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Published in:
European Urology

DOI:
10.1016/j.eururo.2014.12.025

2015

Elsevier Editorial System(tm) for European Urology
Manuscript Draft

Manuscript Number: EURUROL-D-14-01546R2

Title: miR-183 in prostate cancer cells positively regulates synthesis and serum levels of PSA.

Article Type: Original Article

Section/Category: Prostate Cancer (PRO)

Keywords: microRNAs; Prostate Cancer; Prostate-Specific Antigen; Diagnosis; miR-183

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miR-183 in prostate cancer cells positively regulates synthesis and serum levels of PSA.

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Keywords: microRNAs, prostate cancer, Prostate-Specific Antigen, diagnosis, miR-183

Word count of text: 2805

Word count of abstract: 274

Abstract

Background: Factors affecting serum PSA levels in men are clinically important, but, apart from effects mediated through the androgen receptor, poorly understood.

Objective: To investigate whether microRNA (miRNA) affects synthesis and serum levels of PSA.

Design, setting and participants: Reporter assays with PSA and KLK2 3'UTRs to confirm post-transcriptional regulation was followed by high-throughput screening of 1129 miRNAs effect on PSA levels, using reverse phase protein arrays (RPPA), to identify individual regulatory miRNAs. The candidate miRNAs were investigated further *in vitro* by western blot, immunoflourometric, activity assays, qRT-PCR, reporter assays and growth assays, and prostate levels of miR-183 was compared to PSA transcript and serum PSA levels in prostate cancer cohorts.

Outcome measurements and statistical analysis: RankProd was used to evaluate the RPPA and Student's t test was used for the *in vitro* experiments. In the patient material, Spearman's and Cuzick's tests were used and overall survival analysed by the Kaplan-Meier and Log-Rank analysis.

Results and limitations: Gain-of-function screenings identified 32 miRNA increasing PSA levels. One of these, miR-183, was found to directly bind the 3'UTR of PSA and increase both protein and mRNA levels, and prostatic levels of miR-183 and serum PSA showed correlation in a cohort of 74 men. In addition, miR-183 promotes cellular growth *in vitro* and correlates to clinical parameters such as WHO grade and clinical progression.

Conclusions: The synthesis and serum levels of PSA are directly affected by mir-183 and may be a factor to take into consideration when PSA values are evaluated in clinical settings.

Patient summary: These findings give novel insight into the regulation of PSA and may eventually affect the clinical decision making of prostate cancer.

1. Introduction

Each year around 900.000 men are diagnosed with prostate cancer [1]. Most of them are discovered due to raised serum levels of prostate specific antigen (PSA). The PSA gene, *KLK3*, and the related *KLK2* are androgen regulated and highly expressed in the epithelial cells of the prostate. The levels of PSA in the prostate is reported to be over 100 times higher than KLK2, while PSA transcripts are only twice as abundant as *KLK2* [2-4]. The discrepancy in protein-to-transcript ratios has been ascribed to a lower stability of KLK2 [5, 6]. When the PSA test was introduced in clinical practice in the mid-nineties it resulted in an increased prostate cancer incidence, notably without the equivalent decrease in mortality rates. In fact, over detection was estimated to be approximately 50% in the European Randomized Study of Screening for Prostate Cancer [7], leading to overtreatment and decreased quality of life for many men. Thus, there is a need to improve the accuracy of the PSA test both in detection and subsequent prognostication of prostate cancer. This might be achieved by learning more about factors affecting PSA synthesis and secretion.

MicroRNAs (miRNAs) are short regulatory non-coding RNAs reported to be altered in all malignant tissues investigated, including the prostate [8]. Typically, miRNAs bind the 3'UTR of its target mRNA, causing reduction of protein expression. However, there are reports showing that miRNAs can also lead to increased protein expression [9, 10].

In this study, we investigate if miRNAs regulate the expression of PSA and KLK2 using 3'UTR reporter assays and gain-of-function screening with a miRNA library. A miRNA, mir-183, identified in the screen is demonstrated to directly target the 3'UTR of PSA and to co-variate with serum PSA levels in a patient cohorts.

Material and methods

2.1 In vitro manipulations

Prostate cancer cell lines DU145, PC3, 22Rv1, LNCaP clone FGC (ATCC), LNCaP (from DSMZ for the screening), LAPC-4 and LNCaP-ARhi were cultured according to the manufacturer's recommendations. RevertAid cDNA synthesis kit (Thermo Scientific, Wilmington, DE) was used for cDNA production and *AR*, *KLK2*, *KLK3* and *GAPDH* transcripts were quantified by TaqMan qPCR (Life Technologies, primersHs00171172_m1, Hs00428384_g1, Hs02576345_m1 and Hs02758991_g1). free PSA (fPSA) and total PSA (tPSA) were measured with DELFIA Prostatus™ PSAF/T (Perkin-Elmer Life Sciences, Turku, Finland) [11]. KLK2 was measured as described by Vaisanen *et al.* [13]. Total protein

in the cell lysate measured by Bradford protein assay (Bio-Rad, Hercules, CA) was used for normalisation. Cells were transiently transfected with miRIDIAN microRNA Mimic (120 pmole/ml, Dharmacon, Lafayette, CO), miRCURY LNA Knockdown probes (150 pmole/ml, Exiqon, Copenhagen, Denmark) or 160 pmole/ml Dicer ON-TARGETplus SMARTpool siRNA (Dharmacon) using Oligofectamin reagent (Life Technologies, Carlsbad, CA). Control experiments were performed in parallel with miRIDIAN microRNA Mimic Negative Control (Dharmacon) or scramble-miR (Exiqon). Transfections of miRNAs and 600 pmole/ml of LNA target site blockers (TSB) (Exiqon) designed to bind the predicted miR-183 target site in the 3'UTR (TSB-A) and a random site (TSB-B) of PSA, and a site in MET (TSB-C) (Suppl. Fig.1), were done using Lipofectin (Life Technologies) and f and tPSA measured after 72 hours. Western blots were probed with antibodies against PSA (in house rabbit polyclonal [12]), AR (N-20, SC-816, Santa Cruz Biotechnology, TX), GAPDH (MAB374, Chemicon, Temecula, CA) and HRP coupled polyclonal secondary antibodies (mouse or rabbit, Dako). All experiments were performed in triplicate.

2.2 High-throughput screening

LNCaP and LAPC-4 were reverse transfected in duplicate with 20 µmol/mL Pre-miR miRNA Precursor library v2 (Ambion, Austin, TX, 319 molecules) or miRIDIAN microRNA Mimic Libraries v10.1 (Dharmacon, Lafayette, CO, 819 molecules) as described previously [13]. PSA expression was detected at 48 and 72 hours, by staining RRPA slides with PSA antibody (#A0562, Dako Cytomation, Denmark) overnight. The results were normalized using the Loess method and $\log_2$ transformed, after which RankProd was used to identify regulating miRNAs [14].

2.3 Patients and miRNA quantification

Cohort 1 (49 prostate cancer and 25 without) obtained by transurethral resection of the prostate has been described previously [15] and in Suppl. Tab. 1. Cohort 2 consists of FFPE cores obtained from radical prostatectomies from 122 men with prostate adenocarcinoma collected at Malmö University hospital 1999 – 2002. Selected core from 28 patients did not contain cancer cells and were discarded; the remaining 94 specimens are described in Suppl. Tab. 1. Ethical approvals have been obtained from the Regional ethical review board, Lund, Sweden and we adhere to the Helsinki Declaration. RNA was extracted from the prostatic tissue in cohort 1 and 2 and miR-183 quantified as previously described [16, 17]. The miR-183 level was normalised against the geometrical mean of RNU47, RNU48 and RNU66, the selection based on a previous screen [17]. These non-coding RNAs also served as control for the RNA integrity. For external validation of our results we analysed a data set

from Taylor *et al.* constituting 110 prostate cancer samples and 28 non-malignant adjacent benign prostate samples [18].

