The microRNA-3622 family at the 8p21 locus exerts oncogenic effects by regulating the p53-downstream gene network in prostate cancer progression

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Abstract

For human prostate cancer, the chromosome 8p21 locus, which contains NKX3.1 and the microRNA (miR)-3622 family (miR-3622a/b), is a frequently deleted region. Thus, miR-3622 is proposed as a suppressor for prostate cancer, but its role remains debatable. In the present study, we found that expression of miR-3622a was lower, whereas expression of miR-3622b-3p was higher in human prostate cancer tissues than in normal prostate tissues. miR-3622a-3p inhibited cell migration and invasion of human prostate cancer cells, whereas miR-3622b-3p facilitated cell proliferation, migration, and invasion. To address the opposing roles of miR-3622 family members in various human prostate cancer cell lines, we knocked out (KO) endogenous miR-3622, including both miR-3622a/b. Our results showed that miR-3622 KO reduced cell proliferation, migration, and invasion in vitro and tumor growth and metastasis in vivo. Functional analyses revealed that miR-3622 regulated the p53 downstream gene network, including AIFM2, c-MYC, and p21, to control apoptosis and the cell cycle. Furthermore, using CRISPR interference, miRNA/
mRNA immunoprecipitation assays, and dual-luciferase assays, we established that AIFM2, a direct target of miR-3622b-3p, is responsible for miR-3622 KO-induced apoptosis. We identified an miR-3622-AIFM2 axis that contributes to oncogenic function during tumor progression. In addition, miR-3622 KO inhibited the epithelial-mesenchymal transition involved in prostate cancer metastasis via upregulation of vimentin. The results show that miR-3622b-3p is upregulated in human prostate cancers and has an oncogenic function in tumor progression and metastasis via repression of p53 signaling, especially through an miR-3622-AIFM2 axis. In contrast, for human prostate cancer, deletion of the miR-3622 locus at 8p21 reduced the oncogenic effects on tumor progression and metastasis.

**Keywords**

miR-3622b; prostate cancer; tumor progression; AIFM2; p53

**Introduction**

Prostate cancer is a complex disease with multifactorial genetic alterations, including somatic copy number alterations, point mutations, structural rearrangements, and chromosomal number changes [1]. Primary tumors frequently exhibit deletions on chromosomes 6q, 8p, 10q, and 13q, whereas castration-resistant metastatic tumors often have amplified chromosomes X, 7, 8q, and 9q [2]. Key genetic changes include copy number gains of the oncogenes androgen receptor (AR) and c-MYC; losses of the tumor suppressor genes PTEN and NKX3.1; mutation of TP53; and fusions of TMPRSS2 with ETS family genes [1], which define prostate cancer subtypes with the potential for therapeutic stratification [3]. In human prostate cancers, the 8q24.21 locus, containing the oncogene c-MYC, is the most commonly amplified region [4]. Our previous study found amplification of microRNA (miRNA or miR)-1205 at this locus, which contributes to the risk of castration-resistant prostate cancer [5]. Further, the 8p21 locus, containing NKX3.1 and the miR-3622 family (miR-3622a/b), is frequently deleted in human prostate cancers [6, 7], indicating that this locus may contain tumor suppressor genes, such as NKX3.1 and miR-3622a/b.

miRNAs are endogenous, short, non-coding, single-stranded RNA molecules consisting of 19 to 25 nucleotides. These molecules negatively regulate the expression of target genes after transcription, usually binding to the 3’-untranslated regions (UTRs) of the mRNAs [8]. miRNAs may serve as biomarkers for predicting the occurrence and progression of prostate cancer [9, 10]. Notably, in prostate and colorectal cancers, miR-3622a-3p acts as a tumor suppressor by reducing stemness features and the epithelial-mesenchymal transition (EMT) [11, 12]. Likewise, miR-3622b-5p may have a tumor-suppressive role in prostate cancer [7, 13], breast cancer [14], and ovarian cancer [15]. However, miR-3622a may promote proliferation and invasion of bladder cancer cells [16]. In the present study, to address the role of the miR-3622 family and deletion of this miRNA family locus in human prostate cancer, we knocked out miR-3622 and used xenograft models to investigate the effect of miR-3622 knockout (KO) on proliferation, migration, cell cycle, and apoptosis of prostate cancer cells, and on tumor growth and metastasis. Also, for human prostate cancer cells,
we identified the direct targets of miR-3622b-3p and used various technologies to assess its regulatory mechanism.

**Results**

**miR-3622 amplification, deep deletion, and expression in human primary prostate cancers**

The human miR-3622 family includes two members, miR-3622a/b, but transcription of miR-3622a/b occurs in two directions from a single site at the 8p.21.1 locus (Supplementary Fig. S1A), which is frequently deleted in human prostate cancers [6, 7]. First, using multiple public datasets, we analyzed the genetic alterations of miR-3622, including amplification and deep deletion, in 2,730 prostate cancer samples from 7 studies (Supplementary Fig. S1B). Of the samples, 3%–18% had deep deletion, and < 8% showed amplification of the miR-3622 locus. Likewise, the Cancer Genome Atlas (TCGA) dataset analysis revealed that expression of miR-3622a was lower in prostate cancer tissues than in normal prostate tissues, but the difference was not statistically significant (p = 0.2552, Supplementary Fig. S2A). Also, expression of miR-3622a was lower in prostate cancer tissues with high Gleason scores (Supplementary Fig. S2B). Survival analysis showed that low expression of miR-3622a was associated with poor survival, but the difference was not statistically significant (p = 0.14, Supplementary Fig. S2C). However, in TCGA dataset, there was no available information for expression of miR-3622b. To address this question, using laser-capture microdissection in combination with quantitative PCR (qPCR), we measured the expression levels of mature miR-3622b-3p/5p in 46 primary prostate cancer tissues and 10 age-matched normal prostate tissues (Supplementary Table S1). Using micro-dissected tissues, we identified more than 16-fold higher average expression of miR-3622b-3p in prostate cancer tissues than in normal prostate tissues (Fig. 1A, but p = 0.208 by a Mann Whitney test), but no change in expression of miR-3622b-5p (Fig. 1A). Furthermore, expression of miR-3622b-3p increased with Gleason scores (Fig. 1B, p = 0.045 by a Kruskal-Wallis test) and metastasis (Fig. 1C, but p = 0.5481 by a Mann Whitney test). However, expression of miR-3622b-5p was not related to tumor progression (Figs. 1B and 1C). These data suggest that miR-3622b-3p is involved tumor aggressiveness in prostate cancers of patients.

