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miR-145 suppress the androgen receptor in prostate cancer cells and correlates to prostate cancer prognosis

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Abstract

Androgen signalling through the androgen receptor (AR) is essential for prostate cancer initiation, progression, and transformation to the lethal castration resistant state. The aim of this study was to characterize the mechanisms by which miR-145 deregulation contribute to prostate cancer progression. The miR-145 levels, measured by qRT-PCR, were found to inversely correlate with occurrence of metastases, survival and androgen deprivation therapy response in a well-characterised prostate cancer cohort. Introduction of ectopic miR-145 in prostate cancer cells generated an inhibitory effect on the AR at both transcript and protein levels as well as its activity and downstream targets PSA, KLK2, and TMPRSS2. The regulation was shown to be mediated by direct binding using Ago2 specific immunoprecipitation, but there was also indication of synergetic AR activation. These findings were verified in clinical prostate specimens by demonstrating inverse correlations between miR-145 and AR expression as well as serum PSA levels. In addition, miR-145 was found to regulate androgen dependent cell growth *in vitro*. Our findings put forward novel possibilities of therapeutic intervention, as miR-145 potentially could decrease both the stem cells and the AR expressing bulk of the tumour and hence reduce the transformation to the deadly castration resistant form of prostate cancer.

Summary

The miR-145 regulates the Androgen receptor and affect key androgen regulated proteins such as PSA *in vitro* and in patient cohorts. In concordance, miR-145 levels inversely correlate with occurrence of metastases, survival and castration resistance in prostate cancer patients.

Background

Two decades ago, the first microRNA (miRNA) was described in nematodes (1). Today, 2578 mature human miRNAs are annotated in the miRNA database, miRBase (2,3), and it is estimated that 30% of the human gene expression is regulated by miRNAs, including most fundamental intracellular processes (2,4). The miRNAs constitutes a class of highly conserved small, on average 22 nucleotides long, non-coding RNAs, that provide post transcriptional regulation. When the miRNA is incorporated in the RNA inducing silencing complex, it can bind to target transcripts and for example impede translation (5,6). The miRNA profile has been found to be distorted in all types of cancer investigated to date. The resulting changes of the cellular proteome may contribute to cancer pathogenesis, and indeed, miRNAs are strongly implicated in the pathogenesis of numerous malignancies, and thus hold promise both as diagnostic tools and therapeutic targets.

Annually, close to 400,000 new prostate cancer cases are diagnosed in Europe and it is the third leading cause of cancer related deaths in European men (7), with both the highest incidence and mortality found in Northern Europe. The androgen receptor (AR) is a nuclear hormone receptor and a transcription factor that is vital for normal prostate function; it contributes to luminal epithelial growth, inhibits apoptosis, and influence prostate cancer progression. Androgen deprivation therapy, either chemical or surgical castration, is the gold standard treatment for advanced prostate cancer; this induces apoptosis of prostate cancer cells resulting in tumour regression. However, the majority of tumours relapse and develops into castration resistant prostate cancer (CRPC) within 12 – 18 months. In CRPC, the AR or AR signalling has adapted to the lack of androgens or alternative survival and growth pathways have been activated. Mechanisms underlying adaptation of the AR pathway can be increased expression of the AR, increased local production of androgens, hypersensitivity or constitutively active truncated forms of the AR, promiscuity and/or ligand independent activation through kinase crosstalk (8). It is fundamental to increase the knowledge of how CRPC tumours progress so that patient morbidity can be reduced by newly developed therapies.

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In previous studies, we and others have observed downregulation of miR-145 in prostate cancer (9-11). Studying the correlation to clinical parameters in depth, we noted that miR-145 is associated with failure of androgen deprivation treatment, *i.e.* CRPC, why the present study is an investigation of the connection between miR-145 and androgen receptor signalling. Here, we find that miR-145 regulates AR expression and downstream targets, and inhibit androgen-induced cell growth.

Material and Methods

RNA extraction from patient specimen and cell lines

RNA was extracted from prostate specimens of 74 men; 49 with prostate cancer and 25 without evidence of prostate cancer, as has previously been described (9,12) (Suppl. Tab.1). In short, small RNA was extracted by a slight modified protocol of miRVana miRNA Isolation Kit (Life Technology) from formalin fixed paraffin embedded (FFPE) trans urethral of the prostate tissue (TURP) sections collected 1990 – 1999 in Malmö, Sweden. AR immunohistochemistry has been performed on slides adjacent to these and total prostate specific antigen (PSA) was measured in serum from these patients at time of diagnosis (13). The study was approved by the Regional Ethical Review Board in Lund, all patients gave a written informed consent, and we have adhered to the Helsinki Declaration. RNA was also extracted from 28 human tissues of various origins as has been described before (14). Cell lines were obtained from American Type Culture Collection (PC3, DU145, LNCaP clone FGC) and European Collection of Cell Cultures (22Rv1, VCaP and PNT2). LNCaP-ARhi (15) was a kind gift from Professor Tapio Visakorpi, Tampere, Finland. Cell line authentication was performed by DDC Medical, Fairfield, OH. The cells were cultured according to the supplier's recommendations, except VCaP that is grown in RPMI. From the cell lines, total RNA was extracted with Trizol (Life Technology) according to the supplier's protocol. The RNA concentrations were measured using a NanoDrop (ND-1000, Spectrophotometer, Thermo Scientific, Wilmington, DE). For external validation of our results we analysed a microarray data set from Taylor *et al.* constituting 110 prostate cancer tissue samples and 28 non-malignant adjacent benign prostate tissue samples (16) (GEO accession number GSE21032).

Reverse transcription and qRT-PCR

The quantification of miR-145 levels in the prostate cancer cohort was performed according to the TaqMan MicroRNA Assays protocol (Life technology). The results were normalized to the geometrical mean of the endogenous controls RNU47, RNU48, and RNU66 (Life Technology). The expression level of miR-145 in different human tissues was normalized to the geometric mean of the expression of RNU66, RNU48, RNU44 and RNU24 (Life Technology). For gene specific mRNA measurements in the cell lines a total of 500 ng RNA from LNCaP cells and 120 ng from 22Rv1 cells were reverse transcribed using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Thermo Scientific, DE, US) according to the supplier's instructions. Transcript levels of AR (Cat. #4331182), and TMPRSS2 (Cat. # 4331182), were measured using the respective gene specific TaqMan assay (Life technology) according to the supplier's protocol and normalized against the levels of PGK1 (Cat. # 4331182). The expression of miR-145 in cell lines was normalised to the geometric mean of RNU48, RNU66 and RNU24 (Life Technology). No enzyme control monitoring DNA contamination and no template control for observing other contaminators were run along reverse transcriptions and the qRT-PCR processes. All qRT-PCRs were performed on a 7900 HT Real time PCR machine (Life technology). Each sample was run in quadruplicate reactions and final calculations were made according to the $2^{-\Delta\Delta Ct}$ method.

Transient transfections

Cells were transiently transfected with either miRIDIAN microRNA Mimic (80 nM, Dharmacon, Lafayette, CO) or miRCURY LNA Knockdown probes (100 nM, Exiqon, Copenhagen, Denmark) using Oligofectamin reagent (Life Technology, Carlsbad, CA). Control experiments were performed in parallel, transfecting cells with miRIDIAN microRNA Mimic Negative Control for overexpression

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(Dharmacon) and scramble-miR (Exiqon) for knockdown experiments. These will further be denoted as scrambles. Transfections were performed in triplicates and 48 – 96 hours after transfection the endpoint of the experiment was measured or cell were harvested for later analysis.

