FOXP3 Controls an miR-146/NF-κB Negative Feedback Loop That Inhibits Apoptosis in Breast Cancer Cells

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Abstract

FOXP3 functions not only as the master regulator in regulatory T cells, but also as an X-linked tumor suppressor. The tumor-suppressive activity of FOXP3 has been observed in tumor initiation, but its role during tumor progression remains controversial. Moreover, the mechanism of FOXP3-mediated tumor-suppressive activity remains largely unknown. Using chromatin immunoprecipitation (ChIP) sequencing, we identified a series of potential FOXP3-targeted miRNAs in MCF7 cells. Notably, FOXP3 significantly induced the expression of miR-146a/b. In vitro, FOXP3-induced miR-146a/b prevented tumor cell proliferation and enhanced apoptosis. Functional analyses in vitro and in vivo revealed that FOXP3-induced miR-146a/b negatively regulates NF-κB activation by inhibiting the expression of IRAK1 and TRAF6. In ChIP assays, FOXP3 directly bound the promoter region of miR-146a but not of miR-146b, and FOXP3 interacted directly with NF-κB p65 to regulate an miR-146–NF-κB negative feedback regulation loop in normal breast epithelial and tumor cells, as demonstrated with luciferase reporter assays. Although FOXP3 significantly inhibited breast tumor growth and migration in vitro and metastasis in vivo, FOXP3-induced miR-146a/b contributed only to the inhibition of breast tumor growth. These data suggest that miR-146a/b contributes to FOXP3-mediated tumor suppression during tumor growth by triggering apoptosis. The identification of a FOXP3–miR-146–NF-κB axis provides an underlying mechanism for disruption of miR-146 family member expression and constitutive NF-κB activation in breast cancer cells. Linking the tumor suppressor function of FOXP3 to NF-κB activation reveals a potential therapeutic approach for cancers with FOXP3 defects. Cancer Res; 75(4): 1703–13. ©2015 AACR.

Introduction

miRNA-146a (miR-146a) is overexpressed in Foxp3+ Tregs and is critical for Treg function in the immune system (1), suggesting a link between FOXP3 and miR-146a. The miR-146 family includes miR-146a/b in humans, but only miR-146a in mice. Many of the predicted target genes are common to both miR-146a/b, but each miR may have a different posttranscriptional processing mechanism due to genomic location (miR-146a/b on human chromosome 21). miR-146 family members are highly conserved among mammals and are involved in cell proliferation, differentiation, and migration of cancer cells, including EGFR (9, 10), CXCR4 (11), NOTCH1 (12), ROCK1 (13), PRKCE (14), and others, but these targets require further validation. In addition, the regulatory mechanisms controlling miR-146/145 are largely unknown. NF-κB, breast cancer metastasis suppressor 1 (4), p53-binding protein-1 (15), and tumor necrosis factor-related apoptosis-inducing ligand (16) were identified as transactivators of miR-146a/b in breast cancer cells. These proteins induce miR-146a to suppress either NF-κB-dependent tumor growth or chemokine (C-X-C motif) receptor

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4-mediated tumor metastasis in breast cancer cells. However, the mechanism through which miR-146a controls tumor development and/or metastasis remains debated. Given the critical roles for miR-146a/b and FOXP3 in cancer biology (6, 7, 16–18), we tested whether miR-146a/b is involved in FOXP3-mediated tumor suppression in breast cancer cells.

Materials and Methods

Cell lines, antibodies, DNA constructs, and reagents
Breast cancer cell lines MCF7, T47D, BT474, MDA-MB-468, and MDA-MB-231 and the preneoplastic breast epithelial cell line MCF10A were obtained from the 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. GFP- and FOXP3-Tet-off MCF7 cells were established and maintained in 1 μg/mL doxycycline (Dox) as described previously (16, 17, 19). Specific primary antibodies were used to detect the following proteins: FOXP3 (ab450, Abcam; Poly2638b, BioLegend), NF-κB p65 (D14E12, Cell Signaling Technology), IRAK1 (D51G7, Cell Signaling Technology; H-273, Santa Cruz Biotechnology), TRAF6 (D21G3, Cell Signaling Technology; H-274, Santa Cruz Biotechnology), EGFR (D38B1, Cell Signaling Technology), and Bcl2 (B1, Santa Cruz Biotechnology), ERK1/2 (H-72, Santa Cruz Biotechnology), and iNOS (B p65 (D14E12, Cell Signaling Technology), NF-κB activation, and immunoprecipitation
Western blot analysis, quantitative ChIP, and coimmunoprecipitation
Western blotting and chromatin immunoprecipitation (ChIP) were performed as described previously (16–18). For coimmunoprecipitation, collected cells were washed with cold PBS and lysed in ice-cold buffer [20 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1 mmol/L EDTA, and 1% NP-40] supplemented with complete protease inhibitors (Sigma) on ice for 10 minutes. Lysates were aliquoted into two tubes and incubated with the designated antibody or an appropriate IgG control for 16 hours at 4°C. Protein A/G agarose (Invitrogen) was used to precipitate antibody-protein complexes.

