FOXP3–miR-146–NF-κB Axis and Therapy for Precancerous Lesions in Prostate

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Abstract

The tumor-suppressive activity of FOXP3 has been observed in tumor initiation, but the underlying mechanism still remains largely unknown. Here, we identified a FOXP3–miRNA-146 (miR-146)–NF-κB axis in vitro and in vivo in prostate cancer cells. We observed that FOXP3 dramatically induced the expression of miR-146a/b, which contributed to transcriptional inhibition of IRAK1 and TRAF6, in prostate cancer cell lines. Tissue-specific deletion of Foxp3 in mouse prostate caused a significant reduction of miR-146a and upregulation of NF-κB activation. In addition, prostate intraepithelial neoplasia lesions were observed in miR-146a–mutant mice as well as in Foxp3-mutant mice. Notably, the NF-κB inhibitor bortezomib inhibited cell proliferation and induced apoptosis in prostate epithelial cells, attenuating prostatic intraepithelial neoplasia formation in Foxp3-mutant mice. Our data suggest that the FOXP3–miR-146–NF-κB axis has a functional role during tumor initiation in prostate cancer. Targeting the miR-146–NF-κB axis may provide a new therapeutic approach for prostate cancers with FOXP3 defects. Cancer Res; 75(8); 1714–24. ©2015 AACR.

Introduction

FOXP3 is a member of theforkhead box/winged helix transcription factor family. In addition to its well-known function as a transcription factor in regulatory T cells (1), we have identified FOXP3 as an X-linked tumor suppressor gene in prostate cancer (2). Lineage-specific ablation of Foxp3 in mouse prostate epithelial cells leads to prostate hyperplasia and prostatic intraepithelial neoplasia (PIN; ref. 2), suggesting a tumor-repressive function of Foxp3 during tumor initiation. Furthermore, in vitro studies have demonstrated the tumor-suppressive function of FOXP3 during cell growth and proliferation in prostate cancer cells (2). In human prostate cancer, we detected loss of FOXP3 expression in 70% of prostate cancer samples and identified FOXP3 somatic inactivating mutations and gene deletions (2). In addition, FOXP3 inhibits cell proliferation, migration, and invasion in epithelial breast cancer (3–5), ovarian cancer (6), melanoma (7), and glioblastoma (8). Moreover, FOXP3 treatment reduces tumor metastasis in a mouse model of colon cancer (9), which supports a tumor-repressive function of FOXP3 in both tumor initiation and progression. However, clinical observations concerning the role of FOXP3 during tumor progression remain controversial (10, 11).

The mechanism of FOXP3 tumor suppressor activity is still not fully understood. By gene expression array with chromatin immunoprecipitation sequencing (ChIP-seq), more than 800 candidate gene targets of FOXP3 have been identified in cancer cells (12). FOXP3 inactivation leads to overexpression of HER2/Erbb2 and SKP2 and repression of CDKN1A and LATS2 in breast cancer samples (3–5, 13). Notably, FOXP3 can directly target the c-MYC promoter to inhibit its transcription in prostate epithelial cells (3). These FOXP3 target genes are the major contributors to the inhibition of cell proliferation during tumor initiation (2–5, 13), suggesting that FOXP3 regulates multiple target genes and their signaling pathways to achieve tumor suppression. In addition to inhibition of cell proliferation, upregulation of FOXP3 can induce apoptosis of cancer cells and reduce the growth rate in vitro and in vivo (3, 9, 14–16). However, the molecular contributors and their mechanisms of mediating FOXP3-induced apoptosis remain largely unknown.

MicroRNAs (miR) identified as regulated by FOXP3 in cancer cells include miR-7 (17), miR-155 (17), and miR-183 (18). However, an overall assessment of miRs directly targeted by FOXP3 in cancer cells remains undescribed. Recently, we identified a series of FOXP3-target miRs in breast cancer cells (unpublished data). Interestingly, FOXP3 significantly increases the expression levels of miR-146a and -146b (miR-146a/b) in breast cancer cells. Human miR-146a/b have lengths of 22 nt and 91% homology, and many of the predicted target genes are common to both miR-146a/b. Accumulating data suggest that miR-146a/b inhibit cancer cell proliferation, invasion, and metastasis in...
human cancers (19–22), including prostate cancer. Furthermore, genetic studies have indicated a strong association between an miR-146a genetic variant and overall cancer risk, suggesting a potential role of miR-146a in susceptibility to human cancers (23, 24). In addition, NF-kB dysregulation in miR-146a-deficient mice drives the development of myeloid and lymphoid malignancies at a high rate (25, 26).