Protein extraction and measurements

Cells were lysed 72 hours after transfection with M-PER Mammalian Protein Extraction Reagent (Thermo Scientific), containing 1% Halt Protease Inhibitor Cocktail (Thermo Scientific) and 0.005M EDTA. Cell debris was spun down and the protein concentrations were measured on a NanoDrop (Thermo Scientific). SDS-PAGE and immunoblotting were performed as previously described (17). The membranes were incubated with antibodies directed against the AR (N-20, SC-816, Santa Cruz Biotechnology, Inc., TX, US) and GAPDH (a-GAPDH, MAB374, Chemicon, CA, US). Signals from HRP coupled polyclonal secondary antibodies (mouse or rabbit, Dako, Denmark) were generated by ECL (ECL plus, GE Healthcare) and recorded using a CCD camera (LAS-3000, Fujifilm, Tokyo, Japan). Band intensities on western blots were quantified and normalized to the band intensities of GAPDH, using the ImageJ software. Total PSA were measured using a dual label immunofluorometric assay (DELFIA Prostatus™ PSAF/T, Perkin-Elmer Life Sciences, Turku Finland) (18) and concentration of kallikrein-related peptidase 2 (KLK2) was measured as described by Vaisanen *et al.* (19). The result was normalized against the total protein concentrations in the cell lysate that were measured by Bradford protein assay according to the manufacturer's protocol (Bio-Rad, CA, US).

Luciferase Assay

Luciferase assays were performed with pGL3 Enhancer vector (Promega, WI) containing the PSA promoter (20). A pRL Renilla firefly construct (a kind gift from Professor Lars Rönnstrand) was co-transfected to serve as normalizer for variation in transfection efficiency and cell number. Cells were also co-transfected with a mimic-miR-145 and scrambled control as above. Luciferase activity was assayed 72 h after transfection with a Dual-Glo Luciferase Assay System (Promega Corp, Madison, WI) according to the manufacturer's instructions, and measured with a Wallac 1420 Victor2™ Plate-reader (Perkin Elmer Inc., Wellesley, MA).

Growth assay

The Sulforhodamine B (SRB) colorimetric assay was used to assess cell growth indirectly by staining total cellular protein content. In short, 72 hours after transfection, cells were fixed in 10% trichloroacetic acid and stained with 0.4% SRB (SIGMA-Aldrich, St Louis, MO) in 1% acetic acid for 15 min. Bound SRB was dissolved in 10 mM Tris base and absorbance was read at 490 nm using a microplate reader (EI808, BioTek Instruments, Winooski, VT).

Flow cytometry

Transfected cells were incubated for 72 h and treated by a protocol for detection of proliferation or apoptosis. Briefly, for analysing cellular proliferation, cells were incubated with 5-bromo-2 deoxyuridine (BrdU, GE Healthcare, Buckinghamshire, UK) for 1.5 h, and fixed in ice cold 70% ethanol. The fixed cells were incubated in 2 M HCl containing 0.2 mg/ml pepsin and then incubated with monoclonal mouse anti BrdU (Clone MoBU-1) Alexa Fluor® 488 (Invitrogen). Samples were then incubated with 5 µl of 7'AAD Cell Viability Solution (BD, Franklin Lakes, NJ) in PBS, in dark at 4°C overnight. Cells to be analysed for apoptosis were washed with cold PBS, counted and resuspended in Binding buffer (10mM HEPES, pH 7.4, 140mM NaCl, and 2.5mM $CaCl_2$). To 100,000 cells 3 µl of 7-AAD Cell Viability Solution (BD Biosciences) and 5µl of Annexin V-FITC (BD Biosciences) were added and this was incubated for 15 min in the dark. The cells were analysed on a CyFlow® Space (Partec, Münster, Germany) and the data was evaluated with the flow cytometry software FlowJo 7.6.5 (TreeStar Inc., Ashland, OR).

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RNA-IP

22Rv1 cells were transiently transfected with miR-145 or scramble mimics, incubated for one day, and washed in cold PBS before cell lysis (20mM Tris-HCl pH 7.5, 150 mM NaCl, 0.3% Nonidet P-40, 2 mM EDTA, 1mM NaF, 1mM DTT, Protease inhibitor (Pierce), Superase In (Applied Biosystems)). The lysate was kept on ice for 30 min before centrifugation at 10,000 x g for 30 min at 4°C. To reduce background signal, the lysate was pre-cleared with beads blocked with 1 mg/ml tRNA (Applied Biosystems) and BSA (1mg/ml, RNase free) for 30 min on rotation at 4°C. The lysate was then incubated with antibody against human AGO2 (11A9, IgG2a, rat, Ascenion, Germany (21)) on rotation overnight at 4°C. The next day the mixture was incubated with Protein G sepharose 4 fast flow beads (GE Healthcare) on rotation at 4°C for 2 hours. The sample was washed four times with wash buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.3% Nonidet P-40, 1 mM $MgCl_2$, Superase In) and one time with PBS. Thereafter the protein/RNA complexes were digested with 40 µg Proteinase K (Qiagen) in 30 mM Tris-HCl pH 8, 10 mM EDTA, 1% SDS buffer, for 30 min at 50°C. The RNA was isolated by phenol/chloroform extraction and EtOH precipitation, essentially as described earlier (22). The RNA concentration was measured using a NanoDrop (ND-1000, Spectrophotometer, Thermo Scientific, Wilmington, DE).

In situ hybridization

In situ hybridization was performed essentially as described in Hansson *et al*. (23), with some modifications. Non-cancerous prostate FFPE tissues slides, described in Hagman *et al* (24) were deparaffinized, rehydrated and digested with 10 µg/ml proteinase K (Thermo scientific) in proteinase K buffer (30 mM Tris-HCl pH 7.5 and 10 mM CaCl2). The slides were refixed with 4% paraformaldehyde (pH ~ 9), and thereafter fixed further with 1-methylimidazol buffer (0.13 M 1-methylimidazol, 300 mM NaCl, pH 8) for 2 x 10 min and EDC (0.16 M 1-ethyl-3(3-dimethylaminopropyl) carbodiimide) for 1 h in a humified chamber. The slides were acetylated and pre-hybridized in hybridization buffer (50% formamide, 5 x SSC, 5 x Denhardt's, 10% dextran, 10% CHAPS, 20% Tween, 0.4 mg/ml salmon sperm DNA, and 20 mg/ml Roche blocking reagent) at the probes Tm minus 23°C for 1h and then overnight at the same temperature with hybridization buffer containing 250 nM locked nucleic acid (LNA) double DIG (3' and 5') antisense probe (Exiqon). The slides were incubated with blocking reagent (TSA kit #T20915, Life technology) for 1 h and then with 1:100 anti-DIG-AP antibody (#11093274910, Roche, Germany) for 1 h. Slides were washed, equilibrated in AP buffer (100 mM Tris-HCl pH9.5, 50 mM MgCl2, 100 mM NaCl) and developed with NBT/BCIP/levamisol solution (75 mg/ml NBT (nitro-blue tetrazolium), 50 mg/ml BCIP (5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt), 0.25 mg/ml levamisol, 0.05% Tween 20, in AP buffer). The reaction was stopped after colour development and the slides were mounted with ProLong Gold (P36930, Invitrogen). The specificity of the *in situ* hybridization was determined by a negative scramble double DIG labelled LNA probe (Exiqon) that did not give any signal (data not shown).

Statistical analysis

Mann-Whitney U test was used for comparing two groups of clinical samples and the 1-way ANOVA test for more than two groups. Log-Rank test was used for the Kaplan-Meier survival analysis. Spearman's correlation was used to calculate the correlation of PSA to miRNA levels. The software used for statistical analysis were Graph-Pad Prism (San Diego, CA, US) and SPSS 20.0 (SPSS, Inc., 2009, Chicago, IL, US). For the *in vitro* assays, results from three independent experiments were analysed by a two sided Student's t-test. All tests were 2-sided and $p<0.05$ was considered statistically significant.