Next, we determined the expression levels of mature miR-3622b-3p/5p in four human prostate cancer cell lines, PC3, DU145, LNCaP, and RC-77T/E, and one nonmalignant prostate cell line, RC-77N/E (Figs. 1D and 1E). The LNCaP cell line had the highest expression levels of miR-3622b-3p/5p among all cell lines. RC-77T/E had higher expression than RC-77N/E, for which the two cell lines are from the same African-American patient with early-stage prostate cancer [17]. In particular, for these cell lines, the expression levels of miR-3622b-3p were approximately 10-fold higher than that of miR-3622b-5p, suggesting a dominant expression of miR-3622b-3p from miR-3622b in human prostate cancer cells.

**miR-3622a/b have opposite roles in proliferation, migration, and invasion of human prostate cancer cells**

First, we induced or reduced miR-3622a/b expression in DU145, PC3, and LNCaP cells by transfection with miRNA mimics or inhibitors (Supplementary Figs. 3A–L). Next, we
determined the effect of each member of the miR-3622 family on proliferation, migration, and invasion of human prostate cancer cells. For the miR-3622a-5p/3p cluster, in DU145 and PC3 cells, the miR-3622a-3p mimic did not affect cell proliferation but inhibited cell migration and invasion; the miR-3622a-5p mimic did not affect cell proliferation, migration, or invasion (Supplementary Figs. 4A–G). Also, the miR-3622a-5p/3p inhibitor did not affect cell proliferation, migration, or invasion (Supplementary Figs. 4A–G). For the miR-3622b-5p/3p cluster, in DU145, PC3, and LNCaP cells, the miR-3622b-3p mimic induced, but the inhibitor reduced, cell proliferation (Figs. 1F–H). Likewise, for DU145 and PC3 cells, the miR-3622b-3p mimic induced, but the inhibitor reduced, cell migration and invasion by scratch and Transwell invasion assays (Figs. 2A–F). These data indicate that the miR-3622a/b family has opposite roles in proliferation, migration, and invasion of human prostate cancer cells.

To address the integrated role of endogenous miR-3622a/b, in PC3, DU145, LNCaP, and RC-77T/E cells, the miR-3622 locus was knocked out by CRISPR/Cas9 editing. The miR-3622 KO was confirmed by Sanger DNA sequencing (Supplementary Figs. S5A–E). Also, qPCR showed the downregulation of both miR-3622a-3p/5p and miR-3622b-3p/5p in KO cells compared with scramble control cells, except for miR-3622b-5p in PC3 cells (Supplementary Figs. S6A–P). Furthermore, in DU145 and RC-77T/E cells, miR-3622 KO caused less activity of cells, as determined by proliferation and colony formation assays (Figs. 1I–N). Likewise, miR-3622 KO reduced the migration and invasion of DU145 and PC3 cells (Figs. 2G–K). These data indicate that, coordinately, miR-3622a/b acts an oncogenic role in human prostate cancer cells.

miR-3622 KO attenuates tumor growth and metastasis of human prostate cancer cells

To investigate the effect of miR-3622 KO on tumor growth in mice, PC3-luc and LNCaP miR-3622 KO cells were injected separately into the flanks of NOD SCID gamma (NSG) male mice. Xenograft tumor growth was slower for PC3 KO cells than for scrambled cells (Fig. 3A). At 30 days after injection, luciferase imaging and histologic analysis also showed smaller tumor volumes, sizes, and weights of KO xenografts compared to scrambled xenografts (Figs. 3B–D). Moreover, lung metastases were present in 3/4 mice injected with scrambled PC3 cells, but no distant metastases were evident in mice with PC3 KO cells (Figs. 3E and 3F). Likewise, in LNCaP cells, tumor growth was inhibited by miR-3622 KO (Figs. 3G–I). In addition, Ki67+ cells were fewer in KO xenograft tumors than in scrambled xenograft tumors (Figs. 3J–L). These data further indicate that miR-3622a/b may coordinately exert an oncogenic role during prostate cancer progression.

miR-3622 is involved in the p53 signaling pathway and its regulated gene network in human prostate cancer cells

To elucidate the mechanism of miR-3622 KO-mediated tumor suppression, we performed RNA-seq analyses of miR-3622 KO and scrambled LNCaP cells. KEGG enrichment analysis of differentially expressed genes revealed that, in KO cells relative to scramble cells, the top miR-3622-regulated pathway was the p53 signaling pathway (Supplementary Table S2). As shown in Figs. 4A–C, the KEGG enrichment, score, and heatmap of differently expressed genes showed enhanced p53 signaling activation by miR-3622 KO.
Also, using qPCR analysis, we validated the changes of p53-related genes in our RNA-seq data. As shown in Supplementary Fig. S7, miR-3622 KO increased the expression of p53-induced genes (pro-apoptotic genes: BAX, BBC3, PMAIP1, TP53I3, GADD45A, and GADD45B; growth arrest genes: CDKN1A, ZMAT3, SESN1, and RM2B), but decreased the expression of p53-repressed genes (cell cycle genes: CCND1, CCND2, CCNB1, and CCNB2). However, there was no difference in the expression of TP53 between miR-3622 KO and control LNCaP cells. These data support that, in prostate cancer cells, miR-3622 KO activates the p53 signaling pathway, but not directly through p53. Furthermore, RNA-seq data analysis identified 461 coding genes as significantly downregulated targets of miR-3622 in KO cells relative to scramble cells (Fig. 4D). In addition, miR-3622b-3p had an oncogenic role in human prostate cancer cells (Figs. 1F–H and 2A–F), which is consistent with the functional role of miR-3622 KO. Thus, using TargetScan analysis, we predicted 390 direct target genes of miR-3622b-3p (Fig. 4D and Supplementary Table S3). By combining our RNA-seq data with the predicted miR-3622b-3p target data, 16 candidate genes were identified as potential direct targets of miR-3622b-3p (Fig. 4D and Supplementary Table S4).

