraffin-embedded processed to mimic the tissue conditions. miR-182 intensity was objectively quantified using the Vectra imaging system with inForm software version 2.4.1 (PerkinElmer) and higher in RWPE1 miR-183 cluster–expressing lentivirus spheroids (Figure 1A). ISH in formalin-fixed, paraffin-embedded radical prostatectomy tissues revealed that miR-182 was localized to the epithelium and was absent from stroma compared with the positive ISH control, which was present in all cell types (Figure 1B). miR-182 levels appeared higher in areas of cancer compared with adjacent benign epithelium within the same core (Figure 1C). Epithelial specific and high expression of miR-182 in PCa is consistent with previous reports using RT-qPCR and array methods.[14,15,17,20,39]

PCa Has Higher Expression of miR-182 but Low miR-182 Associated with Biochemical Recurrence

The expression of miR-182 was further examined in a TMA of prostatectomy samples (Outcome TMA), which was created by the National Cancer Institute–sponsored Cooperative Prostate Cancer Tissue Resource.[25,26] This TMA used a case-control design in which patients with biochemical recurrence were matched to control patients who did not have disease recurrence by age, Gleason grade sum score, pTNM stage, year of prostatectomy, and ethnicity.[25,26] Usable ISH data were obtained for 133 of the patients on the TMA. Numerous tissue cores had been depleted, limiting the ability for paired case-control analysis. Clinical information for the patients analyzed are provided in Table 2. Using inForm software version 2.4.1 (PerkinElmer) and an H&E reference slide, a pathologist (A.M.A.) segmented the miR-182 ISH images into benign and cancer regions and objectively quantified the mean pixel intensity of miR-182 within these regions. Similar to other reports,[14–18] miR-182 expression was higher in PCa epithelium than benign epithelium by both an unpaired analysis (Figure 2A), and a paired analysis of 56 patients with benign and cancer epithelium available (Figure 2B).

Table 2 Clinical Information for TMA Patients Analyzed from the Outcome and Murphy TMAs

	Outcome TMA				
	All patients measured		Cancer/benign ratio patients		Murphy TMA for cancer/benign ratio patients (n = 55)
Patient information	Recurrence (n = 72)	No recurrence (n = 61)	Recurrence (n = 34)	No recurrence (n = 22)	
Age, means ± SD, years	63.2 ± 5.9	62.7 ± 5.5	63.38 ± 5.8	61.73 ± 6.4	63.15 ± 6.15
Race/ethnicity, % (SNP analysis)					
White for Outcome TMA and >80% EA for Murphy TMA	80.6	83.6	82.4	68.2	21.8
Black for Outcome TMA and >50% AA for Murphy TMA	19.4	16.4	17.6	31.8	45.5
Other	NA	NA	NA	NA	20
Unknown	NA	NA	NA	NA	12.7
Gleason score, %					
Unknown	NA	NA	NA	NA	3.6
3 + 2	1.4	0	0	0	
3 + 3	22.2	21.3	32.4	13.6	5.5
3 + 4	62.5	65.6	61.8	77.3	52.7
4 + 3	12.5	11.5	2.9	9.1	20
4 + 4	1.4	1.6	2.9	0	1.8
4 + 5	NA	NA	NA	NA	10.9
5 + 4	NA	NA	NA	NA	5.5
pT stage, %					
pT2	73.6	73.8	70.6	86.4	41.8
pT3	26.4	26.2	29.4	13.6	58.2
Time to recurrence, means ± SD, mo	39.4 ± 29.4	NA	43.4 ± 32.4	NA	NA

AA, African American; EA, European American; NA, not applicable; SNP, single-nucleotide polymorphism; TMA, tissue microarray.