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Results

Expression of miR-145 correlates to prostate cancer prognosis

The level of miR-145 have previously been found to be significantly decreased in prostate cancer specimens in our Swedish cohort (9). Here, the prognostic value of miR-145 expression levels, as measured by qRT-PCR, was investigated in the same cohort. The levels of miR-145 in patients with metastases (M1) was found to be to be significantly lower than in patients either found not to have metastasis (M0) or not investigated for metastasis since there was no suspicion at the time (Mx), p=0.0001, Mann Whitney U test, Fig. 1A. Men with metastases (M1) was also found to have significantly lower miR-145 levels compared to men found not to have metastases (M0) (p=0.014, Mann Whitney U test, Suppl. Fig. 1). Analysing the miRNA expression levels in the external data set provided by Taylor *et al.* (16), miR-145 ranked as number five of the 373 miRNAs for the ability to separate patients with metastasis from the men with localised prostate cancer (p<0.0001, Mann Whitney U test, Bonferroni adjusted for multiple testing). Further, Kaplan-Meier analysis show that there is a significant difference of patient overall survival based on the miR-145 expression levels in our cohort; patients with low miR-145 expression (50% of the patients) have a median survival of two years while the patients with high miR-145 lived for five years (p=0.0019, Log-Rank test, Fig. 1B), the hazard ratio being 3.0 (95% CI 1.6 – 7). Interestingly, we also observe that low level of miR-145 in prostate cancer tissue is connected to failure of androgen ablation treatment (p=0.007, 1-Way ANOVA, Fig. 1C), which often is linked to increased levels of AR. Subsequently we performed analyses of the distribution of miR-145 expression by qRT-PCR in an array of human tissues. High expression of miR-145 was found in the male reproductive tract; prostate, testis, epididymis, but also remarkably high in skeletal muscle (Fig. 1D), indicating that miR-145 is expressed and has a biological function in tissues dependent on androgen signalling. We then wanted to in detail study the histological expression pattern of miR-145 by performing *in situ* hybridization on prostatic tissues from men without prostate cancer. We found that, miR-145 expression is detected in the basal layer of the epithelial cells in the prostate gland that are devoid of AR, but not in the AR expressing luminal cells. There is no expression detected in the stromal cells (Fig. 1E). These result taken together, could suggest that miR-145 is involved in the regulation of AR, something we further wanted to investigate.

miR-145 effect AR levels in vitro and in vivo

The cell line 22Rv1 has been described to contain several splice variants of the AR (25). After ectopic expression of miR-145 we found that all the three splice forms detectable by our western blot antibody were downregulated; ~112kDa (p=0.030), ~80 (p=0.045) and ~77kDa (p=0.047, Student's t-test, blot shown for the ~112 kDa band Fig. 2A, suppl. Fig. 2A and B). In the other cell lines investigated only the ~110kDa AR variant is present. In LNCaP-ARhi there was a decrease of AR as well, (p=0.034, Student t-test, Fig. 2A, suppl. Fig. 3 A and B), but not in LNCaP (Fig 2A, suppl. Fig. 4 A and B). LNCaP-ARhi overexpresses AR containing a 427 bp part of the 3'UTR (15). We could moreover identify a slight decrease in AR transcript levels in 22Rv1 as measured by qRT-PCR (p=0.03, Student's t test, Fig. 2B), but not in LNCaP cells (data not shown). Our data indicate that the regulation is setting specific and that in 22Rv1 it is occurring at both transcriptional and translational levels, but the main regulation seems to occur at translational level. Next, we analysed the relationship of miR-145 expression and AR levels in prostate cancer patients. The AR protein content in the prostate tissues in our cohort has previously been determined by immunostaining and the staining scored by overall intensity (1; weak, 2; moderate to strong, 3; intense, Fig. 2C, lower panel) (13). When correlating miR-145 expression levels, measured by qRT-PCR on RNA extracted from an adjacent slide, with the semi-quantitative assessment of AR immunostaining in the malignant epithelial cells, a statistically significant inverse association was found (p<0.0001, Mann Whitney U test=0.0025, Student's t-test, Fig. 2C). This could be further confirmed in an external cohort (n=138), where the AR transcript levels are reversely correlated to miR-145 (p<0.0001, Spearman's rho: -0.33, Fig. 2D) (16).

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However, no correlation was found for miR-145 expression and AR staining neither in the benign epithelium nor in stromal tissue (data not shown).

Effects on AR activity and downstream targets

Next, we investigated if the effect of miR-145 on AR levels would affect the activity of AR. As a surrogate endpoint for AR activity we used a luciferase reporter assay containing the PSA promoter, known to be under the control of androgens through AR. Co-transfecting LNCaP, LNCaP-ARhi, or 22Rv1 with the PSA promoter reporter and miR-145 or a scramble control, showed that miR-145 significantly decrease the binding to the PSA promoter by 33%, 63%, and 22% respectively (p=0.0003, p=0.0003, p=0.003, respectively, Student's t-test, Fig. 3A – C). To investigate the effect on downstream targets of the AR, we choose to examine the levels of the two androgen regulated proteins PSA and KLK2. After ectopic transfection with miR-145, total protein was harvested after 96 h and the levels of extracellular PSA and KLK2 was measured using the clinically used Delphia assay. We found both PSA and KLK2 to be significantly down regulated by miR-145 (p=0.0008 and p<0.0001, respectively, Student's t test, Fig. 3D and E). The effect of endogenous expression of miR-145 on another well-known AR regulated transcript, TMPRSS2, was also measured by qRT-PCR and found to be decreased (p=0.0002, Student's t test, Fig. 3F). To verify that the effects on AR signalling also occurs *in vivo* we analysed the correlation of miR-145 expression in the prostate to the serum levels of PSA at diagnosis in our Swedish cohort of prostate cancer patients. We found that miR-145 levels negatively correlates to PSA levels in serum (p=0.0004, rho= -0.5, Spearman, Fig. 3G), indicating that miR-145 also can influence the current detection and monitoring method of prostate cancer.

miR-145 binds directly to AR transcripts and influences androgen-induced cell growth

When miRNAs bind their target they are in stable complexes with Argonaute 2 (Ago2), a catalytic part of the RNA-induced silencing complex (RISC). To study if the AR is a direct target of miR-145, we co-immunoprecipitated Ago2 bound to its mRNA targets in 22Rv1 cells transiently transfected with miR-145 or a scramble control 24 hours earlier. The levels of immunoprecipitated AR mRNA were measured by qRT-PCR and found to be higher in cells transfected with miR-145 (p=0.043, Student's t test, Fig. 3H), suggesting that miR-145 bind to the AR transcript through Ago2, making AR a direct target of miR-145.

