Androgen Receptor and MicroRNA-21 axis down-regulates transforming growth factor beta receptor II (TGFBR2) expression in Prostate Cancer

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Abstract

Prostate cancer cells escape growth inhibition from TGFβ by down-regulating TGFβ receptors. However, the mechanism by which cancer cells down-regulate TGFβ receptors in prostate is not clear. Here, we showed that coordinated action of miR-21 and androgen receptor (AR) signaling played a critical role in inhibiting TGFβ receptor II (TGFBR2) expression in prostate cancer cells. Our results revealed that miR-21 suppresses TGFBR2 levels by binding to its 3'UTR and AR signaling further potentiates this effect in both untransformed and transformed human prostate epithelial cells as well as in human prostate cancers. Analysis of primary prostate cancers showed that increased miR-21/AR expression parallel a significantly reduced expression of TGFBR2. Manipulation of androgen signaling or the expression levels of AR or miR-21 negatively altered TGFBR2 expression in untransformed and transformed human prostate epithelial cells, human prostate cancer xenografts, and mouse prostate glands. Importantly, we demonstrated that miR-21 and AR regulated each other's expression resulting in a positive feedback loop. Our results indicated that miR-21/AR mediate its tumor promoting function by attenuating TGFβ-mediated Smad2/3 activation, cell growth inhibition, cell migration, and apoptosis. Together, these results suggest that the AR and miR-21 axis exerts its oncogenic effects in prostate tumors by down-regulating TGFBR2, hence inhibiting the tumor suppressive activity of TGFβ pathway. Targeting miR-21 alone or in combination with AR may restore the tumor inhibitory activity of TGFβ in prostate cancer.
Keywords
microRNA; androgen receptor; tumor suppressor; prostate cancer

Introduction
TGFβ signaling involves a cooperative interaction between two serine/threonine kinase cell surface receptors, TGFBR1 and TGFBR2. Binding of TGFβ ligand to the constitutively active dimeric TGFBR2 recruits and activates TGFBR1 kinases by phosphorylating serine/threonine residues at a glycine serine (GS) repeat in TGFBR1. Activated TGFBR1 then phosphorylates Smad2 and Smad3 proteins. Translocation of this complex to the nucleus regulates transcription by binding to Smad-binding elements (SBEs) in TGFβ-regulated genes. TGFβ acts as a tumor suppressor in the early stages of tumor progression. The abrogation of TGFβ signaling pathway by either loss or reduction in TGFβ receptor expression imparts a growth advantage to early growing tumors. However, the mechanism of such a loss resulting in decreased sensitivity of prostate cancer cells to TGFβ mediated growth inhibition remains unclear. Human prostate tumors often lose their sensitivity to TGFβ-mediated growth inhibitory responses via down-regulation of TGFBR2. TGFBR2 has been shown to act as a tumor suppressor gene. Silencing of TGFBR2 due to promoter methylation has been reported in some breast, colon and LNCaP prostate cancer cells. However, neither DNA methylation of the TGFBR2 promoter nor any TGFBR2 gene mutations were detected in the primary prostate cancer. These observations suggest that there could be other molecular mechanisms involved in the down-regulation of TGFBR2 that needs further investigation. AR, a 110-kDa zinc finger transcription factor belongs to the nuclear receptor superfamily. The binding of androgen ligand promotes dimerization and nuclear translocation of AR. The activated AR then binds to androgen-responsive elements in the promoters of androgen-regulated genes that are involved in the growth, development, and survival of androgen target tissues such as the prostate. Activated AR is known to trigger epithelial cell growth arrest and differentiation in normal prostate. However, in prostate cancer, AR signaling pathway is modified to promote cell survival and proliferation. One of the important pathways that AR cross talks with is the TGFβ signaling pathway. Several in vitro and in vivo studies show that androgens promote cell survival, partly by blocking TGFβ-induced inhibitory growth responses. However, the underlying molecular mechanism remains to be defined. It has been previously observed in human and rat prostate cell lines that androgen-bound AR protected cells from TGFβ-induced apoptosis by relieving the inhibition of TGFβ on Bcl-xL and cyclin D target genes. Androgens further transcriptionally suppress TGFBR2 expression by down-regulating Sp1 levels, leading to reduced association of Sp1 to the TGFBR2 promoter. During advanced stage of prostate cancer progression, tumor cells overcome the requirement for androgens through a mechanism that does not involve down-regulation of AR expression. Hence, additional mechanism might be operating in the advanced stage aggressive cancer where loss of TGFBR2 and a functional AR signaling pathway is very much retained.
MicroRNAs (miRNAs) have emerged as important regulators of gene expression. miRNAs are 18- to 24-nucleotide RNA polymerase II-transcribed RNA that regulate the translation of mRNAs. Deregulated miRNA expression has been reported in many solid tumors including prostate cancer.\(^\text{17, 18}\) Multiple studies indicate that microRNA-21 (miR-21) is a cancer-related microRNA with oncogenic potential. MiR-21 expression was found to be elevated in early grade prostate cancer patients\(^\text{19}\) and in plasma samples of localized and metastatic cancer patients when compared to the healthy controls\(^\text{20, 21}\). However, some other published reports negate a significant role of miR-21 in prostate cancer tumorigenesis\(^\text{22}\). Hence, it is necessary to further study miR-21 mediated functional responses and downstream cellular targets in prostate cancer. It has been recently reported that activated AR directly interacts with miR-21 regulatory regions, indicating direct transcriptional induction of miR-21\(^\text{23}\). In this study, we have investigated the molecular mechanism of miR-21 mediated attenuation of TGF\(\beta\) signaling in prostate cancer cells. Our results, for the first time, show that the AR and miR-21 by acting through a positive feedback loop drives the down-regulation of TGFBR2, thereby attenuating TGF\(\beta\)-mediated inhibitory growth responses in prostate cancer. Thus, approaches aimed at inhibiting AR and miR-21 expression may serve as a novel therapeutic strategy for treating prostate cancer patients.