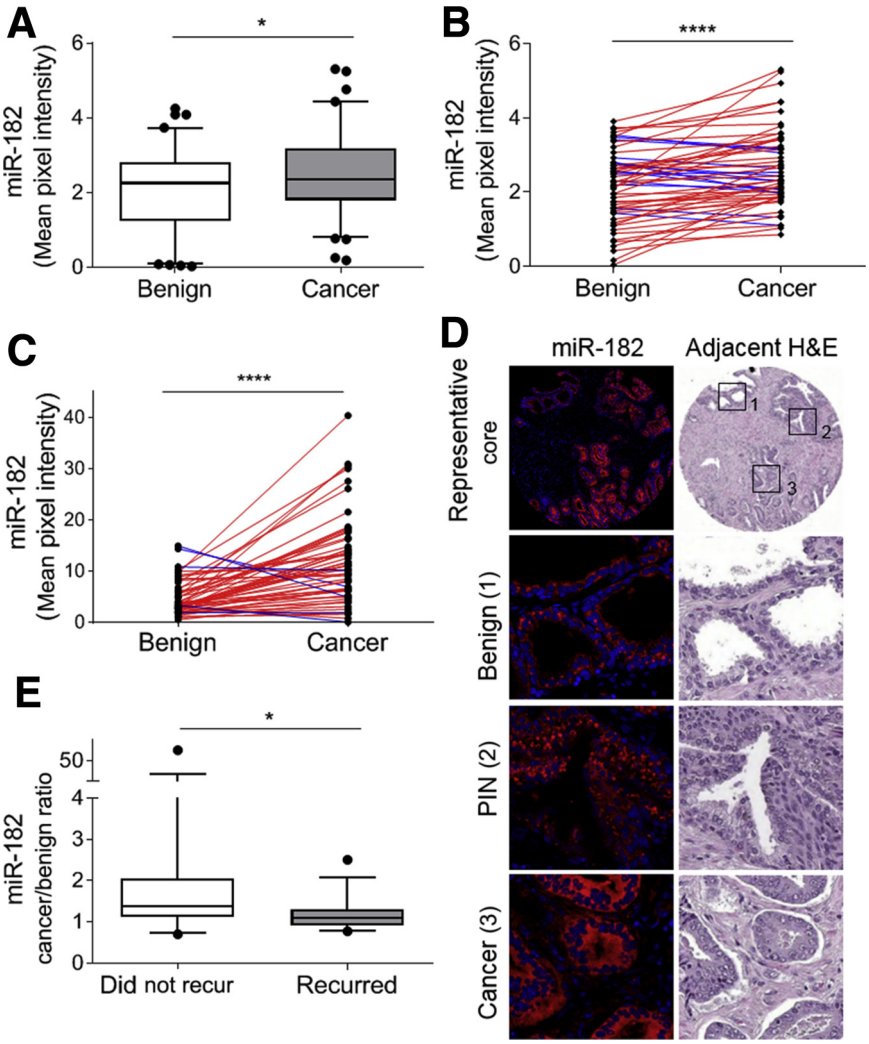

Figure 2 miR-182 levels are higher in prostate cancer epithelium, but low cancer/benign ratio of miR-182 expression is associated with biochemical recurrence. **A** and **B:** miR-182 *in situ* hybridization (ISH) quantification in the prostate by an unpaired analysis (two-tailed *t*-test; **A**) and a paired analysis (two-tailed paired *t*-test; **B**). Lines in **B** connect benign and cancer data points of the same patient, with **red lines** indicating an increase and **blue lines** a decrease. **C:** In the Murphy validation tissue microarray, miR-182 levels increase significantly in cancer epithelium by ISH (two-tailed paired *t*-test). Lines connect benign and cancer data points of the same patient, with **red lines** indicating an increase and **blue lines** a decrease. **D:** miR-182 in a representative core that contains prostatic intraepithelial neoplasia (PIN). **Left column** shows miR-182 ISH. **Right column** shows hematoxylin and eosin (H&E) of adjacent section. **Boxed areas** in **top right panel** are shown in higher magnification in **three rows below**; the **second row** shows benign epithelium; the **third row** shows PIN; the **fourth row** shows cancer. **E:** In the Outcome TMA, cancer/benign ratios of miR-182 expression stratified by biochemical recurrence (*U*-test). Data are expressed as means ± 5th to 95th percentiles. $n = 133$ (**A**); $n = 56$ (**B**); $n = 55$ (**C**); $n = 34$ (**D**, recurred); $n = 22$ (**D**, did not recur). $^{*}P < 0.05$, $^{****}P < 0.0001$. Original magnification: ×5 (**D**, **first row**) ×20 (**D**, **second**, **third**, and **fourth rows**).