In prostate cancer, low expression of miR-146a/b was observed in androgen-independent cancer cell lines (21, 27, 28). Although miR-146a/b are highly expressed in normal prostate tissue, in situ hybridization analysis indicated that the levels of miR-146a/b are significantly downmodulated in prostate cancer tissues (21). Notably, DNA methylation near the FOXP3 and NF-kB binding sites in the miR-146a promoter is significantly reduced by treatment with 5-aza-2’-deoxycytidine, leading to increased miR-146a expression and subsequent tumor inhibition and apoptosis in prostate cancer cells in vitro and in vivo (28). Furthermore, transfection of miR-146a into prostate cancer cells resulted in a marked reduction of cell proliferation, invasion, and metastasis to bone marrow (21, 27). Thus, miR-146a likely functions as a tumor suppressor in prostate cancer cells. Several target genes of miR-146a/b have been identified but require validation. It is well established that miR-146a negatively regulates NF-kB activation by inhibiting expression of IL1 receptor-associated kinase-1 (IRAK1) and TNF receptor-associated factor-6 (TRAF6); however, NF-kB can transcriptionally induce miR-146a, suggesting an miR-146a/NF-kB negative feedback regulation loop (19, 29). Given the critical roles for miR-146a/b in cancer biology (25, 26), we conducted the present study to test whether miR-146a/b are regulated by FOXP3 and to determine the roles of these miRs in prostate cancer cells, with the goal of developing a new therapeutic approach for prostate cancers with FOXP3 defects.

Materials and Methods

Cell lines, antibodies, and reagents

Prostate cancer cell lines PC3, DU145, and LNCaP were obtained from ATCC. Cell lines were authenticated by examination of morphology and growth characteristics and confirmed to be mycoplasma-free. Cells were maintained in DMEM supplemented with 10% FBS (Life Technologies) and cultured for less than 6 months. For Western blotting, the specific primary antibodies indicated were used to detect the following proteins: FOXP3 (ab450, Abcam; hFoxy, ebioscience), NF-kB p65 (D14E12, Cell Signaling), IRAK1 (D51G7, Cell Signalling; H-273, Santa Cruz Biotechnology), and TRAF6 (D21G3, Cell Signaling; H-274, Santa Cruz Biotechnology). For immunohistochemistry (IHC), the indicated specific antibodies were used to detect the following mouse proteins: FOXP3 (Poly2638b; BioLegend), NF-kB p65 (D14E12), IRAK1 (H-273), and TRAF6 (H-274). Scramble control or miR-146a/b inhibitors (Life Technologies) were transfected into PC3 and DU145 cells. lipopolysaccharide (LPS; O111B4; Sigma) and bortezomib (LC Technologies) was used to convert miR to cDNA. The expression levels of miR-146a/b were assessed using the TaqMan MicroRNA Assay (Life Technologies) with the Applied Biosystems 7500 Real-Time PCR System in accordance with the manufacturers’ protocols. Human miR-146a/b and mouse miR-146a TaqMan primers and probes were purchased from Life Technologies. The average relative expression level was determined using the comparative method \(2^{-\Delta\Delta C_t}\) against the endogenous RNU6B (for human) or snoRNA202 (for mouse) controls.

Quantitative real-time PCR

Relative levels of mRNA expression were analyzed by real-time PCR using the Applied Biosystems 7500 Real-Time PCR System, in accordance with the manufacturer’s protocol, and SYBR Green I dye (Life Technologies). The relative expression level was determined using the comparative method \(2^{-\Delta\Delta C_t}\) against the endogenous GAPDH (for human) or Hprt (for mouse) controls. The primer sequences are listed in Supplementary Table S1.