**Results**

**AR and TGFBR2 expression correlate inversely in human prostate samples**

To determine the expression pattern of AR and TGFBR2 in human prostate samples, we checked their expression levels in normal prostate and primary adenocarcinoma datasets available in both the Oncomine and The Cancer Genome Atlas (TCGA) databases. Oncomine database analysis showed a strong inverse correlation between AR and TGFBR2 in prostate tumor (Fig. 1A–B). There was a significant increase in the AR and a down-regulation in TGFBR2 expression in the primary prostate tumors (Fig. 1C–D) when compared to the normal prostate. This inverse correlation was also observed in most untransformed prostate epithelial cell lines, which have relatively higher levels of TGFBR2 and low levels of AR, as well as in most prostate cancer cell lines, which show low TGFBR2 and high AR (Fig. 1E). We next investigated the molecular mechanism behind this inverse correlation observed in prostate cancer.

**DNA demethylation or histone acetylation does not restore complete TGFBR2 expression in prostate cancer cells**

We checked the TGFBR1 and TGFBR2 expression status in different TGF\(\beta\) insensitive prostate cancer cell lines including PacMetUT1, which is a novel cell line established from a metastatic lesion of human prostate tumor to lymph nodes\(^\text{24, 25}\), MDA-PCa-2b, LNCaP and in TGF\(\beta\) responsive, PC-3 cells. While TGFBR1 level was similar among these cells, TGFBR2 expression levels in PacMetUT1 and MDA-PCa-2b cells were significantly reduced when compared to PC-3 (Sup Fig.1A). We found that AR positive cells were less sensitive to TGF\(\beta\) mediated functional effects as revealed by TGF\(\beta\)-mediated cell growth inhibition (Supplementary Fig. 1B), or in a TGF\(\beta\)-responsive promoter luciferase reporter assay (Supplementary Fig. 1C). Previous reports indicated a methylation-mediated silencing of TGFBR2 in LNCaP cells\(^\text{5}\). Similarly, treatment with an HDAC inhibitor, Trichostatin A,
strongly induced TGFBR2 expression in some pancreatic cancer cell lines\textsuperscript{26}. We therefore tested whether TGFBR2 expression may be similarly regulated in the AR positive cells. Surprisingly, Trichostatin A or 5-Aza-2'-deoxycytidine, a DNA demethylating agent treatment did not result in TGFBR2 restoration in PacMetUT1 or MDA-PCa-2b cells as that in the positive control LNCaP cells (Fig. 2A), suggesting that other regulatory mechanisms may be at play in these cells. Since miRNAs are well-established regulators of gene expression and TGFBR2 gene has a long 3' UTR with several potential microRNA-binding sites, we reasoned that miRNAs may play an important role in regulating TGFBR2 expression. Target prediction algorithms predicted several miRNAs to target TGFBR2. Of these, miR-21 was also predicted by other target prediction databases including miRanda, TargetScan and microRNA.org (Fig. 2B). Furthermore, of all the predicted targets, miR-21 showed an inverse expression correlation with TGFBR2 in prostate cancers and cell lines. Interestingly, AR positive cells expressed higher level of miR-21 (22Rv1, PacMetUT1 and MDA-PCa-2b) when compared to AR negative cells (RWPE-1, BPH-1 or PC-3) (Fig. 2C). The meta-analysis of public database from TCGA further confirmed the higher expression of miR-21 and lower expression of TGFBR2 in primary prostate cancers when compared with normal matched control (Fig. 1C and 2D). Consistent with the reduced TGFBR2 expression, the expression of other TGFβ inhibitory genes is also reduced in the primary prostate tumors in comparison with the normal prostate tissues in the same cohort (Fig. 2E).