Validation using a second prostatectomy TMA (Murphy TMA) also found significantly higher miR-182 in cancer by a paired analysis of 55 patients (Figure 2C and Table 2). miR-182 levels consistently appeared to be increased in PIN, equal to that of PCa (Figure 2D), but there were not sufficient regions for statistical analysis. Although the TMAs were not powered to study PIN, it suggests that upregulation of miR-182 is an early event in PCa.

As a measure of data consistency, the interclass correlation coefficient of the mean miR-182 pixel intensity between multiple cores from the same patient on the Outcome TMA was high in both benign epithelium (0.67; 95% CI, 0.51−0.80) and cancer epithelium (0.77; 95% CI, 0.68−0.85). Although intrapatient variance was low, it is unclear whether the higher interpatient variance we observed in miR-182 signal is attributable to true biological variation or differing RNA preservation among the specimens. Importantly, age of the samples did not correlate with ISH signal intensity (Supplemental Figure S2A), but sample fixation and preservation may still differ. Without a robust normalization method available for ISH, the absolute intensities of miR-182 ISH did not correlate with clinical parameters (Supplemental Figure S2B and data not shown). Therefore, internal normalization was applied through the cancer/benign ratio of miR-182 expression per patient, to reduce potential confounding due to interpatient differences in RNA quality.

The expression of miR-182 for both cancer and benign cores was available for 56 patients on the Outcome TMA, and the cancer/benign ratio of miR-182 was significantly associated with recurrence. Patients with a greater increase in miR-182 expression in cancer tissue over benign tissue were significantly less likely to experience biochemical recurrence (Figure 2E). The cancer/benign ratio was not significantly different by patient ethnicity, Gleason score, or stage, although it did trend toward decreasing with increasing preprocedure PSA values (Supplemental Figure S3). The Murphy TMA patients with high cancer/benign ratios tended to not have invasive pathologic findings, although the TMA was not designed with power to assess this statistically (Supplemental Figure S4). The association between high miR-182 cancer/benign ratio in the Outcome TMA and lower

Table 3 Multivariate Logistic Regression for Recurrence

Variable	Odds ratio (95% CI)	*P* value
Cancer/benign Ratio		
Low	Reference	
Medium	0.23 (0.04-1.18)	0.09
High	0.18 (0.03-0.89)	0.04*
Age	1.01 (0.91-1.12)	0.84
Gleason Score		
3 + 3	Reference	
3 + 4	0.37 (0.07-1.70)	0.22
4 + 3 or 4 + 4	0.45 (0.03-5.83)	0.53
Stage		
pT2	Reference	
pT3	3.04 (0.71-16.50)	0.16
Ethnicity/race		
Black	Reference	
White	1.03 (0.24-4.38)	0.97

The miR-182 cancer/benign ratios were segmented into tertiles (low, medium, and high ratios). Gleason scores of 4 + 3 and 4 + 4 were combined into a single predictor because there was only one patient with a Gleason score of 4 + 4.

*$P < 0.05$.

risk of recurrence was also evident in a multivariate logistic regression model for recurrence using clinical data from the Outcome TMA (odds ratio = 0.18; 95% CI, 0.03–0.89; $P = 0.04$) (Table 3). Because of the case selection criteria used for the original matched case-control design of this array, clinical parameters that normally predict recurrence (such as Gleason score and pT stage) were not significantly associated with recurrence.

Gene Targets of miR-182 Expression Are Enriched in Aggressive PCa

To identify potential prostate-specific targets of miR-182, the correlation between miR-182 expression and microarray gene expression profiles from LCM benign prostate epithelium was analyzed in a second cohort of patients with PCa (Figure 3A). Benign tissue was used instead of PCa because PCa exhibits high heterogeneity in gene expression patterns[40] and thus decreases the likelihood of identifying targets of miR-182. The microarray data were previously reported by our group (Gene Expression Omnibus; *https://www.ncbi.nlm.nih.gov/geo*; accession number GSE91037).[27] For this study, miR-182 expression was quantified by RT-qPCR of the RNA from the LCM-collected samples using three reference RNAs for normalization. For 21 patients with both miR-182 and profiling data, Spearman correlation coefficients were calculated between the relative expression of miR-182 (-ΔΔCt method) and each gene ($\log_2$ Robust Multiarray Average) (Figure 3A, Supplemental Table S1).