Western blot

Western blotting was performed as previously described (3–5). For nuclear proteins, the cells were first incubated in buffer A [10 mmol/L HEPES (pH 7.8), 10 mmol/L KCl, 2 mmol/L MgCl2, 0.1 mmol/L EDTA, 1% NP40, and protease inhibitors] and the pellet was suspended in buffer B [50 mmol/L HEPES (pH 7.8), 300 mmol/L NaCl, 50 mmol/L KCl, 0.1 mmol/L EDTA, 10% (v/v) glycerol, and protease inhibitors].

NF-kB activation

NF-kB activation was determined by expression of NF-kB and its target genes. The expression levels of NF-kB target genes Bcl2L1

mice are in the C57BL/6 background. All mice were bred at the animal facilities of the Animal Resources Program at the University of Alabama at Birmingham (UAB; Birmingham, AL). The male F1 mice of given genotypes were used in the study. The pathologic evaluation was performed according to the Bar Harbor meeting guideline (30). All animal experiments were conducted in accordance with accepted standards of animal care and approved by the Institutional Animal Care and Use Committee of UAB.

Xenogenic transplantation

After thawing from liquid nitrogen storage, DU145 cells stably transfected with the empty pEF1 vector or pEF1-FOXP3 (2) were expanded in the presence of antibiotics and used for inoculation within 3 to 4 passages. Before inoculation, cells were cultured in DMEM supplemented with 10% FBS without antibiotics. Subcutaneous injections of cells (5 \(\times\) 10^6 cells; 100 \(\mu\)L) were administered in the left flanks of 8-week-old male NSG mice. Tumor size was measured in the 2 longest dimensions using a Vernier caliper. Tumor volume (V) was calculated with the formula \(V = D_1(D_2)^2/2\), where \(D_1\) is the long dimension and \(D_2\) is the short dimension.

Laser capture microdissection

Laser capture microdissection (LCM) was performed as described previously (2). Five thousand target cells were microdissected from target tissues for gene expression analysis.

TaqMan miR assay

The TaqMan MicroRNA Reverse Transcription Kit (Life Technologies) was used to convert miR to cDNA. The expression levels of miR-146a/b were assessed using the TaqMan MicroRNA Assay (Life Technologies) with the Applied Biosystems 7500 Real-Time PCR System in accordance with the manufacturers’ protocols. Human miR-146a/b and mouse miR-146a TaqMan primers and probes were purchased from Life Technologies. The average relative expression level was determined using the comparative method \(2^{-\Delta\Delta C_t}\) against the endogenous RNU6B (for human) or snoRNA202 (for mouse) controls.
and Traf1/2 were determined by qPCR. At the same time, activation of NF-κB was also determined by Western blot analysis of the accumulated nuclear p65 subunit, which is commonly used as a distinct marker for NF-κB activation (31). In addition, LPS treatment in mice enhances NF-κB signaling in cells, leading to increases in p65 loading (32). Thus, our analysis of NF-κB activation in mice was performed at 12 hours after intraperitoneal injection of LPS (100 μL of a 3 mg/ml solution per mouse).

**Immunohistochemistry**

The Vectastain Elite ABC Kit (Vector Laboratories) was used for immunostaining according to the manufacturer’s protocol as described previously (2).

**Electrophoretic mobility shift assay**

Mice were injected with 2 × LPS at 12 and 0.5 hours before sacrifice. Nuclear extracts from mouse prostate were prepared as described previously (33). Supershift analysis was performed using the Chemiluminescent EMSA Kit (Pierce Biotechnology) according to the manufacturer’s protocol. The probe sequences are listed in Supplementary Table S1.

**TUNEL analysis for detection of apoptosis**

Prostate tissues were fixed overnight in 4% paraformaldehyde, paraffin-embedded, and sectioned for TUNEL staining, which was performed using the Fluorescein In Situ Cell Death Detection Kit (Roche) according to the manufacturer’s protocol. The apoptotic index (%) was calculated as the number of TUNEL-positive cells divided by the total number of cells.

**MRI of prostate**

Prostate size was measured using MRI as described previously (2).