**MicroRNA-21 targets TGFBR2 gene**

MicroRNA’s are known to down-regulate target gene expressions by binding to their 3' UTR and causing either mRNA decay or translation repression\textsuperscript{27}. Bioinformatic analyses revealed that TGFBR2 3' UTR contained one putative binding site for miR-21. To examine whether miR-21 indeed binds to the TGFBR2 3' UTR, we transfected BPH-1 cells with pMIR-reporter construct containing TGFBR2 3' UTR in the presence of miR-21 mimic and luciferase activity was measured. As shown in Fig. 3A, luciferase activity was significantly reduced in pMIR-TGFBR2 3' UTR-transfected cells compared to scramble-transfected cells. Similarly, luciferase activity was significantly increased when PacMetUT1 and 22Rv1 cells were transfected with pMIR-TGFBR2 3' UTR construct in the presence of anti-miR-21 inhibitor. These results suggested that miR-21 may regulate TGFBR2 expression by binding to its 3’-UTR. This is consistent with the previous study in human adipose tissue-derived mesenchymal stem cells demonstrating the abrogation of miR-21 effect on TGFBR2-3’UTR in a similar luciferase assay when the putative miR-21 site was mutated\textsuperscript{28}. To further substantiate these findings, we assessed the effect of miR-21 mimic and inhibitor on TGFBR2 mRNA and protein levels. Transfection of miR-21 mimic in BPH-1 and PC-3 prostate epithelial cells resulted in significantly reduced levels of mRNA (Fig. 3B) and TGFBR2 protein (Fig. 3C). On the other hand, transfection of 22Rv1 and MDA-PCa-2b cells with anti-miR-21 inhibitor increased the TGFBR2 expression (Fig. 3C).

**MicroRNA-21 attenuated TGFβ signaling in prostate cancer cells**

To determine whether miR-21 can functionally alter the sensitivity of prostate cancer cells to TGFβ treatment, we tested the phosphorylation of TGFβ downstream mediator, Smad-3, after miR-21 mimic transfection in PC-3 and BPH-1 cells. MiR-21 decreased the level of phosphorylated Smad3 in both the cell lines (Fig. 4A). MiR-21 also reduced the sensitivity...
of PC-3 cells to TGFβ-induced Smad3 phosphorylation when compared to control siRNA-transfected cells (Fig. 4B). Furthermore, miR-21 decreased TGFβ-induced transcriptional activity of the TGFβ-responsive promoter in both PC-3 and BPH-1 cells (Fig. 4C). Inhibition of endogenous miR-21 levels with an anti-miR-21 inhibitor in MDA-PCa-2b cells enhanced TGFβ1 signaling activity (Fig. 4D). Next, we examined effect of miR-21 on TGFβ-mediated cell growth, apoptosis and migration in vitro using BPH-1 cells. MiR-21 mimic transfection increased BPH1 cell growth and attenuated TGFβ-mediated cell growth inhibition (Fig. 4E), apoptosis (Fig. 4F), and migration (Fig. 4G) when compared to negative control siRNA transfected cells. These findings are highly significant as it suggests that miR-21 dependent regulation of TGFBR2 may be an important mechanism for the prostate tumor cells to proliferate, survive and escape TGFβ1-mediated tumor suppressive effects during early tumor progression.