This approach to identifying miR-182 targets was validated by our observation that genes negatively correlated with miR-182 in prostate epithelium were enriched for predicted targets of miR-182 from TarBase, TargetScan, and miRDB by gene-set enrichment analysis (Figure 3B).[32–35] The genes most strongly negatively correlated with miR-182 in benign prostate epithelium that are also predicted targets are listed in Table 4. Kyoto Encyclopedia of Genes and Genomes version 6.1 pathways enriched in miR-182 negatively correlated genes included protein degradation, protein export, oxidative phosphorylation, neurologic disorders, and bacterial infection (Figure 3C).[36] Genes negatively correlated with miR-182 were strongly enriched for a gene set published by Chandran et al[37] that contains genes up-regulated in metastatic PCa compared with primary PCa, which further links lower miR-182 to aggressive disease (Figure 3D). Many of the predicted miR-182 target genes from Table 4, *ELL2*, *CD164*, *RNF152*, and *PRKAR1A*, were validated in two different patient-derived primary prostate basal epithelial cells overexpressing miR-182 (Supplemental Figure S5, Figure 3E). To investigate whether the correlations identified were also present in PCa, TCGA prostate adenocarcinoma whole prostate tumor sequencing data were used. Despite a stromal bias in TCGA prostate adenocarcinoma samples,[40] a few of the predicted targets and nearly a third of the top negatively correlated genes with miR-182 in our data set were negatively correlated with miR-182 in TCGA (Supplemental Tables S2 and S3). *PRKAR1A* and *NR3C1*, which have been reported to have higher expression in aggressive PCa,[41,42] were negatively correlated with miR-182 in TCGA and validated as miR-182 targets in two patient-derived prostate cell lines (Figure 3E).

miR-182 Expression in Benign Prostate Epithelium Associated with Gleason Grade of the Tumor and with Patient Ancestry

The clinical data for the LCM cohort used for the miR-182 target discovery was limited but did contain single-nucleotide polymorphism quantified West African ancestry[27] and Gleason grade (Supplemental Table S4). Notably, this cohort lacked outcome data and PCa samples. Benign epithelial levels of miR-182 trended toward being higher in patients with lower Gleason grade tumors but did not reach significance (Figure 4A). In this cohort, the expression of miR-182 was significantly twofold lower in benign epithelium from African American patients compared with white men of European ancestry (Figure 4B). Because African American patients with PCa have an elevated risk of developing aggressive PCa and recurrence,[43] whether the predicted miR-182 targets overlap with genes highly expressed in primary PCa tumors from African American patients was examined. The genes up-regulated in PCa tumors from African American men compared with PCa tumors from European men from the gene sets in the studies by Wallace et al[38] and Powell et al[44] were enriched in the predicted miR-182 targets, consistent with lower miR-182 associating with aggressive PCa (Figure 4, C and D). Neither gene set was significantly enriched in benign epithelium from African American patients in the microarray data set, suggesting these findings are not an artifact of the