Next, we selected the top three coding genes, CYB5D2, TMEM220, and AIFM2, for further analysis (Fig. 4E). qPCR analysis confirmed that, in LNCaP cells, the miR-3622b-3p mimic inhibited the expression of CYB5D2, TMEM220, and AIFM2 (Figs. 4F–H). However, in these cells, miR-3622 KO increased the expression of AIFM2 but not that of CYB5D2 or TMEM220 (Fig. 4H). AIFM2 is a p53-inducible gene downregulated in tumors and may have a tumor-suppressive function [18]. In TCGA dataset, compared to normal tissue controls, mRNA expression of CYB5D2, TMEM220, and AIFM2 was downregulated in human primary prostate cancers (Supplementary Fig. S8). Higher expression levels of AIFM2 mRNA were also validated in miR-3622 KO DU145 cells compared to scrambled DU145 cells (Fig. 4I). Also, immunohistochemistry (IHC) analysis showed higher expression levels of AIFM2 protein in PC3 and LNCaP KO xenograft tumors compared to scrambled xenograft tumors (Fig. 4J). These data suggest that, for human prostate cancer cells, miR-3622b-3p most likely targets AIFM2.

**AIFM2 is a direct target of miR-3622b-3p in human prostate cancer cells**

To observe the dynamic regulation of the AIFM2 transcript by miR-3622b-3p, we generated a Dox-inducible CRISPRi (CRISPR-dead Cas9 (dCas9)-KRAB with miR-3622b single guide RNA (sgRNAs)) cell model using the prostate cancer cell line DU145 (Fig. 5A). At 72 hours after Dox addition, the expression of dCas9-KRAB (GFP) with miR-3622b sgRNA (mIFP) was validated in the cells (Fig. 5B). After Dox treatment, expression of miR-3622b-3p was gradually downregulated in the cells, but expression of miR-3622a-5p/3p and miR-3622b-5p was not significantly changed from day 0 to 7 (Fig. 5C). Simultaneously, after Dox treatment, expression of AIFM2 and CYB5D2 was upregulated (Figs. 5D and 5E). Although expression of TMEM220 was elevated on day 4, its expression was lower on day 7 after Dox treatment (Fig. 5F). Next, we performed, with LNCaP cells, an miRNA/mRNA immunoprecipitation (IP) with Ago1/2/3 antibody and the miR-3622b-3p mimic to see if there is direct binding between miR-3622b-3p and AIFM2 mRNA (Fig. 5G). As shown in Fig. 5H, The AIFM2 transcript was more than 3-fold higher in the Ago-IP group.
compared to the IgG control group. However, CYB5D2 and TMEM220 transcripts were not significantly changed between the Ago-IP and IgG control groups (Fig. 5H). To address the transcriptional regulation of AIFM2 by miR-3622b-3p, we performed a dual luciferase assay with HEK293 cells (Fig. 5I). Luciferase activity linked with the AIFM2 3’-UTR was suppressed by the miR-3622b-3p mimic relative to an miRNA negative control (Fig. 5J). These data suggest that, in human prostate cancer cells, miR-3622b-3p directly regulates the expression of AIFM2.

An miR-3622-AIFM2 axis controls the cell cycle, the EMT, and apoptosis in human prostate cancer cells

To determine the role of the miR-3622-AIFM2 axis in cell proliferation and migration, we generated miR-3622 and AIFM2 double-KO cell models in PC3 and LNCaP cells (Figs. 6A and 6B). Although miR-3622 KO reduced cell proliferation, AIFM2 KO or knockdown restored the proliferation of miR-3622 KO PC3 and LNCaP cells, as determined with proliferation and colony formation assays (Figs. 6C–6H and Supplementary Figs. S9A and S9B). However, in LNCaP cells but not PC3 cells, the miR-3622 KO-mediated suppression of cell proliferation disappeared under androgen-depleted situations (Supplementary Figs. S10A and S10B). Next, with PC3 and LNCaP cells, we assessed the effect of miR-3622 KO and AIFM2 KO on the cell cycle by use of flow cytometry (Supplementary Figs. S11A and S11B). To arrest the cells at G0-G1 phase, the cells were starved under serum-free situations for 48 hours. After serum stimulation, the cell cycle progression was slower for miR-3622 KO cells than for scramble cells (Figs. 6I and 6J). However, in miR-3622 KO cells, AIFM2 KO rescued the cell cycle progression (Figs. 6I and 6J). Likewise, in a scratch assay or a Transwell assay, AIFM2 KO blocked the miR-3622 KO-mediated suppression of PC3 cell migration (Figs. 6K and 6L). To investigate the molecular mechanism of miR-3622-AIFM2 axis-regulated cell migration, we examined the effect of miR-3622 KO and AIFM2 KO on the EMT in PC3 cells by use of Western blots (Fig. 6M). Although expression of E-cadherin was not changed, vimentin expression was lower in miR-3622 KO cells but was restored in miR-3622 and AIFM2 double-KO cells (Fig. 6M). Also, expression of twist was high in miR-3622 KO cells but low in miR-3622 and AIFM2 double-KO cells (Fig. 6M).