**Androgen receptor and miR-21 axis down-regulate TGFBR2 expression**

Next, we determined whether reduced levels of TGFBR2 in prostate cancer cells is due to suppression of TGFBR2 by AR signaling and whether miR-21 plays any role in this signaling cascade. To address this, we first checked the endogenous TGFBR2 level in AR silenced prostate cancer cells. Interestingly, AR inhibition resulted in decreased levels of miR-21 and consequently increases in TGFBR2 expression in 22Rv1 and MDA-PCa-2b cells (Fig. 5A–B) suggesting that miR-21 may mediate AR regulation of TGFBR2. AR knockdown further increased the sensitivity of 22Rv1 cells as measured from TGFβ1 mediated p-Smad2 levels (Fig. 5C). Conversely, AR overexpression in BPH-1 cells decreased the TGFβ1 mediated p-Smad2 levels (Fig. 5C). To study whether AR and miR-21 together is involved in the down-regulation of TGFBR2, we stably transfected full length human AR in BPH-1 cells (BPH-1/AR) and showed that it becomes androgen responsive as measured from a PSA-promoter luciferase assay (Fig. 5D). We also observed that the transfected AR in BPH-1 cells is predominantly localized in nucleus and hence is transcriptionally active when compared to BPH-1/C cells (Fig. 5E). We found a decrease in the TGFBR2 mRNA level in BPH-1/AR cells and a concomitant increase in miR-21 level when compared to BPH-1/C cells (Fig. 5F). When we inhibited this increase in miR-21 expression in BPH-1/AR cells with an anti-miR-21 inhibitor, the repression of TGFBR2 level by AR was relieved (Fig. 5G) further demonstrating that miR-21 mediates AR-induced repression of TGFBR2.

**MicroRNA-21 inversely correlates with TGFBR2 expression in vivo**

We used CWR22 xenograft mouse model to study the effect on miR-21 and TGFBR2 expression under androgen depletion with castration. CWR22 is an androgen dependent xenograft model derived from a primary human prostatic carcinoma that responds very well to castration. After implanting the tumor subcutaneously in male nude mice with a testosterone pellet, we surgically castrated the mice 30 days after tumor implantation with the removal of testosterone pellet and harvested tumor for miR-21 and TGFBR2 expression analysis at 14 days post castration. Our results showed an increase in TGFBR2 expression at 14 days after castration when compared to control non-castrated mice (Fig. 6A). Interestingly, we observed a significant decrease in miR-21 expression levels in the castrated mice showing an inverse correlation between miR-21 and TGFBR2 expression in vivo (Fig.
In a separate experiment, we surgically castrated male BALB/c mice and harvested prostate for miR-21 and TGFBR2 expression measurement. After 15 days of castration, we observed down-regulation in miR-21 expression and a concomitant increase in TGFBR2 level in the prostate (Fig. 6B). To investigate whether the increased TGFBR2 level was indeed caused by the decreased miR-21, we injected an anti-miR-21-liposomal complex into the ventral lobe of mouse prostate glands and checked for the TGFBR2 expression. We observed that anti-miR-21 inhibitor injection significantly reduced endogenous miR-21 and increased TGFBR2 mRNA in the injected prostate lobe in comparison to the negative control miScript-injected group (Fig. 6C). All these results suggest that androgen signaling and miR-21 act in concert to down-regulate TGFBR2 expression in vivo.

**MicroRNA-21 and AR form a positive feedback loop in regulating each other’s expression**

As described above, AR can positively regulate miR-21 expression, however, it is not clear whether miR-21 can regulate AR expression. Interestingly, transfection of the AR negative BPH-1 cells with miR-21 mimic, resulted in a significant upregulation of the AR mRNA level (Fig. 7A). This was confirmed with immunocytochemical staining revealing nuclear and cytoplasmic localization of AR (Fig. 7B). This miR-21-mediated increase of AR is further confirmed using androgen-responsive transcriptionally active PSA-promoter luciferase assay (Fig. 7C). MiR-21 mimic transfection also restored the AR expression in other AR negative prostate epithelial cells including RWPE-1 and in PC-3 prostate cancer cell line (Fig. 7D). Furthermore, inhibition of endogenous miR-21 in 22Rv1, MDA-PCa-2b and PacMetUT1 cells also significantly reduced the AR expression (Fig. 7D and E) and AR mediated PSA-promoter luciferase activity (Fig. 7F). Inhibition of endogenous mouse miR-21 in BALB/c mouse prostate gland with an anti-miR-21 inhibitor also inhibited AR expression (Fig. 7G) indicating that the regulation of AR by miR-21 can be achieved in vivo. These results show that miR-21 and AR form a positive feedback loop in prostate epithelial cells. We thus propose that in prostate cancer, AR and miR-21 is involved in a positive feedback loop with the AR-miR-21 axis down-regulating TGFBR2 expression in prostate epithelial cells (Fig. 7H).