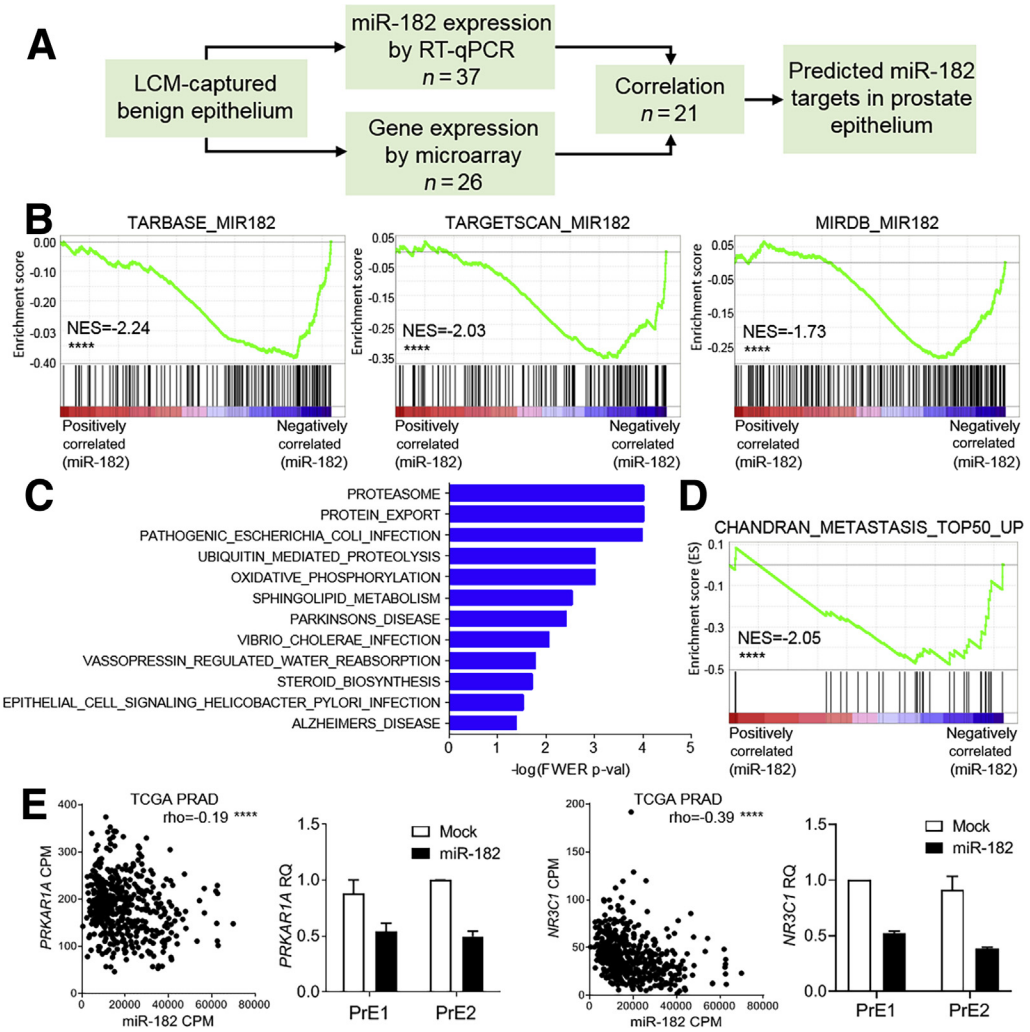

Figure 3 miR-182–regulated genes identified by correlation with expression profiling are enriched for genes up-regulated in prostate cancer (PCa) metastasis. **A:** Analysis scheme to identify miR-182–regulated genes in prostate epithelial tissue. Gene expression profiling and miR-182 levels were examined in laser-capture microdissected (LCM) benign epithelium from 21 patients. Gene expression by Affymetrix GeneChip 1.0 Human Gene ST arrays and miR-182 by quantitative RT-PCR (RT-qPCR) normalized to *RNU60*, *RNU44*, and *RNU48*. Genes negatively correlated with miR-182 by Spearman analysis contain our predicted miR-182 targets. **B:** The predicted miR-182 targets are enriched for computationally predicted targets of miR-182 by gene-set enrichment analysis: TargetScan version 7.1 cumulative-weighted context score <-0.50, miRDB target score >85, and TarBase v8 prediction score >85. **C:** Kyoto Encyclopedia of Genes and Genomes version 6.1 pathways enriched in genes negatively correlated with miR-182 in prostate epithelium. The -$\log_{10}$ family-wise error rate corrected *P* value is shown. **D:** Predicted targets of miR-182 are enriched for the most strongly up-regulated genes in metastatic PCa compared with primary PCa from the gene set in the study by Chandran et al.[37] **E:** *PRKAR1A* (left) and *NR3C1* (right) correlation with miR-182 in The Cancer Genome Atlas (TCGA) prostate adenocarcinoma tumor samples and RT-qPCR in two prostate epithelial (PrE) cell lines transfected with miR-182 pre-miRNA (PrE1: 50 μmol/L, PrE2: 25 μmol/L) or mock transfected. Data are expressed as means ± SD. $****P < 0.0001$. CPM, counts per million; NES, normalized enrichment score; RQ, relative quantity; TCGA PRAD, TCGA prostate adenocarcinoma.

lower miR-182 levels in African American patients in our cohort (Supplemental Figure S6). In summary, our findings support that both lower miR-182 expression in benign epithelium and a smaller increase in miR-182 expression in PCa are associated with more aggressive PCa (Figure 4E).