Because AIFM2 is a pro-apoptotic gene [19], we considered the possibility that miR-3622 suppresses prostate cancer cell apoptosis through AIFM2. Thus, we investigated the effect of the miR-3622-AIFM2 axis on cell apoptosis using flow cytometry with PC3 and LNCaP cells. The apoptosis assay showed that the percentage of apoptotic cells was higher in miR-3622 KO cells than scramble cells but rescued in miR-3622 and AIFM2 double-KO cells (Figs. 7A–7C). Also, compared to scrambled cells, expression of Bax and cleaved caspase 3 was high in miR-3622 KO cells but low or not changed in miR-3622 and AIFM2 double-KO cells (Figs. 7D and 7E). These data suggest that, for human prostate cancer cells, the miR-3622-AIFM2 axis controls cell proliferation via regulation of the cell cycle, cell migration via regulation of the EMT, and cell apoptosis via anti-apoptotic factors (Supplementary Fig. S12).
miR-3622 KO enhances the p53 signaling pathway in human prostate cancer cells

To further elucidate the molecular mechanism of the miR-3622-regulated p53 signaling pathway, we examined the expression of upstream and downstream proteins on p53 signaling pathways in miR-3622 KO and/or AIFM2 KO or knockdown cells. As shown in Fig. 7E and Supplementary Fig. S9C, expression of MDM2 and p53 proteins was not changed after miR-3622 KO or miR-3622 and AIFM2 double-KOs or miR-3622 KO with AIFM2 knockdown. As a p53 downstream target gene, p21 expression was high in miR-3622 KO cells but low in miR-3622 and AIFM2 double-KO cells or miR-3622 KO cells after AIFM2 knockdown compared to scrambled cells (Fig. 7E and Supplementary Fig. S9C); expression of c-MYC was low in miR-3622 KO cells but rescued in miR-3622 and AIFM2 double-KO cells or miR-3622 KO cells after AIFM2 knockdown (Fig. 7E and Supplementary Fig. S9C). In addition, IHC analysis validated high expression levels of p21 protein in miR-3622 KO cells compared to scrambled cells (Figs. 7F–H). These data suggest that, in human prostate cancer cells, miR-3622 KO-mediated tumor suppression is through the p53-downstream gene network, including direct targeting of AIFM2 and indirectly targeting p21 and c-MYC (Supplementary Fig. S12).

Discussion

Previous studies and analysis of public data reveal that the miR-3622 locus at 8p21 is frequently deleted in human prostate cancers [7, 13]. Also, miR-3622a-3p and miR-3622b-5p are proposed as tumor suppressors [7, 11–15] but miR-3622a as a tumor promoter [16] in human cancers, including prostate cancer. In the present study, miR-3622a-3p and miR-3622b-3p were dominantly (>10-fold) expressed in human prostate cancer cells compared to miR-3622a-5p and miR-3622b-5p. Also, miR-3622a-3p and miR-3622b-3p, but not miR-3622a-5p and miR-3622b-5p, affect the behavior of prostate cancer cells. However, in tumor growth and progression, there are opposite roles between miR-3622a-3p and miR-3622b-3p. The former acts as a tumor suppressor, whereas the latter acts as an oncogene. Here, we were first to investigate the integrated role of the miR-3622a/b family in human prostate cancer cells using CRISPR genomic editing. Our data suggest that, in human prostate cancer cells, the miR-3622 family at the 8p21 locus exerts oncogenic effects by regulating the p53-downstream gene network.

The miR-3622a-3p mimic reduced cell migration and invasion, but the miR-3622a-3p inhibitor had no impact on the behavior of prostate cancer cells. However, miR-3622b-3p mimic induced, but the miR-3622b-3p inhibitor reduced, the proliferation, migration, and invasion of human prostate cancer cells. Furthermore, miR-3622 KO reduced miR-3622a/b in various human prostate cancer cell lines. Using these KO cell models, we observed the miR-3622 KO-mediated suppression of tumor growth and metastasis in cell culture and xenograft models, suggesting that the miR-3622a/b family together contributes to prostate cancer progression. Also, this oncogenic contribution is likely to be caused by miR-3622b-3p. In addition, miR-3622 KO-mediated suppression of tumor growth was AR-dependent in AR-positive LNCaP cells, but miR-3622 KO also inhibited growth of AR-negative PC3 cells in an AR-independent manner. These data indicate that, in prostate
cancer cells, the function of miR-3622 may involve both AR-dependent and independent oncogenic regulation.

*Alfml2* (also known as *Fsp1* or *AMID*), as a target gene of p53, induces the apoptosis of cancer cells under toxicological stress [20]. Alfml2 is downregulated in a variety of cancers, suggesting its anticancer effect [18, 21]. Our study described the regulatory link between miR-3622b-3p and *Alfml2* and established, by use of various technologies, that, in human prostate cancer cells, *Alfml2* is a direct target of miR-3622b-3p. Also, miR-3622b-3p negatively regulated apoptosis via inhibition of expression of *Alfml2*. Although, in mitochondria, Alfml2 is a traditional inducer of apoptosis, recent studies show that Alfml2 is an antioxidant regulator in ferroptosis, regardless of its mitochondrial function [22, 23]. Thus, it is of interest to investigate, in a future study, the effect of an miR-3622-Alfml2 axis in ferroptosis.

Our RNA-seq data analysis showed that, in *TP53* wild-type (WT) LNCaP cells, miR-3622 KO upregulated activation of the p53 signaling pathway. P53 induces apoptosis through induction of a set of apoptotic genes, including Bax, NOXA, PUMA, and Alfml2 [18], and causes cell cycle arrest and senescence through induction of a set of cell cycle regulators, such as p21 and c-MYC [24]. Although p21 is not targeted directly by miR-3622b-3p, miR-3622 KO upregulates p21 expression, which may cause miR-3622 KO-mediated cell cycle arrest and inhibition of proliferation of prostate cancer cells. Also, p53-dependent transcriptional repression of c-MYC can lead to cell cycle arrest [25]. Our data showed, for LNCaP cells, downregulation of c-MYC by miR-3622 KO. However, expression of p53 and MDM2 was not changed after miR-3622 KO. Also, miR-3622 KO-mediated upregulation of p21 was evident in both *TP53* WT LNCaP cells and *TP53*-null PC3 cells. Likewise, miR-3622 KO upregulates the expression of p53-induced pro-apoptotic genes and growth arrest genes and downregulates the expression of p53-repressed cell cycle genes, but does not change *TP53* expression. These data support that, in prostate cancer cells, miR-3622 KO activates the p53 signaling pathway but not directly through p53. These data suggest that, to control proliferation of human prostate cancer cells, miR-3622b-3p regulates the p53-downstream gene network through direct targeting of *Alfml2* and various indirect targets, such as c-MYC and p21.