**Discussion**

Down-regulation of TGFBR2 is a frequent event observed in a wide variety of cancer including prostate. In humans, TGFBR2 silencing due to DNA methylation of its promoter CpG islands has been observed in a limited number of cell lines but has not been found in any primary cancers. Yamashita et al. also failed to find TGFBR2 methylation silencing and gene mutations in human primary prostate tumors. Our initial experiments showed that DNA demethylation or chromatin acetylation did not fully restore TGFBR2 expression in some AR positive cells. These observations suggested that there could be other molecular mechanisms involved in the down-regulation of TGFBR2. Here we provide first evidence demonstrating miR-21-mediated suppression of TGFβ signaling pathway by down-regulation of TGFBR2 in prostate cancer. Our analysis of Oncomine and TCGA database's further showed an inverse correlation between AR and TGFBR2 in normal as well as prostate cancer cells and in human prostate tumor specimens. In this paper, we have
delineated the molecular mechanism of this inverse correlation implicating microRNA-21 as one of the central mediators.

Although miR-21 is widely accepted as a tumor promoter, its function in prostate cancer is undefined because of limited studies and known targets in prostate cancer. Some studies suggested that miRNA-21 is not differentially expressed in tumor and normal prostate tissues or in localized and metastatic prostate cancer\textsuperscript{22, 34–36}. However, some recent published studies do report an elevated miR-21 expression in the plasma and serum samples from patients with localized and metastatic prostate cancer compared to healthy controls\textsuperscript{19, 20, 37}, and its high expression also predicted the risk of biochemical recurrence after radical prostatectomy\textsuperscript{38}. Thus, it is imperative to understand the role of miR-21 and its downstream mediators for understanding its contribution to prostate cancer pathogenesis.

MiR-21 was shown previously to regulate adipogenic differentiation through the down-regulation of TGFBR2 in mesenchymal stem cells derived from human adipose tissue\textsuperscript{28}. However, the role of miR-21 in the regulation of TGFBR2 in prostate cells has not been reported. We observed that AR positive cancer cell lines expressed relatively higher level of miR-21 when compared to AR negative cells. We found that miR-21 was targeting 3’-UTR region of TGFBR2 gene thereby decreasing both mRNA and protein levels of TGFBR2. We also observed that miR-21 was able to abrogate the responsiveness of prostate cells to TGFβ mediated downstream Smad phosphorylation, cell growth suppression, apoptosis and migration in vitro. We hypothesized that miR-21 might be acting downstream of AR and this signaling axis could further down-regulate TGFBR2 expression thereby attenuating TGFβ signaling in prostate cancer. We observed an increase in TGFBR2 expression that coincided with a decrease in miR-21 after a transient knockdown of AR in 22Rv1 and MDA-PCa-2b cells. Furthermore, BPH-1 cells stably overexpressing AR showed a decreased level of TGFBR2 and an increase in miR-21 when compared to the non-AR expressing cells. Interestingly, we observed a positive feedback loop operating between AR and miR-21 in the prostate cells wherein miR-21 was seen to increase the AR expression and activity. It is possible that the oncogenic signals from miR-21 in the early growing tumor can drive AR mediated initiation of prostate neoplasia. This miR-21-AR positive feedback signaling axis can make the tumor cells remain addicted to AR signaling for their malignant growth. It has been previously reported that the interaction between PTEN and AR inhibits the AR nuclear translocation and promotes its protein degradation that results in the suppression of AR transactivation\textsuperscript{39, 40}. PTEN has been shown to be a downstream target of miR-21 in a variety of tumors\textsuperscript{41–43}. The up-regulation of AR via decreased PTEN expression in prostate cancer cells by miR-21 however needs further investigation. Androgen ablation induced by castration in rat prostate has been shown to activate TGFβ signaling not only by inducing the expression of TGFβs but also by elevating the levels of TGFBR2\textsuperscript{44, 45}. These papers measured TGFBR2 expression only after one week of castration. In our study we have checked the TGFBR2 expression at longer time point of 15 days. Our data is consistent with these observations wherein we show an increase in TGFBR2 expression after castration using a CWR22 xenograft mouse model as well as in normal mouse prostate. We for the first time show that this increase in TGFBR2 expression coincides very well with a decrease in miR-21 suggesting that castration-mediated down-
regulation of miR-21 resulted in the release of its inhibition on TGFBR2. We further inhibited the endogenous miR-21 expression using a mouse specific anti-miR-21, and observed an induction in TGFBR2 expression and a significant AR decrease in vivo.