Discussion

Dysregulated miRs in PCa have been proposed to contribute to disease progression, as well as act as potential biomarkers for the presence of PCa or to predict PCa outcomes.[11,13,45–47] We examined the association of miR-182 expression with clinical markers of PCa aggressiveness in a TMA cohort and proposed gene and pathway targets of miR-182 in prostate epithelium using LCM-collected tissue. This study yielded expected and unexpected expression patterns for miR-182 in PCa, which prompted us to examine its mRNA targets, ultimately determining that PCa with high levels of miR-182 behave less aggressively than tumors with less overexpression of this oncomiR.

In line with previous reports by our group and others, miR-182 expression was higher in PCa epithelium than benign epithelium.[14–18,39] However, the negative

Table 4 Top Predicted Targets of miR-182 in Prostate Epithelium

Gene	Spearman's ρ	Unadjusted *P* value	TarBase score >85	TargetScan score <−0.5	miRDB score >85
ELL2	−0.6866	0.00059	x		x
PPM1A	−0.6805	0.00094	x		
CD164	−0.6675	0.00126	x	x	
MAPK1IP1L	−0.6610	0.00146		x	
RAB10	−0.6571	0.00158		x	x
SPPL2A	−0.6351	0.00249		x	
NUFIP2	−0.6338	0.00255	x		
RNF152	−0.6325	0.00262		x	
ARF4	−0.6312	0.00269	x	x	x
PRKAR1A	−0.6299	0.00276		x	
RAB2A	−0.6221	0.00320		x	
ARHGDIA	−0.6175	0.00587	x		
RTN4	−0.6169	0.00353			x
ARHGEF12	−0.6156	0.00361		x	

Genes that are strongly negatively correlated with miR-182 expression in benign prostate epithelium and are predicted targets by either TargetScan version 7.1 cumulative-weighted context score <−0.50, miRDB target score >85, or TarBase v8 prediction score >85.

association detected between miR-182 expression and recurrence has not been reported before in PCa and was unexpected. The results in PCa are supported by studies in breast, lung, and bone cancers in which low expression of miR-182 has been associated with metastasis.[48–50] Conversely, studies in PCa cell lines have found high miR-182 as increasing *in vitro* and *in vivo* phenotypes of aggressiveness.[17,18,20,21] Measurement of miR-182 by RT-qPCR in prostate tumor specimens, not microdissected, have found either no significant association[21,47] or a positive association of miR-182 expression with recurrence.[39] In the study by Casanova-Salas et al,[39] medium, but not high levels of miR-182 were significantly associated with recurrence, which is somewhat supported by our findings. The discrepancies between our results and previous studies may be attributable to differing measurement methods.[21,39,47] Our findings using ISH and LCM-collected prostate epithelium remove stromal contamination as a bias that is unavoidable in the whole tissues used in the other studies. In addition, the cancer miR-182 levels were normalized to benign levels within each patient for the analysis, which is in contrast to the other studies of miR-182 in recurrence that did not consider benign tissues. It is plausible that the change in expression of miR-182 in PCa per patient is more physiologically important than its standalone expression level.

Overall, our results suggest that the function of miR-182 changes during PCa, possibly promoting early oncogenic changes but becoming protective or selected against in high-risk PCa. A limitation to our study is the lack of matched metastases in which to determine the levels in advanced stages of disease. A previous study that supported the hypothesis of a dual role of miR-182 in PCa found that miR-182 promoted resistance to apoptosis on growth factor withdrawal in an immortalized primary prostate cell line but reversed the epithelial to mesenchymal transition that is observed in that cell line.[51] This expression paradox is not unusual in PCa. α-Methylacyl CoA racemase is also up-