**Bortezomib treatment in Foxp3cKO mice**

To test the role of NF-κB on Foxp3-mediated tumor suppression, 100 μL of PBS or NF-κB inhibitor bortezomib (1 mg/kg) was injected into the Foxp3cKO mice by intraperitoneal injection every 4 days starting at 37 weeks of age (34). After 3 weeks (5 doses), prostate in the mice were scanned by MRI. After 45, 50, 55, and 60 weeks of age, 5 mice per group were sacrificed and their prostates were used for histologic analysis to assess PIN development. Tumor growth rates were compared by Fisher protected least significant difference (PLSD) test. All data were entered into an access database and analyzed using Excel 2010, StatView (version 5.0; SAS), and SPSS (version 20; IBM).

**Results**

**FOXP3 induces miR-146a/b expression in prostate cancer cells**

To determine whether miR-146a/b are induced by FOXP3 in prostate cancer cells, FOXP3 was transfected into PC3, DU145, and LNCaP cells, all of which normally express a low level of endogenous FOXP3 (2). MiR-146a/b expression increased dramatically at 48 hours after FOXP3 transfection in PC3 cells (~4- and 6-fold, respectively), DU145 cells (~7- and 16-fold, respectively), and LNCaP cells (~3- and 8-fold, respectively; Fig. 1A). Likewise, the expression levels of miR-146a/b target genes IRAK1 and TRAF6 were repressed at 48 hours after the induction of miR-146a/b mediated by transfection with FOXP3 but rescued by co-transfection with miR-146a/b inhibitors (Fig. 1B). In immunodeficient NSG mice subcutaneously injected with DU145 cells, the growth rate of FOXP3+/−DU145 tumors was substantially less than that of FOXP3−/−DU145 tumors (Fig. 1C). This is substantiated by a significantly lower terminal weight for FOXP3−/− tumors compared with FOXP3+/+ tumors (Fig. 1D). Consistent with the in vitro observations, higher miR-146a/b levels were also observed in microdissected FOXP3−/− tumor cells than in microdissected FOXP3+/+ tumor cells (Fig. 1E).

**Identification of a FOXP3–miR-146–NF-κB axis in vivo**

To determine whether endogenous FOXP3 regulates miR-146a/b, we analyzed the expression of miR-146a in mice with or without the prostate-specific Foxp3cKO (miR-146a is the only miR-146 in mouse and is highly homologous to human miR-146a/b). For the generation of Foxp3cKO mice, we crossed mice carrying the floxed Foxp3 allele (35) to a transgenic line that expresses PB-Cre(4). We observed more than 80% deletion of the Foxp3 locus with reduction of the Foxp3 mRNA and protein in the dorsal, lateral, and ventral prostate lobes in microdissected prostate epithelial cells in 12-week-old Foxp3cKO mice (2). We removed the anterior lobes from total prostate tissues to avoid complications associated with the incomplete Foxp3 deletion in this lobe (2, 36). We measured the expression level of miR-146a in both PB-Cre+/− and Foxp3cKO mice at 12 (pre-hyperplasia), 24 (hyperplasia), and 43 (PIN) weeks of age (2). MiR-146a was significantly reduced in microdissected prostate epithelial cells of the Foxp3cKO mice compared with those of PB-Cre+/− control mice (2.7-fold at 12 weeks of age and 3.5-fold at both 24 and 43 weeks of age, Fig. 2A), suggesting a downregulation of miR-146a by Foxp3cKO in mouse prostate in vivo.

We then tested the effects of prostate-specific deletion of Foxp3 on the expression of Ira1 and Traf6 and subsequent NF-κB activation. Our analysis of NF-κB activation was performed at 12 hours after intraperitoneal injection of LPS in the mice at 43 weeks of age. Both mRNA and protein levels of Ira1 and Traf6 were significantly increased in the Foxp3cKO prostate compared with PB-Cre+/− control prostate (Fig. 2B and C). IHC analysis revealed obvious increases of IRAK1, TRAF6, and nuclear p65 in the Foxp3cKO prostate compared with PB-Cre+/− control prostates (Fig. 2D), suggesting a FOXP3–miR-146a/NF-κB axis in the Foxp3cKO prostate. Furthermore, increased expression levels of 2 NF-κB target genes, Bcl2l1 and Traf1/2, were also observed in the
miR-146a/b expression levels as a percentage of the mean and SD of the tumor volumes and the experiments were repeated 2 times. D, weights of tumors at day 27 after injection. E, quantification of miR-146a/b expression as a percentage of the mean and SD of triplicates and the experiments were repeated 3 times. B, quantification of IRAK1 and TRAF6 mRNA expression as a percentage of GAPDH expression in PC3, DU145, and LNCaP cells at 48 hours after cotransfection with FOX3 and scramble miR or miR-146a/b inhibitors. Data are presented as the mean and SD of triplicates and the experiments were repeated 3 times. C, tumor growth in NSG mice subcutaneously injected with DU145 cells stably transfected with vector or FOX3. Data are presented as the mean and SD of the tumor volumes and the experiments were repeated 2 times. D, weights of tumors at day 27 after injection. E, quantification of miR-146a/b expression levels as a percentage of RNU6B expression in the microdissected tumor cells from xenotransplanted NSG mice at day 27 after injection. *, P < 0.05, 2-tailed t test.