One of the major clinical challenges in prostate cancer is the current inability to distinguish indolent from aggressive tumors in patients who present with low Gleason grade tumors upon biopsy. This lack of prognostic information leads to overtreatment of patients, who may not require treatment if their tumors are relatively indolent. Our preliminary analysis in a limited set of normal and human prostate adenocarcinoma samples shows an increased AR and miR-21 expression with an associated decrease in TGFBR2 (Sup. Fig2). Our finding provides an opportunity to test whether the three-gene expression signature (AR\textsuperscript{hi}miR-21\textsuperscript{hi}TGFBR2\textsuperscript{low}) can serve as a novel prognostic biomarker to distinguish patients with a high risk of developing aggressive disease in our future studies.

In summary, we show that AR and miR-21 form a positive feedback loop in driving each other’s expression leading to down-regulation of TGFBR2 expression in prostate cancer cells thereby making the cells less susceptible to TGFβ mediated growth inhibition and apoptosis. Our findings provide a novel mechanism that is likely involved in driving prostate carcinogenesis. These findings provide a strong rationale to perform preclinical testing of miR-21 inhibitor alone or in combination with AR for the effective management of prostate cancer.

Materials and Methods

Cell Cultures

RWPE-1, MDA-PCa-2b, 22Rv1, PC-3 and LNCaP cells were from the American Type Culture Collection (ATCC). PacMetUT1 was isolated from the lymph node metastasis of a prostate tumor of a 57-year old male in our institution\textsuperscript{24}. The cells were cultured in medium recommended by ATCC. Cells were maintained at 37°C in a 5% CO\textsubscript{2} humidified incubator.

Oncomine and The Cancer genome Atlas (TCGA) analysis

Microarray data from oncomine and TCGA analysis were downloaded for different studies and log\textsubscript{2} median centered intensity was plotted on the Y-axis for Oncomine studies. Heat maps were created with MultiExperiment Viewer software with data obtained from TCGA database. The gene expression data were downloaded from TCGA as normalized counts calculated using the the SeqWare framework via the RSEM algorithm\textsuperscript{46}.

Cell Proliferation Assay

Cells were plated in 96-well plate at 2000 cells/well in 4 well replicates. After 3 days, cell proliferation quantified at 595nm wavelength on a Biotek plate reader as described previously\textsuperscript{25}.

Western Blot Analysis

Cells after harvesting were lysed in Laemmli buffer containing protease inhibitors and resolved as described previously\textsuperscript{25}. Antibodies to T-Smad2, T-Smad3, p-Smad2, p-Smad3
were from Cell signaling, Actin (Sigma), GAPDH (CalBioChem), Tubulin, TGFBR2 and AR antibodies were from Santa Cruz.

5-aza-dC or Trichostatin A treatment

For treatment with 5-aza-dC, 2×10^5 cells were seeded on day 0, and treated with 2ug/ml of 5-aza-dC (Sigma) on day 2 and day 5. Cells were harvested on day 7 for RNA isolation. For treatment with Trichostatin A (TSA), cells were seeded at a half confluent density and treated with 100ng/ml of TSA (Sigma). After 24 h, cells were harvested for RNA isolation.

DNA Construction, Transient Transfection and In Vitro Luciferase Assay

Primers were designed to amplify the 3'UTR sequence of human TGFBR2 gene, and Mlu1 and Spe1 sequences were added to the end of primers. Cloning of 3'-UTR was performed in pMIR reporter vector (Ambion) according to manufacturer's instruction. For transient transfection, 0.5 × 10^5 cells were plated in 24 well plates 12 hours prior to transfection. pMIR-TGFBR2 3’ UTR reporter vector (100ng) was cotransfected with 50ng of β-Gal and miRNA-21 mimic or anti-miR-21 with lipofectamine 2000 (Invitrogen) transfection reagent. All Stars negative control siRNA (Qiagen) and miScript Inhibitor Negative Control (Qiagen) were used as respective controls for mimic or inhibitor transfection. After 24 h of incubation, luciferase assay was performed as described previously.

Generation of stable AR expressing cells

Human AR cDNA was cloned in pSDM101 lentiviral vector. The empty pSDM101 and AR-pSDM101 vectors containing a GFP-expressing cassette were transfected into HEK293 cells, together with two other plasmids, which helps in the packaging of the virus. Viral supernatant were harvested for infection into target cells. Almost all the cells expressed GFP indicating stable transfection of cells. The AR activity in the overexpressed cells was further confirmed using the PSA-promoter luciferase assay. The PSA-luciferase reporter construct was kindly provided by Dr. Paramita Ghosh at University of California, Davis. It contains −1 to −631 bp of human PSA promoter.