In human prostate cancer cells, miR-3622b-3p promoted cell migration, apparently through the miR-3622-Alfml2 axis. Also, miR-3622 KO reduced the expression of vimentin but induced the expression of twist, leading to inhibition of the EMT and cell migration and invasion. Of note, in LNCaP cells, Alfml2 KO rescued the miR-3622 KO-changed expression of vimentin and twist. However, the mechanism by which Alfml2 regulates vimentin and twist, as well as the EMT, remains elusive. Likewise, it is unknown why Alfml2 KO reduces the expression of p21. Indeed, miR-3622b-3p may target multiple genes and signaling pathways, although the p53 signaling pathway is the top candidate as identified in our RNA-seq data. Thus, in human prostate cancer cells, miR-3622b-3p may target a gene network more complex than the p53 signaling pathway.

In summary, in human prostate cancers, the miR-3622a/b family coordinately facilitates tumor progression. In these cancers, miR-3622b-3p may contribute to the oncogenic role
of the miR-3622 family via regulation of the cell cycle and apoptosis and by targeting the p53-downstream gene network. In particular, an miR-3622-AIFM2 axis is associated with progression of prostate cancer and may become a therapeutic target for patients with prostate cancer.

**Materials and Methods**

**Cell lines, antibodies, and reagents**

The human prostate cancer cell lines PC3, DU145, and LNCaP were obtained from the American Type Culture Collection (Manassas, VA). The PC3-luc cell line was obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). The isogenic human prostate cell lines RC-77N/E (nonmalignant cells) and RC-77T/E (malignant cells) were a kind gift from Dr. Clayton Yates at Tuskegee University [17]. All cell lines were cultured for less than 6 months, authenticated by examination of morphology and growth characteristics, and confirmed to be mycoplasma-free. Short tandem-repeat analysis for DNA fingerprinting was also used to verify the cell lines. PC3 and DU145 cells were maintained in Dulbecco’s Modified Eagle’s medium plus 10% fetal bovine serum (FBS). LNCaP cells were maintained in Roswell Park Memorial Institute 1640 medium plus 10% FBS. RC-77N/E and RC-77T/E cells were maintained in keratinocyte serum-free medium. For experiments associated with hormones, cells were cultured in phenol red-free medium plus 10% charcoal-stripped FBS. Antibodies used were specific for the following targets: AIFM2 (Cat No. 20886–1-AP, 1:3000 for Western blots, 1:100 for IHC staining, Proteintech, Rosemont, IL), Bax (Cat No. 2774, 1:5000 for Western blots, Cell Signaling, Danvers, MA), c-Myc (Cat No. ab32072, 1:5000 for Western blots, Abcam, Cambridge, MA), E-cadherin (Cat No. 3195, 1:3000 for Western blots, Cell Signaling), GAPDH (Cat No. 5174, 1:5000 for Western blots; Cell Signaling), Ki67 (Cat No. ab15580, 1:200 for IHC staining; Abcam), p21 (Cat No. 2947, 1:200 for IHC staining, 1:5000 for Western blots, Cell Signaling), p53 (Cat No. sc-126, 1:5000 for Western blots, Santa Cruz, Dallas, TX), pro- and cleaved-caspase3 (Cat No. 9662 and 9661, 1:5000 for Western blots, Cell Signaling), twist (Cat No. ab175430, 1:5000 for Western blots, Abcam), vimentin (Cat No. sc-6260, 1:200 for IHC staining, 1:3000 for Western blots, Santa Cruz). The information for miR-3622a/b mimics/inhibitor, sgRNAs, primers, and DNA constructs is shown in Supplementary Table S5. Transient transfection of miRNA scramble control (50 nM), mimics (50 nM), inhibitor (100 nM), and plasmids (2 mg) into cells was accomplished in 6-well plates using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s protocol.

**qPCR**

Total RNA was extracted from cultured cells using Trizol reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions. For miRNA expression, 5 μl of RNA in 20-μl reactions was reverse-transcribed using miScript II RT Kits (QIAGEN, Germantown, MD) according to the manufacturer’s protocol. Then, 2 μl of cDNA was used as a template for real-time PCR using a LightCycler 480 Real-Time PCR System (Roche Applied Sciences, Indianapolis, IN) with miScript SYBR Green PCR kits (QIAGEN). The preparations were incubated in 96-well optical plates at 95°C for 10 min, followed by 40 cycles of 95°C
for 15 sec and 60°C for 1 min. After the reaction, the cycle threshold (CT) data were determined with fixed threshold settings, and the mean CT was determined from triplicate PCRs. Quantification was performed using the 2−△△CT method; GAPDH and U6 were used as the reference genes for mRNA and miRNA, respectively. The qPCR primer sequences are listed in Supplementary Table S5.

Proliferation assay

Cell proliferation analysis was performed using cells transfected with scrambled miRNA control or miRNA mimics/inhibitor and scramble or KO cells. The cells were plated in 24 well plates in 1,000 μl of the medium at about 5 × 10^3 cells per well. After incubation for 1 to 6 days, wells were trypsinized, and total viable cells were determined using trypan blue exclusion on a Countess automated cell counter. Results are expressed as the numbers of cells from seeding to counting.

Western blots

Cells were harvested at 48 hours after transfection and lysed in lysis buffer [10 mM Tris-HCl (pH 7.4), 1% SDS, 10% glycerol, 5 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 5 μg/mL leupeptin, and 21 μg/mL aprotinin]. A total of 30 μg of each protein lysate was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The proteins were blocked with 5% non-fat milk and incubated with the primary antibodies. After being washed in phosphate-buffered saline buffer 3 times, signals were probed with a secondary antibody. The bands were then visualized by chemiluminescence.

Colony formation assay

Cells were seeded onto 6-well plates (400 or 600 cells/well) and cultured at 37°C in a humidified 5% CO₂ atmosphere for 12 days, and the plates were shaken to distribute the cells equally. When the colonies were visible by eye, culturing was terminated by removing the medium and washing the cells twice with PBS. The colonies were fixed with 95% ethanol for 30 min, dried, and stained with a 0.1% crystal violet solution for 30 min, and the plates were washed three times with water. Then, images of the stained plates were captured, and colonies containing more than 50 cells were counted.