2.4 Functional assays

The 3'UTRs of *KLK3* (637 nt) and *KLK2* (672 nt) were cloned into pMIR-REPORTER (Life Technologies) and the identity of the inserts confirmed by DNA sequencing. 22Rv1 and LNCaP cells were transfected as described above, and a pRL Renilla firefly construct served as normaliser. Luciferase activity was measured 72 h after transfection with Dual-Glo Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's instructions, and measured in triplicate on a Wallac 1420 Victor2^{TM} (Perkin Elmer). The Sulforhodamine B (SRB) colorimetric assay was performed as described previously [19].

2.5 Statistical analysis

Student's t test was used for the *in vitro* experiments and the Rank Product method was used to evaluate the RPPA. Spearman's rank was used to compare PSA to miR-183 expression, Cuzick's trend test was used to analyse miR-183 expression and WHO status, and overall survival was analyzed using Kaplan-Meier curves, and statistical significance was assessed with the Log-Rank method. Levels of statistically significance were set at $p < 0.05$.

3. Results

3.1 PSA is post transcriptionally regulated

We could verify and expand earlier findings that the protein/transcript ratios were drastically higher for PSA than KLK2 in several cell lines (Fig. 1A). To test whether translational blocking by miRNAs decrease protein levels, the 3'UTRs of PSA and KLK2 were cloned to reporter vectors and introduced into LNCaP cells. Surprisingly, the introduction of KLK2 3'UTR had no effect, whereas the PSA 3'UTR significantly increased luciferase levels ($p=0.002$, Student's t test, Fig. 1B). To determine if the increased translation was due to miRNAs, the cells were treated with siRNAs directed towards Dicer, as all miRNAs (except miR-451) undergo Dicer-dependent maturation. The increased luciferase levels caused by the PSA 3'UTR was significantly decreased when adding siRNAs against Dicer, implicating post-transcriptional regulation by miRNAs (Fig 1C).

3.2 Systematic analyses of miRNAs regulating PSA levels

High-throughput RPPA miRNA screens was conducted to identify individual miRNAs influencing the levels of PSA in two androgen dependent prostate cancer cell lines at two time points. The cells were transfected with two miRNA mimic libraries containing a total of 1129 molecules. The result is consistent over time in LNCaP cells while more varying in the LAPC-4, as expected as the PSA levels are close to the detection limit in the latter. The subsequent analyses were hence based on the results from the LNCaP cell line. We identified a total of 62 unique miRNAs having effect on PSA expression; 32 upregulated and 30 downregulated PSA levels (FDR p-value < 0.05; Fig. 2, Supp. Table 2 and 3). None of the miRNAs increasing PSA had been identified to target AR in an earlier RPPA screen performed in the same setting [20]. However, among the miRNAs decreasing PSA levels, 40% had been identified to target AR, indicating that the reduction in PSA levels could be secondary to an effect mediated by the AR regulation.

3.4 miR-183 increase mRNA and protein levels of PSA

Three miRNAs increasing PSA levels and predicted to target KLK3; miR-183, predicted to target the 3'UTR and upregulated in prostate cancer patients [17], miR-650 predicted to target the CDS and promotes tumourigenicity in gastric cancer [21] and miR-423 predicted to target the promoter and increased in metastatic breast cancer patients [22] were selected for further validation. Both secreted and intracellular levels of tPSA and fPSA were increased in prostate cancer cells upon transfection with mimic-miR-183, mimic-miR-423, mimic-miR-650 compared to the scramble control (Fig 3A-B and Suppl. Tab. 4). The effect was reversed when blocking miR-183 (tPSA p = 0.004, Fig. 3C and Suppl. Tab. 4), but not miR-423 nor -650 (results not shown),indicating that the latter two does not regulate PSA levels in this setting. Based on this, only miR-183 was selected for further investigation. In Western blotting miR-183 significantly upregulated PSA in 22Rv1 and LNCaP cells (p = 0.04 and 0.0002 respectively, Fig 3D). Further, miR-183 does not change the proteolytic activity of PSA (Suppl. Fig. 2), also supported by the unchanged f/tPSA ratio (Suppl. Table 4). Ectopic expression of miR-183 also increased the transcript levels of PSA, (p = 0.01 in 22Rv1 and p = 0.0001 in LNCaP, Fig. 3E). This effect is not mediated through the AR, as miR-183 does not affect AR transcript nor protein levels (Suppl. Fig. 3), confirming that miR-183 act though post-transcriptional mechanisms. A luciferase reporter assay verified that miR-183 bind directly to the 3'UTR of PSA in both LNCaP and 22Rv1 cells (Fig.4A and B). Next, a TSB was designed to prevent miR-183 to access the site predicted using the algorithm RNA22 (TSB-A). The increased levels of PSA induced by miR-183 was abolished

when co-transfecting with TSB-A (tPSA, p = 0.02, Fig. 4C, fPSA p= 0.01, Suppl. Fig. 4). Introduction of the TSB-A on its own did not have effect on the PSA levels compared to the control TSBs or non-transfected cells indicating that the effect is specific. In the KLK2 3'UTR the seeding site of the miR-183 binding is mutated, hence it is possible that miR-183 binding to this site can explain at least part of PSAs higher protein/transcript ratio.

3.5 Correlation to serum PSA and clinical parameters

Next, we set out to investigate miR-183 regulation of PSA in clinical material. In cohort 1, the miR-183 expression level in the prostate was positively correlated to patient serum PSA level (Spearman rho = 0.35, p = 0.005, Fig.5A). It is noteworthy that in this cohort, two men with untreated prostate cancer have miR-183 levels below the median and PSA levels less than 3.0 ng/ml and hence could have been missed in a PSA screen. To support the correlation found in cohort 1, an external dataset constituting a cohort of 138 prostate specimens was analysed. The analysis of this dataset showed a positive correlation between miR-183 and the PSA encoding transcript (Spearman rho = 0.2, p = 0.02, Fig. 5B) [18]. Further, there is no correlation between miR-183 and AR in the patient cohort 1 supporting the *in vitro* findings that miR-183 is acting by post-transcriptional regulation. The expression of miR-183 is significantly increased with WHO grade (p = 0.03, Cuzick's trend test, Fig. 5C). In line with this miR-183 induced cell growth in LNCaP, PC3, and DU145 (p = 0.046, 0.00085, and 0.0016, Fig. 6). Conversely, inhibiting endogenous miR-183 decreased cell growth in all cell lines (Fig. 6E-H). We have identified one of the targets of miR-183 contributing to this phenotype: transient expression of miR-183 decrease FOXO1 as shown by western blot in LNCaP (p = 0.006, Suppl. Fig 5). The ability of miR-183 to predict clinical progression, defined as biochemical recurrence, prostate cancer related death or adjuvant therapy after diagnosis was further analysed in the larger cohort 2. Patients were divided into two groups according to expression of miR-183 and clinical progression analysed by Kaplan-Meyer curves. We found a hazard ratio of 2.9 for earlier clinical recurrence in patients with high miR-183. For patients with low to medium miR-183 levels, less than 50% experienced clinical progression, whereas the median time to clinical progression for patients with high levels was 11.15 years (p = 0.02, Log-Rank, Fig. 5D). As comprehensive pre-treatment PSA data was not available, the independence to PSA could not conclusively be investigated.

4. Discussion

In this study we address the post-transcriptional regulation of PSA by miRNA. We show a 3'UTR dependent upregulation of PSA, mediated by miRNA, as demonstrated by decreased reporter activity

upon siRNA targeting of Dicer. In a systematic gain-of-function screening 62 individual miRNAs were identified to regulate PSA levels. Of these, miR-183 was further studied, and confirmed to increase PSA levels, target the 3'UTR and to correlate to serum PSA. To our knowledge, this is the first report of miRNAs directly regulating PSA levels. As PSA is currently used in clinical practice throughout the world for detection and monitoring of prostate cancer, it is of high importance to enhance the understanding of the mechanisms regulating this molecule, as *e.g.* men with prostate cancer and low miR-183 levels might go undetected in a PSA-based screening.

