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Regulation of cancer-specific miRNAs by MDA-7/IL-24

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

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Table of Contents

List of Figures……..iv

List of Abbreviations……v

Abstract……vii

Introduction……1

Materials and Methods……..12

Results……16

Figures……19

Discussion……..29

References……35

List of Figures

Figure 1 miScript PCR plate array……..19

Figure 2 Validation of PCR plate array……21

Figure 3 miR-330 and miR-183 changes are specific to DU145……23

Figure 4 Three miRNAs were downregulated in three prostate cancer cell lines……24

Figure 5 Gene targets of miR-125a, miR-145, and miR-23b respectively……26

Figure 6 Regulation of NLRC5 expression by miR-125a……27

Figure 7 miR-125a is regulated by DICER……28

Abbreviations

Metastatic castration-resistant prostate cancer mCRPC

Androgen deprivation therapy ADT

Prostate-specific membrane antigen PSMA

Melanoma differentiation associated gene -7 MDA-7

interleukin 24 IL-24

interleukin 10 IL-10

endoplasmic reticulum ER

Reactive oxygen species ROS

focal adhesion kinase FAK

matric metalloproteinases MMP

microRNA miRNA

untranslated region UTR

messenger RNA mRNA

primary miRNA pri-miRNA

precursor miRNA (pre-miRNA

anti-miRNA oligonucleotides AMO

locked nucleic acids LNA

toll-like receptor TLR

lipopolysaccharide LPS

NOD-like receptor NLR

NOD-like receptor family, caspase recruitment domain containing 5 NLRC5

pattern-recognition receptors PRRs

major histocompatibility complex MHC

class I transactivator CITA

class II transactivator CIITA

epithelial-mesenchymal transition EMT

Krüppel-like factor 4 KLF4

Krüppel-like factor 15 KLF15

Abstract

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Melanoma differentiation associated gene 7/Interleukin-24 (MDA-7/IL-24) is a secreted cytokine which acts as a tumor suppressor. It is capable of selectively killing cancer cells, regardless of anatomic origin, while sparing normal cells. miRNAs are master regulators of gene expression that can play two roles in cancer: tumor-suppression and oncogenesis. We identified a number of miRNAs that are regulated by MDA-7/IL-24 using a PCR plate array containing probes for miRNAs known to play a role in prostate cancer. We independently validated the array with qRT-PCR to identify three miRNAs which are downregulated by MDA-7/IL-24 treatment in DU145, PC3, and PC3ML prostate cancer lines. These miRNAs were miR-125a, miR-145, and miR-23b. Their gene targets were identified using TargetScan and confirmed to be regulated in our prostate cancer model. NLRC5, KLF4, and KLF15, respectively, were upregulated after treatment with MDA-7/IL-24. We focused on NLRC5 as a novel target of MDA-7/IL-24, which plays a role in immune evasion by cancer cells. NLRC5 is upregulated following inhibition of miR-125a. It is not downregulated by overexpression of miR-125a which suggests that more than one miRNA may be acting to regulate its expression. Finally, we determined that miR-125a is downregulated by MDA-7 through DICER, an important processing enzyme for miRNA biogenesis.

Introduction

Prostate Cancer

Prostate cancer is the most common cancer among men in industrialized parts of the world including North America, Western and Northern Europe, and Australia. Incidence varies with ethnicity and geography, but the risk increases with age. In the early stages, the cancer is localized and the 5-year survival rate with early detection is almost 100%. When tumors metastasize, often to bone, and become metastatic castration-resistant prostate cancer (mCRPC), the disease becomes high risk and lethal for most men[1,2].

The occurrence of prostate cancer is first evident at about age 45 in men. The mean age of diagnosis is 67 with 35.3% of diagnoses being in men from age 65 to 74[2]. African American men are affected at the highest rate and have the highest mortality rate as well. East, Southeast, and South-Central Asian men have the lowest rate of incidence and lowest mortality rate. Prostate cancer is heritable, but identifying genes which contribute to disease progression has proven difficult[3]. *HPC1*[4,5]*, PCAP*[5]*, HPCX*[4]*, CAPB*[6]*, HPC20*[7]*,* and *HOXB13*[8] have been identified in European populations and validated in other ethnicities[1] . However, these genes are not included in genetic testing as their risk and the effect of treatment have not been determined. However, mutations in *BRCA2* can be detected through genetic testing and have been found to be associated with aggressive prostate cancer[9]. Age, ethnicity, and genetic predisposition all play a role in the development of prostate cancer, but, as with many cancers, environmental factors such as diet and smoking also play a role[1,2].

Prostate cancer can be diagnosed with a digital rectal examination, trans-rectal ultrasound, and serum prostate-specific antigen with a confirmation by biopsy[2]. Currently, treatment options include prostatectomy for the local tumor, and radiotherapy with or without androgen-deprivation therapy (ADT) for high-risk tumors and metastasis. ADT inhibits the binding of testosterone with the androgen receptors in order to inhibit cellular proliferation[10]. Prior to 2004, chemotherapy only provided a palliative effect with no increase in survival. It is at this point that docetaxel, a new chemotherapeutic, came on the scene. Treatment with docetaxel increased patient survival and improved quality of life. It became the standard for treatment of metastatic castration-resistant prostate cancer[11]. Combining chemotherapy with ADT has led to a greater improvement of patient outcomes, but the question became how long to continue the ADT. Long-term treatment up to 3 years improved disease-free survival[12]. However, some physicians feel differences between short-term and long-term treatment are not significant. Long-term ADT also comes with side effects such as hot flushes, insomnia, decreased sexual interest, anemia, development of insulin resistance, and bone density loss[13,14], thus, leaving the field without a consensus on the best length of treatment with ADT.

Although there is a standard course of treatment for prostate cancer, 10-20% of patients develop resistance to treatment[15]. New strategies for the detection and treatment of mCRPC are coming into the field in order to minimize side effects and increase efficiency of treatment. Many researchers are taking advantage of the prostate-specific membrane antigen (PSMA) for targeted therapy. PSMA is overexpressed on prostate cancer cells making it an ideal marker for targeted therapies. Clinical trials using radiolabeled anti-PSMA antibody such as J591 demonstrated improved targeting of prostate cancer[16]. Many radiolabeled drugs using a PSMA

ligand for targeting have been developed. Many of them have been used for diagnosis and non-invasive imaging through positron emission tomography (PET) or single photon emission computed tomography (SPECT). Combining the PSMA ligand with a radiotherapy agent or small molecule inhibitor enables direct targeted treatment as PSMA is internalized by endocytosis leading to enhanced uptake and retention of a drug in the cancer cells[17,18].

MDA-7/IL-24

Melanoma differentiation associated gene-7 (MDA-7) which is also known as interleukin-24 (IL-24) is a secreted cytokine and a member of the interleukin 10 (IL-10) gene family which plays an anti-cancer role in a wide range of cancers[4,19]. The initial designation of *mda-7* emanates from the discovery of this gene through subtraction hybridization of cDNA libraries from melanoma cells induced to terminally differentiate. MDA-7/IL-24 was found to be upregulated in melanoma cells that were terminally differentiating[20,21,22]. The Human Gene Organization named it IL-24 because it shares a chromosomal region with IL-10 genes as well as containing an IL-10 signature sequence[23].

The *mda-7/IL-24* gene contains seven exons and six introns which encodes a 206-amino acid protein[23]. At the N-terminus, there is a 49-amino acid hydrophobic signal peptide that is cleaved to enable protein secretion. MDA-7/IL-24 can be glycosylated at three amino acids: 85, 99, and 126. The IL-10 signature sequence is at amino acids 101 and 121. In addition, there are three protein kinase C consensus phosphorylation sites and three casein kinase II consensus phosphorylation sites. MDA-7/IL-24 functions through its receptors: IL-20R1/IL-20R2, IL-22R1/IL-20R2, or IL-22R1/IL-20R1[24]. MDA-7/IL-24 is then internalized and localizes to the

endoplasmic reticulum (ER) where it induces an ER stress response in a cancer-selective manner[25,26,27]. MDA-7/IL-24 protein can be secreted from cells after cleavage and then induce MDA-7/IL-24 expression in surrounding cells[28]. This bystander effect is how MDA-7/IL-14 induces apoptosis in distant cancer cells[25,29].

MDA-7/IL-24 is expressed in immune cells and melanocytes and can be expressed in keratinocytes after immune stimulation. It plays a role in proinflammatory, infectious, autoimmune diseases, but the function that is most pertinent to this work is the cancer-specific killing[30]. One hallmark of cancer is resistance to cell death. Cancer cells can accomplish this through avoidance of apoptotic pathways[31,32] and increasing protective autophagy[33,34,35]. MDA-7/IL-24 overcomes this hurdle by inducing ER stress and mitochondrial apoptotic pathways through the production of reactive oxygen species (ROS). MDA-7/IL-24 induces expression of pro-apoptotic proteins such as PUMA, Bim, Bax, and Bak while inhibiting anti-apoptotic proteins such as BCL-2. MDA-7/IL-24 tips the scale from protective autophagy to toxic autophagy through the induction of Beclin-1 and PERK[26,36–38].

The anti-cancer activity of MDA-7/IL-24 involves more than apoptosis and autophagy. Another hallmark of cancer is invasion and metastasis to distant sites. MDA-7/IL-24 can prevent both processes through the downregulation of focal adhesion kinase (FAK) and matrix metalloproteinases (MMPs), as well as proteins such as TGF-β, CD44, and upregulation of E-cadherin[39–41]. In total, these gene regulated by MDA-7/IL-24 lead to effective inhibition of metastasis. Beyond these functions, MDA-7/IL-24 also blocks angiogenesis, the formation of new blood vessels[42,43]. Vascular endothelial growth factor, basic fibroblast growth factor, and

PI3K/Akt signaling are all downregulated by MDA-7/IL-24, which inhibits angiogenesis and starves the tumor of oxygen and essential nutrients[26,37,44].

The major benefit of MDA-7/IL-24 is that it is effective against a wide-variety of cancers regardless of anatomic origin or p53 status[45]. MDA-7/IL-24 spares normal cells from its killing effects, making MDA-7/IL-24 an ideal treatment for cancer. As such, MDA-7/IL-24 has already entered the clinic for a phase I clinical trial. Repeat intratumoral injections of MDA-7/IL-24 were found to be well-tolerated and 44% of lesions demonstrated induction of apoptosis in local and distant sites. One patient demonstrated complete regression of the tumor and was still alive >600 days after the treatment[46,47,20].

Finally, treatment for prostate cancer is currently to be limited to surgery, chemotherapy, and hormone therapy. MDA-7/IL-24 has been found to be effective against prostate cancer *in vitro* and *in vivo. In vitro*, MDA-7/IL-24 induces apoptosis marked by caspase 3 cleavage and an inhibition of cellular proliferation[48]. In orthotopic and transgenic mouse models, MDA-7/IL-24 is effective at inducing regression of primary tumors and prevention/treatment of metastases[49–51]. Even if MDA-7/IL-24 alone is not enough to induce complete regression, it can be used in conjunction with other pharmacological agents such as Sabutoclax or other BH3 mimetics to enhance cancer cell-killing[52,53].

MDA-7/IL-24 plays a role in stimulation of the immune system as another method of anticancer activity[30]. A study in human PBMCs found MDA7/IL-24 could upregulate proinflammatory proteins such as IFNγ, IL-6, IL-1β, and GM-CSF, TNF-α[54]. Upregulation of these proteins can induce cancer cell death through the activation of cytotoxic T-cells[55], JAK/STAT

signaling to induce apoptosis[56], and sensitization to chemotherapeutics[57]. For this study, signaling pathways involved in T-cell activation are the most intriguing.

miRNAs

miRNAs are small noncoding RNAs that are about 22 nucleotides long[58]. They function as post-transcriptional gene silencers through degradation of mRNA or inhibition of translation[58]. lin-4 and let-7 were the first miRNAs discovered in *Caenorhabditis elegans* and were found to regulate the timing of larva development[59]. Since the initial discovery, it has come to light that miRNAs are evolutionarily conserved regulators of gene expression. Humans, zebrafish, mice, fruit flies, worms, and even plants encode and make use of miRNAs[60–62].

miRNAs function to silence gene expression by targeting the 3' untranslated region (UTR) in the mRNA of a target gene[58]. In some cases, miRNAs can bind to 5' UTRs as well[59]. miRNAs bind to 2-8 nucleotide sequences which are complementary to the miRNA sequence[59]. miRNAs that bind perfectly to this sequence trigger mRNA degradation while miRNAs that bind imperfectly inhibit translation[59]. In mammalian cells, the inhibition of translation is the predominant mechanism of gene silencing as perfect matches between miRNAs and mRNAs are rare. In animal models, it is estimated that miRNAs can target up to 60% of protein-coding mRNAs and single miRNAs can potentially have more than one hundred targets[61,63].

There are two pathways from which miRNAs are generated: the canonical and non-canonical pathways[64]. In both cases, miRNAs are transcribed by RNA polymerase II from the coding gene. This produces the primary-miRNA (pri-miRNA) which is an mRNA complex which contains a hairpin loop structure. In the canonical pathway, the hairpin is cleaved into the

precursor miRNA (pre-miRNA) by the Drosha-DGCR8 complex[65,66]. In the non-canonical pathway, the pri-miRNA is cleaved by the spliceosome[64]. From there the pathways converge and follow the same route. The pre-miRNA hairpin structure is exported into the cytoplasm by Exportin-5[67]. The pre-miRNA is then cleaved by DICER into two mature single-stranded miRNAs which are referred to as -5p or -3p. While the mature miRNAs came from the same encoding gene, the -5p and -3p variants have complementary sequences and thus will target different mRNAs[68]. The single-stranded mature miRNA is loaded into the RNA-induced silencing complex which contains AGO2 and TRBP[68]. These proteins guide the miRNA to the gene target and suppress gene expression through translation inhibition or mRNA degradation[61–63,69].