Wound healing assay

The capacity for cell migration was examined by a wound healing assay. Cells were cultivated to 90% confluence on 6-well plates and transfected with a scramble control or miRNA mimics/inhibitor. Then, after transfection for 48 hours in triplicate, a 200-μl pipette tip was used to scratch the confluent cells. To avoid the effect of cell proliferation on migration analysis, cells were incubated with 10 μg/ml mitomycin C (Sigma-Aldrich) for 2 hours prior to the wound healing assay. Images of the cell migration were captured at 0 and 24 hours.
Transwell migration assay

The capacity for cell migration was examined by Transwell migration assay kits (Corning, Cleveland, TN). At 48 hours post-transfection, the concentration of cells in each group was adjusted to 1 × 10^5 cells/ml. Also, cells were incubated with 10 μg/ml mitomycin C (Sigma-Aldrich) for 2 hours prior to the Transwell migration assay. The upper chamber of a 24-well Transwell permeable support with an 8-μm pore size was loaded with 200 μl of cell suspension, and the lower chamber was filled with 500 μl of medium containing 10% FBS. Cells were then incubated at 37°C/5% CO₂ for 24 hours. After incubation, the medium was removed from the upper chamber, and cells were scraped out of the upper chamber with a cotton swab. Cells migrating to the other side of the membrane were fixed with methanol and stained with a 0.1% crystal violet solution for 30 min. The numbers of cells invading the Matrigel were counted in three randomly selected fields using an inverted microscope (200× magnification).

Establishment of miR-3622 KO cells

The miR-3622 sgRNAs were designed using the online CRISPR design tool (Benchling, San Francisco, CA, https://benchling.com). The pri-miR-3622 locus was selected to be targeted by CRISPR/Cas9 editing. A ranked list of sgRNAs was generated with specificity and efficiency scores. In two flanking sites of the mature miR-3622 sequence, paired sgRNAs with >30% specificity and efficiency scores were selected (Supplementary Table S5). The pair of oligos for two targeting sites was annealed and ligated to the BbsI-digested pSpCas9(BB)−2A-GFP (pX458) vector (Addgene, Cambridge, MA). The pX458 plasmid containing each target sgRNA sequence or pX458 empty vector was transfected into cells with Lipofectamine 3000. After flow cytometric sorting with green fluorescent protein (GFP), 200 GFP+ cells were seeded into 10-cm dishes. After colony selection, miR-3622 KO colonies were determined by Sanger sequencing of isolated genomic DNA, and miR-3622a/b expression levels in each clone were validated by qPCR. Likewise, cells were transfected with no sgRNA target-Cas9 to generate scrambled control colonies, which were confirmed by qPCR and Sanger sequencing after single-cell sorting with GFP. All sgRNAs were accessed using the online off-target searching tool (Cas-OFFinder, Daejeon, South Korea, http://www.rgenome.net/cas-offinder) [26] (Supplementary Tables S6a and S6b). All selected colonies of miR-3622 scramble and KO cells were validated by PCR and Sanger sequence analysis to exclude off-target effects in potential off-target regions of sgRNAs, as described previously [5, 27].

Generation of inducible CRISPR interference (CRISPRi) cell lines

CRISPRi was designed by co-expression of piggyBac (PB)-based dCas9 effector and sgRNA in the cells. The PB-based dCas9 constructs [KRAB-SpdCas9, PiggyBac-Tre3g-KRAB-dCas9 (Bstxl)-2NLS-p2A-sfGFP-SV40polyA/R (EF1a-Zeocin-f2A-rtTA-1), a gift from Dr. Stanley Qi, Stanford] were used for CRISPR-dCas9-mediated transcription repression of miR-3622b. sgRNAs were cloned into the pLenti U6-spsgTRE3G CMV-miFP (a gift from Dr. Qi, Stanford), as described previously [28], to generate sgRNA lentiviral expression constructs. sgRNA target sequences are listed in Supplementary Table S5. To generate stable CRISPRi cell lines, reporter cells were seeded at a density of 5x10^5 per well.
in a 6-well plate and transfected with 3.6 μg PB-based dCas9 construct and 1.4 μg Super PB Transposase construct (Systems Biosciences, Palo Alto, CA) using Lipofectamine 3000. Then, 100 μg/ml zeocin (Thermo Fisher Scientific) was added for the selection of a DU145 cell line stably expressing KRAB-SpdCas9. Next, the CRISPR/dCas9 stably expressing cell lines were transduced with sgRNA lentiviruses. After transduction, cells were sorted by fluorescence-activated cell sorting (FACS) using a BD FACS Aria2 with GFP (dCas9) and mIFP (sgRNA). Finally, the CRISPRi stably expressing DU145 cell line was established. For the CRISPRi cell lines, doxycycline (DOX, 1 μg/ml) was supplemented daily to induce expression of dCas9-KRAB. The plasmids are summarized in Supplementary Table S5.

Tumor xenograft assay

NSG male mice (6–8 weeks old, 18–20 g) were purchased and maintained in a specific pathogen-free environment. The experimental protocol was approved by the University of Alabama at Birmingham Animal Care Committee, and all procedures were performed in compliance with the institutional guidelines. In experiments involving tumor growth using PC3 and LNCaP cells, male mice were randomly split into two groups (miR-3622 scramble and KO). $1.0 \times 10^6$ cells in 100 μl PBS were inoculated subcutaneously into the two flanks of mice. Tumor size was measured with Vernier calipers, and the tumor volume was calculated with the formula: $length \times (width)^2 \times 1/2$ [29, 30]. The mice were observed over 30 days for tumor formation. The xenograft tumors were collected, and tumor weights were measured. The lung tissues were collected for analysis of metastasis.