The regulatory effect of miR-183 on PSA is mediated through binding a specific site in the 3'UTR*,* but the exact mechanism, whether it pertains to mRNA half-life or translation efficiency, remains to be elucidated. It is of interest to note that the effect on PSA mRNA level is less pronounced than the effect on protein level, and the correlation between miR-183 and PSA encoding transcript is also weaker than to the protein. These findings indicate that possibly both mechnisms are affected. Several miRNAs has been reported to bind the 3'-UTRs causing increased translation their targets [9, 23, 24], a mechanism that has been suggested is that the miRNA-Argonaute complex can recruit the RNA binding protein FXR1 to the mRNA and activate translation during cell cycle arrest. Previously, we and others have shown that miR-183 is upregulated in prostate cancer tumours [17, 25, 26]. Here, we show that miR-183 levels significantly correlates with increasing WHO grade and that high levels correspond to earlier clinical progression, suggesting oncogenic properties and confirming earlier findings [26]. In line with this, we found that miR-183 increase cell growth *in vitro*, and we identified a potential mechanism through FOXO1 regulation, confirming earlier findings in endometrial cancer and Hodgkin lymphoma [27, 28]. It is possible that in addition to directly regulate PSA, miR-183 also indirectly increases the PSA levels by increasing the proliferation of the prostate cancer cells.

Not only is the intracellular PSA level regulated by miR-183, but also the secreted portion is affected, suggesting that miR-183 can influence serum PSA levels. Substantiating the functional data, we found a positive correlation between miR-183 and serum PSA levels in a prostate cancer cohort, something that is also confirmed in an earlier study [26]. In current clinical practice the level of tPSA is used to monitor prostate cancer, but decreased fPSA/tPSA has been proposed to be associated with more aggressive cancer [29], therefore it is noteworthy that miR-183 does not change this ratio.

Conclusions: To identify factors that influence the levels of PSA in serum and thus increase the accuracy of this test can lead to less unnecessary invasive diagnostic procedures and reduce over diagnosis. This study identifies miR-183 as a regulator of PSA levels in prostate cancer cells and in serum. As such, the individual's miR-183 levels might influence current prostate cancer detection and monitoring.

Acknowledgement

We thank Margareta Persson for her work on the second cohort of prostate cancer patients and Elise Nilsson for preparing prostatic tissue samples. Rami Mäkelä and Suvi-Katri Leivonen are acknowledged for participation in the miRNA gain-of-function functional screening of prostate cancer cell lines. We are grateful to Professor Charles Sawyer, Los Angeles for the LAPC-4 cells, Professor Tapio Visakorpi, Tampere, Finland for the LNCaP-ARhi, Professor Rönnstrand, Lund University for the pRL Renilla firefly construct, Dr. Carlos Rovira, Lund University for the siRNA against Dicer. The research leading to these results has received funding from the European Union Seventh Framework Programme (*FP7/2007-2013*) under grant agreement n°*201438*, the Swedish Research council, Gunnar Nilssons Cancer foundation, and Jeanssons foundation.

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Figure legends

Figure 1. A) Protein and transcript levels of PSA (tPSA) and KLK2 were measured by immunofluorometry (tPSA/total protein) and qRT-PCR (*KLK3*/*GAPDH*) in prostate cell lines and presented as a protein/transcript ratio. **B)** When comparing firefly luciferase activity in LNCaP cells transfected with the empty vector, the vector containing the 3'UTR of PSA, or KLK2, only the 3'UTR of

PSA upregulate luciferase activity. **C)** The increase in protein level is significantly reduced upon cotransfection with siRNA against Dicer, suggesting involvement of miRNA.

Figure 2. Lysate micro array, LMA. LAPC-4 and LNCaP cells were reverse transfected with 1129 miRNA molecules. The lysates was collected at 48 and 72 hours after transfection and printed on nitro-cellulose covered glass slides and then stained with a PSA antibody. The staining intensity was normalized to total protein content (Sybro blot staining). Here, the data is presented using median and MAD from R/Bioconductor (v.2.9) with standard options. Red represents increases in PSA, whereas blue indicates decrease. The scale is Z-score standardized values (+/−4 *SD*).

Figure 3. A) To confirm the LMA, three miRNAs were selected (miR-183, 423, and 650) for further *in vitro* experiments. In LNCaP cells all three miRNAs upregulate secreted tPSA and **B)** intracellular tPSA. **C)** By blocking miR-183 in LNCaP cells intracellular tPSA decrease. **D)** 22Rv1 and LNCaP were transiently transfected with mimic-miR-183 or a scramble control. By western blot we find that miR-183 upregulate PSA in both cell lines. GAPDH was used as loading control. **B)** The PSA encoding transcript was also increased as measured by qRT-PCR. The result was normalized against *GAPDH*. Along with the reverse transcription and the qRT-PCR, a no enzyme negative control and a no template control were run to exclude PCR or genomic DNA contamination.

Figure 4. 22Rv1 **(A)** and LNCaP **(B)** were co-transfected with a luciferase reporter vector containing the 3'UTR of PSA and mimic-miR-183 or scramble control. In both cell lines miR-183 upregulate the firefly luciferase protein level suggesting miR-183 binding to the 3'UTR. **C)** Target site blockers (TSB) were designed to bind a predicted miR-183 binding site (TSB-A), a random control site in the coding part of PSA (TSB-B), and MET mRNA (TSB-C). Here, we show that in LNCaP, only miR-183 on its own upregulate the tPSA expression. Co-transfecting mimic-miR-183 with TSB-A rescue this effect. All results are normalised to a co-transfected Renilla firefly construct to compensate for variation in transfection efficiency and cell number.

Figure 5. Clinical parameters. **A)** We find that patient serum tPSA levels are correlated to miR-183 expression in prostatic tissue (cohort 1) as measured by by immunofluorometry and qRT-PCR respectively. **B)** As an external validation we downloaded the dataset conducted by Taylor et al. [18] and found that the transcript of *KLK3* and miR-183 significantly correlates. **C)** In cohort 1, there is a significant trend of increasing miR-183 related to WHO grading. **D)** In cohort 2, constituting 94 men

with adenocarcinoma, we find that clinical progression occurs earlier in patients with high miR-183 compared to low.

Figure 6. Cell growth was measured by staining transfected cells with SRB three days after transfection. We compared mimic-miR-183 to a scramble control, and anti-miR-183 to anti-scramble control. Transient overexpression of miR-183 lead to an increased cell growth in LNCaP **(A)**, DU145 **(B)**, and PC3 **(C)**, but in 22Rv1 **(D)** there was no difference compared to the scramble control. Blocking miR-183 decreased cell growth in all cell lines **(E – H)**.

Supplementary figure 1. The cloned 3'UTR of *KLK3* consist of 637 nucleotides. An *in silico* prediction of miR-183 binding site was done and is underlined in the sequence. The details are shown below the sequence. The design of the target site blockers is shown as well, underlined nucleotides are LNA modified. Binding site of TSB-A is shown in bold in the sequence.

Supplementary figure 2. 22Rv1 cells were transfected with mimic-miR-183 or a scramble control. PSA activity is analyzed by measuring the consumption of a flourogenic substrate consisting of a tetrapeptide conjugated to aminomethylcoumarin (SSYY-AMC).

Supplementary figure 3. 22Rv1 and LNCaP cells were transfected with mimic-miR-183 or a scramble control. RNA was collected three days after transfection and AR levels measured by qRT-PCR and normalized to GAPDH transcript levels. miR-183 do not have an effect on AR levels in neither cell line.

Supplementary figure 4. Target site blockers (TSB) were designed to bind a predicted miR-183 binding site (TSB-A), a random control site in the coding part of PSA (TSB-B), and MET mRNA (TSB-C). In LNCaP, only miR-183 on its own upregulates the fPSA expression, but co-transfecting mimic-miR-183 with TSB-A rescue this effect. All results are normalised to a co-transfected Renilla firefly construct to compensate for variation in transfection efficiency and cell number.