Apoptosis ELISA Assay

BPH-1 cells were transfected for 48 hours with control siRNA (20nM) and miR-21 mimic (20nM). Cells were then replated and treated with TGFβ1 (2ng/ml) in complete medium for 5 days. Cells were trypsinized and lysed in apoptosis lysis buffer followed by cell death detection ELISA assay according to manufacturer's protocol (Roche).

Immunofluorescence Cytochemical Staining

Cells were seeded in 24-well plate on sterile glass coverslips. After 24h, cells were fixed with 2% paraformaldehyde for 15min, followed by permeabilization in 0.2% Triton-X-100. After blocking with 1% BSA in PHEM buffer for 1h, cells were incubated with AR primary antibody for overnight in 4°C. AR staining was analyzed with Alexa 568 incubation for 1h at room temperature. The slides were mounted with Vectashield mounting media (Vector) and viewed under an Olympus Fluoview FV1000 confocal fluorescence microscope.
Migration Assay

Migration assays were performed in 24-well Boyden chambers with 8-μm polycarbonate membrane pores (BD Biosciences). Cells were seeded in the upper chamber at the indicated number in serum-free medium with or without treatment. Complete medium was added to the lower chamber. After 18h, cells that had migrated through the membrane were stained with Hema 3 Stain 18 kit (Fischer Scientific) according to the manufacturer’s protocol. Stained cells were counted under a microscope with 100X magnification.

RNA Extraction and qRT-PCR

TRIzol reagent (Sigma) was added to the cells for cell lysis and dissociation of any RNA-protein complexes. RNA was isolated using chloroform-isopropanol extraction. Total RNA (2ug) was reverse transcribed to make cDNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems) with random primers. After reverse transcription, quantitative real time-PCR was performed using Step One Plus Real Time PCR System (Applied Biosystems) with the gene specific forward and reverse primers after diluting the cDNA (1:5) using SYBR Green dye (ABI). Primer sequences are available upon request. Total RNA containing microRNA was isolated using miRNeasy kit (Qiagen) according to the manufacturer's protocol. β-Actin and U6B were used as endogenous controls for mRNA and miRNA quantification respectively.

Animal Experiments

Four- to five-week-old male athymic nude mice and 6–7 week-old BALB/c mice were purchased from Harlan Sprague-Dawley, Inc. Animals were maintained under the care and supervision of the Laboratory Animal Research facility at the University of Texas Health Science Center, San Antonio, Texas. The animal protocol was approved and monitored by the Institutional Animal Care and Use Committee.

Subcutaneous injections and castration

CWR22 human xenograft tumors were maintained by serial passaging in male nude mice implanted with a 90-day slow release testosterone pellet. Tumor cell suspension (0.07g) was mixed with an equal volume of matrigel, and was subcutaneously implanted on the lower back of male nude mice. After four weeks, testosterone pellet was removed and mice were surgically castrated. Control mice were left on the testosterone pellet and not castrated. After 14 days of castration, tumors from two mice each were harvested for RNA isolation using RNeasy Plus kit (Qiagen, Valencia, CA). The flow through was used for microRNA isolation using miRNeasy kit (Qiagen). Three control mice were also sacrificed for tumor isolation. Six BALB/c mice each were also surgically castrated and sham treated. After 15 days of castration, mouse prostates were isolated from sham and castrated groups for RNA isolation as described above.

Mouse anti-miR-21 injection

miScript Inhibitor negative control (Qiagen) and mouse anti-miR-21 (Qiagen) was mixed with liposome 2000 (Invitrogen) and incubated at room temperature for 20min. Negative control and anti-miR-21 complex was injected into the ventral lobes of five Balb/c mice.
each with a final concentration of 200nM. After 48h of injection, prostate was isolated and processed for both RNA and microRNA isolation as described above.