IHC

The xenograft tumors were immersed in 10% formalin for 8–12 hours and embedded in paraffin. Antigens were retrieved by microwaving in 1 x target retrieval buffer (Agilent, Santa Clara, CA) for 12 min. ABC kits (Vector Laboratories, Burlingame, CA) were used for immunostaining according to the manufacturer’s protocol.

miRNA/mRNA IP assay

miRNA/mRNA IP was performed by using miRNA Target IP kits (Active Motif, Carlsbad, CA) according to the manufacturer’s protocol. Briefly, cells (1.5 × 10^7) were transfected with miRNA mimics (50 nM) using Lipofectamine 3000 for 48 hours. Transfected cells of each sample were lysed in 150 μl of complete lysis buffer and incubated on ice for 5 min and then at −80°C for 2 hours. Protein G magnetic beads (50 μl) were mixed with 200 μl of BSA solution for 10 min, and then the tubes were placed on a magnet to pellet the beads. After removal of the supernatant, 100 μl of wash buffer containing 5 μg of anti-Ago1/2/3 antibody or negative control anti-IgG antibody was incubated for 30 min. The lysate, mixed with 1,000 μl of IP buffer, was added to the protein G magnetic beads, incubated overnight at 4°C, and treated with proteinase K to digest protein for 30 min at 55°C. Purification and measurement of mRNA and miRNA are described above. The results of Ago-IP were normalized to that of negative control IgG-IP.
**Dual luciferase assay**

Cells transfected with an indicated plasmid and miRNA mimics were harvested and subjected to luciferase reporter assays using the dual luciferase assay reporter system (Promega, Madison, WI) according to the manufacturer’s instructions [30–32]. Lipofectamine 3000 was used for co-transfection of cells with miRNA mimics and a reporter plasmid containing a luciferase gene fused to the target sequence of the WT or mutant 3’-UTR of *Apoptosis Inducing Factor Mitochondria Associated 2 (AIFM2)*. At 36 hours after transfection, cells were collected and analyzed using a Veritas Microplate Luminometer (TD-20/20 Luminometer, Turner Designs).

**Site-directed mutagenesis**

Site-directed mutagenesis of the *AIFM2* 3’-UTR-luciferase reporter plasmid was accomplished according to the protocol from the Site-Directed Mutagenesis System (Thermo Fisher Scientific). The primers for mutagenesis are shown in Supplementary Table S5. The miR-3622b-3p targeting motif sequences (868 to 875: UCAGGUGA) in the pMIR-AIFM2-3’-UTR construct were mutagenized to generate the targeting motif deletions.

**Cell cycle analysis**

After two days of starvation, cells were fixed with ice-cold 80% ethanol for > 24 hours and then dyed with propidium iodide/RNase buffer. After 30 min of incubation at 4°C, while protected from light, the cells were measured by flow cytometry [33, 34].

**Apoptosis analysis**

Quantification of apoptotic cells was performed with Annexin-V-APC Apoptosis Detection Kits (Thermo Fisher Scientific) according to the manufacturer’s instructions. Early apoptotic cells were defined as Annexin-V-positive, PI-negative cells. Analyses were performed on a FACScan flow cytometer (Becton–Dickinson, Sunnyvale, CA).

**RNA-seq**

RNA libraries were prepared using a TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA) according to the manufacturer’s protocol. Integrity was assessed with an Agilent 2200 Tapestation instrument (Agilent). First-strand cDNA syntheses were performed using random hexamers and ProtoScript II Reverse Transcriptase (New England Biolabs, Ipswich, MA). The libraries were normalized, pooled, and subjected to cluster and pair read sequencing performed for 150 cycles on a HiSeqX10 instrument (Illumina), according to the manufacturer’s instructions. The RNA-seq data are submitted to NCBI GEO (Accession no. GSE179399).

**Prediction of miR-3622b target genes and analysis of public datasets**

The potential miR-3622b target genes were selected from our RNA-Seq data and TargetScan (http://www.targetscan.org/) [35]. In brief, the candidate target genes (log2(fold change) ≥ 0.67 or ≤−0.67; q value < 0.001) of miR-3622b were selected from the TargetScan due to the total context score ≤ 0.15. The overlapping genes between the TargetScan dataset and our RNA-seq data were regarded as the target genes. TCGA data were obtained from
the UALCAN portal (http://ualcan.path.uab.edu/) [36]. The genetic alteration analysis in the prostate cancer dataset was performed using cBioPortal (http://www.cbioportal.org) [37].

**Statistical analyses**

Student’s t-tests or the Mann-Whitney U test were used to measure the differences between two independent groups. Analysis of variance (ANOVA), one- and two-way, was used to test for overall differences, followed by a Dunnett post hoc test for differences between groups. All data were expressed as means ± standard deviation (SD). \( p < 0.05 (*) \) and \( p < 0.01 (***) \) were considered statistically significant. All statistical analyses were performed using IBM SPSS 25.0 (Armonk, NY) and GraphPad Prism 8 (San Diego, CA) software.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

We thank Dr. Donald Hill for editorial assistance in preparing this manuscript. This work was supported by grants from the National Cancer Institute (CA118948 for L. Wang), the Department of Defense (W81XWH-21-1-0100 for L. Wang and W81XWH-15-1-0323 and W81XWH-20-1-0426 for R. Liu) and the Mike Slive Foundation for Prostate Cancer Research (R. Liu). Results are based, in part, upon data generated by TargetScan (http://www.targetscan.org/), the UALCAN portal (http://ualcan.path.uab.edu/), and the cBioPortal (http://www.cbioportal.org).

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Figure 1. Expression of miR-3622b in primary prostate cancer and the effect of miR-3622b and miR-3622 KO on proliferation of human prostate cancer cells.