Supplementary figure 5. By western blot we find that transiently overexpressed miR-183 decrease the levels of FOXO1 in LNCaP cells. GAPDH was used as loading control.

Supplementary table. 1 Patient and clinicopathologic information about cohort 1 and 2. ND = no data.

Supplementary table 2. miRNAs decreasing (down) or increasing (up) the expression of PSA. Abbreviations: RP/Rsum, RankProduct/Ranksum; FC, Fold Change; FDR q-value, False Discovery Rate multiple testing corrected q-value. Cut-off used in the analysis was FDR q-value < 0.05.

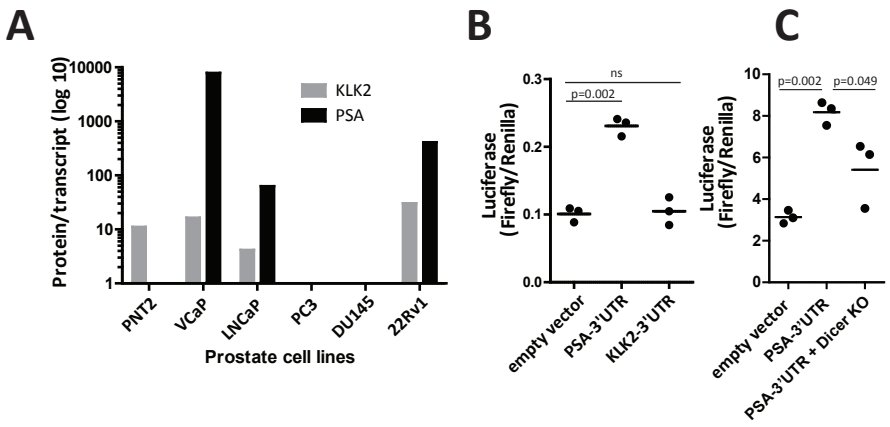
Supplementary table 3. Raw and processed data of PSA protein levels and Sypro (total protein expression standard) using the Dharmacon miRIDIAN miRNA mimic library (mirBase v. 10.1). Columns: ID_ref, identifier; MIMAT_id, miRbase mature miRNA identifier; miRNA_Gene_Name, official name of the miRNA (mirbase v10.1), miRNA_Mature_Name, official miRbase mature name; mirbase10.1_seq, miRBase v. 10.1mature miRNA sequence; Product Id, Dharmacon product identifier; Product Name, Dharmacon product name; Library Name, Dharmacon library name; Plate Description, plate description including the lot number; Well, 384-well plate well location; Library-Plate, library and plate identifiers together. Descriptions of the data column data types are as follows. Raw: measured values from staining the slides with PSA antibody (#A0562, Dako Cytomation) overnight and the Sypto total protein stain. Int: log2 of the average level the raw values for PSA and Sypro (used for loess normalization). Rat: log2 loess-normalized ratio between PSA and Sypro stains, positive indicates a higher than average level of PSA, negative lower than average level. Zrat: z-score of the ratio calculated using median absolute deviation (MAD) to calculate standard deviation. Z-scores are used in further analysis and visualizations.

Supplementary table 4. Free and total PSA (PSA and tPSA) in LNCaP and 22Rv1 cells transfected with mimic-miRNAs or antisense-miRNAs. The average and one standard deviation (STDV) of triplicate experiment are shown. Student's t test p-values are comparing the transiently overexpressed or blocked miRNAs to respectively control. ND = no data

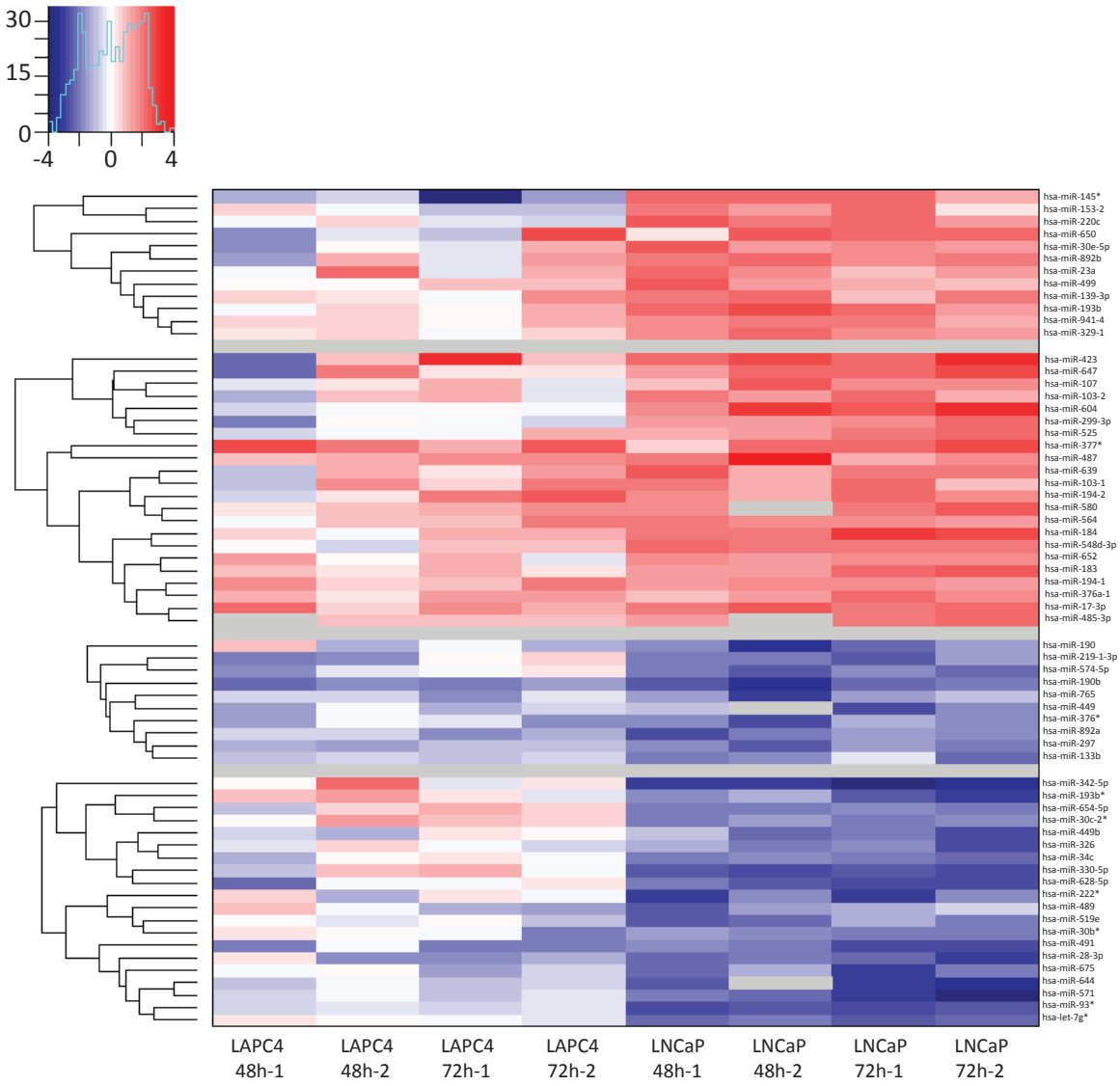
***Take Home Message**

The synthesis and serum levels of PSA are directly affected by mir-183 in prostate cancer tissue, thus the levels of this miRNA in the prostate may affect the clinical decision making of prostate cancer.