Since one of the main functions of the AR in prostate epithelial cells is to stimulate cell growth, we investigated how miR-145 influences androgen-induced cell growth. In the androgen dependent cell lines VCaP, LNCaP, and LNCaP-ARhi, overexpression of miR-145 lead to decreased cell growth (p=0.046, p=0.00078, and 0.019 respectively, Student's t-test, Fig. 4A – C). In accordance with this, blocking miR-145 in VCaP cells lead to increased cell growth (p=0.03, Student's t-test, Fig. 4D). To further investigate this, the effect of ectopic overexpression of miR-145 was studied in the androgen independent cell lines PC3 and DU145. Strikingly, miR-145 exerted a growth stimulating effect in these cells (p=0.026 and p=0.017 respectively, Student's t-test, Fig. 4E and F). In line with this, blocking of miR-145 in the androgen independent PNT2 lead to decrease of cell growth (p=0.0001, Student's t-test, Fig. 4G). This implies that miR-145 also targets another pathway with growth inhibiting effects. In androgen dependent cells, miR-145 predominantly targets the growth promoting effect mediated through the AR, but in androgen independent cells, the growth inhibition pathway is targeted. This is supported by the finding that miR-145 induce cell growth in LNCaP cells cultured in androgen deprived medium. There was a trend three days after transfection (p=0.06, data not shown), but after five days the difference was even more pronounced (p=0.005, Student's t-test, Fig. 4H). However, there was no effect on growth in the AR expressing, but androgen independent cell line, 22Rv1 (data not shown). This cell line has a constitutively active AR splice form and it is possible that this balances the levels of the unknown pathway with opposite effect.

miR-145 acts through proliferation or apoptosis in different cellular setting

To investigate if the effect of miR-145 on androgen-induced and androgen independent cell growth is mediated through effects of the cellular proliferation and/or apoptosis, FACS analysis was performed. In the androgen dependent LNCaP-ARhi cells the cellular proliferation rate was reduced when miR-145 was introduced (p=0.01, Student's t test, Fig. 5A). In addition, we found that the percentage of cells in the G1 phase was significantly higher in miR-145 transfected cells (p<0.0001, Student's t test, Suppl. Fig. 5) and lower in S and G2 phase (p=0.016 and 0.001 respectively, Student's t test, Suppl. Fig. 5). Next, we found that miR-145 do not induce apoptosis in LNCaP-ARhi (Fig. 5B). This indicated that the growth reduction exerted by miR-145 in this androgen dependent cell line is mediated through a decreased proliferation. When the androgen independent cell line DU145 was transfected with miR-145 no effect on proliferation could be detected (Fig 5C). However, ectopic expression of miR-145 reduced apoptosis (p<0.0001, Student's t test, Fig 5D); 34% less in early apoptosis and 49% less in late apoptosis (p=0.04 and p=0.0002 respectively, Student's t test). This suggests that the increase of cells instigated by miR-145 in this androgen independent cell line is mediated through a reduced apoptosis rate.

Discussion

The miRNA miR-145 has consistently been found to be down regulated in several types of cancer including colorectal, breast, AML, non-small lung cancer, and prostate cancer (9,10,26-28). A possible mechanism for the decrease of miR-145 in cancer is methylation of its promoter as has been described for the prostate cancer cell lines PC3, DU145 and LNCaP (29). In line with this, Zaman *et al.* detected only low levels of miR-145 in these cell lines but high levels in PWR-1E; an immortalized normal prostate cell line (30). miR-145 has also been suggested to be transcriptionally activated by p53, which frequently is mutated in advanced prostate cancer cells including PC3, DU145 and 22Rv1, and repressed by IL-6, that is known to be up regulated in prostate cancer (29-31). In addition, we show that miR-145 expression is primarily localised to the basal epithelial cells in the prostate. These cells are usually not detected in progressed tumours, conceivably resulting in a loss of miR-145 expression. Reasonably, a combination of these events is responsible for the decreased miR-145 level in prostate cancer progression that is found in this study. The prognostic potential of miR-145 have also been highlighted by the finding that it is possible to detect in serum and that in combination with miR-20a, miR-21, and miR-221 could distinguish high versus low risk PCa (32). As we found miR-145 to correlate to metastases, overall survival and treatment response the possibility of non-invasive detection of this marker merits further investigation.

In order to elucidate its therapeutic potential of miR-145 we investigated the phenotypic consequences of deregulation of this molecule. We found that miR-145 binds directly to the AR transcript through the RISC complex and that it reduces the transcript and protein levels as well as the activity and downstream signalling of AR. The finding that miR-145 regulates AR in LNCaP-AR high further suggests that the binding site is present within the first 427 bp part of the 3'UTR. This part does contain a predicted miR-145 binding site (13); however we have not been able to verify this by luciferase assays. The AR 3'UTR has been cloned in eight pMIR-Reporter vectors each containing around 1 kb long pieces. These vectors were individually co-transfected with miR-145 or scramble mimics, but no significant reduction in luciferase signal was registered (data not shown). This could be due to miR-145 binding to other regions of the AR except the 3'UTR or that coordinated binding is necessary.

The discrepancy between the effect of miR-145 on AR in LNCaP and LNCaP-ARhi cells is intriguing. The observation that miR-145 is not regulating AR in LNCaP cells is supported by the LMA screening of miRNAs affecting AR in LNCaP cells where miR-145 is not identified (13). However, AR activity was found to be regulated by miR-145 in LNCaP cells, possibly indicating that miR-145 in addition to AR also regulates an AR cofactor or chaperon acting in synergy to influence the activity of AR. The latter could possibly explain the cell-type specific effects. This should be further investigated in a future study. Another speculation could be that in LNCaP cells miR-145 also regulates a factor

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counteracting the decreased level of AR, but when the AR levels are raised, AR regulation is dominating.

Since androgen signalling in high degree induces both proliferation and survival of prostate epithelial cells, the effect of miR-145 on cell growth was investigated. We found that miR-145 decreased cell growth in the androgen dependent cell lines, but in the androgen independent cell lines miR-145 increased cell growth. In agreement with this we found that cell growth was induced by miR-145 in LNCaP cells grown in charcoal stripped medium, suggesting that androgen signalling through the AR is needed for miR-145 inhibited cell growth. We are aware of the work performed by Zaman *et al*. and Fuse *et al.* who contrary to us register a decrease of cell growth in DU145 and PC3 cells transfected with miR-145 (30,33). It is possible that this can be explained by difference in cell line batches or by methodology. However, miRNAs do not only act through one target, the phenotypic outcome is the sum of the effect on all its targets. In the androgen dependent cell lines we would have expected the decrease of AR to induce apoptosis (34), but it did not. This can suggest that miR-145 also in these cell lines targets something else with pro-apoptotic features that counteracts this expected effect mediated through AR, possibly the same as in the androgen independent cell lines. We have not identified the mechanisms for miR-145 reduction of apoptosis. However, a search for miR-145 targets was performed *in silico* using PicTar, Target Scan, MiRanda and miRBase. The combined list of all predicted miR-145 targets was analysed using DAVID Bioinformatics Database functional-annotation tools (http://david.abcc.ncifcrf.gov/) to identify biological pathways that they are involved in. Strikingly, the top BBID functional annotation is 14-3-3 apoptosis. This effect could be mediated through several targets, one being BAX, a pro-apoptotic member of the Bcl-2 family that is predicted to contain a mir-145 binding site. In metastatic colorectal cancers, miR-145 has been suggested to have oncogenic features (35). There are several examples of miRNAs having different mechanisms of action in different cellular settings *e.g.* miR-205 (24).

Several miRNAs have been linked to the acquisition of CRPC, for example miR-34c and miR-205 have similarly to miR-145 been shown to target the AR. Other miRNAs has been shown to affect AR levels indirectly, *e.g.* let-7c that effect the transcript levels of AR through c-Myc or miR-221 that facilitated onset of androgen independence through HECTD2 and RAB1A (36,37). Reducing the AR activity by anti-androgens is the primary mode of treatment for advanced prostate cancer. However, the AR signalling pathway can adapt to the lack of androgens resulting in CRPC. If instead, the AR itself was removed, the risk of progressive disease would be reduced. The data presented here suggests that miR-145 is an important regulator of AR levels during prostate cancer progression, both directly regulating the AR and possibly also regulating AR activity independent of AR this could make the adaptation of the AR signalling pathway more difficult compare to treatment with anti-androgens. A way for the tumour to circumvent androgen deprivation therapy is through a subpopulation of prostate epithelial stem cells that are androgen independent, which are reported to be activated during androgen depleted conditions (38). Finding drugs that targets both the bulk of the tumour and the prostate stem cell-like cells would be ideal. Interestingly, miR-145 has previously been shown to inhibit stem cell renewal and pluripotency by targeting OCT4, SOX2 and KLF4 (39). It is noteworthy that the natural setting of miR-145 is in the AR-devoid basal cells, and not in the stem cells or the adjacent AR expressing luminal cells. It is possible that if exogenous miR-145 was used therapeutically, it could decrease both the stem cells and the AR expressing bulk of the tumour and hence represents a more effective means to delay the recurrence of the deadly androgen independent form of prostate cancer. In conclusion, our results indicate that the therapeutic reintroduction of miR-145 should be further explored as it might provide novel treatment opportunities for advanced prostate cancer.