A. The normalized expression levels of miR-3622b-5p/3p in human primary prostate cancer tissues and normal prostate tissues. B and C. Expression of miR-3622b-5p/3p in prostate cancer tissues with tumor grades (Gleason scores) and tumor stages (localized and metastatic tumors). D and E. Expression of miR-3622b-5p/3p in PC3, DU145, LNCaP, RC-77T/E and RC-77N/E cells. F, G, and H. Growth of PC3, DU145, and LNCaP cells transfected with a scramble control, an miR-3622b-5p mimic, an miR-3622b-5p inhibitor, an miR-3622b-3p mimic, or an miR-3622b-3p inhibitor. I and L. Growth of miR-3622 scramble, KO DU145, and RC-77T/E cells. J, K, M, and N. Colony formation and quantitative analysis of miR-3622 scramble, KO DU145, and RC-77T/E cells. * p < 0.05; ** p < 0.01. P values were determined by a two-way ANOVA test or a two-tailed t test. Scr, scramble; KO, knockout. All experiments were repeated three times.
Figure 2. Effect of miR-3622b and miR-3622 KO on migration and invasion of human prostate cancer cells.
A-F. Cell migration (scratch assay) and invasion (Transwell assay) of PC3 and DU145 cells, and quantitative analysis of cells transfected with a scramble control, an miR-3622b-5p mimic, an miR-3622b-5p inhibitor, an miR-3622b-3p mimic, or an miR-3622b-3p inhibitor. 
G and H. Migration (scratch assay) and quantitative analysis of miR-3622 scramble and KO DU145 cells. I-K. Cell invasion (Transwell assay) and quantitative analysis of miR-3622 scramble and KO PC3 and DU145. ** p < 0.01. P values were determined by a two-tailed t test. Scr, scramble; KO, knockout. All experiments were repeated three times.
Figure 3. Effect of miR-3622 KO on tumor growth and metastasis of human prostate cancer cells in NSG mice.
A. Xenograft tumor growth of miR-3622 scramble and KO PC3-luciferase (luc) cells. B. Representative luc imaging of scramble and KO PC3-luc xenograft tumors at day 30. C and D. Tumor sizes and weights of scramble and KO PC3-luc xenografts. E and F. Lung metastasis of scramble and KO xenograft tumors. G, H, and I. Tumor growth, sizes, and weights of miR-3622 scramble and KO LNCaP xenografts. J, K, and L. Ki67 staining and quantitative analysis of scramble and KO PC3 and LNCaP xenograft tumors. ** p < 0.01. P values were determined by a two-tailed t test or a two-way ANOVA test. Scr, scramble; KO, knockout. All experiments were repeated three times.
Figure 4. miR-3622 KO enhances the p53 signaling activation and its regulated genes in LNCaP cells.

(A) KEGG enrichment plot, (B) Random Enrichment score (ES) and (C) heatmap of gene expression for the top candidate p53 signaling pathways identified by our RNA-seq data in the miR-3622-KO group versus a control group. D. Venn diagram showing the intersection of differentially up-regulated genes in our RNA-seq and target genes of miR-3622b-3p predicted by Targetscan. E. RNA-seq data for CYB5D2, TMEM220, and AIFM2. F, G, and H. mRNA expression of CYB5D2, TMEM220, and AIFM2 in miR-3622 scramble, miR-3622 KO, and miR-3622b-3p mimic-transfected LNCaP cells. I. mRNA expression of AIFM2 in miR-3622 scramble and KO DU145 cells. J. AIFM2 staining of miR-3622 scramble and KO PC3 and LNCaP xenograft tumors. * p < 0.05; ** p < 0.01. P values were determined by a two-tailed t test. Scr, scramble; KO, knockout. All experiments were repeated three times.
Figure 5. Identification of AIFM2 as a direct target gene of miR-3622b-3p in human prostate cancer cells.

A. Schematic illustration of the CRISPRi system. B. Expression of dCas9-KRAB (GFP) and sgRNA (miFP) determined by IF analysis in CRISPRi DU145 cells. C-F. Dynamic expression changes of mi-R3622a/b, AIFM2, CYB5D2, and TMEM220 measured by qPCR in CRISPRi cells after Dox induction (day 0–7). G. Schematic representation of miRNA interaction with the 3′-UTRs of its corresponding targets. Association with AGO proteins, key components of the RNA-induced silencing complex, is characteristic of functional miRNA. H. Interaction analysis of miR-3622b-3p with the 3′-UTRs of CYB5D2, TMEM220, and AIFM2 mRNAs by miRNA/mRNA IP assay of LNCaP cells. I. miR-3622b-3p target motif of the AIFM2 3-UTR and deletion mutagenesis of miR-3622b-3p target motif (AIFM2-MT). The miRNA binding site was predicted by TargetScan. J. Luciferase activity of LNCaP cells transfected with an miRNA negative control or an miR-3622b-3p mimic. A dual-luciferase reporter assay was performed on aliquots of the samples, and firefly luciferase activity normalized to Renilla luciferase was plotted. ** p < 0.01. P values were determined by a two-tailed t test. All experiments were repeated three times.
Figure 6. Effect of the miR-3622-AIFM2 axis on cell proliferation and migration in human prostate cancer cells.

A and B. Protein expression of AIFM2 by Western blots in scramble, miR-3622 KO, and AIFM2 KO PC3 and LNCaP cells.

C and F. Cell growth of scramble, miR-3622 KO, and miR-3622/AIFM2 KO PC3 and LNCaP cells.

D, E, G, and H. Colony formation and quantitative analysis of scramble, miR-3622 KO, and miR-3622/AIFM2 KO PC3 and LNCaP cells.

K and L. Cell migration and quantitative analysis of scramble, miR-3622 KO, and miR-3622/AIFM2 KO PC3 cells.

I. Quantitative analysis of cell cycle progression (0–20 hours) in scramble, miR-3622 KO, and miR-3622/AIFM2 KO PC3 and LNCaP cells.

M. Protein expression of E-cadherin, vimentin, and twist by Western blots in scramble, miR-3622 KO, and miR-3622/AIFM2 KO PC3 cells. ** p < 0.01. P values were determined by a two-tailed t test or two-way ANOVA test. Scr, scramble; KO, knockout. All experiments were repeated three times.
Figure 7. Effect of the miR-3622-AIFM2 axis on apoptosis and p53 signaling activation in human prostate cancer cells.
A-C. Cell apoptosis and quantitative analysis of scramble, miR-3622 KO, and miR-3622/AIFM2 KO PC3 and LNCaP cells. D. Protein expression of Bax, caspase 3, and cleaved caspase 3 by Western blots in scramble, miR-3622 KO, and miR-3622/AIFM2 KO LNCaP cells. E. Protein expression of MDM2, p53, p21, and c-MYC by Western blots in scramble, miR-3622 KO, and miR-3622/AIFM2 KO LNCaP cells. F-H. Protein expression of p21 by IHC in miR-3622b scramble and KO PC3 and LNCaP xenograft tumors. ** p < 0.01. P values were determined by a two-tailed t-test. Scr, scramble; KO, knockout. All experiments were repeated three times.