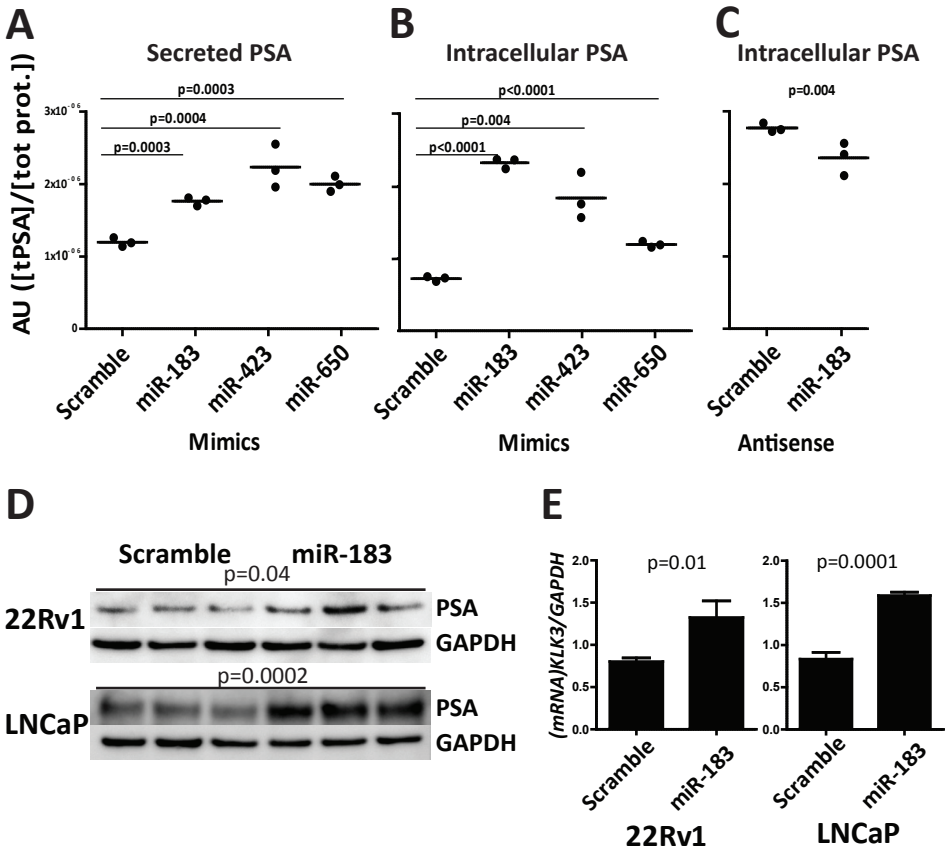
Illustration



Illustration



Illustration



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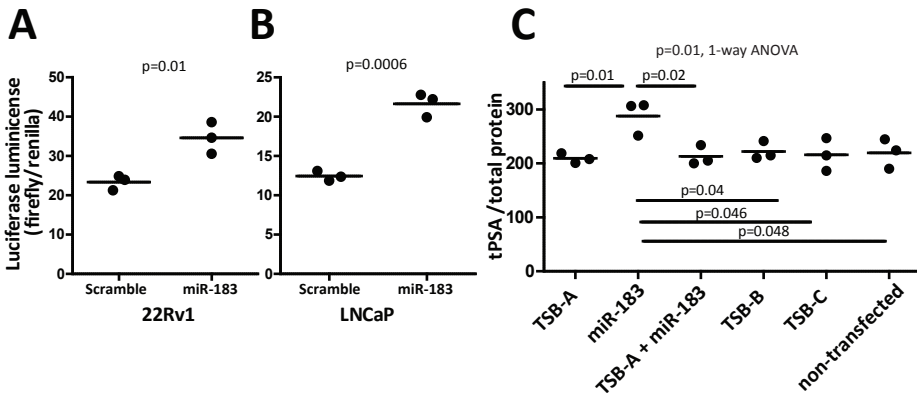
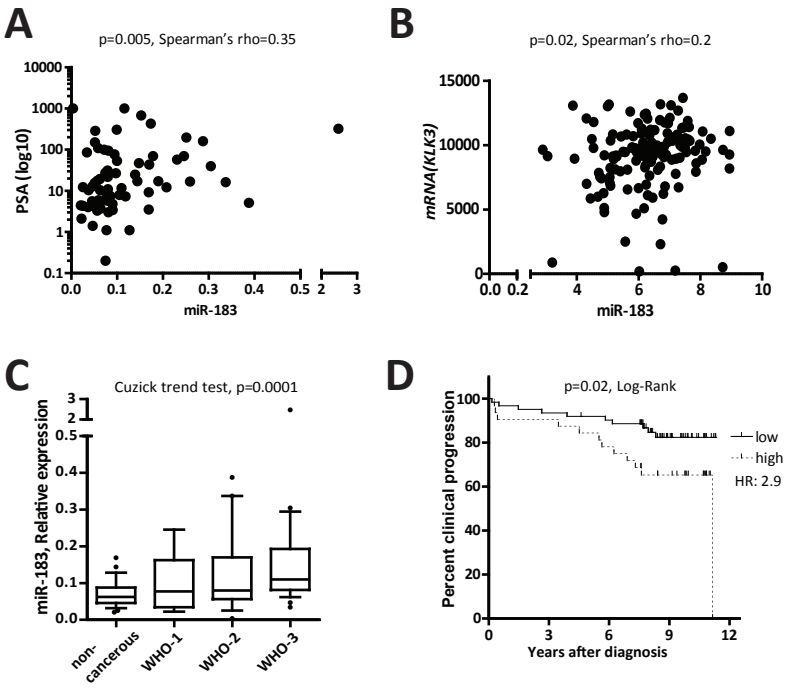


Illustration



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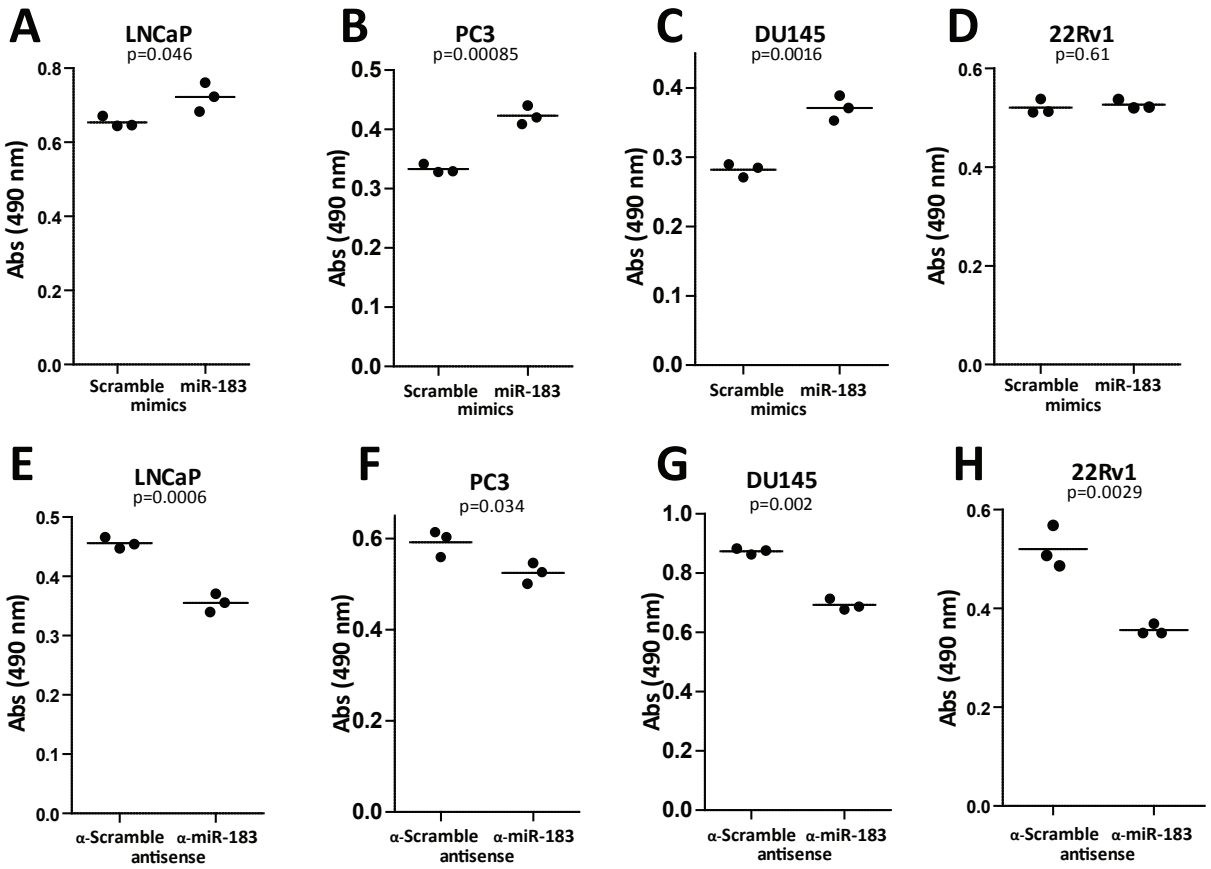