TaqMan miR assay
Expression levels of miR-146a/b were assessed using TaqMan MicroRNA Assay (Life Technologies). Human miR-146a/b and mouse miR-146a TaqMan primers and probes were purchased from Life Technologies. The average relative expression was determined using the comparative method (2^ΔCt) against the endogenous RNU6B (for human) or snoRNA202 (for mouse) controls.

Cell proliferation and apoptosis assays
Cell morphology, viability, and number of GFP- and FOXP3-Tet-off MCF7 cells were monitored at 0, 3, 5, 7, 10, and 14 days without doxycycline using a microscope and flow-cytometric assays based on cell binding to Annexin V (561012, BD Biosciences) and 7-AAD (7-AAD; 555816, BD Biosciences). Because miR-146a/b inhibitors were effective for at least 4 days as tested (Supplementary Fig. S1), transfection with miR-146a/b inhibitors was repeated every 4 days during cell proliferation.

qRT-PCR
Relative mRNA expression levels were determined using the comparative method (2^ΔCt) against endogenous GAPDH (for human) or Hprt (for mouse) controls. Primer sequences are listed in Supplementary Table S2.
In vivo tumor metastasis assay

A total of 1 × 10^6 control MDA-MB-231 cells or MDA-MB-231 cells stably expressing exogenous FOXP3 were implanted intravenously into 8-week-old female NSG mice. At 7 weeks after implantation, the mice were euthanized for histologic examination and miR analysis. The number of surface lesions over all lobes of the liver and lungs was scored before pathologic analysis. Tumor burden in the liver and lungs was quantified in two-step sections from each lobe (liver, left and right lobes; lung, left two lobes and right three lobes) in a blinded fashion by calculating the area of tumor tissue as a percentage of the total tissue area (27).

Human tissue specimens

Tumor specimens were collected from 20 patients who underwent primary surgery between January 2012 and June 2014. All patients had histologically confirmed breast cancer and had not received hormone or radiation therapy. Twenty breast cancer tissues were used for this study.

Statistical analysis

We compared the means of the variable using a two-tailed t-test or a Mann–Whitney test between two groups and Fisher protected least significant difference (PLSD) test among multiple groups. All data were entered into an access database using Excel 2010 and analyzed with SPSS (version 20; IBM) and StatView (version 5.0.1; SAS Institute Inc.).

Results

Regulation of miR-146a/b by FOXP3 contributes to tumor suppression through apoptosis in breast cancer cells

There are two subtypes of miRs. Intragenic miRs located within exons of host genes can be cotranscribed with their host genes by the same promoters (28–30), but transcriptional regulation of intergenic miRs is poorly understood because miR promoters are poorly characterized (28–30). However, the −2.0 kb region from 5′ pre-miR transcripts is considered the most likely promoter locus. Thus, we screened the FOXP3-binding peaks near the 5′ locus. Thus, we screened the FOXP3-binding peaks near the 5′/0 kb to 0 kb) of intragenic miRs or near transcriptional start sites (−2.0 kb to 0 kb) of intragenic miR host genes. ChIP-seq analysis identified 43 candidate FOXP3-target miRs in the FOXP3-Tet-off MCF7 cells (Supplementary Table S1). To test whether these miRs are regulated by FOXP3, we conducted TaqMan miR assays at 2 and 4 days after FOXP3 induction. FOXP3 expression increased dramatically 2 days after doxycycline removal (16, 17, 19) and was observed predominately in cell nuclei (Supplementary Fig. S2). As shown in Supplementary Table S1, a cutoff of at least a 1.5-fold change in miR expression after FOXP3 induction and no significant change (≤1.5-fold) in the GFP-Tet-off MCF7 control cells was applied to identify potential FOXP3-target miRs.