Drosha, DICER, and DGCR8 are the primary regulators of miRNA processing, but miRNA expression can be affected a number of ways. miRNA expression can be regulated at the transcriptional level through transcription factors. C-MYC, E2F1, and p53 are important transcription factors that have roles in cell growth/death, genome stability, and cancer progression[61,70,71]. These three transcription factors also directly regulate a number of miRNAs. Upregulation of these transcription factors leads to aberrant miRNA expression. miRNA expression is affected by mutations in the genes by which they are encoded[72]. If there is loss of the miRNA gene, it will not be transcribed leading to absence of gene silencing. On the opposite end of the spectrum, if there is amplification of the gene, the miRNA may also be transcribed in greater levels leading to enhanced gene silencing[73]. Chromosomal abnormalities can often result in the gain or loss of miRNA expression as well[63,74,75].

Like any other gene, miRNAs can be regulated through epigenetics[76]. Hypermethylation of CpG islands in the promoters of miRNA genes can inhibit miRNA expression[76]. Meanwhile,

hypomethylation in the promoter can result in an increase of miRNA expression. In addition, histone modifications can enhance or inhibit transcription of miRNAs[76]. These miRNAs are subject to epigenetic regulation, but can also regulate the epigenetic machinery, adding another layer of gene regulation to miRNAs[77,78]. They can influence global DNA methylation by targeting DNA methyltransferases and tet methylcytosine dioxygenases as well as histone modifications through targeting genes for proteins such as HDAC1[79,80,81].

In cancer, miRNAs have conflicting and sometimes complicated roles[82]. To begin, miRNA expression is often dysregulated due to increased expression of oncogenic transcription factors, or loss of expression of tumor suppressing transcription factors[83]. Amplification of oncogenes and loss of tumor suppressors can result in disruption of normal miRNA processing or transcription. Within the field of cancer, miRNAs can fall into two categories: oncomiRs or tumor-suppressor miRNAs[61,63,69].

As the name suggests, oncomiRs function to promote oncogenesis through inhibiting tumor suppressor genes[84,85]. These miRNAs are generally upregulated in cancers. One such oncomiRs is miR-221 which functions to suppress cell cycle arrest, apoptosis, and toxic autophagy and promote tumor progression[86,87]. It is frequently upregulated in a number of cancers including prostate[88]. MDA-7/IL-24 downregulates this miRNA and induces cancer cell death and when this miRNA is forcibly overexpressed in combination with MDA-7/IL-24, it can prevent cell death[89]. This shows how potent just one oncomiR can be in the protection and progression of cancer cells.

On the other side of the spectrum, tumor suppressor miRNAs work to silence oncogenes, anti-differentiation, and/or anti-apoptosis genes[90–92]. The expression of these miRNAs can be lost in cancer resulting in increased cell growth and evasion of apoptosis. *Let-7* is an example of a tumor suppressor miRNA which targets *Ras*[93]. When *let-7* is overexpressed in cancer cells there is a reduction of cell growth through suppression of RAS as well as other oncogenes and cell-cycle associated genes[94–96]. This effect has been seen *in vitro* as well as *in vivo* and the enormous potential of *let-7* as a tumor suppressing miRNA is still being explored today[61,63,69].

Their prevalent role in cancer makes miRNAs interesting targets for the treatment of the disease. In order to inhibit the activity of miRNAs, researchers have used a number of strategies[97]. The first is a miRNA sponge which is a synthetic mRNA which acts as a decoy for miRNAs[98]. It is homologous to a target mRNA and contains the target sequence to which the miRNA will bind. This prevents gene silencing of the true mRNA. This technique is easy to use *in vitro*, but *in vivo* it is limited to transgenic animals which express the sponge in specific tissues[69,99,100]. Anti-miRNA oligonucleotides (AMOs) are another popular technique for inhibiting miRNA function[101]. AMOs have high complementarity with miRNAs and tightly bind them so they cannot bind with the mRNA target[102]. However, AMOs have to be chemically modified in order to prevent degradation by exonucleases before they can be used *in vivo*. One example is locked nucleic acids (LNA) which are RNA analogs which are locked in an RNA-mimicking conformation by an extra bridge between the 2’ carbon and 4’ oxygen[101]. These LNAs are thermally stable upon binding with miRNAs, aqueously soluble, compatible with RNase H cleavage, and have low toxicity. They are also resistant to nuclease activity which makes their

use possible for *in vivo* applications[62,69]. However, despite their increased activity AMOs and LNAs require a method of delivery *in vivo.* Some delivery methods include conjugation with cholesterol, liposome-mediated delivery, nanoparticle delivery, and antibody-based delivery[103,104]. Careful selection of a delivery route, and method of inhibition is extremely important as inhibition of an off-target miRNA could potentially lead to deleterious effects[69,105].

miRNAs in Prostate Cancer

miRNAs can potentially serve as biomarkers to assess prostate cancer risk and progression[106]. PSA, which was frequently used for disease diagnosis, is subject to variability because of infections or inflammation[107]. Therefore, there is a poor correlation between PSA levels and disease aggressiveness[108,109]. Tumor grade and clinical stage can also be taken into account, but they also have limitations in predicting metastasis and disease progression[110,111].

miRNA profiling of prostate cancer tissues has been used to identify a number of miRNAs that appear to be associated with disease progression. For instance, let-7f, miR-19b, and miR-184 are associated with advanced disease[112]. miR-101, miR-200a, and miR-200b are differentially expressed in the localized tumors versus a tumor which has metastasized[113]. This indicates these miRNAs could be biomarkers for the formation of lethal metastases. In some studies, circulating miRNAs were observed in the serum of prostate cancer patients[114,115]. These miRNAs include: miR-141, miR-26, miR-195, miR-30c. Some of these circulating miRNAs were found to be inversely associated with the Gleason score of the tumor[110,111]. However, using serum miRNAs as biomarkers remains controversial due to a lack of relevant data and conflicting results in serum compared to tissues[111]. miRNAs could even serve as a biomarker for

androgen sensitivity which can lead to difficult-to-treat tumors[116]. Androgen receptors are under control of miR-130, miR-203, and miR-205 which are expressed at low levels in prostate cancer. miR-34a and miR-34c have an inverse correlation with androgen receptor expression[117,118]. Monitoring these miRNAs can also enable clinicians to better determine how long to continue ADT as treatment for mCRPC.

In this study, we seek to identify miRNAs regulated by MDA-7/IL-24 which may play a role in specifically regulating anti-cancer activity.

Materials and Methods

Cell Lines

RWPE-1, DU145, PC3, and PC3-ML were purchased from American Type Culture Collection (Manassas, VA). RWPE-1 was maintained in Keratinocyte serum-free media with provided supplements and 1% penicillin and streptomycin. DU145 was maintained in Eagle's Minimum Essential Media (EMEM) with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. PC3 was maintained in F-12K media with 10% FBS and 1% penicillin and streptomycin. PC3ML was maintained in Dulbecco's Modified Eagle Media (DMEM) with 10% FBS and 1% penicillin and streptomycin. Cells were recovered from liquid nitrogen stock provided by Praveen Bhoopathi and Anjan Pradhan which were frozen at passage 2 and were not used beyond passage 9. Cells were maintained at 37°C in 5% CO_2 humified incubators. Cells were maintained in T-75 flasks (Thermofisher) at 50-60% confluency. For downstream experiments, cells were seeded in 6 cm dishes at 60% confluency. RWPE-1 was used as the control cell line for all experiments.

Viral Infection

For the plate array, Ad.5-*CMV-mda-7* and Ad.5-*null* were used to treat cells for the PCR plate arrays. For all other experiments, Ad.5/3-*CMV-mda-7* was used to transduce MDA-7/IL-24 following viral infection and Ad.5/3-*null* was used as a control. Ad.5/3 vector was used to control for low infectibility of PC3 by Ad.5 adenovirus. Cells were treated with 2000 vp/cell in serum-free DMEM media for 4 hours. This dose was chosen because it is sublethal. After 4 hours, complete media was added to the plates. 72 hours after infection, cells were collected for experiments.

miRNA and total RNA isolation

miRNA and total RNA was isolated using the miRNeasy kit purchased from Qiagen (Hilden, Germany). Cells were lysed directly in the 6 cm dish with 700 µL QIAzol lysis reagent and homogenized by shaking for 5 minutes. The QIAzol was collected from the plate and transferred to a 1.5 µL centrifuge tube. 140 µL chloroform was added and the tube was vortexed for 20 seconds. The tube was centrifuged 15,000 rpm for 15 minutes at 4°C. The upper aqueous phase was transferred to a new 1.5 µL centrifuge tube and 1.5 volumes of 100% ethanol was added to precipitate the RNA. The solution was transferred to an RNeasy Mini column and centrifuged at >8,000xg for 15 seconds. The flow-through was discarded. 700 µL RWT was added to the column and centrifuged at >8,000xg for 15 seconds. The flow-through was discarded. 500 µL RPE was added to the column and centrifuged at >8,000xg for 15 seconds. This step was repeated but the column was centrifuged at >8,000xg for 2 minutes. The membrane was dried by centrifuging at 15,000 rpm. 30 µL of RNase free water was added directly to the membrane and then centrifuged at 15,000 rpm for 15 seconds. The concentration of RNA was measured using the nanodrop. RNA was stored at -80°C.

miRNA inhibition and overexpression

miExpress™ expression vector and miArrest™ inhibitor against miR-125a-5p were purchased from Genecopoeia (Rockville, MD). Cells were transfected in serum free media with respective vectors using the FuGene(6) transfection reagent purchased from Promega (Madison, WI). Cells were incubated with the transfection reagents and vectors for 4 hours. After 4 hours complete media was added. 72 hours later positive transfection was observed by expression of GFP and mCherry, respectively. Pure populations of transfected cells were isolated by fluorescent-activated cell sorting in the Flow Cytometry Core lab by gating for cells strongly expressing the reporter. miRNA and total RNA expression were collected from these populations for further analysis.

qRT-PCR

Gene and miRNA specific TaqMan primers were purchased from Thermofisher (Waltham, MA). 2 µg RNA was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit purchased from Thermofisher. For miRNAs, cDNA was generated using the miRNA specific primers supplied with the TaqMan primers. For gene targets, cDNA was generated with random primers supplied in the kit. cDNA synthesis was performed using the thermocycler and the following conditions: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes, and then hold at 4°C. If not used immediately for downstream applications, cDNA was stored at -20°C for up to one week. q-RT-PCR was performed using TaqMan Master Mix, cDNA, and RNase free water for a final volume of 20 µL. The parameters for the thermocycler are as follows: 50°C for 2 minutes, 95°C for 10 minutes, then 95°C for 15 second and 60°C for 1 minute repeated for 40 cycles. qRT-PCR experiments were done in triplicate and repeated at least three times.

miRNA Plate Array - Prostate Cancer

miScript miRNA PCR Plate Array specifically for prostate cancer (Catalog #: MIHS-112Z) was purchased from Qiagen. The miScript II RT Kit purchased from Qiagen was used for reverse transcription. cDNA was synthesized using 2 µg of RNA template, the miScript HiSpec buffer and oligo-dT primers for amplification of mature miRNAs. The reaction mix was incubated at 37°C for 1 hour and then 95°C for 5 minutes. Immediately after cDNA synthesis, we proceeded with the PCR plate array. The total 20 µL cDNA mix was diluted in 200 µL of RNAse-free water for a final concentration of 10-20 ng of cDNA per well. The miScript SYBR Green PCR Kit purchased from Qiagen was used to prepare the master mix for the RT-PCR reactions containing cDNA (10-20 ng per well), QuantiTect SYBR green Master mix, and miScript Universal Reverse Primer. The plate was centrifuged before adding the PCR master mix to ensure the probes were at the bottom of the wells. 25 µL of the master mix was added to each well in

the PCR array plate using a multichannel pipettor. Results were analyzed using Qiagen's GeneGlobe Data Analysis Center. The PCR plate arrays were not repeated.

Statistical Analysis

Statistical analysis was done by t-test for two samples and ANOVA for more than two samples in GraphPad Prism. P-values less than .05 were considered to be statistically significant.

Results

miRNAs are regulated by MDA-7/IL-24 in prostate cancer cell line

The miScript PCR plate array contained probes for 84 miRNAs that are found to be differentially regulated in prostate cancer. miRNAs let-7c and 34c were differentially regulated in RWPE-1, an immortalized normal prostate epithelial cell line, after treatment with Ad.5-*mda-7* (Fig. 1A). In DU145, a prostate cancer cell line, eight miRNAs were downregulated: 125a, 145, 146b, 183, 23b, 27b, 330, 455. Four miRNAs were upregulated: 20b, 494, 616, 7-5p (Fig. 1B). In comparing the effect of MDA-7/IL-24 between DU145 and RWPE-1, more miRNAs were regulated in DU145 than RWPE-1 (Fig. 1C).

Validation of PCR plate array

Two miRNAs were selected to independently validate the results of the PCR plate array based on an interest in their predicted gene targets which included the Wnt signaling pathway and genes related to DNA damage. miR-330 was significantly downregulated in DU145 after Ad.5-*mda-7* treatment for 72 hours (Fig. 2A). This miRNA is upregulated in DU145, PC3, and PC3ML prostate cancer cell lines compared to RWPE-1 (Fig.2B) miR-183 is significantly downregulated after treatment with Ad.5-*mda-7* (Fig. 2C). miR-183 is also upregulated in the three prostate cancer cells lines compared to RWPE-1 (Fig. 2D).

miR-330 and miR-183 changes are specific to DU145

To confirm that these miRNAs are relevant to the prostate cancer context, their expression was evaluated in PC3 and PC3ML. Cells were treated with 2000 vp/cell dose of Ad.5/3-*mda-7* to control for low expression of CAR receptors on PC3. This ensures the cells are being equally infected. miR-330 (Fig. 3A) and miR-183 (Fig. 3B) were significantly downregulated in DU145 and were not downregulated by MDA-7/IL-24 in PC3 and PC3ML.