Illustration

>*(mRNA)KLK3* 3′UTR
GCACCCCTATCAACCCCCTATTGTAGTAAACTTGGAACCTTGGAAATGACCAGGCCAAGACTCAAGCC
TCCCCAGTTCTACTGACCTTTGTCCTTAGGTGTGAGGTCCAGGGTTGCTAGGAAAAGAAATCAGCAGA
CACAGGTGTAGACCAGAGTGTTTCTTAAATGGTGTAATTTTGTCCTCTCTGTGTCCTGGGGAATACTG
GCCATGCCTGGAGACATATCACTCAATTTCTCTGAGGACACAGATAGGGATGGGGTGTCTGTGTTATT
TGTGGGTACAGAGATGAAAGAGGGGTGGGATCCACACTGAGAGAGTGGAGAGTGACATGTGCTGGACA
CTGTCCATGAAGCACTGAGCAGAAGCTGGAGGCACAACGCACCAGACACTCACAGCAAGGATGGAGCT
GAAAACATAACCCACTCTGTCCTGGAGGCACTGGGAA**GCCTAGAGAAGGCTGTGAGCC**AAGGAGGGAG
GGTCTTCCTTTGGCATGGGATGGGGATGAAGTAAGGAGAGGGACTGGACCCCCTGGAAGCTGATTCAC
TATGGGGGGAGGTGTATTGAAGTCCTCCAGACAACCCTCAGATTTGATGATTTCCTAGTAGAACTCAC
AGAAATAAAGAGCTGTTATACTGTG

GG-GAAGCCTAGAGAAG-GCTGTG (underlined in sequence above)
   ||| ||| || ||||||
UCACUUAAGAUGG--UCACGGUAU miR-183 Folding energy = -21.400000 Kcal/mol (RNA22)

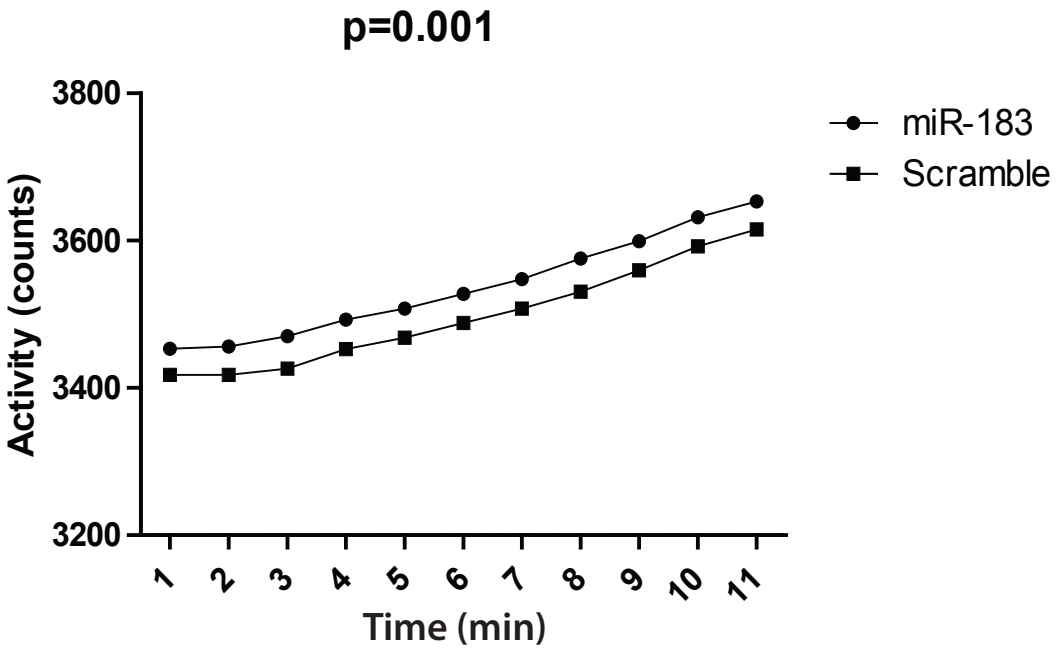
>TSB-A (underlined=LNA modified, binding site in **bold** in sequence above)
GGCACACAGCCTTCTCTAGGC

>TSB-B (underlined=LNA modified, binding site in coding region)
CAGGATGAGGGGTGCAGCAC

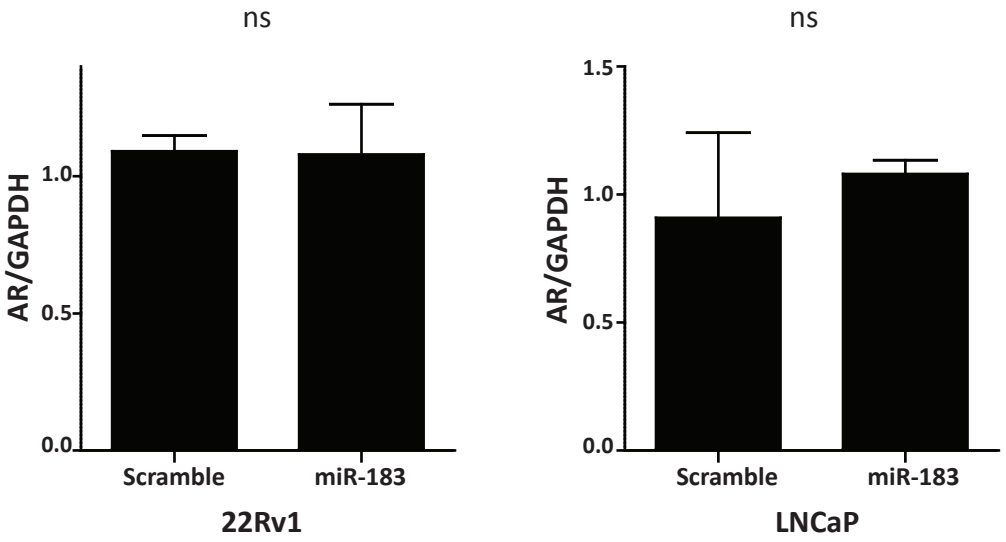
>TSB-C (underlined=LNA modified, binding site in MET mRNA)
TTAAAGGTCAGGCAGTGAA

Supplementary Figure 1.

Illustration

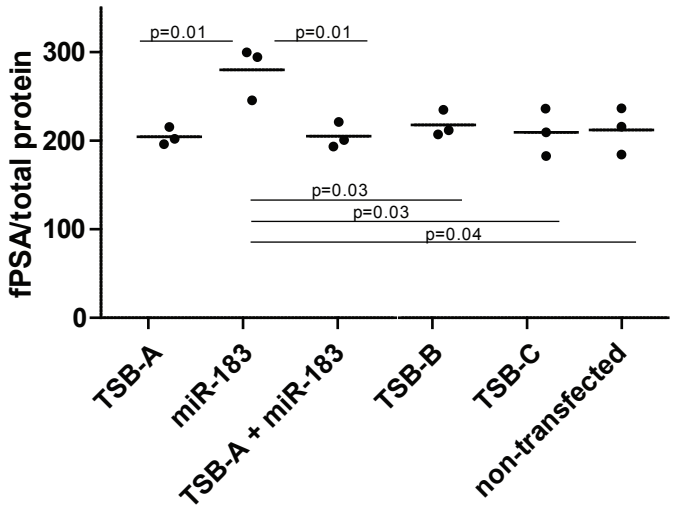


Illustration



Supplementary Figure 3.