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Conflict of Interest Statement: Dr. Hans Lilja who holds patents for free PSA, intact PSA, and hK2 assays.

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Figure legends

Figure 1. ***Clinical material.*** miR-145 levels in TURP material was measured by qRT-PCR. The levels were normalized to RNU47, RNU48, and RNU66. Here, we show that **A)** the metastatic status is related to miR-145 expression, M0=no metastatic events, M1=the patients have metastases, MX=no suspicion of metastases, n(M0+MX)=27, n(M1)=18, **B)** patients with low miR-145 n=25) have a three years shorter median survival than patients with high miR-145 (n=24) (HR=3.0), **C)** There is a difference of miR-145 expression among hormone naïve patients (n=22), patients with ongoing androgen deprivation therapy (n=6) and patients with CRPC (n=14); men with CRPC have low miR-145. ***Distribution of miR-145.*** **D)** miR-145 levels were screened in an array of tissues consisting of 28 different tissues. Highest levels were found in skeletal muscle followed by testis and prostate. High levels are found in the male genital tract (marked with dark grey). **E)** , miR-145 is located in the basal part of the epithelial cell layer of the prostate here shown by *in situ* hybridization using double DIG-labeled antisense. A scrambled probe was used as negative control, and shown no signal.

Figure 2. ***AR measurements.*** **A)** Introduction of miR-145 decrease the levels of AR in 22Rv1 and LNCaP-ARhi compared to scrambled control, as shown by western blot. The data is normalized to the band intensities of GAPDH. The blot on LNCaP cells were from the same gel, but an empty well in the middle was cut out. The experiment was performed in triplicate. **B)** Also at transcript levels miR-145 decrease AR in 22Rv1 cells (qRT-PCR) compared to scrambled control. The experiment was performed in triplicate and the data is normalized to GAPDH mRNA expression. **C)** Prostatic malignant tissue was immunohistochemicaly stained for AR and the intensity and quantity were scored from 1 – 3. From adjacent tissue RNA was extracted and miR-145 expression was measured by qRT-PCR. The qRT-PCR data is normalized against RNU47, RNU48, and RNU66. Here, we show that low miR-145 levels is associated to high levels of AR, n(1+2)=37, n(3)=10. **D)** In the data set generated by Taylor *et al.* (16), we find that miR-145 expression and AR transcript levels are correlated (n=138).

Figure 3. ***AR activity, downstream targets and PSA in vivo.*** To measure the AR activity a PSA promoter containing firefly luciferase construct was co-transfected with miR-145 or a scramble control. We found that miR-145 decrease the firefly luciferase in LNCaP **(A)**, LNCaP-ARhi **(B)**, and in 22Rv1 **(C)**. The experiment was performed in triplicate and the firefly luciferase levels were normalized to a renilla control. Further we show that downstream targets of AR were down regulated by miR-145 in 22Rv1; secreted tPSA **(D),** secreted KLK2 **(E)**, and TMPRSS2 mRNA **(F)**. The experiment was performed in triplicate and tPSA and KLK2 levels were normalized against total protein concentration, TMPRSS2 levels against GAPDH. **G)** The miR-145 levels in prostatic tissue and prostate cancer patient serum PSA was found to correlate (n=46). The miR-145 levels were measured by qRT-PCR and normalized against RNU47, RNU48, and RNU66. **H)** ***RNA-IP.*** Ago2 was pulled down in 22Rv1 cells that were transfected with miR-145 or a scramble control. RNA was extracted and by qRT-PCR AR mRNA levels were measured and found up regulated in miR-145 transfected cells. The experiment was performed in triplicate and the qRT-PCR result was normalized against input RNA levels.

Figure 4. ***Cell growth.*** To measure effects of miR-145 on growth miR-145 was either transiently overexpressed or blocked in prostate cell lines. Three days later the cells were examined by the colorimetric SRB assay that stains all available protein. The results were compared to cells transfected with scramble controls. We find that miR-145 reduce cell growth in the androgen dependent AR positive cell lines LNCaP **(A)**, LNCaP-ARhi **(B)**, and VCaP **(C)**. Also, blocking of miR-145 increase cell growth in VCaP cells **(D)**. In the AR negative cells we find that miR-145 increase cell growth; PC3 **(E)** and DU145 **(F)**, and that blocking decreases cell growth in PNT2 **(G).** When LNCaP cells were grown in charcoal stripped medium, five days after transfection the cell growth induction was significant. **(H)**. Dark grey bars denote AR positive cell lines and light grey AR negative. All the experiment was performed in triplicate.

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Figure 5. ***Flow Cytometry.*** Proliferation was analyzed by detecting BrdU incorporation into newly synthesized DNA. Apoptosis was analyzed by detecting cells that were Annexin-V or Annexin-V and 7'AAD positive. **A)** We find that LNCaP AR-hi cells proliferate less when transfected with miR-145, **B)** but no difference is seen on the apoptotic level. **C)** In the AR negative DU145 cells miR-145 does not affect proliferation, **D)** but the apoptotic rate is inhibited. Dark grey bars denote AR positive cell lines and light grey AR negative. All the experiment was performed in triplicate.

Supplementary figure 1. ***Metastasis versus miR-145.*** RNA was extracted from TURP FFPE tissue and miR-145 levels were measured by qRT-PCR and normalized against RNU47, RNU48, and RNU66. Here, we show that in patients with metastasis miR-145 levels are lower compared to patients without metastasis.

Supplementary figure 2. ***Western blot*** with the ladder in the first lane followed by protein from 22Rv1 transfected with a scramble control in lanes 2-4, and miR-145 in lane 5-7. **A)** The membrane treated with AR-N20 antibody. Three AR bands are showing; ~112, ~80 and ~77 kDa. **B)** GAPDH (~37kDa).

Supplementary figure 3. ***Western blot*** with the ladder in the first lane followed by protein from LNCaP-ARhi transfected with a scramble control in lanes 2-4, and miR-145 in lane 5-7. **A)** The membrane treated with AR-N20 antibody (the ~110 kDa) and **B)** GAPDH (~37kDa).

Supplementary figure 4. ***Western blot*** with the ladder in the first lane followed by protein from LNCaP transfected with a scramble control in lanes 2-4, and miR-145 in lane 5-7. **A)** The membrane treated with AR-N20 antibody (~110k Da) and **B)** GAPDH (~37kDa).The upper band represents the AR still glowing through.

Supplementary figure 5. ***Cell cycle.*** The level of pulse BrdU and 7'AAD DNA incorporation were analyzed after measured on a flow cytometer. In LNCaP-ARhi, we found that miR-145 increase the percentage of cells in G1 phase **(A),** and decrease the percentage of cells in S **(B)** and G2 phase **(C)**.