Three miRNAs were downregulated in three prostate cancer cell lines

There were 10 other miRNAs to choose from that were regulated in DU145. We evaluated all ten and found 3 which were consistently downregulated in all three prostate cancer cell lines. miR-125a (Fig 4A), miR-145 (Fig 4B), and miR-23b (Fig. 4C) were significantly downregulated after treatment with Ad.5/3-*mda-7* for 72 hours. TargetScan (Release 7.2) was used to identify potential gene targets of these miRNAs. NOD-Like receptor family caspase recruitment domain containing 5 (NLRC5) was predicted to be a target of miR-125a and would be a novel target of MDA-7/IL-24. After 72 hours treatment with Ad.5/3-*mda-7,* it was significantly upregulated in prostate cancer cell lines DU145 and PC3 while its expression was unchanged in RWPE-1 (Fig. 5A). KLF4 is a predicted target of miR-145. This gene was significantly upregulated in DU145, but not PC3 and RWPE-1 (Fig. 5B). KLF15 is another potentially novel target of MDA-7/IL-24 predicted by TargetScan. It was significantly upregulated in both prostate cancer cell lines, but not RWPE-1 (Fig 5C).

Regulation of NLRC5 by miR-125a

NLRC5 is an interesting gene target because it has not been studied with regards to MDA-7/IL-24 and it may play a role in immune evasion. To evaluate if miR-125a is regulates NLRC5 expression, we used the miArrest™ plasmid to inhibit miR-125a expression. After inhibition, NLRC5 expression was significantly increased (Fig. 6A). To determine if the opposite is true, we used the miExpress™ plasmid to induce miR-125a expression. While miR-125a was significantly upregulated, NLRC5 expression did not change (Fig. 6B). This indicates that miR-125a expression alone may not be sufficient to downregulate NLRC5. This is likely due to NLRC5 already being expressed at very low levels or not expressed at all in prostate cancer cell lines. As such, miR-125a cannot drive down the expression any lower.

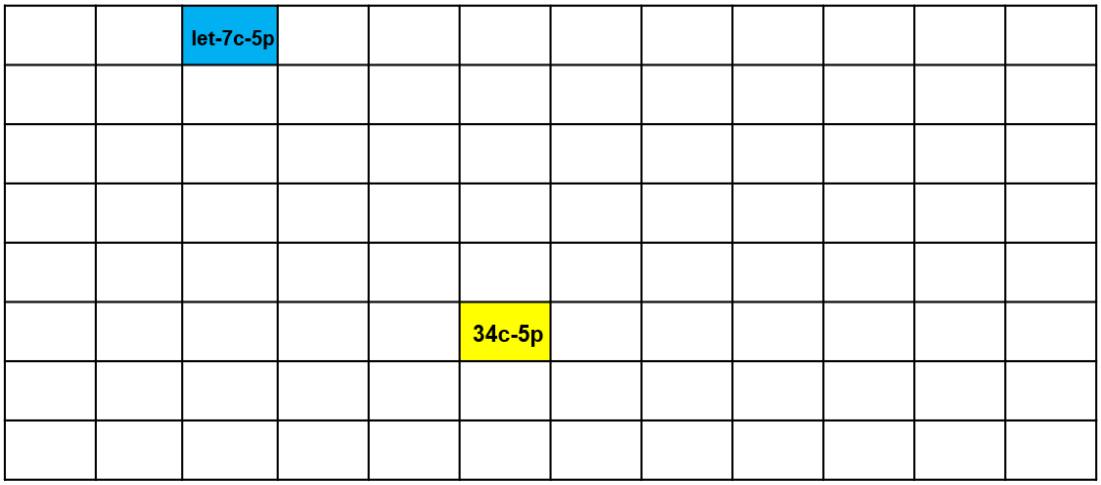
MDA-7/IL-24 regulates miR-125a expression through DICER.

Finally, to determine how miR-125a is regulated by MDA-7/IL-24 we looked at the expression of miR-125a precursors and the expression of miRNA processing enzymes. 72 hours after treatment, there is no change to the precursor miRNA in DU145 or PC3, indicating that miRNA expression is regulated after export to the cytoplasm (Fig. 7A). In these cell lines, treatment with Ad.5/3-*mda-7* for 72 hours resulted in the downregulation of DICER, a key enzyme for processing miRNAs into their mature and active forms (Fig 7B). Lastly, a DU145 cell line which stably overexpressed DICER was treated with Ad.5/3-*mda-7*. There was no change in miR-125a expression which indicates miR-125a is regulated by MDA-7/IL-24 through DICER.

Figures

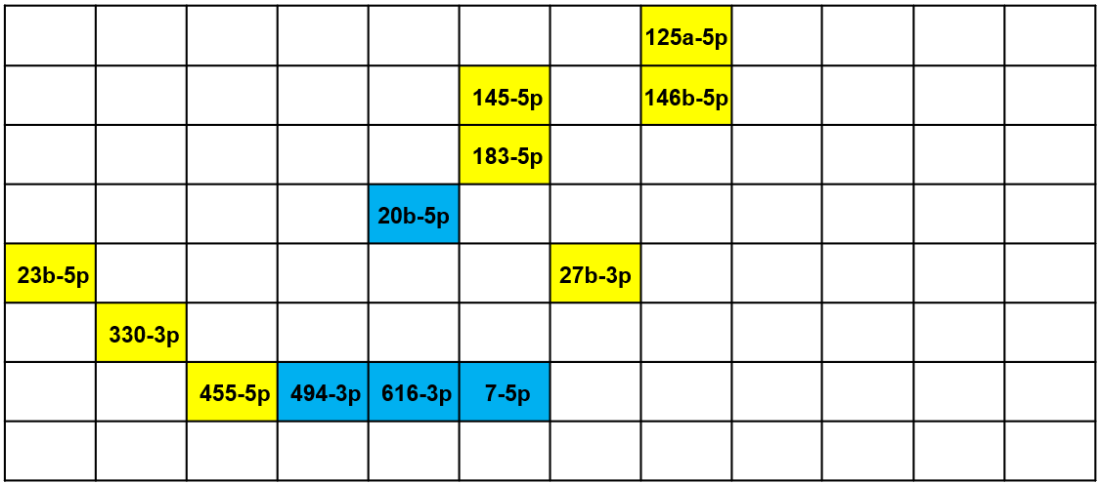
1A

RWPE-1 - Ad.5-null vs. Ad.5-*mda-7*



1B

DU145 - Ad.5-null vs. Ad.5-*mda-7*



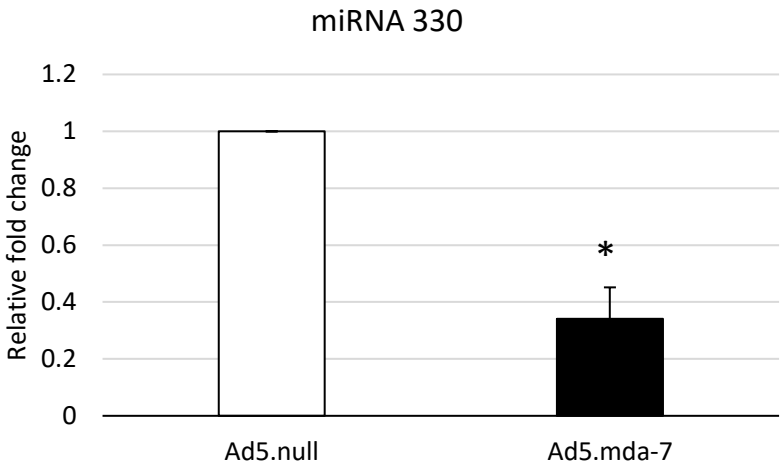
MDA-7 Treatment – DU145 vs. RWPE-1

1C

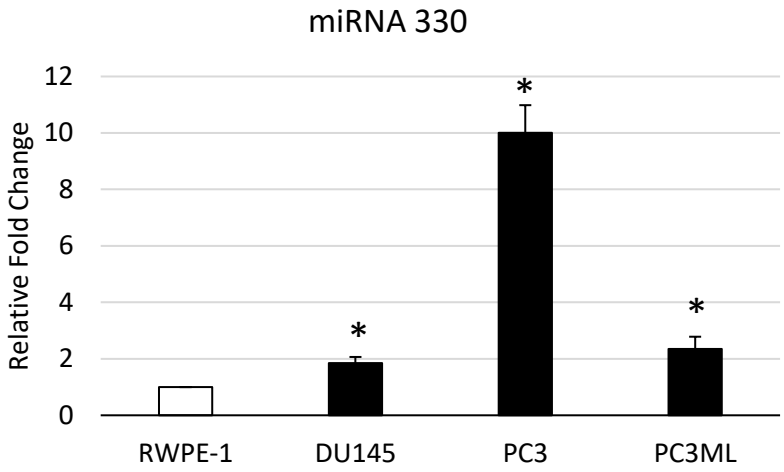
				100-5p					126-3p	126-5p	128-3p
						146a-5p		148a-3p		15b-5p	
17-5p	17-3p	181a-5p	181b-5p	182-5p	183-5p		194-5p		196a-5p	19b-3p	200b-3p
	203a-3p	205-5p	20a-5p	20b-5p	21-5p	218-5p					224-5p
					27a-3p		296-5p	29b-3p			
	330-3p	331-3p		34b-5p	34c-5p	361-5p	365b-3p	3662		374c-5p	375
425-5p					7-5p				96-5p		

Figure 1. miScript PCR Plate Array. (A) RWPE-1 were treated with 2000 vp/cell dose of Ad.5-null or Ad.5-*mda-7* for 72 hours. miRNAs highlighted in blue are greater than 2-fold upregulated. miRNAs highlighted in yellow are greater than 2-fold downregulated. (B) DU145 were treated with 2000 vp/cell dose of Ad.5-null or Ad.5-*mda-7* for 72 hours. miRNAs highlighted in blue are greater than 2-fold upregulated. miRNAs highlighted in yellow are greater than 2-fold downregulated. (C) Comparison of miRNAs differentially regulated in DU145 compared to RWPE-1 after MDA-7/IL-24 treatment.

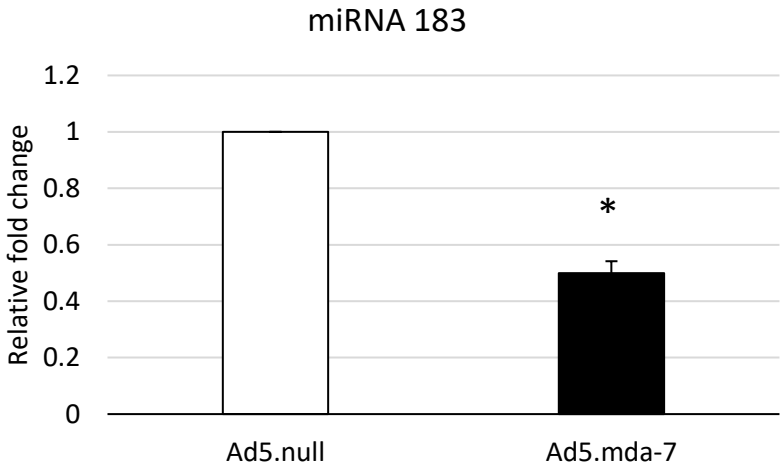
2A


miRNA 330
Relative fold change
1.2
1
0.8
0.6
0.4
0.2
0
*
Ad5.null
Ad5.mda-7

2B


miRNA 330
Relative Fold Change
12
10
8
6
4
2
0
*
*
*
RWPE-1
DU145
PC3
PC3ML

2C


miRNA 183
Relative fold change
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1
0.8
0.6
0.4
0.2
0
*
Ad5.null
Ad5.mda-7

2D

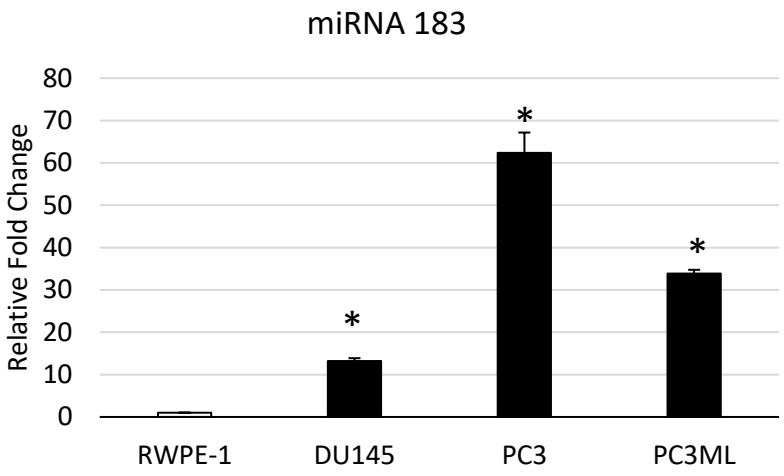
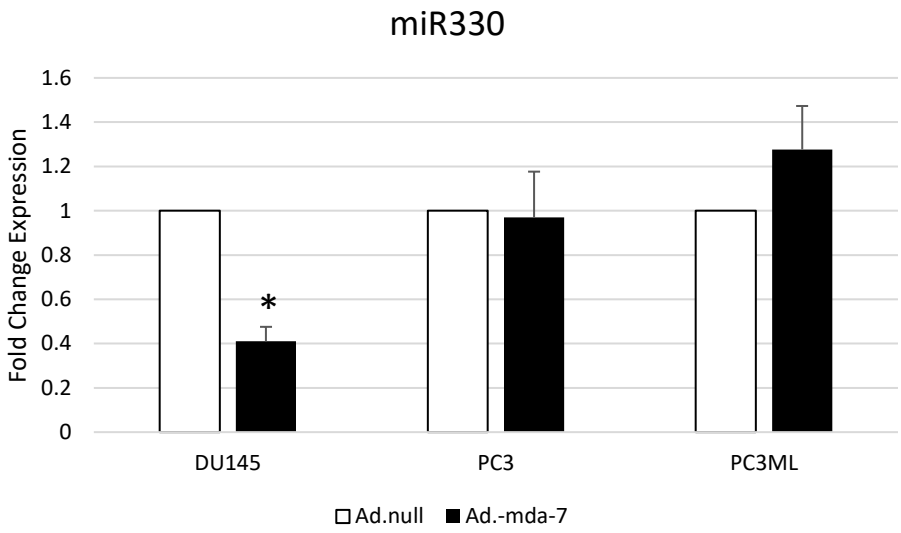


Figure 2. Validation of PCR plate array. (A) miR-330 expression in DU145 after treatment with 2000 vp/cell dose of Ad.5-null or Ad.5-*mda-7* for 72 hours. (B) Basal expression of miR-330 in three prostate cancer cell lines compared to RWPE-1. (C) miR-183 expression in DU145 after treatment with 2000 vp/cell dose of Ad.5-null or Ad.5-*mda-7* for 72 hours. (D) Basal expression of miR-183 in three prostate cancer cell lines compared to RWPE-1. * denotes $p<0.05$. Each experiment was repeated three times.