Foxp3Cre+/− control prostate and those of the Bortezomib-treated group were sacrificed at the ages of 45, 50, 55, and 60 weeks. Histologic analysis of prostate revealed PIN development after 45 weeks of age in the untreated mice but a more than 60% reduction of PIN incidence in the mice treated with bortezomib (Figs. 3C and 4A), although miR-146a/miR-146b expression was not changed by this treatment (Fig. 3D). Of the 15 bortezomib-treated mice, 5 developed PIN, but their PIN lesions were limited to the ventral prostates. Reduced cell proliferation was also observed in prostate epithelial cells of bortezomib-treated mice by Ki67 staining analysis (Fig. 4A). Furthermore, lack of nuclear p65 was significantly reduced in bortezomib-treated prostate cancer cells (Fig. 2F).

**Targeting FOXP3–miR-146–NF-κB Axis for Cancer Therapy**

Figure 1. FOXP3–miR-146–NF-κB axis in prostate cancer cells. A, PC3, DU145, and LNCaP cells were transfected with a pEF1 control vector or pEF1/FOX3 vector for 48 hours. Top, representative Western blot analyses showing FOX3 expression at 24 and 48 hours after transfection. Bottom graphs, quantification of miR-146a/b expression as a percentage of GAPDH expression in PC3, DU145, and LNCaP cells at 48 hours after cotransfection with FOX3 and scrambled miR or miR-146a/b inhibitors. Data are presented as the mean and SD of triplicates and the experiments were repeated 3 times. C, tumor growth in NSG mice subcutaneously injected with DU145 cells stably transfected with vector or FOX3. Data are presented as the mean and SD of the tumor volumes and the experiments were repeated 2 times. D, weights of tumors at day 27 after injection. E, quantification of miR-146a/b expression levels as a percentage of RNU6B expression in the microdissected tumor cells from xenotransplanted NSG mice at day 27 after injection. *, P < 0.05, 2-tailed t test.

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observed in the bortezomib–treated prostate at 12 hours after LPS injection (Fig. 4B). To verify the impact of bortezomib treatment on NF-κB activation, we observed the expression of NF-κB targets in the mouse prostate. In contrast to the prostate-specific expression in untreated Foxp3Cre+ mice, the expression levels of Bcl2l1 and Traf1/2 were significantly downregulated in the prostates of treated mice (Fig. 4C). Importantly, the percentage of apoptotic prostate epithelial cells increased after bortezomib treatment (Fig. 4D). In addition, this in vivo observation was supported by results of in vitro transfections with FOXP3 or NF-κB p65-siRNA (Supplementary Table S1) or treatment with the NF-κB inhibitor bortezomib in DU145 cells. As shown in Supplementary Fig. S1, compared with empty vector–transfected cells, cell proliferation and nuclear p65 were observably reduced by NF-κB p65-siRNAs, bortezomib treatment, or FOXP3 expression. No significant differences in cell proliferation were detected between the treatments, suggesting that bortezomib is an effective approach for tumor suppression in prostate cancer cells with FOXP3 defect.