Supplementary table 1. ***Patient information and clinicopathological description of the patient cohort.***

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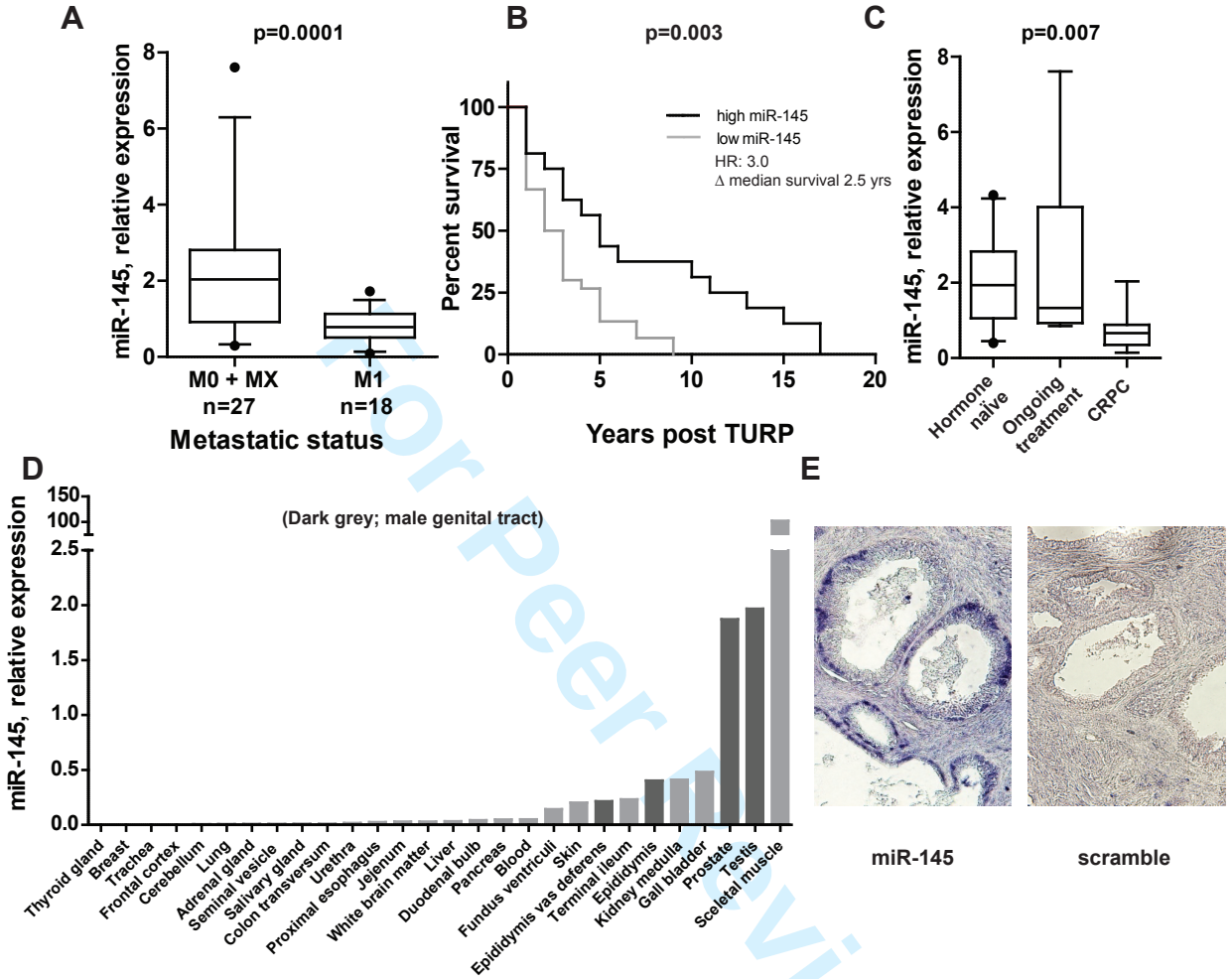

Figure 1.

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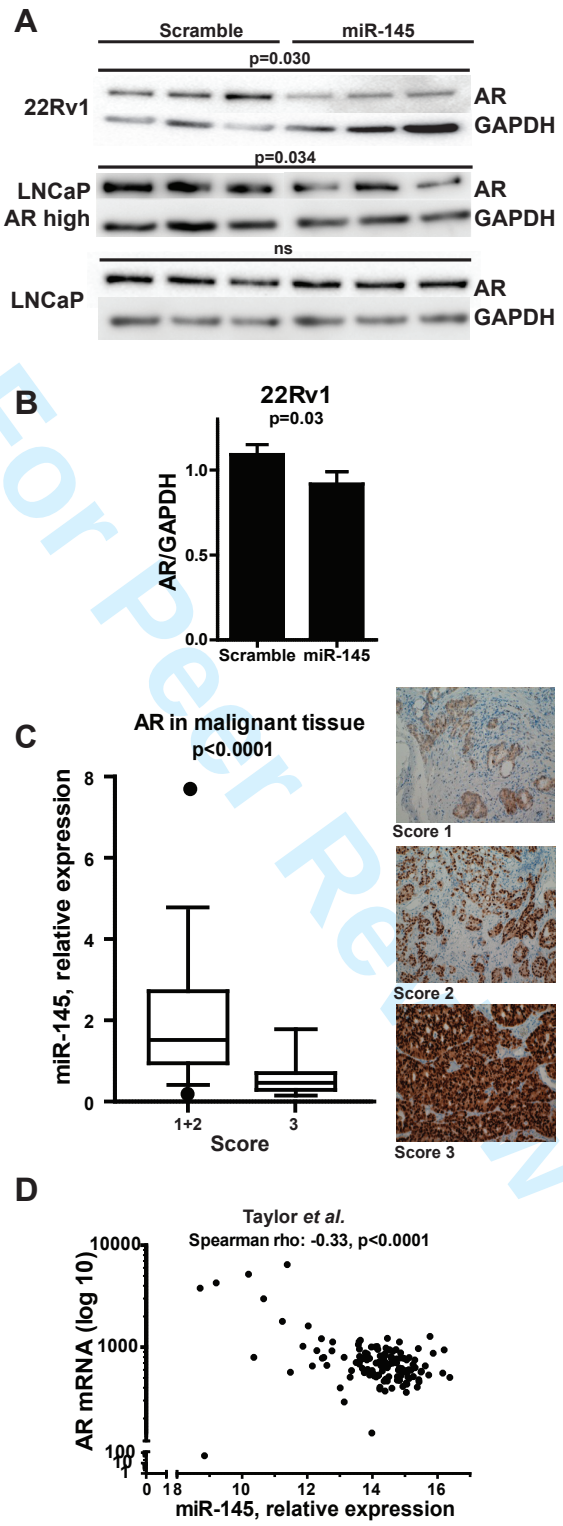

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Figure 2.