Illustration



Supplementary Figure 4.

Table

Supplementary table 2. miRNAs influencing the level of PSA.

Name	Type	RP/Rsum*	FC	FDR q-value
hsa-miR-423	up	3.3504	1.773	0.0000
hsa-miR-604	up	3.3636	1.7588	0.0000
hsa-miR-184	up	6.5935	1.7032	0.0000
hsa-miR-193b	up	12.3203	1.5781	0.0000
hsa-miR-17-3p	up	12.5364	1.5761	0.0000
hsa-miR-647	up	12.5994	1.6056	0.0000
hsa-miR-220c	up	12.7104	1.5329	0.0000
hsa-miR-580	up	13.0757	1.5858	0.0000
hsa-miR-548d-3p	up	13.5381	1.5659	0.0000
hsa-miR-183	up	16.8104	1.5308	0.002
hsa-miR-487	up	17.0674	1.6722	0.0027
hsa-miR-145*	up	17.1446	1.5056	0.0025
hsa-miR-485-3p	up	19.0131	1.5288	0.0038
hsa-miR-892b	up	20.3034	1.5244	0.0043
hsa-miR-377*	up	20.3619	1.5344	0.0047
hsa-miR-639	up	21.2555	1.4884	0.0056
hsa-miR-650	up	21.3143	1.5048	0.0053
hsa-miR-103-2	up	26.5575	1.4668	0.0133
hsa-miR-30e-5p	up	26.9393	1.4536	0.0137
hsa-miR-194-2	up	27.7734	1.4679	0.015
hsa-miR-329-1	up	29.6362	1.4694	0.02
hsa-miR-139-3p	up	29.7318	1.4539	0.0191
hsa-miR-525	up	29.9519	1.4708	0.0191
hsa-miR-107	up	30.8096	1.4519	0.0204
hsa-miR-941-4	up	34.4416	1.4495	0.0288
hsa-miR-299-3p	up	36.9653	1.4441	0.0365
hsa-miR-103-1	up	37.6747	1.4163	0.0378
hsa-miR-652	up	38.0896	1.4351	0.0382
hsa-miR-564	up	38.3268	1.427	0.0372
hsa-miR-499	up	39.3062	1.3528	0.0398
hsa-miR-23a	up	40.5447	1.3857	0.0419
hsa-miR-153-2	up	41.3826	1.3833	0.0434
hsa-miR-376a-1	up	41.9894	1.4122	0.0439
hsa-miR-194-1	up	43.4205	1.4074	0.0482
hsa-miR-342-5p	down	2.3403	0.4771	0.0000
hsa-miR-644	down	2.9907	0.5134	0.0000
hsa-miR-571	down	6.0344	0.5418	0.0000
hsa-miR-93*	down	7.0682	0.569	0.0000
hsa-miR-222*	down	7.2257	0.597	0.0000
hsa-miR-330-5p	down	8.0301	0.5675	0.0000
hsa-miR-190b	down	9.118	0.6027	0.0000
hsa-miR-628-5p	down	12.1175	0.5854	0.0012
hsa-miR-28-3p	down	12.2031	0.602	0.0011
hsa-miR-491	down	15.839	0.6009	0.001
hsa-miR-190	down	16.1275	0.6354	9e-04
hsa-let-7g*	down	16.3465	0.6176	8e-04
hsa-miR-574-5p	down	19.6528	0.6445	0.0023
hsa-miR-675	down	20.0811	0.6442	0.0021
hsa-miR-519e	down	21.0021	0.6613	0.0033
hsa-miR-193b*	down	21.8113	0.6265	0.0038
hsa-miR-34c	down	22.1778	0.6503	0.0053
hsa-miR-892a	down	22.5654	0.6762	0.0078
hsa-miR-219-1-3p	down	25.4028	0.6576	0.0121
hsa-miR-367*	down	27.5606	0.6834	0.0165
hsa-miR-449	down	27.673	0.6576	0.0157
hsa-miR-326	down	28.0842	0.6576	0.0164
hsa-miR-449b	down	28.1555	0.651	0.0157
hsa-miR-297	down	30.026	0.6841	0.0183
hsa-miR-654-5p	down	31.1663	0.6843	0.0204
hsa-miR-765	down	31.375	0.6999	0.02
hsa-miR-30b*	down	33.0392	0.6849	0.0237
hsa-miR-30c-2*	down	37.8886	0.7003	0.0357
hsa-miR-133b	down	39.662	0.714	0.041
hsa-miR-489	down	42.2545	0.7335	0.0493

*RankProduct/Ranksum, FC; Fold Change, FDR q-value; False Discovery Rate q-value. Cut-off used FDR q-value < 0.05