Deletion of miR-146a causes precancerous lesions in mouse prostate

To observe the prostates in miR-146a−/− mice by histologic analysis, we sacrificed 5 miR-146a−/− and 5 wild-type (WT)
mice at 3, 6, 9, 12, and 15 months of age. As shown in Fig. 5A and B, miR-146a−/− mice displayed signs of prostate hyperplasia as early as 9 months of age (9 months: 2 of 5 mice; 12 months: 3 of 5 mice; 15 months: 4 of 5 mice). Early PIN was detectable at 12 months (1 of 5 mice) in a small fraction of the anterior prostate lobes characterized by stratification of atypical epithelial cells with focal cribriform growth pattern (Fig. 5C). The epithelial cells in this region had significantly enlarged nuclei with occasional prominent nucleoli in comparison with the nearby single-layered epithelial cells in the same glandular structure (Fig. 5D). IHC analysis revealed the atypical epithelial cells with a focal cribriform growth pattern (Fig. 5C). The cytoplasmic localization of FOXP3 is associated with the loss of tumor infiltration, such as lymphocyte infiltration and reactive epithelial proliferation or atypia (Fig. 5A and C).

Validation of a FOXP3–miR-146–NF-κB axis in human prostate cancer

Nuclear FOXP3 is expressed in normal prostate epithelial cells but is lost in prostate cancer cells in 70% of human prostate cancer samples (2). In cancer cells that do express FOXP3, it localizes predominately to the cytoplasm. In contrast, FOXP3 localizes predominately to the nuclei of normal cells (2–5). The cytoplasmic localization of FOXP3 is associated with the loss of tumor inhibition (11); thus, we used IHC analysis with a specific antibody against human FOXP3 to divide prostate cancer samples into two groups, one group with 10 nuclear FOXP3+ samples and one group with 12 FOXP3− samples (Fig. 7A). To precisely determine the expression levels of miR-146a/b and their target genes IRAK1 and TRAF6, we isolated the prostate cancer cells from tumor tissues by LCM. To determine whether downregulation of nuclear FOXP3 contributes to miR-146/NF-κB signaling in prostate cancer, we analyzed the expression of miR-146a/b, IRAK1, and TRAF6 in microdissected cancer cells. As shown in Fig. 7B, expression levels of miR-146a/b are significantly higher in nuclear FOXP3+ cancer cells (~2.0- and 1.7-fold, respectively) than in FOXP3− cancer cells. Because IRAK1 and TRAF6 can lead to activation of NF-κB signaling (19, 29), we analyzed the mRNA levels of IRAK1 and TRAF6 in the 22 microdissected tumor
Discussion

The mechanism by which FOXP3 functions as a tumor suppressor is not fully characterized, although several genes and pathways have been identified as potential FOXP3 targets. An miR-146/NF-κB negative feedback regulation loop has been suggested (19, 29), and we have now identified a functional FOXP3–miR-146–NF-κB axis during tumor initiation in prostate cancer cells. Notably, the function of this axis was validated in Foxp3cKO mice. MiR-146a−/− mice, as well as Foxp3cKO mice, developed PIN but not prostate cancer, suggesting that miR-146a has a tumor-suppressive role during tumor initiation.

Previous studies have demonstrated that deletion of miR-146a in C57BL/6 mice leads to histologically and immunophenotypically defined myeloid sarcomas (40%) and lymphomas (20%) in 18- to 22-month-old mice (25, 26). Indeed, we found the expression of the antiapoptotic factors, Bcl2l1, reduced by miR-146a expression in vivo. Bcl2l1 encodes a well-characterized apoptotic inhibitor that can help cells evade autophagic cell death (42). Interestingly, Bcl2l1 is likely regulated by p65 (43, 44). In fact, our data showed that nuclear p65 increased in Foxp3cKO samples. In contrast to miR-146a/b expression levels, mRNA expression of IRAK1 and TRAF6 was significantly lower in nuclear FOXP3+ cancer cells (~2.9- and 2.8-fold downregulation, respectively) than in FOXP3− cancer cells (Fig. 7C). Therefore, FOXP3 defects are likely a major determinant of miR-146a/b levels and their target signaling in human prostate cancer.
prostates, suggesting a p65-dependent manner of FOXP3/miR-146–induced apoptosis. Likewise, NF-κB targeting genes Traf1/2, which can suppress caspase-8 activation to inhibit apoptosis (45), were also transcriptionally upregulated in both Foxp3cKO and miR-146a–/– prostates. As supportive evidence, the NF-κB inhibitor bortezomib induced apoptosis in prostate epithelial cells of Foxp3cKO mice by transcriptional downregulation of Bcl2l1 and Traf1/2, suggesting that NF-κB–related apoptosis is involved in FOXP3–mediated tumor suppression. In a parallel study published in this issue, we report that miR-146a–induced apoptosis also contributes to FOXP3–mediated tumor suppression in breast cancer cells (unpublished data). In addition, we identified the mechanism underlying regulation of miR-146a by FOXP3 in both normal breast epithelial cells and breast cancer cells. FOXP3 can directly bind to the proximal promoter region of miR-146a where it functionally interacts with NF-κB to induce miR-146a transcriptional activity, suggesting a relationship between FOXP3 and NF-κB in breast cancer cells and prostate cancer cells. Given the pathologic relevance of the miR-146–NF-κB axis in a variety of human solid tumors (19, 20, 46, 47), the apoptosis induced by the FOXP3–miR-146–NF-κB axis may be important during tumor initiation, which advances our understanding of FOXP3–mediated tumor suppression.