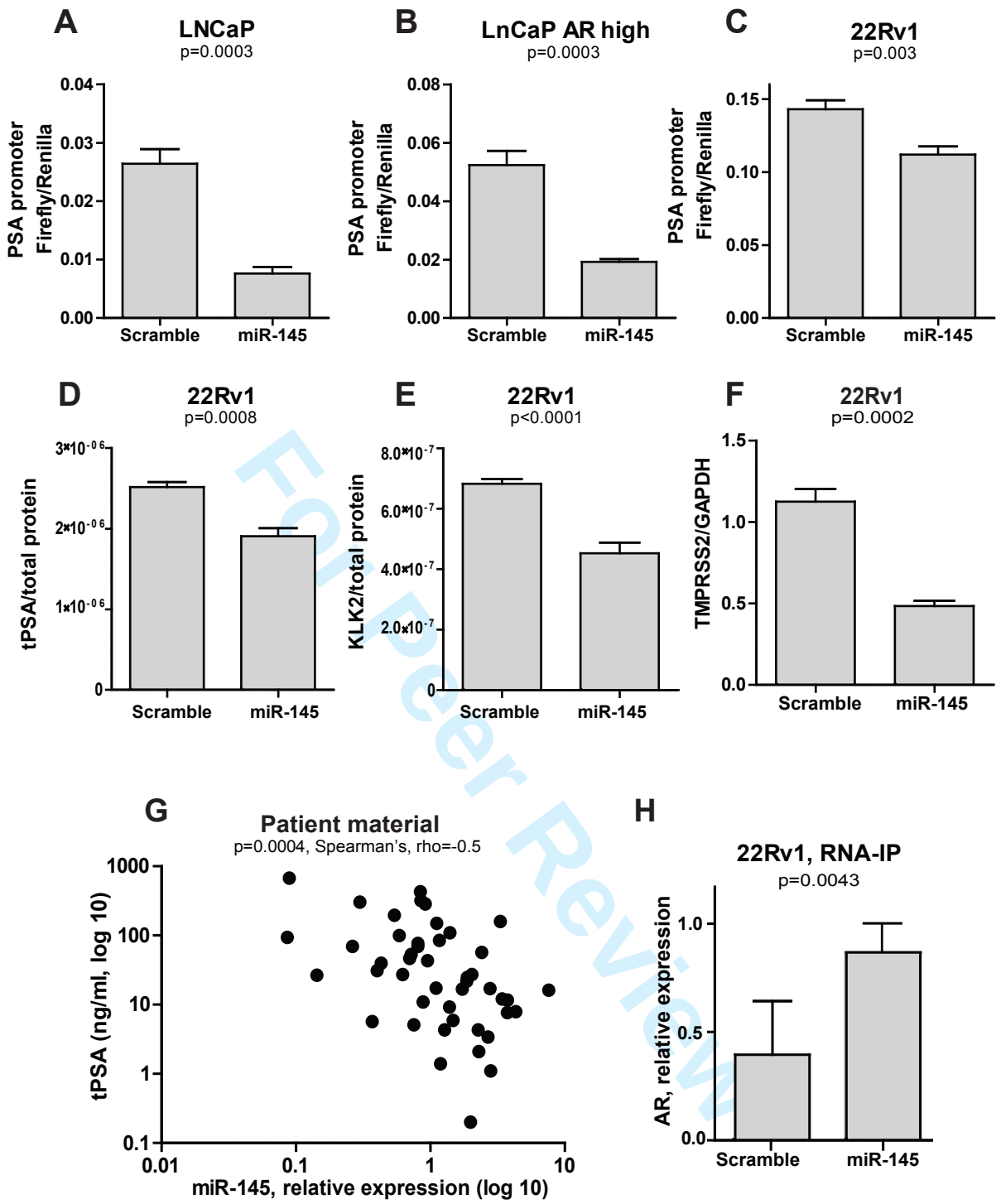

Figure 3.

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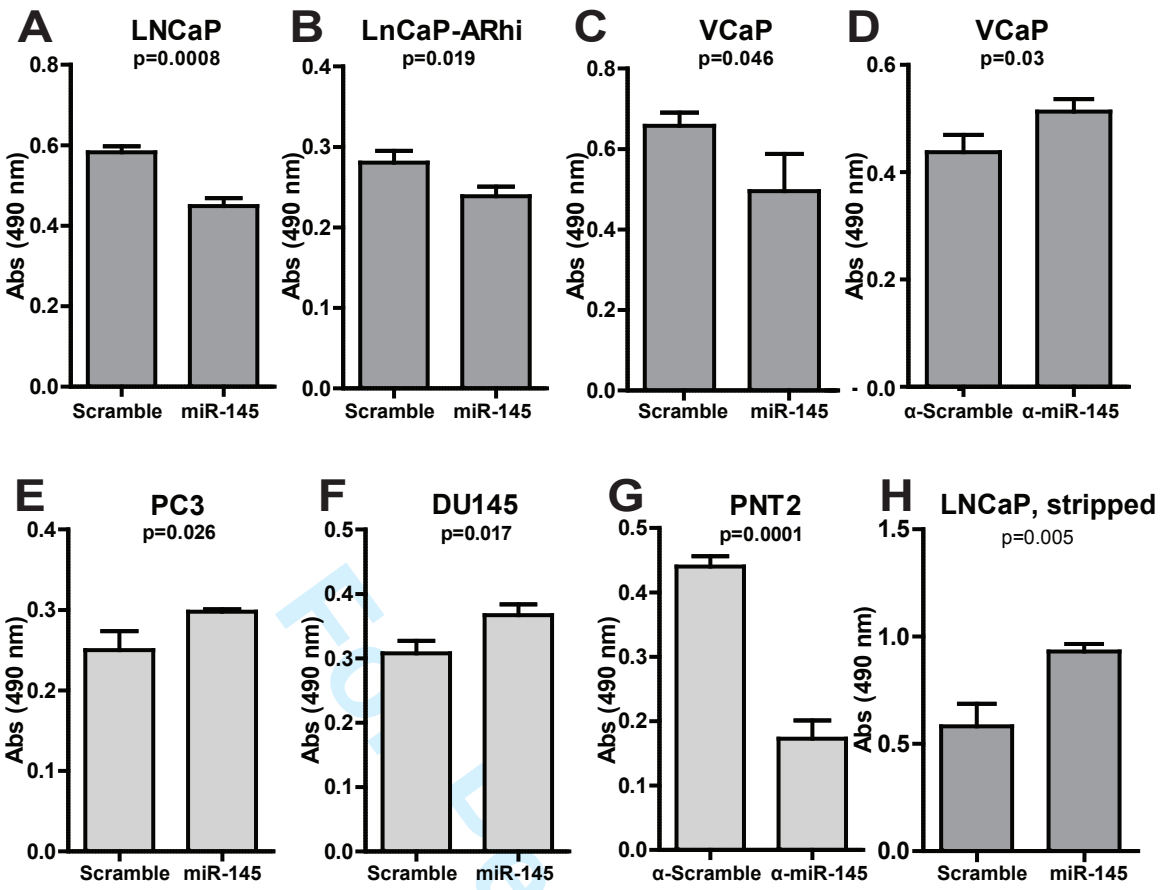

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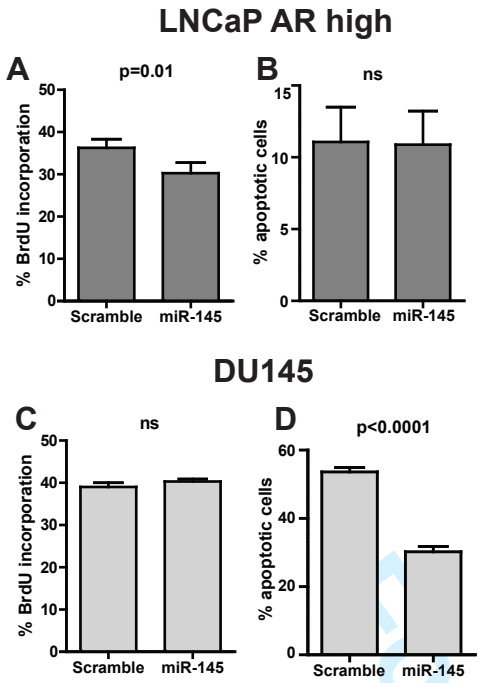

Figure 5

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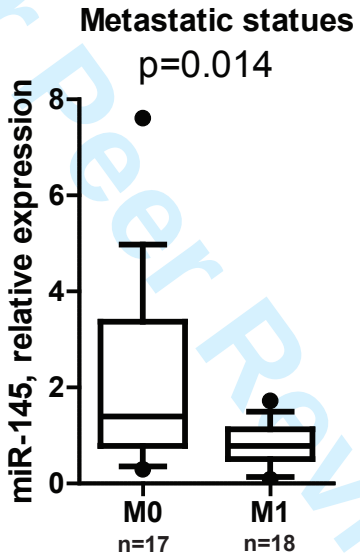