**Statistical Analysis**

One-way ANOVA was used for analysis when more than two groups of data sets were analyzed with Tukey-Kramer post hoc test. Two-tailed Student’s t-tests were used to compare two groups. Results are expressed as mean ± SEM. P<0.05 was considered as statistically significant.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Expression of AR and TGFBR2 in human prostate samples

(A and B) AR and TGFBR2 are inversely correlated in human tumor samples from Oncomine database. Representative AR (C) and TGFBR2 (D) gene expressions in normal prostate and prostate tumor from Oncomine and TCGA databases were plotted. AR and TGFBR2 levels correlate inversely in normal prostate epithelial and prostate cancer cell lines (E). Each whisker represents the highest and lowest value in the given data set. The horizontal bar represents the median value in each group. *Indicates statistically significant difference between the two groups with a student's t-test at $P<0.05$, **$P<0.01$, ****$P<0.0001$. 

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Figure 2. Inverse association of miR-21 and TGFBR2 in prostate tumors

(A) 50% confluent PCa cell lines, were treated with Trichostatin A (100ng/ml) and 5-aza (2ug/ml) followed by qRT-PCR for TGFBR2 quantification. Each bar is presented as mean ± SEM from triplicate measurements. Statistically significant difference among the cells is indicated with P<0.05 using one-way ANOVA analysis. (B) miRNA's targeting the 3'-UTR of TGFBR2 was analyzed using indicated prediction databases. Venn diagram shows miR-21 to be the common miRNA. (C) MiR-21 analysis in prostate epithelial cells by quantitative real-time PCR was performed. (D) Bar graph analysis of miR-21 expression in normal and prostate tumor from TCGA database. The boxes cover 25–75 percentile of data and whiskers cover 10–90 percentile of data. Significant increase (P<0.001) of miR-21 was observed. (E) Heat map depicting the expression of miR-21, TGFBR2 and TGFβ-modulated gene expression in 37 normal and 138 primary prostate tumors derived from TCGA database.
Figure 3. MiR-21 targets TGFBR2 gene
(A) Luciferase assay was performed with miR-21 mimic or anti-miR-21 inhibitor transfection in cells after cotransfection with pMIR-3’-UTR-R2I and a β-gal expression construct. ***P<0.001 with a student’s t-test. (B) BPH-1 and PC-3 cells were transfected with negative control siRNA and miR-21 mimic (20nM) for 48h. Total RNA was isolated and reverse transcribed to check for TGFBR2 and actin genes. (C) The cell lysates were used for western analysis to measure the level of TGFBR2 and GAPDH after miR-21 mimic or anti-miR-21 inhibitor (50nM and 100nM) transfection for 48h.
Figure 4. MicroRNA-21 attenuates TGFβ signaling in prostate cancer cells
Both basal level of p-Smad3 (A) as well as TGFβ1 mediated Smad-3 phosphorylation (B) was decreased by miR-21 in cells. MiR-21 abrogates TGFβ1 mediated SBE-luciferase activity (C–D), cell growth inhibition (E), apoptosis (F) and migration (G).
Figure 5. AR and microRNA-21 axis down-regulate TGFBR2 expression

(A and B) AR knockdown increases TGFBR2 and decreases miR-21 expression. (C) Knockdown in 22Rv1 or overexpression of AR in BPH-1 modulates sensitivity of TGFβ1 to Smad-2 phosphorylation. (D) Immunoblotting analysis for AR expression in BPH-1 cells stably transfected with AR (BPH-1/AR). PSA-luciferase assay was performed in BPH-1/AR after R1881 (10nm) treatment for 18h. Data represent mean ± SEM from triplicates normalized to β-galactosidase activity. *P<0.05. (E) Immunocytochemical analysis was performed to check for AR localization in BPH-1/C and BPH-1/AR cells. (F) AR overexpression decreases TGFBR2 and increases miR-21 expression. (G) Inhibition of miR-21 in AR-overexpressing cells, rescues TGFBR2 decrease.
Figure 6. MicroRNA-21 inversely correlates with TGFBR2 expression in vivo
TGFBR2 and miR-21 was analyzed in CWR22 tumor (A) and in BALB/c mouse prostate (B) after 14 and 15 days of castration respectively. (C) Inhibition of endogenous miR-21 by anti-miR-21 in BALB/c mouse prostate increased TGFBR2 expression.
Figure 7. MicroRNA-21 increases AR expression and activity
(A and B). MiR-21 increases AR mRNA expression and nuclear localization. (C) MiR-21 increases AR mediated PSA-luciferase reporter activity. (D) MiR-21 increases AR protein expression in prostate cancer cells. Inhibition of miR-21 decreases AR mRNA expression in cells (E), PSA-reporter activity (F) and AR mRNA expression in mouse prostate (G). (H) A flowchart depicting the androgen receptor and miR-21 axis in the attenuation of TGFβ signaling in prostate cancer cells.