The FOXP3–miR-146–NF-κB axis is a potential therapeutic target for cancers with FOXP3 defects. Although PIN formation was not completely eliminated by bortezomib, this treatment inhibited mouse prostate growth and reduced PIN incidence, most likely as a result of the observed increase in apoptotic cells.
Notably, knockout of miR-146a in mice led to hyperplasia, and by 15 months of age, most miR-146a−/− mice had early PIN lesions. These results suggest that miR-146a is necessary to prevent early prostate cancer development, and our future studies will assess the effects of miR-146a-knockout on prostate cancer development in older mice.

Figure 6. Upregulation of IRAK1, TRAF6, and nuclear p65 and its target gene in miR-146a−/− mice. A, representative images of Western blot analyses showing the protein expression of IRAK1, TRAF6, p65, and nuclear p65 (N-p65) in mouse prostates at 12 hours after LPS injection. B and C, quantification of Irak1, Traf6, Bcl2l1, and Traf1/2 mRNA expression as a percentage of Hprt expression in microdissected mouse prostate epithelial cells measured by qPCR at 12 hours after LPS injection in miR-146a−/− and WT mice. Horizontal lines represent the average values. *P values were determined by 2-tailed t test. All experiments were repeated 2 times.

miR-146a−/−/WT, ratio of the expression in miR-146a−/− cells to the expression in WT cells.

Figure 7. Effect of FOXP3 defects on the expression of miR-146a/b, IRAK1, and TRAF6 mRNA in human prostate cancer samples. A, representative IHC analysis with a specific antibody against human FOXP3 used to identify nuclear FOXP3+ and FOXP3− primary prostate cancer samples. Red arrows, regulatory T cells with FOXP3 staining. B, quantification of miR-146a/b expression as a percentage of RNU6B expression in microdissected nuclear FOXP3+ cancer cells and FOXP3− cells. C, quantification of IRAK1 and TRAF6 mRNA expression as a percentage of GAPDH expression in microdissected nuclear FOXP3+ cancer cells and FOXP3− cells. All P values were calculated by 2-tailed t test. All experiments were repeated 2 times.
abundance of a notable inflammatory response in the prostates of miR-146a−/− mice suggest that miR-146a deletion promotes prostate tumorigenesis through NF-kB–related proliferation and apoptosis but not inflammation. Overall, these observations not only support a role for the FOXP3–miR-146–NF-kB axis during tumor initiation but also inform the development of a potential therapeutic approach for tumors with defective FOXP3. Because the efficiency of treatment with miRs or miR mimics has not been fully validated in vivo, bortezomib treatment, which is already approved by the FDA for use in humans, may provide a more immediate therapeutic approach for cancers with either FOXP3 or miR-146 defects.

In summary, FOXP3 induces miR-146a/b to inhibit NF-kB activation by repressing IκB and Traf6, leading to apoptosis during tumor initiation and tumor suppression in prostate epithelial cells. The identification of the FOXP3–miR-146–NF-kB axis provides a potential mechanism for disruption of miR-146 family member expression and constitutive NF-kB activation in prostate cancer cells and reveals a new therapeutic approach for cancers with FOXP3 defects.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
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References


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