Supplementary figure 1

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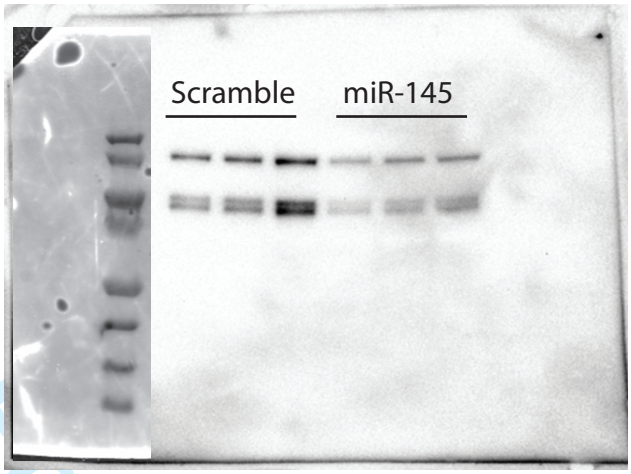
A



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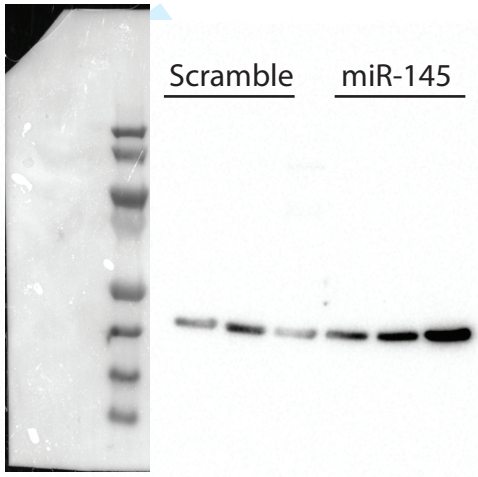
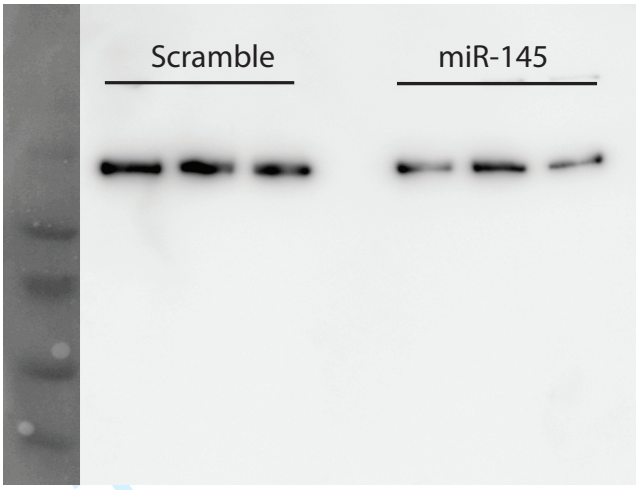


Supplementary figure 2.

A



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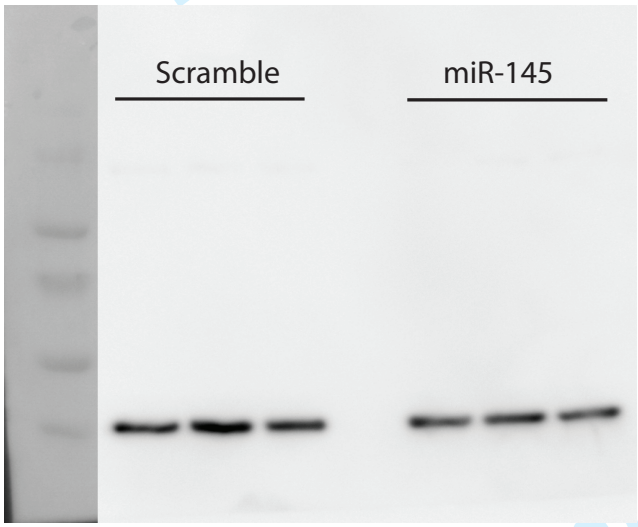

Supplementary figure 3.

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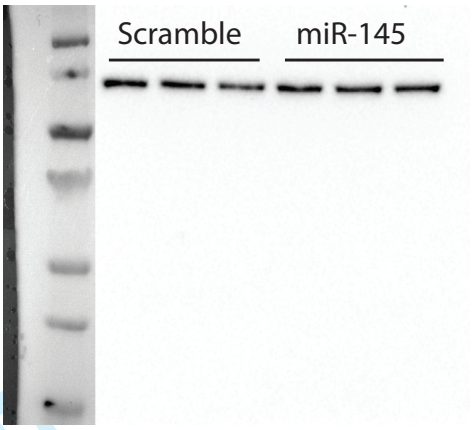

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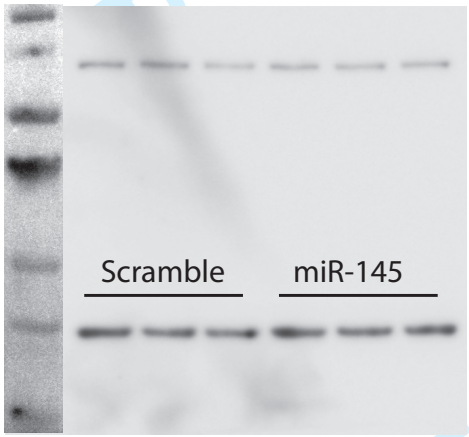

Supplementary figure 4.

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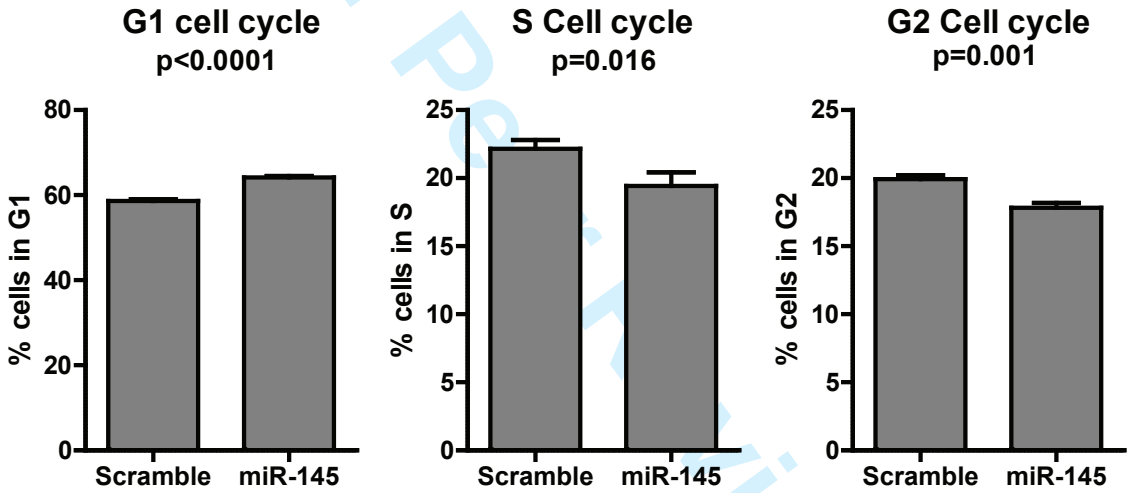

Supplementary figure 5

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Supplementary table 1

WHO grade	(n)
Grade I	5
Grade II	19
Grade III	25
Unknown	-
Clinical stage	(n)
T1	9
T2	22
T3	14
T4	3
Unknown	1
PSA at diagnosis	n=46,ng/ml
Median (range)	26.95 (0.2 – 672)
Occurrence of metastasis	(n)
M0 = no metastasis	17
M1 = having metastasis	18
Mx = not investigated since no suspicion at the time	10
Unknown	4
Cancer in samples	(%) Mean (range) 42 (5 – 90)
Non-cancerous patients (n)	25
PSA at diagnosis (n=19)	ng/ml
Median (range)	5.9 (1.1-19.4)

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