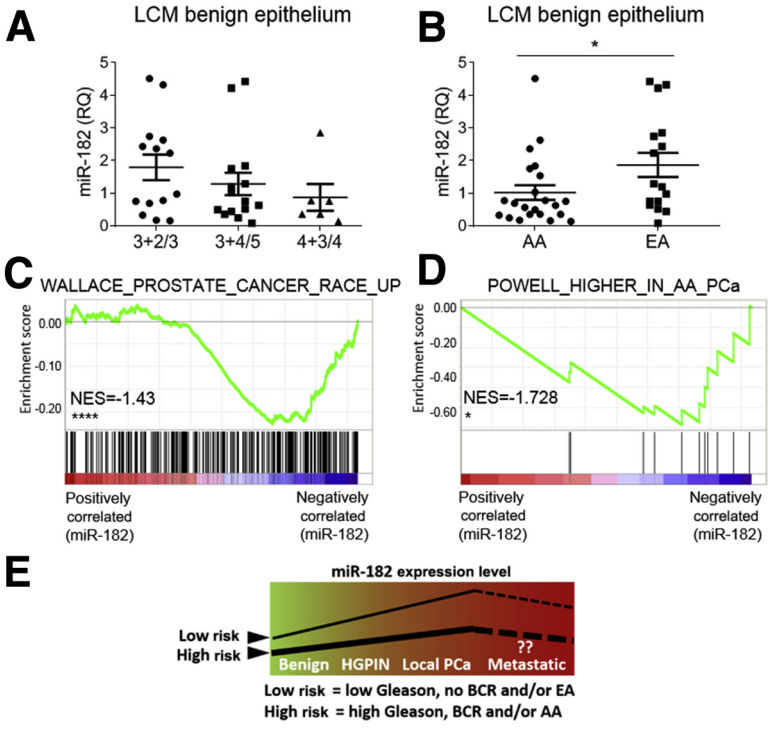

Figure 4 Benign epithelial expression of miR-182 is lower in prostate that contains high Gleason grade and in African American (AA) men. **A:** The expression of miR-182 in benign epithelium trends toward being higher in patients with lower Gleason grade tumors. Normalization for quantitative RT-PCR was performed using the mean expression of *RNU44*, *RNU48*, and *RNU66* Kruskal-Wallis test, not significant. **B:** miR-182 expression in patients by ancestry were designated by single-nucleotide polymorphism (SNP) analysis showing >50% West African ancestry (AA) or >80% European ancestry (EA), respectively. SNP data were not available for two patients, and self-reported race was used (*U*-test). **C** and **D:** Predicted miR-182 target genes are enriched for genes with higher expression in prostate cancer (PCa) tumors from AA men than in PCa tumors from EA men from the gene sets in the studies by Wallace et al[38] (**C**) and from Powell et al[44] (**D**). **E:** Proposed model of miR-182 expression patterns at different disease stages in patients at high risk and low risk for PCa based on the data in this study. The expression of miR-182 in metastatic PCa was not measured in this study, but because miR-182 was negatively correlated with genes expressed in metastasis in prostate epithelium (Figure 3D), it is hypothesized to decrease. Data are expressed as means ± SD. $n = 14$ (**A**, Gleason 3+ 3/2); $n = 15$ (**A**, Gleason 3+ 4/5); $n = 6$ (**A**, Gleason 4+ 3/4); $n = 22$ (**B**, AA men); $n = 16$ (**B**, EA mean). $^*P < 0.05$, $^{****}P < 0.0001$. BCR, biochemical recurrence; HGPIN, high-grade prostatic intraepithelial neoplasia; LCM, laser-captured microdissected; NES, normalized enrichment score; RQ, relative quantity.

regulated in PCa and may functionally promote PCa growth,[52] but lower levels of α-methylacyl CoA racemase are associated with more advanced disease.[53,54] In addition, androgen receptor is a well-established driver of prostate proliferation, but higher expression of several androgen receptor target genes predicts indolent disease in the Oncotype DX genomic assay.[55] miR-182 is regulated by androgen receptor in lymph node carcinoma of the prostate PCa cells,[18,56] suggesting that miR-182 levels may be a passenger change that results directly or indirectly from crosstalk with androgen signaling. However, no correlation was observed between miR-182 expression and the expression of transcripts for *AR*, AR-target *KLK3* (PSA), or *AMACR* in benign prostate epithelium in our study (Supplemental Table S1). Potentially of interest, the transcript for glucocorticoid receptor, which is down-regulated in primary PCa tumors and re-expressed in metastases, is strongly negatively correlated with miR-182 in our array data set (*NR3C1*, $\rho = -0.64$, $P = 0.002$).[42] Glucocorticoid receptor may promote AR independence of advanced PCa, and biochemical relapse patients with high expression of glucocorticoid receptor have significantly shorter progression-free survival.[42]