3A



3B

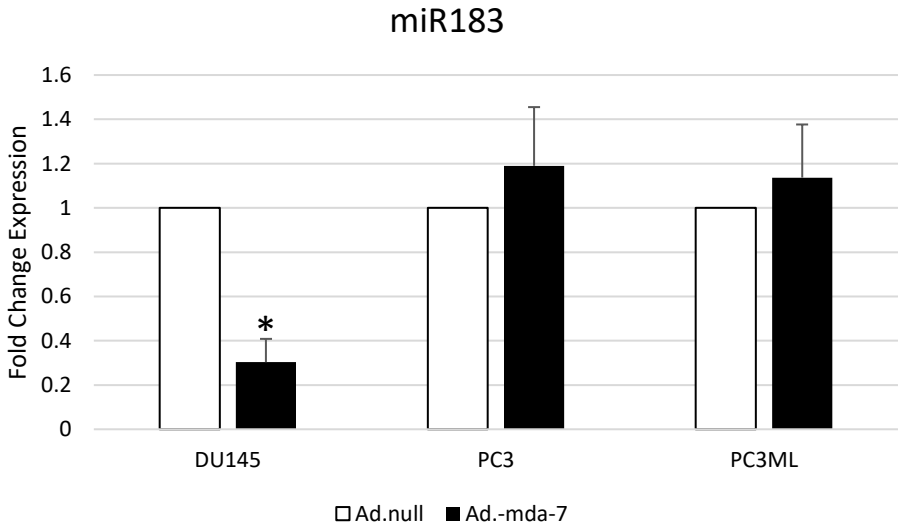
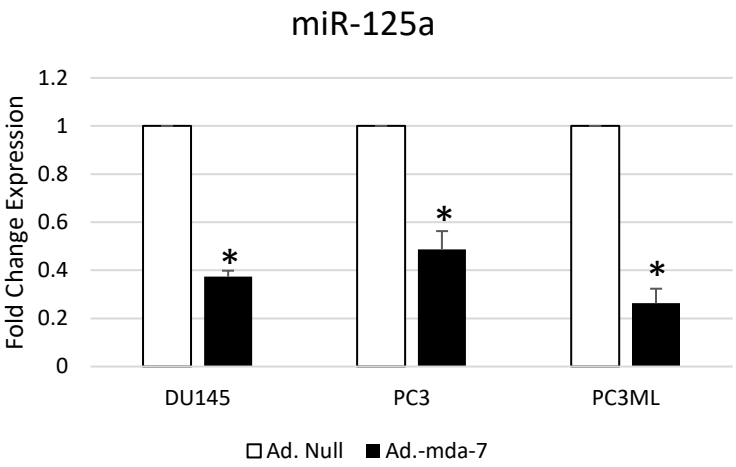
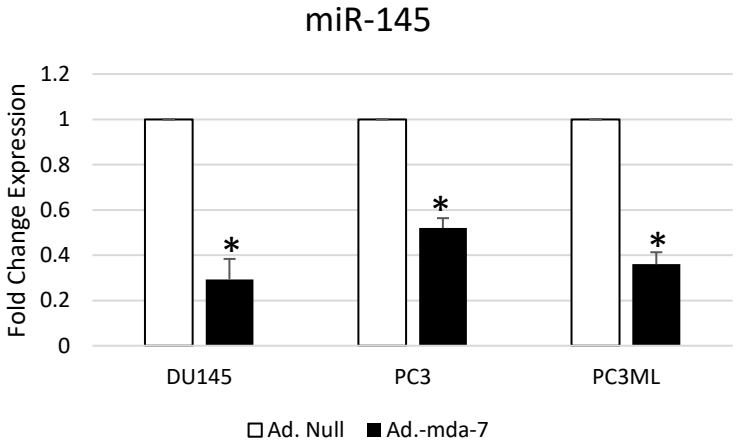


Figure 3. miR-330 and miR-183 changes are specific to DU145. (A) miR-330 expression in DU145, PC3, and PC3ML 72 hours after treatment with 2000 vp/cell dose of Ad.5/3-*mda-7* (B) miR-183 expression in DU145, PC3, and PC3ML 72 hours after treatment with 2000 vp/cell dose of Ad.5-*mda-7.* * denotes $p<0.05$. Each experiment was repeated three times.

4A


miR-125a
Fold Change Expression
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
DU145
PC3
PC3ML
Ad. Null
Ad.-mda-7

4B


miR-145
Fold Change Expression
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
DU145
PC3
PC3ML
Ad. Null
Ad.-mda-7

4C

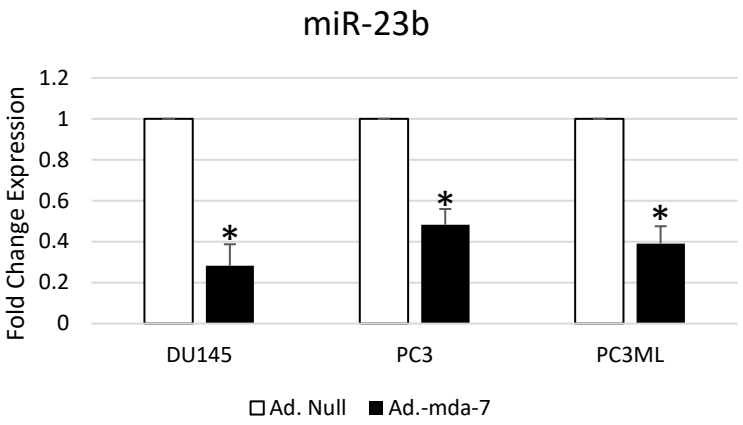
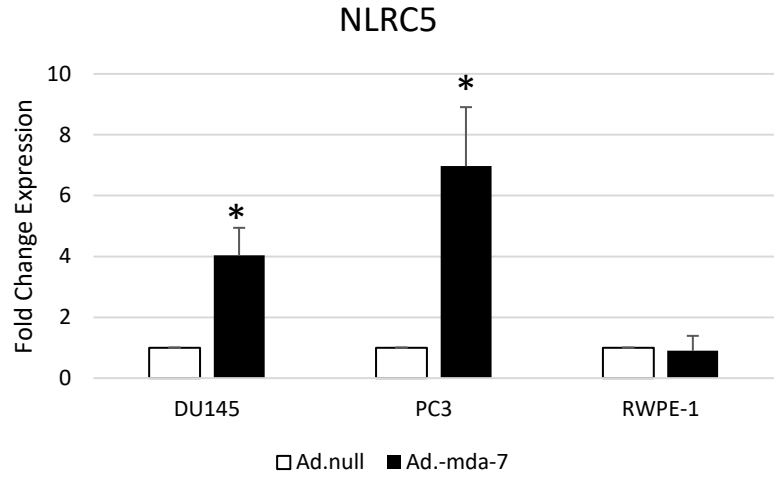
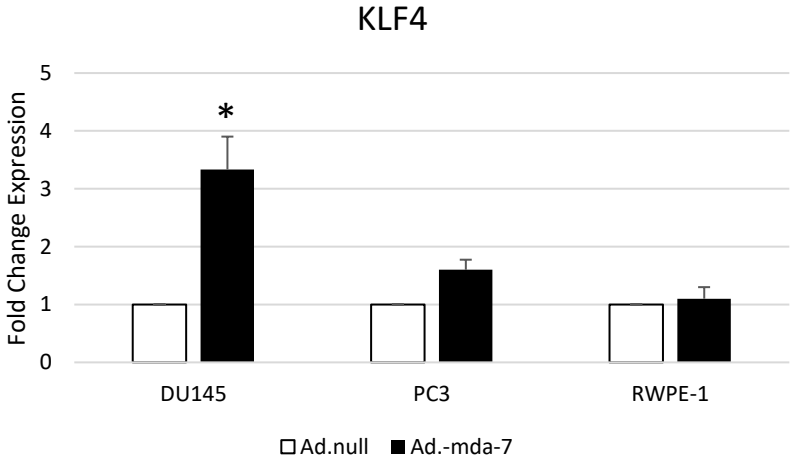


Figure 4. Three miRNAs were downregulated in three prostate cancer cell lines. (A) miR-125a (B) miR-145 (C) miR-23b expression after treatment with 2000 vp/cell dose of Ad.5/3-null or Ad.5/3-*mda-7* for 72 hours. * denotes $p<0.05$. Each experiment was repeated three times.

5A



5B



5C

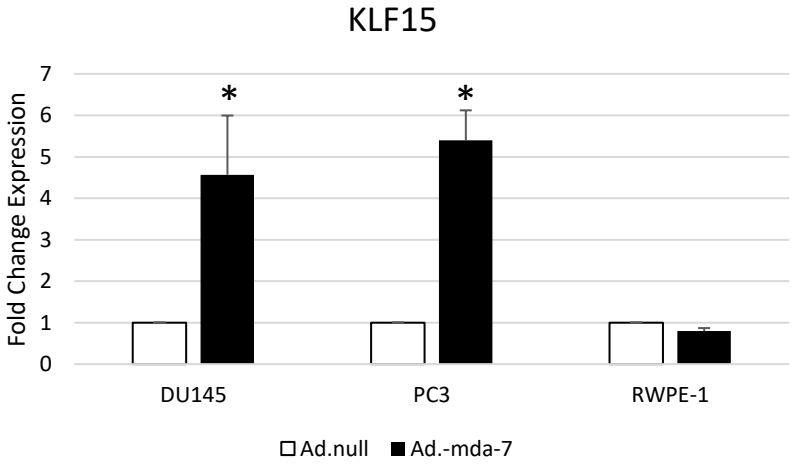
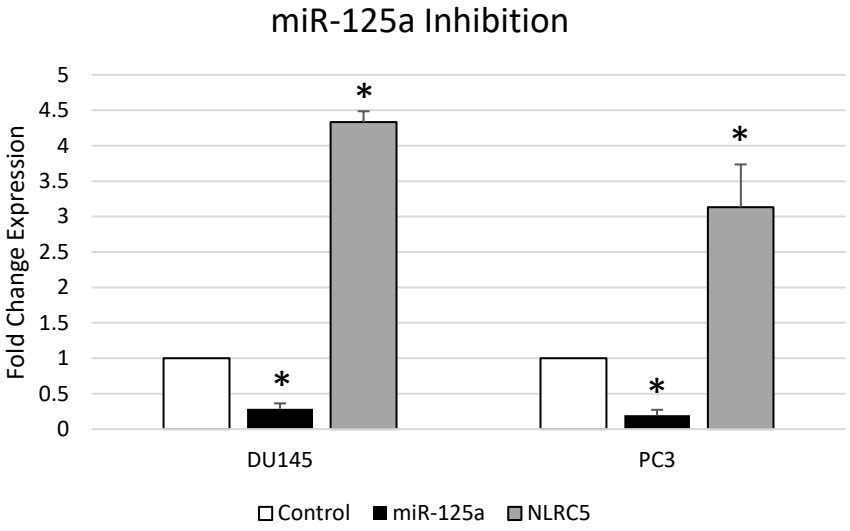


Figure 5. Gene targets of miR-125a, miR-145, and miR-23b respectively. (A) NLRC5 (B) KLF4 (C) KLF15 expression after treatment with 2000 vp/cell dose of Ad.5/3-null or Ad.5/3-*mda-7* for 72 hours. * denotes $p<0.05$. Each experiment was repeated three times.