FOXP3 induction by doxycycline removal significantly increased the expression levels of miR-146a (2.1-fold at 2 days, 2.8-fold at 4 days) in FOXP3-Tet-off MCF7 (ER−) cells but not in GFP-Tet-off MCF7 control cells (Fig. 1A and Supplementary Table S1). MCF7 cells express low levels of endogenous miR-146a under basal conditions (4, 5, 31). FOXP3 induction led to even greater induction of miR-146b (4.4-fold at 2 days, 8.0-fold at 4 days) in FOXP3-Tet-off MCF7 cells (Fig. 1A). These observations were validated in FOXP3-transfected T47D (ER+) and BT474 (ER−) cells and MDA-MB-468 (ER+) cells (2.6- to 6.5-fold miR-146a induction, 3.0- to 4.6-fold miR-146b induction; Fig. 1B), which also express low levels of endogenous miR-146a/b (4, 5). We then observed the effects of inhibiting miR-146a/b on cell morphology, viability, and proliferation in FOXP3-Tet-off MCF7 cells during FOXP3 induction. Transfection with miR-146a/b inhibitors, individually or combined, partially mitigated the decrease in cell proliferation mediated by FOXP3 induction (Fig. 1C). Notably, FOXP3 induction promotes apoptosis in MCF7 cells (16), and miR-146a/b inhibitors also dramatically reduced the apoptosis observed upon FOXP3 induction for 7 days (Fig. 1D).

FOXP3-miR-146/NF-kB axis in breast epithelial cells in vitro

Because miR-146a/b represses NF-kB through a negative feedback loop involving downregulation of IRAK1 and TRAF6 (2, 3), we tested whether FOXP3-induced miR-146a/b represses NF-kB activation in breast cancer cells. In FOXP3-Tet-off MCF7 cells, the expression levels of IRAK1 and TRAF6 decreased 3 days after FOXP3 induction, as did the level of nuclear p65 (Fig. 2A and B). Treatment with inhibitors of either miR-146a or miR-146b significantly blocked this repression (Fig. 2A and B). Furthermore, we analyzed FOXP3 and miR-146a/b transcriptional regulation of NF-kB target genes selected from the potential FOXP3-regulated genes identified in our previous study (19). From day 1 to day 4 after FOXP3 induction, CDKN1A expression increased, similar to our previous results (16, 19), whereas CXC4R1 and MMP9 expression decreased. MiR-146a/b inhibitors did not interfere with the expression of these genes (Fig. 2C). The expression levels of BCL2L1 and TRAF1/2 were significantly downregulated from day 2 to day 5 after FOXP3 induction, but expression of these genes was rescued by miR-146a/b inhibitors (Fig. 2C). Because the cells undergo apoptosis after FOXP3 induction, all genes were downregulated from day 5 onward after doxycycline removal in FOXP3-Tet-off MCF7 cells, but not in GFP-Tet-off MCF7 cells (Figs. 1D and 2C). The expression levels of other antiapoptotic genes, including BCL2, BCL10, and BIRC2/3, in FOXP3-Tet-off MCF7 cells did not change in response to miR-146a/b inhibition during FOXP3 induction (Fig. 2C and Supplementary Fig. S3). EGR3 is a potential target of miR-146a/b (9, 10), but the expression of this gene and its downstream target ERK1/2 did not change after FOXP3 induction or treatment with miR-146a/b inhibitors (Fig. 2A and B).

To validate the endogenous FOXP3-miR-146a/b–NF-kB axis in breast epithelial cells, we tested the effect of the FOXP3 shRNAs on miR-146a/b expression in the normal breast epithelial cell line MCF10A. FOXP3 shRNAs caused a substantial reduction in the expression of miR-146a/b (Fig. 3A). Correspondingly, IRAK1 and TRAF6 transcripts were significantly elevated by FOXP3 shRNAs, and this increase was suppressed by cotransfection of miR-146a/b mimics (Fig. 3B). Similarly, IRAK1 and TRAF6 protein expression increased after FOXP3 inhibition, before or after stimulation with TNFα to activate NF-kB (Fig. 3C). Furthermore, expression of miR-146a/b was dramatically induced by TNFα at 8 hours, especially in FOXP3-expressing cells (Fig. 3C and D). Although nuclear p65 levels did not increase after FOXP3 silencing, p65 was present in nuclei for up to 8 hours after treatment with TNFα (Fig. 3C). In addition, the mRNA and protein expression levels of
NF-κB-target genes BCL2L1 and TRAF1/2 were significantly elevated after FOXP3 silencing when cells were stimulated with TNFα (Fig. 3E and F).

**FOXP3–miR-146–NF-κB axis in breast epithelial cells in vivo**

To validate the FOXP3–miR-146–NF-κB axis in breast epithelial cells *in vivo*, we created breast-specific Foxp3cKO mice (Fig. 4A). We observed approximately 90% deletion of the Foxp3 locus (Fig. 4B) and >90% reduction of Foxp3 mRNA in microdissected breast epithelial cells of 12-week-old nongestational Foxp3cKO mice compared with MMTV-Cre control mice (Fig. 4C). Reduced expression of FOXP3 protein was confirmed by IHC (Fig. 4E). This deletion also caused a 2-fold decrease in miR-146a expression in the microdissected breast epithelial cells of both 12-week-old nongestational and 20-week-old gestational mice (Fig. 4D).