Table

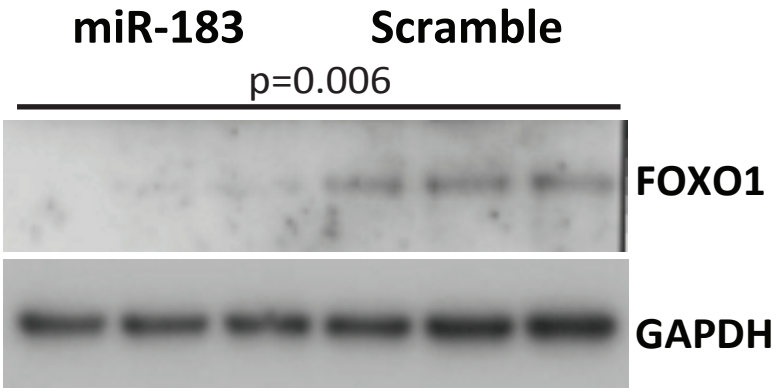
ID_ref	MIMAT_id	miRNA_Gene_Nan	miRNA_Mature_N	mirbase10.1_seq
DPM-1-A03	MIMAT0000062	hsa-let-7a-2	hsa-let-7a	UGAGGUAGUAGGUU
DPM-1-A04	MIMAT0001541	hsa-miR-449	hsa-miR-449	UGGCAGUGUAUUGU
DPM-1-A05	MIMAT0000064	hsa-let-7c	hsa-let-7c	UGAGGUAGUAGGUU
DPM-1-A06	MIMAT0001618	hsa-miR-191*	hsa-miR-191*	CAACGGAAUCCCAAA
DPM-1-A07	MIMAT0000068	hsa-miR-15a	hsa-miR-15a	UAGCAGCACAUAAUG
DPM-1-A08	MIMAT0001621	hsa-miR-369-5p	hsa-miR-369-5p	AGAUCGACCGUGUU
DPM-1-A09	MIMAT0000069	hsa-miR-16-1	hsa-miR-16	UAGCAGCACGUAAAU
DPM-1-A10	MIMAT0001625	hsa-miR-431	hsa-miR-431	UGUCUUGCAGGCCG
DPM-1-A11	MIMAT0000073	hsa-miR-19a	hsa-miR-19a	UGUGCAAAUCUAUG
DPM-1-A12	MIMAT0001627	hsa-miR-433	hsa-miR-433	AUCAUGAUGGGCUC
DPM-1-A13	MIMAT0000074	hsa-miR-19b-1	hsa-miR-19b	UGUGCAAAUCCAUG
DPM-1-A14	MIMAT0001629	hsa-miR-329-1	hsa-miR-329	AACACACCUGGUUA
DPM-1-A15	MIMAT0000075	hsa-miR-20a	hsa-miR-20a	UAAAGUGCUUAUAG
DPM-1-A16	MIMAT0001629	hsa-miR-329-2	hsa-miR-329	AACACACCUGGUUA
DPM-1-A17	MIMAT0000076	hsa-miR-21	hsa-miR-21	UAGCUUAUCAGACU
DPM-1-A18	MIMAT0002170	hsa-miR-412	hsa-miR-412	ACUUCACCUGGUCC
DPM-1-A19	MIMAT0000077	hsa-miR-22	hsa-miR-22	AAGCUGCCAGUUGA
DPM-1-A20	MIMAT0002171	hsa-miR-410	hsa-miR-410	AAUAUAACACAGAUG
DPM-1-A21	MIMAT0000078	hsa-miR-23a	hsa-miR-23a	AUCACAUUGCCAGG
DPM-1-A22	MIMAT0002172	hsa-miR-376b	hsa-miR-376b	AUCAUAGAGGAAAA
DPM-1-B03	MIMAT0003242	hsa-miR-577	hsa-miR-577	UAGAUAAAAUAUUG
DPM-1-B04	MIMAT0003302	hsa-miR-632	hsa-miR-632	GUGUCUGCUUCCUG
DPM-1-B05	MIMAT0003243	hsa-miR-578	hsa-miR-578	CUUCUUGUGCUCUA
DPM-1-B06	MIMAT0003303	hsa-miR-633	hsa-miR-633	CUAAUAGUAUCUAC
DPM-1-B07	MIMAT0003245	hsa-miR-580	hsa-miR-580	UUGAGAAUGAUGAA
DPM-1-B08	MIMAT0003304	hsa-miR-634	hsa-miR-634	AACCAGCACCCCAAC
DPM-1-B09	MIMAT0003246	hsa-miR-581	hsa-miR-581	UCUUGUGUUCUCUA
DPM-1-B10	MIMAT0003305	hsa-miR-635	hsa-miR-635	ACUUGGGCACUGAA
DPM-1-B11	MIMAT0003247	hsa-miR-582	hsa-miR-582	UUACAGUUGUUCAA
DPM-1-B12	MIMAT0003307	hsa-miR-637	hsa-miR-637	ACUGGGGGCUUUCG
DPM-1-B13	MIMAT0003248	hsa-miR-583	hsa-miR-583	CAAAGAGGAAGGUC
DPM-1-B14	MIMAT0003308	hsa-miR-638	hsa-miR-638	AGGGAUCGCGGGCG
DPM-1-B15	MIMAT0003249	hsa-miR-584	hsa-miR-584	UUAUGGUUUGCCUG
DPM-1-B16	MIMAT0003309	hsa-miR-639	hsa-miR-639	AUCGCUGCGGUUGC
DPM-1-B17	MIMAT0003250	hsa-miR-585	hsa-miR-585	UGGGCGUAUCUGUA
DPM-1-B18	MIMAT0003310	hsa-miR-640	hsa-miR-640	AUGAUCCAGGAACCU
DPM-1-B19	MIMAT0003251	hsa-miR-548a-3p-1	hsa-miR-548a	CAAAACUGGCAAUUA
DPM-1-B20	MIMAT0003311	hsa-miR-641	hsa-miR-641	AAAGACAUAGGAUA
DPM-1-B21	MIMAT0003252	hsa-miR-586	hsa-miR-586	UAUGCAUUGUAUUU
DPM-1-B22	MIMAT0003312	hsa-miR-642	hsa-miR-642	GUCCCUCUCCAAAUC
DPM-1-C03	MIMAT0000080	hsa-miR-24-2	hsa-miR-24	UGGCUCAGUUCAGC
DPM-1-C04	MIMAT0002174	hsa-miR-484	hsa-miR-484	UCAGGCUCAGUCCCC
DPM-1-C05	MIMAT0000084	hsa-miR-27a	hsa-miR-27a	UUCACAGUGGCUAA
DPM-1-C06	MIMAT0002175	hsa-miR-485-5p	hsa-miR-485-5p	AGAGGCUGGCCGUG
DPM-1-C07	MIMAT0000087	hsa-miR-30a-5p	hsa-miR-30a-5p	UGUAAACAUCCUCGA
DPM-1-C08	MIMAT0002176	hsa-miR-485-3p	hsa-miR-485-3p	GUCAUACACGGCUCU
DPM-1-C09	MIMAT0000088	hsa-miR-30a-3p	hsa-miR-30a-3p	CUUUCAGUCGGAUG
DPM-1-C10	MIMAT0002177	hsa-miR-486	hsa-miR-486	UCCUGUACUGAGCU
DPM-1-C11	MIMAT0000097	hsa-miR-99a	hsa-miR-99a	AACCCGUAGAUCCGA

Table

Supplementary table 2.

	Cell line	LNCaP			22Rv1		
	Transfected miRNA or control	Average	STDV	p-value	Average	STDV	p-value
Secreted free PSA / Tot. protein	Mimic-scramble	1,04E-06	2,31E-08	-	6,42E-06	3,57E-07	-
	Mimic-miR-183	1,65E-06	9,70E-09	<0.0001	9,31E-06	1,17E-06	0.01
	Mimic-miR-423	2,09E-06	2,64E-07	0.003	ND	ND	-
	Mimic-miR-650	1,77E-06	9,09E-08	0.0002	ND	ND	-
	α-scramble	2,09E-06	3,05E-08	-	ND	ND	-
	α-miR-183	1,67E-06	2,74E-07	0.056	ND	ND	-
Intracellular free PSA/ Tot. protein	Mimic-scramble	6,28E-07	5,27E-08	-	8,09E-05	4,64E-06	-
	Mimic-miR-183	2,23E-06	5,48E-08	0.0003	1,25E-04	1,25E-05	0.004
	Mimic-miR-423	1,72E-06	2,98E-07	<0.0001	ND	ND	-
	Mimic-miR-650	1,12E-06	6,78E-08	0.0006	ND	ND	-
	α-scramble	2,52E-06	5,37E-08	-	ND	ND	-
	α-miR-183	2,11E-06	1,86E-07	0.02	ND	ND	-
Secreted total PSA / Tot. protein	Mimic-scramble	1,19E-06	6,16E-08	-	1,53E-05	8,71E-07	-
	Mimic-miR-183	1,76E-06	5,88E-08	0.0003	2,22E-05	3,36E-06	0.03
	Mimic-miR-423	2,24E-06	2,97E-07	0.0004	ND	ND	-
	Mimic-miR-650	2,00E-06	1,03E-07	0.0003	ND	ND	-
	α-scramble	2,34E-06	1,31E-08	-	ND	ND	-
	α-miR-183	1,88E-06	1,89E-07	0.07	ND	ND	-
Intracellular total PSA/Tot. protein	Mimic-scramble	7,22E-07	3,31E-08	-	1,71E-04	9,24E-06	-
	Mimic-miR-183	2,32E-06	7,20E-08	<0.0001	2,63E-04	2,20E-05	0.003
	Mimic-miR-423	1,84E-06	3,21E-07	0.004	ND	ND	-
	Mimic-miR-650	1,19E-06	3,94E-08	<0.0001	ND	ND	-
	α-scramble	2,77E-06	6,05E-08	-	ND	ND	-
	α-miR-183	2,36E-06	2,25E-07	0.004	ND	ND	-

Illustration



Table

Supplementary Table 1.

	Cohort 1	Cohort 2
WHO grade	(n)	(n)
Grade I	5	6
Grade II	19	59
Grade III	25	28
Unknown		1
Gleason Sum	(n)	(n)
5		16
6		35
7		35
8		2
9		5
Unknown	49	1
Clinical stage	(n)	(n)
T1	9	44
T2	22	49
T3	14	1
T4	3	-
Unknown	1	-
Pre-treatment PSA (ng/ml)	n=46	n=73
Median (range)	27 (0.2 – 428)	12 (2.6-36.5)
Occurrence of metastasis	(n)	(n)
M0 = no metastasis	17	89
M1 = having metastasis	18	5
Mx = not investigated since no suspicion at the time	10	-
Unknown	4	-
Cancer in tissue (%)		
Mean (range)	42 (5 – 90)	ND
Clinical Recurrence	ND	(n) 22
Age at treatment		
Mean (range)	76 (63-89)	62 (48-73)
Non-cancerous patients	(n) 25	(n) 0
PSA (n=19)		
Median (range) ng/ml	5.9 (1.1-19.4)	