6A



6B

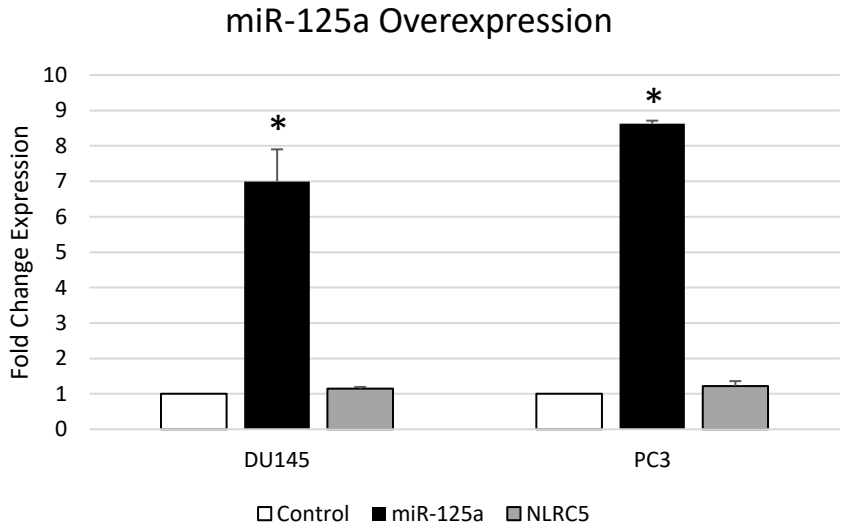
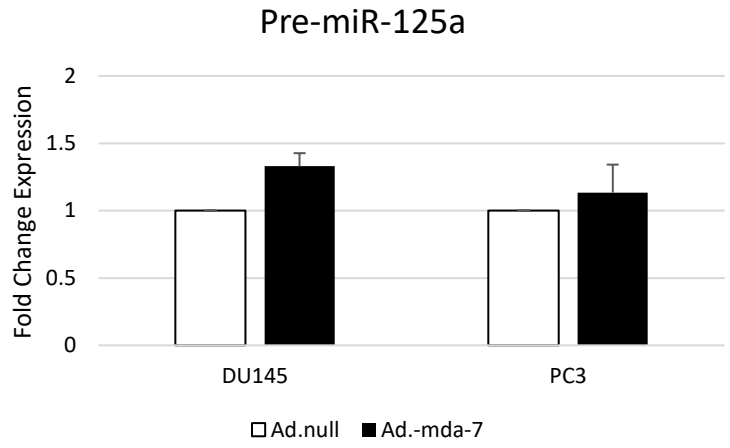
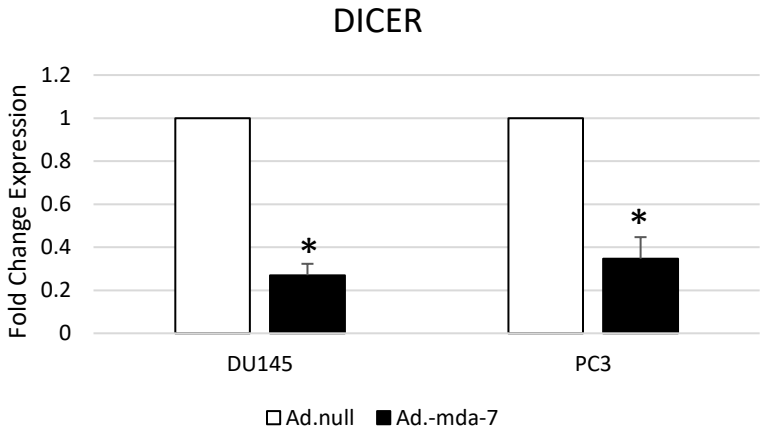


Figure 6. Regulation of NLRC5 expression by miR-125a. (A) miR-125a was inhibited by transfection with miR-125a miArrest plasmid in DU145 and PC3. Control cells were treated with scramble control plasmid. Cells were treated for 72 hours. (B) mir-125a was overexpressed by transfection with miR-125a miExpress plasmid in DU145 and PC3. Controls were treated with scramble control plasmid. Cells were treated for 72 hours. * denotes $p<0.05$. Each experiment was repeated three times.

A



B



C

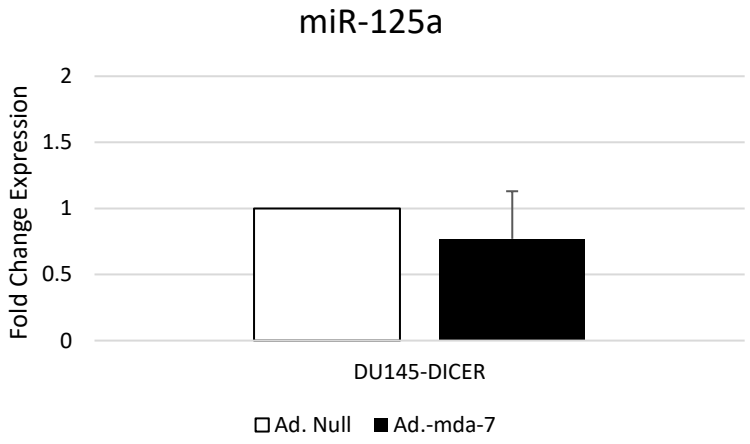


Figure 7. MDA-7/IL-24 regulates miR-125a expression through DICER. (A) Expression of the precursor RNA for miR-125a product prior to exportation to the cytoplasm after treatment with 2000 vp/cell dose of Ad.5/3-null or Ad.5/3-*mda-7* for 72 hours. (B) Expression of miRNA processing enzyme DICER after treatment with 2000 vp/cell dose of Ad.5/3-null or Ad.5/3-*mda-7* for 72 hours. (C) Expression of miR-125a in a DICER-stable DU145 cell line. * denotes $p<0.05$. Each experiment was repeated three times.

Discussion

The current study and a recent study by Pradhan *et al.* indicate that MDA-7/IL-24 regulates miRNA expression through the processing enzyme DICER. Downregulation of DICER leads to global dysregulation of all miRNAs that are processed through this enzyme. Upregulation of DICER can confer resistance to the anticancer effects of MDA-7/IL-24. This provides strong evidence that miRNAs play a role in the anti-cancer activity of MDA-7/IL-24. The reason for MDA-7/IL-24's cancer-specificity is still unknown and miRNAs may be the key to determining the mechanism. Here, we focused on miRNAs that were only regulated in cancer cells, but remained unchanged in the normal cell line. It is most likely these types of miRNAs that play a role in cancer-specificity. However, miRNAs that are regulated in the normal cell type may also be interesting targets. miRNAs could potentially confer susceptibility or resistance to MDA-7/IL-24 depending on their gene targets. For instance, in addition to upregulation of DICER, it has been previously reported that blocking the p38 MAPK pathway pharmacologically can protect cancer cells from MDA-7/IL-24-mediated apoptosis[119]. In future studies, we hope to explore and determine which miRNAs can make cancer cells resistant to MDA-7/IL-24 and/or identify miRNAs that can make normal cells susceptible to MDA-7/IL-24 treatment. Identifying such miRNAs could enhance the efficacy of MDA-7/IL-24 through combination treatments. MDA-7/IL-24 is a potent anticancer agent[20,26,37] and a combination treatment with miRNAs could give hope of a cure for many types of cancer including those that are difficult to treat.

miRNAs regulated by MDA-7/IL-24 were studied to determine if they were regulated across multiple cell lines. Some miRNAs, such as miR-183 and miR-330, appeared to be only regulated by MDA-7/IL-24 in one cell line, but not the others. This is likely due to differences in the genetic backgrounds across these cell lines. Going forward, miR-125a was downregulated by MDA-7/IL-24 across all the prostate cancer cell lines and was unchanged in normal prostate cell lines. A known target of miR-125a is BAK, a proapoptotic protein which is regulated by MDA-7/IL-24 which indicated that this could be a

miRNA that is relevant to the anticancer effects of MDA-7/IL-24 [26,120]. The role of miR-125a in cancer is somewhat controversial. Several reports indicate it acts as a tumor suppressor with expression being lost in cancers such as breast[121], ovarian[122], hepatocellular carcinoma[123], colon[124], and cervical[125]. Meanwhile, other reports indicate that it can act as an oncomiR in cancers such as pancreatic[126]. In the case of prostate cancer, the miR-125 family which includes miR-125b have been found to act as oncomiRs[126]. One study by Yi Fu and Fuhua Cao found that miR-125a, is specifically upregulated in prostate cancer cell lines and downregulating it has anti-proliferative effects[127]. With minimal information available on the role of miR-125a in prostate cancer and its known target being BAK, we chose to explore novel gene targets of this miRNA which might be relevant to the cancer-specificity of MDA-7/IL-24.

miR-125a has also been found to play a role in regulation of the immune system[128,129]. It works in combination with two other miRNAs to regulate toll-like receptor (TLR) signaling. TLR signaling plays a vital role in balancing the scales between pro-inflammatory and anti-inflammatory cascades which are triggered by pathogens[130]. In this context, miR-125a is upregulated by interleukin 10 (IL-10) which acts as an inhibitor of TLR signaling and also targeted genes associated with inflammation. As a result, the cluster of miRNAs including miR-125a act as inhibitors of the TLR signaling pathway[131]. TLR signaling plays a role in the activation of macrophages as well. Macrophage activation falls into two categories: classical and alternative. Macrophages activated by ligands such as lipopolysaccharide (LPS) of IFNγ are considered classically activated while macrophages activated by cytokines such as IL-10, IL-4, and IL-13 are alternatively activated[132–134]. One study found that miR-125a suppressed the classical activation and enhanced alternative activation which falls in line with the regulation of miR-125a by IL-10[129]. Another study found that miR-125a in combination with miR-125b can activate NF-κB signaling by suppressing its negative regulator *TNFAIP3*[135]. Constitutive NF-κB signaling is reported to promote tumor initiation,

metastasis, and angiogenesis by inducing chronic inflammation in the tumor microenvironment[136]. This suggests that miR-125a may be a potential oncomiR with the ability to shape the immune responses.

In this study, NLRC5 was predicted by TargetScan to be a target of miR-125a. NLRC5 belongs to a family of leucine-rich containing proteins which function as pattern-recognition receptors (PRRs)[137]. PRRs play a role in innate and adaptive immunity by sensing microbe-associated patterns and danger-associated molecular patterns[137]. Not all NLRs function as PRRs and in fact, they can fall into one of three categories: PRRs which sense microbes and activate transcriptional changes in the host, inducers of the formation of the inflammasome which triggers cell death, or nuclear-translocators that act as transcription transactivators[138]. NLRC5, sometimes referred to as class I transactivator (CITA), falls into the class of transcriptional activators[139]. NLRC5's primary function is to transcriptionally activate major histocompatibility complex (MHC) class I genes[139]. NLRC5 shares this function with class II transactivator (CIITA) which can also activate MHC class II genes[140]. MHC class I and II genes play a role in immune evasion which is another hallmark of cancer[35,141]. MHC class I genes present peptides derived from the cytosol to CD8+ cytotoxic T-cells[142]. MHC class II genes present vesicular-derived peptides to CD4+ helper T-cells[143]. Loss of expression of the MHC class I genes has been seen in a variety of cancers including breast, pancreatic, cervical, and non-small cell lung cancer. Researchers found that inducing expression of MHC class I and class II genes induces tumor rejection and induced a vaccine effect against new MHC-negative cancer cells[144–146]. Regulation of NLRC5 could be a new mechanism for MDA-7/IL-24 to stimulate the immune system. By restoring, NLRC5 and MHCI expression to cancerous cells, cytotoxic T-cells could now target them for destruction. This could enhance the efficacy of MDA-7/IL-24 treatment.

The down-regulation of miR-145 by MDA-7/IL-24 is a conflicting result which is both expected and unexpected. miR-145 is well established in the literature to act as a tumor suppressor. Loss of miR-145 expression has been reported in multiple cancers including prostate[111,147–150]. Its expression inhibits

invasion and metastasis by regulating proteins such as cadherins, fibronectin, and SMAD3[151]. One could envision MDA-7/IL-24 upregulating this miRNA to enhance cancer cell-killing and prevent metastasis. However, it is likely that MDA-7/IL-24 is downregulating miR-145 because this miRNA is dependent on DICER for processing. Although it is downregulated in this study, it is still possible for miR-145 to contribute to cancer cell-killing by MDA-7/IL-24 because some of its predicted targets include tumor suppressors. Expression of these tumor suppressors would be upregulated after miR-145 inhibition.

miR-23b is another miRNA that has contrasting roles in different cancers. In bladder[152] and cervical[153] cancer it has been reported to function as a tumor suppressor. This miRNA inhibits cellular proliferation by targeting the PI3K-AKT pathway and inhibits epithelial-mesenchymal transition (EMT) by targeting cytoskeletal remodeling genes[154]. In glioma[155] and renal[156] cancer, miR-23b is upregulated and acts as an oncomiR. The most unfavorable consequence of miR-23b oncomiR function is the downregulation of PTEN which in turn, promotes cancer cell proliferation[157]. Its function is further complicated in breast and prostate cancer where it has been reported to act as both a tumor-suppressor and an oncomiR[158–161]. Interestingly, miR-23b is also reported to play a role in the production of ROS which is a mechanism by which MDA-7/IL-24 mediates cell killing[4]. In the context of miR-23b, inhibition results in the generation of reactive oxygen species through the upregulation of NOX4 which is a member of the NADPH oxidase family. Inhibition also increases proline oxidase expression to generate ROS[162]. Taken together, we categorized miR-23b as an oncomiR in this study and focused on potential new gene targets that function as tumor suppressors after MDA-7/IL-24 treatment.

Krüppel-like factor 4 and 15 (KLF4 and KLF15) emerged as targets of the miR-145 and miR-23b, respectively. Both were found to be upregulated by MDA-7/IL-24 treatment. These genes belong to a family of transcription factors which contain zinc-finger domains in the C-terminal which target DNA with GC-rich sequences, preferably a 5'-CACCC-3' motif[163]. These transcription factors are unique because they contain three $Cysteine_2/Histidine_2$ containing zinc fingers located at the extreme C-

terminus, share a highly conserved seven residue sequence between zinc fingers, and can also bind to the GT box[164–166]. KLF4 has been extensively studied in cancer, while KLF15 is a newcomer to the field.