miR-182 targets that may explain its observed association with recurrence were identified from genes that contain predicted miR-182 binding sites and negatively correlate with miR-182 in benign prostate epithelium. These genes include *CD164*, which promotes circulating cancer cell adherence to bone[57]; *ARF4*, which contributes to a metastatic increased ER-Golgi trafficking phenotype in breast cancer[58]; *PRKAR1A*, which was higher expressed in patients with PCa who developed metastasis[41]; and *NUFIP2*, a stress granule protein and member of the metastasis gene set used in the study by Chandran et al.[37] Kyoto Encyclopedia of Genes and Genomes pathways enriched in the miR-182 negatively correlated genes have also been linked to aggressive PCa, including protein degradation, sphingolipid synthesis, and inflammation.[59–61] Interestingly, *ELL2*, one of the most strongly negatively correlated genes with miR-182, induces a PIN phenotype in murine prostate when conditionally deleted,[62] and we observed high expression of miR-182 in PIN. There may be limitations to our miR-182 target-identification method, however, because several previously reported targets of miR-182 were not strongly negatively correlated with miR-182 in our data set.[17,18,20,21] Discrepancies between our target predictions and published targets of miR-182 in prostate cell lines are likely a result of our approach, including using LCM-collected total epithelium rather than cell cultures. Our RNA expression–based associations would not detect targets that are primarily down-regulated by miR-182 by translation blockade rather than mRNA instability. Another limitation is that differences in miR-182 gene targets may also exist between benign prostate and PCa. Although many predicted targets also negatively correlated with miR-182 in TCGA tumor samples, others did not or had positive correlations. This might support different regulation in tumors; however, because miR-182 in prostate is epithelial, the results are confounded by the varying epithelial/stromal composition of TCGA samples. In future studies, predicted prometastatic targets of miR-182 in LCM-collected prostate epithelium should be tested for correlation to miR-182 in LCM-collected PCa specimens and validated functionally.

Although only a small number of patients with LCM-collected tissue were analyzed in this study, the observation that miR-182 expression was significantly lower in benign prostate epithelium from African American men than European men is of potential interest. African ancestry is considered a risk factor for PCa,[43] and both the incidence and mortality of PCa is approximately twice as high in African American men compared with other men.[63] Genes that were negatively correlated with miR-182 in our LCM microarray data significantly aligned with gene set signatures of genes highly expressed in PCa from African American patients,[38,44] suggesting that miR-182 levels may also be lower in PCa in African American men. Because low miR-182 was associated with recurrence in this study, lower levels of miR-182 may drive a gene expression profile from which aggressive prostate tumors arise in African American men. Importantly, our analysis was limited to benign epithelium, and it remains to be tested if the lower expression of miR-182 observed in African American men persists in cancer. By ISH, the cancer/benign ratio of miR-182 was not significantly different between African American and European men (Supplemental Figure S3), a finding limited by the lack of outcome data available for these men.

In summary, we conclude that the role of miR-182 in PCa is bimodal. Increased expression of miR-182 in PCa may promote early or localized disease, but higher magnitude changes in miR-182 expression in PCa may protect against disease recurrence or not be selected for in aggressive disease. A strength of this study is the use of ISH and LCM, which allowed for precise analysis of an epithelial miR, in contrast to previous similar studies that use whole or macrodissected prostate tissue.[21,39,40,47] A limitation to this study is the small sample size of patients with recurrence data analyzed ($n = 56$) and that patients with recurrence data lacked data for modern clinical risk estimators. Lower expression of miR-182 in prostate biopsy specimens may be a clinically useful indicator of high-risk PCa in conjunction with existing risk estimators, and future studies in a larger and more ethnically diverse cohort are needed to confirm that miR-182 is associated with recurrence.

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B.B. and L.N. conceived and designed the study, analyzed data, and wrote the manuscript; B.B., A.M.A., Z.R., and A.S. acquired and analyzed data; R.D. performed TMA scanning and assisted with inForm software analysis; P.H.G. provided advice on statistics and interpreted data; A.K.-B. provided the Outcome TMA; A.M. provided the Murphy TMA; the manuscript was reviewed and revised by all authors.

Supplemental Data

Supplemental material for this article can be found at *https://doi.org/10.1016/j.ajpath.2018.12.014*.

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