KLF4, also known as gut enriched Krüppel-like factor, is well-known for its role in self-renewal and pluripotency in embryonic stem cells, hematopoietic cells, and bone marrow stromal cells through activation of genes which maintain these processes such as NANOG, OCT4, and SOX2[167]. In cancer, KLF4 has conflicting roles as an oncogene and as a tumor suppressor. For instance, in triple-negative breast cancer KLF4 has been found to be upregulated and silencing it inhibits cell migration and viability[168]. Some studies draws a connection between miR-145 and KLF4 in bladder cancer[169,79]. miR-145 is downregulated and KLF4 is upregulated in bladder cancer cells. Restoration of miR-145 drove KLF4 expression down and induced apoptosis by downregulating aerobic glycolysis[170]. However, there is substantial evidence that supports KLF4's function as a tumor suppressor. In pancreatic[171], colorectal[172], and prostate cancer[173], KLF4 expression is downregulated in cell lines and patient samples. Notably, in prostate cancer, restoration of KLF4 inhibited cell proliferation, migration, and induced cell cycle arrest[174]. Here, MDA-7/IL-24 upregulated KLF4 while downregulating miR-145. Considering the literature, miR-145 inhibition would have oncogenic effects. Even though miR-145 is inhibited in this context, MDA-7/IL-24 is still capable of killing cancer cells. miR-145 inhibition would allow for the upregulation of KLF4 and thus enable its tumor-suppressing actions. Combined with the anti-cancer function of MDA-7/IL-24, this observation suggests that miR-145 inhibition is not sufficient to induce tumorigenesis. It is also possible that miR-145 is not directly targeting KLF4 as a number of miRNAs have been reported to act on this gene.

KLF15, also known as kidney-enriched Krüppel-like factor (KKLF), is among one of the KLFs which has yet to have its protein interaction domains characterized. It is expressed in metabolically active endothelial cells found in liver, kidney, and heart tissue[163,175]. KLF15 has established itself as a regulator of insulin-sensitivity, glucose uptake[176], and lipid metabolism[177]. In disease, KLF15 has also been

implicated in kidney disease as the loss of KLF15 is associated with podocyte differentiation and renal fibrosis[175]. It functions to suppress cardiac hypertrophy as well[178]. Studies in KLF15 knockout mice found increased blood pressure, increased cardiac mass, and dysfunction of the left ventricle[179]. In studies of heart failure in humans, KLF15 mRNA is down-regulated in left ventricle tissue[178]. In recent years, KLF15 has emerged as a potential novel tumor suppressor. Studies in breast, lung, and gastric cancer models found KLF15 expression is downregulated in cancer tissue compared to normal tissue. Overexpression of KLF15 suppressed proliferation of cancer cells by upregulating p21 and p27 which induce cell cycle arrest[180–182]. MDA-7/IL-24 has previously been shown to induce p27 expression[183] and here, KLF15 expression. KLF15 was not upregulated in normal cells and this may indicate a potential mechanism to explain cancer-specific killing by MDA-7/IL-24. Here, we did not go in depth to explore the connection between KLF15 and MDA-7/IL-24. Further studies would include blocking KLF15 expression in combination with MDA-7/IL-24 treatment to determine if p27 expression can be inhibited to save cancer cells from cell-cycle arrest.

While pondering how miRNAs play a role in MDA-7/IL-24 efficacy, there was also a question that lingered in the back of our minds: what makes normal cells normal? Inevitably, the question ended up being far outside of the scope of this study. Fortunately, there is an ever-growing breadth of knowledge that seeks to determine what truly differentiates cancer and normal cells. miRNAs have firmly established their place as regulators of gene expression so it is not far-fetched to believe that miRNAs may play a role in maintenance of normal physiology. The best way to discern which miRNAs contribute to a normal phenotype would be through RNAseq. This would allow researchers to identify known and new miRNAs. The goal would be to identify miRNAs that are only expressed in normal cells, but not in any type of cancer cells. Alternatively, we could also identify miRNAs which are never expressed in normal cells. It is not likely that one miRNA is what decides if a cell is normal so a strategy using RNAseq

would give us numerous potential miRNAs. From there, overexpression or inhibition experiments could be done to determine if groups of miRNAs can transform a cancer cell into a “normal” cell or vice versa.

References

1. Rebbeck, T. R. Prostate Cancer Genetics: Variation by Race, Ethnicity, and Geography. *Semin Radiat Oncol* **27**, 3–10 (201AD).

2. Grozescu, T. & Popa, F. Prostate cancer between prognosis and adequate/proper therapy. *J. Med. Life* **10**, 5–12 (2017).

3. DeRycke, M. S. *et al.* An expanded variant list and assembly annotation identifies multiple novel coding and noncoding genes for prostate cancer risk using a normal prostate tissue eQTL data set. *PLoS One* **14**, 1–15 (2019).

4. Agalliu, I. *et al.* Contribution of HPC1 (RNASEL) and HPCX variants to prostatecancer in a founder population. *Prostate* **70**, 1716–1727 (2010).

5. Berry, R. *et al.* Linkage Analyses at the Chromosome 1 Loci 1q24-25 (HPC1), 1q42.2-43 (PCAP), and 1p36 (CAPB) in Families with Hereditary Prostate Cancer. *Am. J. Hum. Genet.* **66**, 539–546 (2002).

6. Datta, M. W. *et al.* Perlecan, a candidate gene for the CAPB locus, regulates prostate cancer cell growth via the Sonic Hedgehog pathway. *Mol. Cancer* **5**, 1–15 (2006).

7. Cunningham, J. M. *et al.* Genome linkage screen for prostate cancer susceptibility loci: Results from the Mayo Clinic familial prostate cancer study. *Prostate* **57**, (2003).

8. Kim, Y. R. *et al.* HOXB13-mediated suppression of p21WAF1/CIP1 regulates JNK/c-Jun signaling in prostate cancer cells. *Oncol. Rep.* **35**, 2011–2016 (2016).

9. Oh, M. *et al.* The association of BRCA1 and BRCA2 mutations with prostate cancer risk,

frequency, and mortality: A meta-analysis. *Prostate* (2019). doi:https://doi.org/10.1002/pros.23795

10. Perlmutter, M. A. & Lepor, H. Androgen Deprivation Therapy in the Treatment of Advanced Prostate Cancer. *MedReviews* **44**, 3–8 (2007).

11. Boulos, S. & Mazhar, D. The evolving role of chemotherapy in androgen-independent (hormone-refractory) prostate cancer. *Futur. Oncol.* **13**, 1091–1095 (2017).

12. Bolla, M. *et al.* Long-term results with immediate androgen suppression and external irradiation in patients with locally advanced prostate cancer (an EORTC study): a phase III randomised trial. *Lancet* **360**, 103–108 (2002).

13. Chang, A. J., Autio, K. A., Roach, M. & Scher, H. I. High-risk prostate cancer-Classification and therapy. *Nat. Rev. Clin. Oncol.* **11**, 308–323 (2014).

14. Ahmadi, H. & Daneshmand, S. Androgen deprivation therapy: Evidence-based management of side effects. *BJU Int.* **111**, 543–548 (2013).

15. Wade, C. A. & Kyprianou, N. Profiling prostate cancer therapeutic resistance. *Int. J. Mol. Sci.* **19**, 1–19 (2018).

16. Tagawa, S. T. *et al.* Phase II study of lutetium-177-labeled anti-prostate-specific membrane antigen monoclonal antibody J591 for metastatic castration-resistant prostate cancer. *Clin. Cancer Res.* **19**, 5182–5191 (2013).

17. Haberkorn, U., Eder, M., Kopka, K., Babich, J. W. & Eisenhut, M. New strategies in prostate cancer: Prostate-specific membrane antigen (PSMA) ligands for diagnosis and

therapy. *Clin. Cancer Res.* **22**, 9–15 (2016).

18. Kretschmer, A. & Tilki, D. Biomarkers in prostate cancer – Current clinical utility and future perspectives. *Crit. Rev. Oncol. Hematol.* **120**, 180–193 (2017).

19. Dash, R. *et al.* mda-7/IL-24: A unique member of the IL-10 gene family promoting cancer-targeted toxicity. *Cytokine Growth Factor Rev.* **21**, 381–391 (2010).

20. Lin, J. J. *et al.* The melanoma differentiation associated gene mda-7 suppresses cancer cell growth. *Proc. Natl. Acad. Sci.* **93**, 9160–9165 (1996).

21. Jiang, H., Lin, J., Su, Z., Goldstein, N. & Fisher, P. Subtraction hybridization identifies a novel melanoma differentiation associated gene, mda-7, modulated during humanmelanoma differentiation, growth and progression. *Oncogene* **11**, 2477–2486 (1995).

22. Jiang, H. & Fisher, P. Use of a sensitive and efficient sub-traction hybridization protocol for the identification of genesdifferentially expressed during growth, differentiation in hu-man melanoma cells. *Mol Cell Differ.* **1**, 285–299 (1993).

23. Huang, E. Y. *et al.* Genomic structure, chromosomal localization and expression profile of a novel melanoma differentiation associated (mda-7) gene with cancer specific growth suppressing and apoptosis inducing properties. *Oncogene* **20**, 7051–7063 (2001).

24. Wang, M., Tan, Z., Zhang, R., Kotenko, S. V. & Liang, P. Interleukin 24 (MDA-7/MOB-5) signals through two heterodimeric receptors, IL-22R1/IL-20R2 and IL-20R1/IL-20R2. *J. Biol. Chem.* **277**, 7341–7347 (2002).

25. Sauane, M. *et al.* Autocrine regulation of mda-7/IL-24 mediates cancer-specific apoptosis. *Proc. Natl. Acad. Sci.* **105**, 9763–9768 (2008).

26. Menezes, M. E. *et al. Role of MDA-7/IL-24 a Multifunction Protein in Human Diseases*. *Advances in Cancer Research* **138**, (Elsevier Inc., 2018).

27. Sieger, K. A. *et al.* The tumor suppressor activity of MDA-7/IL-24 is mediated by intracellular protein expression in NSCLC cells. *Mol. Ther.* **9**, 355–367 (2004).

28. Su, Z. *et al.* Unique aspects of mda- 7 / IL-24 antitumor bystander activity : establishing a role for secretion of MDA-7 / IL-24 protein by normal cells. *Oncogene* **24**, 7552–7566 (2005).

29. Chada, S. *et al.* Bystander Activity of Ad-mda7 : Human MDA-7 Protein Kills Melanoma Cells via an IL-20 Receptor-Dependent but STAT3-Independent Mechanism. *Mol. Ther.* **10**, 1085–1095 (2004).

30. Chada, S. *et al.* MDA-7 / IL-24 is a unique cytokine – tumor suppressor in the IL-10 Family. *Int. Immunopharmacol.* **4**, 649–667 (2004).

31. Mohamed, M., Bishr, M., Almutairi, F. & Ali, A. Inhibitors of apoptosis: clinical implications in cancer. *Apoptosis* **22**, 1487–1509 (2017).

32. Mohammad, R. M. *et al.* Broad targeting of resistance to apoptosis in cancer. *Semin. Cancer Biol.* **35**, S78–S103 (2015).

33. Liu, H., He, Z. & Simon, H.-U. Protective role of autophagy and autophagy-related protein 5 in early tumorigenesis. *J. Mol. Med.* **93**, 159–164 (2015).

34. Arroyo, D. *et al.* Autophagy in inflammation, infection, neurodegeneration and cancer. *Int. Immunopharmacol.* **18**, 55–65 (2014).

35. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: The next generation. *Cell* **144**, 646–674 (2011).

36. Park, M. A. *et al.* PERK-dependent regulation of MDA-7/IL-24-induced autophagy in primary human glioma cells. *Autophagy* **4**, 513–515 (2008).

37. Gupta, P. *et al.* mda-7/IL-24: Multifunctional cancer-specific apoptosis-inducing cytokine. *Pharmacol. Ther.* **111**, 596–628 (2006).

38. Su, Z. *et al.* A cancer terminator virus eradicates both primary and distant human melanomas. *Cancer Gene Ther.* **15**, 293–302 (2008).

39. Mhashilkar, A. M. *et al.* MDA-7 Negatively Regulates the B-Catenin and PI3K Signaling Pathways in Breast and Lung Tumor Cells. *Mol. Ther.* **8**, 207–219 (2003).

40. Huo, W. E. I., Li, Z., Zhu, X., Bao, Y. & An, L. MDA-7 / IL-24 suppresses tumor adhesion and invasive potential in hepatocellular carcinoma cell lines. *Oncol. Lett.* **30**, 986–992 (2013).

41. Ramesh, R. *et al.* Ectopic Production of MDA-7 / IL-24 Inhibits Invasion and Migration of Human Lung Cancer Cells. *Mol. Ther.* **9**, 510–518 (2004).

42. Ramesh, R. *et al.* Melanoma Differentiation-associated Gene 7/Interleukin (IL)-24 Is a Novel Ligand That Regulates Angiogenesis via the IL-22 Receptor. *Cancer Res.* **63**, 5105–5113 (2003).

43. Nishikawa, T., Ramesh, R., Munshi, A., Chada, S. & Meyn, R. E. Adenovirus-Mediated

mda-7 (IL24) Gene Therapy Suppresses Angiogenesis and Sensitizes NSCLC Xenograft Tumors to Radiation. *Mol. Ther.* **9**, 818–828 (2004).

44. Azab, B. M. *et al.* Enhanced Prostate Cancer Gene Transfer and Therapy Using a Novel Serotype Chimera Cancer Terminator Virus (Ad.5/3-CTV). *J. Cell. Physiol.* **229**, 34–43 (2014).

45. Su, Z. *et al.* Melanoma differentiation associated gene-7 , mda -7 / IL-24 , selectively induces growth suppression , apoptosis and radiosensitization in malignant gliomas in a p53 -independent manner. *Oncogene* **22**, 1164–1180 (2003).

46. Cunningham, C. C. *et al.* Clinical and local biological effects of an intratumoral injection of mda-7 (IL24; INGN 241) in patients with advanced carcinoma: a phase I study. *Mol. Ther.* **11**, 149–159 (2005).

47. Tong, A. W. *et al.* Intratumoral Injection of INGN 241 , a Nonreplicating Adenovector Expressing the Melanoma-Differentiation Associated Gene-7 (mda-7 / IL24): Biologic Outcome in Advanced Cancer Patients. *Mol. Ther.* **11**, 160–172 (2005).

48. Yacoub, A. *et al.* Caspase-, cathepsin-, and PERK-dependent regulationof MDA-7 / IL-24-induced cell killing in primary human glioma cells. *Mol. Cancer Ther.* **7**, 297–314 (2008).

49. Gopalan, B., Shanker, M., Chada, S. & Ramesh, R. MDA-7 / IL-24 suppresses human ovarian carcinoma growth in vitro and in vivo. *Mol. Cancer* **6**, 1–10 (2007).

50. Yacoub, A. *et al.* Melanoma Differentiation-associated 7 (Interleukin 24) Inhibits Growth and Enhances Radiosensitivity of Glioma Cells in Vitro and in Vivo 1. *Clin. Cancer Res.* **9**,

3272–3281 (2003).

51. Yang, C. *et al.* Inhibition of autophagy induced by overexpression of mda-7 / interleukin-24 strongly augments the antileukemia activity in vitro and in vivo. *Cancer Gene Ther.* **17**, 109–119 (2010).

52. Wang, X.-Y. *et al.* Therapy of prostate cancer using a novel cancer terminator virus and a small molecule BH-3 mimetic. *Oncotarget* **6**, (2015).

53. Wang, X.-Y. *et al.* Apogossypol derivative BI-97C1 (Sabutoclax) targeting Mcl-1 sensitizes prostate cancer cells to mda-7/IL-24-mediated toxicity. *Proc. Natl. Acad. Sci.* **108**, 8785–8790 (2011).

54. Caudell, E. G. *et al.* The Protein Product of the Tumor Suppressor Gene, Melanoma Differentiation-Associated Gene 7, Exhibits Immunostimulatory Activity and Is Designated IL-24. *J. Immunol.* **168**, 6041–6046 (2002).

55. Wei, X. X. *et al.* Systemic GM-CSF Recruits Effector T Cells into the Tumor Microenvironment in Localized Prostate Cancer. *Cancer Immunol. Res.* **4**, 948–958 (2016).

56. Wee, Z. N. *et al.* EZH2-mediated inactivation of IFN-γ-JAK-STAT1 signaling is an effective therapeutic target in MYC-driven prostate cancer. *Cell Rep.* **8**, 204–216 (2014).

57. Smith, P. C., Hobisch, A., Lin, D. L., Culig, Z. & Keller, E. T. Interleukin-6 and prostate cancer progression. *Cytokine Growth Factor Rev.* **12**, 33–40 (2001).

58. Bartel, D. P., Lee, R. & Feinbaum, R. MicroRNAs : Genomics , Biogenesis , Mechanism ,

and Function Genomics : The miRNA Genes. *Cell* **116**, 281–297 (2004).

59. Ørom, U. A., Nielsen, F. C. & Lund, A. H. MicroRNA-10a Binds the 50UTR of Ribosomal Protein mRNAs and Enhances Their Translation. *Mol. Cell* **30**, 460–471 (2008).

60. Agarwal, V., Bell, G. W., Nam, J. W. & Bartel, D. P. Predicting effective microRNA target sites in mammalian mRNAs. *Elife* **4**, 1–38 (2015).

61. Pradhan, A. K., Emdad, L., Das, S. K., Sarkar, D. & Fisher, P. B. *The Enigma of miRNA Regulation in Cancer*. *Advances in Cancer Research* **135**, (Elsevier Inc., 2017).

62. Li, C., Feng, Y., Coukos, G. & Zhang, L. Therapeutic MicroRNA Strategies in Human Cancer. *AAPS J.* **2**, 747–757 (2010).

63. Tong, A. W. & Nemunaitis, J. Modulation of miRNA activity in human cancer: A new paradigm for cancer gene therapy? *Cancer Gene Ther.* **15**, 341–355 (2008).

64. Ramalingam, P. *et al.* Biogenesis of intronic miRNAs located in clusters by independent transcription and alternative splicing. *RNA* **20**, 76–87 (2013).

65. Yeom, K., Lee, Y., Han, J., Suh, M. R. & Kim, V. N. Characterization of DGCR8 / Pasha , the essential cofactor for Drosha in primary miRNA processing. *Nucleic Acid Res.* **34**, 4622–4629 (2006).

66. Han, J. *et al.* The Drosha – DGCR8 complex in primary microRNA processing. *Genes Dev.* **18**, 3016–3027 (2004).

67. Bohnsack, M. T., Czaplinski, K. & Go, D. Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. *RNA* **10**, 185–191 (2004).

68. Chendrimada, T. P. *et al.* TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature* **436**, 740–744 (2005).

69. Li, Z. & Rana, T. M. Therapeutic targeting of microRNAs: Current status and future challenges. *Nat. Rev. Drug Discov.* **13**, 622–638 (2014).

70. Adams, C. M., Hiebert, S. W. & Eischen, C. M. Myc Induces miRNA-Mediated Apoptosis in Response to HDAC Inhibition in Hematologic Malignancies. *Cancer Res.* **76**, (2016).

71. O'Donnell, K., Wentzel, E., Zeller, K., Dang, C. & Mendell, J. c-myc-regulated microRNAs modulate E2F1 expression. *Nature* **435**, 839–843 (2005).

72. Clop, A. *et al.* A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep. *Nat. Genet.* **38**, 813–818 (2006).

73. Bray, I. *et al.* Widespread Dysregulation of MiRNAs by MYCN Amplification and Chromosomal Imbalances in Neuroblastoma : Association of miRNA Expression with Survival. *PLoS One* **4**, (2009).

74. Kiefer, Y., Schulte, C., Tiemann, M. & Bullerdiek, J. Chronic lymphocytic leukemia-associated chromosomal abnormalities and miRNA deregulation. *Appl. Clin. Genet.* **2**, 21–28 (2012).

75. Rossi, S. *et al.* microRNA fingerprinting of CLL patients with chromosome 17p deletion identify a miR-21 score that stratifies early survival. *Blood* **116**, 945–953 (2019).

76. Saito, Y. & Jones, P. M. Epigenetic Activation of Tumor Suppressor MicroRNAs in Human Cancer Cells ND ES SC. *Cell Cycle* **5**, 2220–2222 (2006).

77. Majid, S. *et al.* miRNA-34b Inhibits Prostate Cancer through Demethylation, Active Chromatin Modifications, and AKT Pathways. *Clin. Cancer Res.* **19**, 73–85 (2013).

78. Noonan, E. J. *et al.* miR-449a targets HDAC-1 and induces growth arrest in prostate cancer. *Oncogene* **28**, 1714–1724 (2009).

79. Yao, Q., Chen, Y. & Zhou, X. The roles of microRNAs in epigenetic regulation. *Curr. Opin. Chem. Biol.* **51**, 11–17 (2019).

80. Fabbri, M. *et al.* MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. *PNAS* **104**, 15805–15810 (2007).

81. Pan, W. *et al.* MicroRNA-21 and MicroRNA-148a Lupus CD4+ T Cells by Directly and Contribute to DNA Hypomethylation in Indirectly Targeting DNA Methyltransferase 1. *J. Immunol.* **184**, 6773–6781 (2010).

82. Jansson, M. D. & Lund, A. H. MicroRNA and cancer. *Mol. Oncol.* **6**, 590–610 (2012).

83. Ha, M. & Kim, V. Regulation of microRNA biogenesis. *Nat. Rev. Mol. Cell Biol.* **15**, 509–524 (2014).

84. Hayashita, Y. *et al.* Priority Report A Polycistronic MicroRNA Cluster , miR-17-92 , Is Overexpressed in Human Lung Cancers and Enhances Cell Proliferation. 9628–9633 (2005). doi:10.1158/0008-5472.CAN-05-2352

85. Lewis, B. P., Shih, I., Jones-rhoades, M. W., Bartel, D. P. & Burge, C. B. Prediction of Mammalian MicroRNA Targets. **115**, 787–798 (2003).

86. Pineau, P. *et al.* miR-221 overexpression contributes to liver tumorigenesis. (2010).

doi:10.1073/pnas.0907904107

87. Sage, C. *et al.* Regulation of the p27 tumor suppressor by miR-221 and miR-222 promotes cancer cell proliferation. *EMBO J.* **26**, 3699–3708 (2007).

88. Galardi, S. *et al.* miR-221 and miR-222 Expression Affects the Proliferation Potential of Human Prostate Carcinoma Cell Lines by. **282**, 23716–23724 (2007).

89. Pradhan, A. K. *et al.* Mda-7/IL-24 mediates cancer cell-specific death via regulation of miR-221 and the beclin-1 axis. *Cancer Res.* **77**, 949–959 (2017).

90. Fabbri, M. *et al.* miR-15 and miR-16 induce apoptosis by targeting BCL2. *PNAS* **103**, 13944–13949 (2005).

91. Takamizawa, J. *et al.* Advances in Brief Reduced Expression of the let-7 MicroRNAs in Human Lung Cancers in Association with Shortened Postoperative Survival. 3753–3756 (2004).

92. Zhang, B., Pan, X., Cobb, G. P. & Anderson, T. A. microRNAs as oncogenes and tumor suppressors. **302**, 1–12 (2007).

93. Johnson, S. M. *et al.* RAS Is Regulated by the let-7 MicroRNA Family. *Cell* **120**, 635–647 (2005).

94. Chen, Z. *et al.* Down-regulation of let-7 microRNA increased K-ras expression in lung damage induced by radon. *Environ. Toxicol. Pharmacol.* **40**, 541–548 (2015).

95. Yu, F. *et al.* let-7 Regulates Self Renewal and Tumorigenicity of Breast Cancer Cells. 1109–1123 (2007). doi:10.1016/j.cell.2007.10.054

96. Zhao, B. *et al.* MicroRNA let-7c inhibits migration and invasion of human non-small cell lung cancer by targeting ITGB3 and MAP4K3. *Cancer Lett.* **342**, 43–51 (2014).

97. Svoboda, P. A toolbox for miRNA analysis. *FEBS Lett.* **589**, 1694–1701 (2015).

98. Militello, G. *et al.* Screening and validation of lncRNAs and circRNAs as miRNA sponges. *Brief. Bioinform.* **18**, 780–788 (2017).

99. Migault, M., Donnou-fournet, E. & Galibert, M. Definition and identification of small RNA sponges : Focus on miRNA sequestration. *Methods* **117**, 35–47 (2017).

100. Broderick, J. A. & Zamore, P. D. Previews Competitive Endogenous RNAs Cannot Alter MicroRNA Function In Vivo. *Mol. Cell* **54**, 711–713 (2014).

101. Lima, J. F. *et al.* Anti-miRNA oligonucleotides : A comprehensive guide for design. *RNA Biol.* **15**, 338–352 (2018).

102. Ariyoshi, J. *et al.* Effective Anti-miRNA Oligonucleotides Show High Releasing Rate of MicroRNA from RNA-Induced Silencing Complex. *Nucleic Acid Ther.* **27**, 303–308 (2017).

103. Trang, P. *et al.* Systemic Delivery of Tumor Suppressor microRNA Mimics Using a Neutral Lipid Emulsion Inhibits Lung Tumors in Mice. *Mol. Ther.* **19**, 1116–1122 (2011).

104. Pirollo, K. F. *et al.* Cancer Therapy : Preclinical argeting Nanocomplex Delivery of Novel T umor Suppressor RB94 Chemosensitizes Bladder Carcinoma Cells In vitro and In vivo. **14**, 2190–2199 (2008).

105. Zhang, Y., Wang, Z. & Gemeinhart, R. A. Progress in microRNA delivery. *J. Control. Release* **172**, 962–974 (2013).

106. Fabris, L. *et al.* The Potential of MicroRNAs as Prostate Cancer Biomarkers Title. *Eur. Urol.* **70**, 312–322 (2016).

107. Umbehr, M. *et al.* Intraprostatic inflammation is positively associated with serum PSA in men with PSA <4 ng ml−1, normal DRE and negative for prostate cancer. *Prostate Cancer Prostatic Dis.* **18**, 264–269 (2015).

108. Penney, K. L. *et al.* Association of KLK3 (PSA) genetic variants with prostate cancer risk and PSA levels. *Carcinogenesis* **32**, 853–859 (2011).

109. Nam, R. *et al.* A Novel Serum Marker, Total Prostate Secretory Protein of 94 Amino Acids, Improves Prostate Cancer Detection and Helps Identify High Grade Cancers at Diagnosis. *J. Urol.* **4**, 1291–1297 (2006).

110. Luu, H. N. *et al.* miRNAs associated with prostate cancer risk and progression. *BMC Urol.* **17**, 1–18 (2017).

111. Song, C. J. *et al.* The potential of microRNAs as human prostate cancer biomarkers: A meta-analysis of related studies. *J. Cell. Biochem.* **119**, 2763–2786 (2018).

112. Porkka, K. P. *et al.* MicroRNA Expression Profiling in Prostate Cancer. *Cancer Res.* **67**, 6130–6136 (2007).

113. Ambs, S. *et al.* Genomic Profiling of MicroRNA and Messenger RNA Reveals Deregulated MicroRNA Expression in Prostate Cancer. *Cancer Res.* **68**, 6162–6171 (2008).

114. Mitchell, P. S. *et al.* Circulating microRNAs as stable blood-based markers for cancer detection. *PNAS* **105**, 10513–10518 (2008).

115. Lodes, M. J. *et al.* Detection of Cancer with Serum miRNAs on an Oligonucleotide Microarray. *PLoS One* **4**, (2009).

116. Chen, C. *et al.* Molecular determinants of resistance to antiandrogen therapy. *Nat. Med.* **10**, 33–39 (2004).

117. Rokhlin, O. *et al.* MicroRNA-34 mediates AR-dependent p53-induced apoptosis in prostate cancer. *Cancer Biol Ther* **7**, 1288–96 (2008).

118. Culig, Z. & Santer, F. R. Androgen receptor signaling in prostate cancer. *Cancer Metastasis Rev.* **33**, 413–427 (2014).

119. Sarkar, D. *et al.* mda-7 (IL-24) mediates selective apoptosis in human melanoma cells by inducing the coordinated overexpression of the GADD family of genes by means of p38 MAPK. *Proc. Natl. Acad. Sci.* **99**, 10054–10059 (2002).

120. Koetz-ploch, L. *et al.* MicroRNA-125a promotes resistance to BRAF inhibitors through suppression of the intrinsic apoptotic pathway. *Pigment Cell Melanoma* **30**, 328–338 (2017).

121. Guo, X., Wu, Y. & Hartley, R. S. MicroRNA-125a represses cell growth by targeting HuR in breast cancer. *RNA Biol.* **6**, 575–583 (2009).

122. Lee, M., Kim, E. J. & Jeon, M. J. MicroRNAs 125a and 125b inhibit ovarian cancer cells through post-transcriptional inactivation of EIF4EBP1. *Oncotarget* **7**, (2015).

123. Potenza, N. *et al.* Molecular mechanisms governing microRNA-125a expression in human hepatocellular carcinoma cells. *Sci. Rep.* **7**, (2017).

124. Tong, Z. *et al.* miR-125a-5p inhibits cell proliferation and induces apoptosis in colon cancer via targeting BCL2, BCL2L12 and MCL1. *Biomed. Pharmacother.* **75**, 129–136 (2015).

125. Fana, Z. *et al.* MiR-125a suppresses tumor growth, invasion and metastasis in cervical cancer by targeting STAT3. *Oncotarget* **6**, 25266–25280 (2015).

126. Yang, J. *et al.* An androgen-regulated miRNA suppresses Bak1 expression and induces androgen-independent growth of prostate cancer cells. *Proc. Natl. Acad. Sci.* **104**, 19983–19988 (2007).

127. Fu, Y. & Cao, F. MicroRNA -125a-5p regulates cancer cell proliferation and migration through NAIFI in prostate carcinoma. *Onco. Targets. Ther.* **8**, 3827–3835 (2015).

128. Niu, W. *et al.* TLR-4/microRNA-125a/NF-κB signaling modulates the immune response to Mycobacterium tuberculosis infection. *Cell Cycle* **17**, 1931–1945 (2018).

129. Banerjee, S. *et al.* MiR-125a-5p regulates differential activation of macrophages and inflammation. *J. Biol. Chem.* **288**, 35428–35436 (2013).

130. Korneev, K. *et al.* TLR-signaling and proinflammatory cytokines as drivers of tumorigenesis. *Cytokine* **89**, 127–135 (2017).

131. Bazzoni, F. *et al.* Multi-Step Regulation of the TLR4 Pathway by the miR-125a~99b~let-7e Cluster. *Front. Immunol.* **9**, 1–16 (2018).

132. Bhattacharjee, A. *et al.* IL-4 and IL-13 employ discrete signaling pathways for target gene expression in alternatively activated monocytes / macrophages. *Free Radic. Biol. Med.*

54, 1–16 (2013).

133. Li, C. *et al.* Macrophage polarization and meta-inflammation. *Transl. Res.* **191**, 29–44 (2017).

134. Gordon, S. & Martinez, F. O. Alternative Activation of Macrophages : Mechanism and Functions. *Immunity* **32**, 593–604 (2010).

135. Kim, S.-W. *et al.* MicroRNAs miR-125a and miR-125b constitutively activate the NF- B pathway by targeting the tumor necrosis factor alpha-induced protein 3 (TNFAIP3, A20). *Proc. Natl. Acad. Sci.* **109**, 7865–7870 (2012).

136. Xia, Y., Shen, S. & Verma, I. M. NF- B, an Active Player in Human Cancers. *Cancer Immunol. Res.* **2**, 823–830 (2014).

137. Shekarian, T. *et al.* Pattern recognition receptors : immune targets to enhance cancer immunotherapy. *Ann. Oncol.* **28**, 1756–1766 (2017).

138. Benko, S., Kovács, E. G., Hezel, F. & Kufer, T. A. NLRC5 functions beyond MHC I regulation-What do we know so far? *Front. Immunol.* **8**, 1–7 (2017).

139. Kobayashi, K. S. *et al.* NLR family member NLRC5 is a transcriptional regulator of MHC class I genes. *Proc. Natl. Acad. Sci.* **107**, 13794–13799 (2010).

140. Ting, J. & Trowsdale, J. Genetic Control of MHC Class II Expresion. *Cell* **109**, S21–S33 (2002).

141. Neerincx, A. *et al.* The N-Terminal Domain of NLRC5 Confers Transcriptional Activity for MHC Class I and II Gene Expression. *J. Immunol.* **193**, 3090–3100 (2014).

142. Germain, R. MHC-dependent antigen processing and peptide presentation: Providing ligands for T lymphocyte activation. *Cell* **76**, P287–P299 (1994).

143. Viville, S. *et al.* Mice lacking the MHC class II-associated invariant chain. *Cell* **72**, 635–648 (1993).

144. Eddine, F. B. N. *et al.* CIITA-driven MHC class ii expressing tumor cells can efficiently prime naive CD4+TH cells in vivo and vaccinate the host against parental MHC-II-negative tumor cells. *Oncoimmunology* **6**, 1–10 (2016).

145. Ekkirala, C. R. *et al.* Class II transactivator-induced MHC class II expression in pancreatic cancer cells leads to tumor rejection and a specific antitumor memory response. *Pancreas* **43**, 1066–1072 (2014).

146. Wilcox, T. & Hirshkowitz, A. MHC Class I Loss is a Frequent Mechanism of Immune Escape in Papillary Thyroid Cancer that is Reversed by Interferon and Selumetinib Treatment in vitro. **85**, 1–27 (2015).

147. Mo, D. *et al.* MiRNA-145 suppresses lung adenocarcinoma cell invasion and migration by targeting N-cadherin. *Biotechnol. Lett.* **39**, 701–710 (2017).

148. Johannessen, C. *et al.* Expression and function of the miR-143/145 cluster in vitro and in vivo in human breast cancer. *PLoS One* **12**, 1–22 (2017).

149. Zaman, M. S. *et al.* The functional significance of microRNA-145 in prostate cancer. *Br. J. Cancer* **103**, 256–264 (2010).

150. Baradaran, B., Shahbazi, R. & Khordadmehr, M. Dysregulation of key microRNAs in

pancreatic cancer development. *Biomed. Pharmacother.* **109**, 1008–1015 (2019).

151. Zeinali, T., Mansoori, B., Mohammadi, A. & Baradaran, B. Regulatory mechanisms of miR-145 expression and the importance of its function in cancer metastasis. *Biomed. Pharmacother.* **109**, 195–207 (2019).

152. Majid, S. *et al.* MicroRNA-23b Functions as a Tumor Suppressor by Regulating Zeb1 in Bladder Cancer. *PLoS One* **8**, (2013).

153. Au Yeung, C. L., Tsang, T. Y., Yau, P. L. & Kwok, T. T. Human papillomavirus type 16 E6 induces cervical cancer cell migration through the p53/microRNA-23b/urokinase-type plasminogen activator pathway. *Oncogene* **30**, 2401–2410 (2011).

154. Salvi, A., De Petro, G., Baiocchi, G., Portolani, N. & Grossi, I. Functional Role of microRNA-23b-3p in Cancer Biology. *MicroRNA* **7**, 156–166 (2018).

155. Chen, L. *et al.* VHL regulates the effects of miR-23b on glioma survival and invasion via suppression of HIF-1a/VEGF and b-catenin/Tcf-4 signaling. *Neuro. Oncol.* **14**, 1026–1036 (2012).

156. Tanaka, Y. *et al.* Inhibition of PTEN Gene Expression by Oncogenic miR-23b-3p in Renal Cancer. *PLoS One* **7**, e50203 (2012).

157. Wang, W., Wang, Y., Liu, W. & van Wijnen, A. J. Regulation and biological roles of the multifaceted miRNA-23b (MIR23B). *Gene* **642**, 103–109 (2018).

158. Tian, L., Fang, Y. xiang, Xue, J. lun & Chen, J. zhong. Four microRNAs promote prostate cell proliferation with regulation of PTEN and its downstream signals in vitro. *PLoS One* **8**,

1–19 (2013).

159. Stebbing, J. *et al.* miR-23b regulates cytoskeletal remodeling, motility and metastasis by directly targeting multiple transcripts. *Nucleic Acids Res.* **41**, 5400–5412 (2013).

160. Saini, S. *et al.* miR-23b Represses Proto-oncogene Src Kinase and Functions as Methylation-Silenced Tumor Suppressor with Diagnostic and Prognostic Significance in Prostate Cancer. *Cancer Res.* **72**, 6435–6446 (2012).

161. Jin, L. *et al.* Prooncogenic factors miR-23b and miR-27b are regulated by Her2/Neu, EGF, and TNF-A in breast cancer. *Cancer Res.* **73**, 2884–2896 (2013).

162. Donadelli, M., Dando, I., Fiorini, C. & Palmieri, M. Regulation of miR-23b expression and its dual role on ROS production and tumour development. *Cancer Lett.* **349**, 107–113 (2014).

163. Fan, L., Hsieh, P. N., Sweet, D. R. & Jain, M. K. Krüppel-like factor 15: Regulator of BCAA metabolism and circadian protein rhythmicity. *Pharmacol. Res.* **130**, 123–126 (2018).

164. Turner, J. & Crossley, M. Basic Krüppel-like factor functions within a network of interacting haematopoietic transcription factors. *Int. J. Biochem. Cell Biol.* **31**, 11691174 (1999).

165. Dang, D., Zhao, W., Mahatan, C., Geiman, D. & Yang, V. Opposing effects of Krüppel-like factor 4 (gut-enriched Krüppel-like factor) and Krüppel-like factor 5 (intestinal-enriched Krüppel-like factor) on the promoter of the Krüppel-like factor 4 gene. *Nucleic Acids Res.* **30**, 2736–2741 (2002).

166. Bieker, J. Isolation, genomic structure, and expression of human erythroid Krüppel-like factor (EKLF). *DNA Cell Biol.* **15**, 347–352 (1996).

167. Park, C. S., Lewis, A., Chen, T. & Lacorazza, D. Concise Review: Regulation of Self-Renewal in Normal and Malignant Hematopoietic Stem Cells by Krüppel-Like Factor 4. *Stem Cells Transl. Med.* **00**, 1–7 (2019).

168. Nagata, T. *et al.* KLF4 and NANOG are prognostic biomarkers for triple-negative breast cancer. *Breast Cancer* **24**, 326–335 (2017).

169. Zhuang, J. *et al.* TGFβ1 promotes gemcitabine resistance through regulating the LncRNA-LET/NF90/miR-145 signaling axis in bladder cancer. *Theranostics* **7**, 3053–3067 (2017).

170. Minami, K. *et al.* MiR-145 negatively regulates Warburg effect by silencing KLF4 and PTBP1 in bladder cancer cells. *Oncotarget* **8**, 33064–33077 (2017).

171. Shi, M. *et al.* A novel KLF4/LDHA signaling pathway regulates aerobic glycolysis in and progression of pancreatic cancer. *Clin. Cancer Res.* **20**, 4370–4380 (2014).

172. Yang, V. W. *et al.* Identification of Krüppel-like factor 4 as a potential tumor suppressor gene in colorectal cancer. *Oncogene* **23**, 395–402 (2004).

173. Wang, X. *et al.* Prognostic Value and Function of KLF4 in Prostate Cancer: RNAa and Vector-Mediated Overexpression Identify KLF4 as an Inhibitor of Tumor Cell Growth and Migration. *Cancer Res.* **70**, 10182–10191 (2010).

174. Zhang, N. *et al.* Downregulated Krüppel-like factor 4 expression is associated with aggressiveness of prostate cancer. *Oncol. Rep.* **41**, 1789–1796 (2019).

175. Rane, M. J., Zhao, Y. & Cai, L. Krüppel-like factors (KLFs) in renal physiology and disease. *EBioMedicine* **40**, 743–750 (2019).

176. Haspel, R. *et al.* The Krüppel-like Factor KLF15 Regulates the Insulin-sensitive Glucose Transporter GLUT4. *J. Biol. Chem.* **277**, 34322–34328 (2002).

177. Shelkay, S. *et al.* Kruppel-like Factor 15 Is a Critical Regulator of Cardiac Lipid Metabolism. *J. Biol. Chem.* **289**, 5914–5924 (2014).

178. Patel, S. K., Ramchand, J., Crocitti, V. & Burrell, L. M. Kruppel-like factor 15 is critical for the development of left ventricular hypertrophy. *Int. J. Mol. Sci.* **19**, 1–12 (2018).

179. Fisch, S. *et al.* Kruppel-like factor 15 is a regulator of cardiomyocyte hypertrophy. *Proc. Natl. Acad. Sci.* **104**, 7074–7079 (2007).

180. Wang, X., He, M., Li, J., Wang, H. & Huang, J. KLF15 suppresses cell growth and predicts prognosis in lung adenocarcinoma. *Biomed. Pharmacother.* **106**, 672–677 (2018).

181. Yoda, T. *et al.* KLF15 in breast cancer: a novel tumor suppressor? *Cell. Oncol.* **38**, 227–235 (2015).

182. Ma, P. *et al.* KLF15 Inhibits Cell Proliferation in Gastric Cancer Cells via Up-Regulating CDKN1A/p21 and CDKN1C/p57 Expression. *Dig. Dis. Sci.* **62**, 1518–1526 (2017).

183. Zheng, M. *et al.* Human interleukin 24 (MDA-7/IL-24) protein kills breast cancer cells via the IL-20 receptor and is antagonized by IL-10. *Cancer Immunol. Immunother.* **56**, 205–215 (2007).