To observe the effect of breast-specific Foxp3 deletion on NF-κB activation, we injected the mice with LPS 12 hours before sacrifice. Expression of IRAK1, TRAF6, and p65 substantially increased in the breast epithelial cells of Foxp3cKO mice with those of MMTV-Cre control mice (Fig. 4E). Microdissected breast epithelial cells from Foxp3cKO mice also had a >3-fold increase in the mRNA levels of *Irak1* and *Traf6* and NF-κB target genes Bcl2l1 and *Traf1/2* but no change in *RelA* mRNA levels (Fig. 4F).

**The mechanism of miR-146a/b induction by FOXP3 in breast cancer cells**

Using our previous ChIP-seq data (19), we found that FOXP3 directly binds to the promoter region of the human miR-146a host gene DQ658414 (Supplementary Fig. S4A). MiR-146a, located within exon 2 of DQ658414 (Fig. 5A), shares the promoter of this gene and is thus transcriptionally expressed with it (2). Interestingly, the FOXP3-binding sites are adjacent to NF-κB-binding elements 1 and 2 (Fig. 5C; ref. 2). To confirm the FOXP3-binding sites, we performed a ChIP assay with qPCR analysis (16–18). FOXP3 bound the proximal promoter region...
of the miR-146a host gene in MCF7 cells (Fig. 5B). However, no FOXP3-binding signal was identified in the intergenic miR-146b locus (~20 kb) + 10 kb; Supplementary Fig. S4B). Although an miR promoter locus can occasionally be far more than 10 kb from the 5’ pre-miR transcript (28, 29), no significant FOXP3-binding sites were identified between ~17 kb and +1 kb from the 5’ pre-miR-146b transcript (Supplementary Fig. S5).

We continued to verify the regulation of miR-146a by direct binding of FOXP3 to its promoter using a dual-luciferase reporter assay in MCF7 cells (17, 18) and found that transfection of FOXP3 significantly induced the transcriptional activity of the miR-146a promoter (Fig. 5C). We have theoretically predicted the potential forkhead-binding motifs (RYMAAYA) in the miR-146a promoter region (Fig. 5C and D; ref. 19). Sequence alignment analysis revealed two conserved regions in the miR-146a proximal promoter that contain two forkhead-binding motifs surrounding the highest ChIP signal (Fig. 5B) and two NF-κB-binding sites (Fig. 5C and D). Deletion of either forkhead-binding motif abrogated miR-146a promoter activity in the luciferase assay (Fig. 5C). To test whether FOXP3 interacts with NF-κB to regulate the transcriptional activity of the miR-146a promoter, we analyzed luciferase expression after stimulation with TNFα in MCF7 and T47D cells. TNFα significantly increased the promoter activity of miR-146a in the absence of exogenous FOXP3 and dramatically enhanced the FOXP3-mediated induction of promoter activity. This induction was significantly blocked by treatment with the NF-κB inhibitor Bay 11-7082 (Fig. 5E). In addition, FOXP3 silencing significantly reduced the TNFα-induced promoter activity of miR-146a in MCF10A cells (Fig. 5E). The specific binding of FOXP3 to two forkhead-binding motifs in the miR-146a promoter region was also validated by a gel-shift assay using either recombinant FOXP3 or nuclear extract from FOXP3-induced MCF7 cells (Fig. 5F and Supplementary Fig. S6). Furthermore, a direct interaction of FOXP3 with NF-κB p65 was observed after stimulation of FOXP3-Tet-off MCF7 cells with TNFα and nuclear extract from FOXP3-induced MCF7 cells (Fig. 5F, and Supplementary Fig. S6). This interaction was attenuated by exogenous miR-146a/b mimics (Fig. 5G).

FOXP3-induced miR-146a/b inhibits tumor growth but not tumor metastasis

We next investigated the effects of FOXP3-induced miR-146a/b on tumor cell migration, invasion, and metastasis. In in vitro scratch assays with FOXP3-Tet-off MCF7 cells, cell migration was inhibited by FOXP3 induction, but this inhibition was not reversed by miR-146a/b inhibitors (Fig. 6A). Similarly, cell
migration was significantly reduced after FOXP3 induction and was not rescued by miR-146a/b inhibitors (Fig. 6B).

MDA-MB-321 metastatic breast cancer cells express endogenous miR-146a/b (4, 5, 31) but only a low level of endogenous FOXP3 (18). Expression of exogenous FOXP3, which localized in nuclei, significantly increased the expression of miR-146a/b in these cells (Supplementary Fig. S7). To test the role of FOXP3-induced miR-146a/b in tumor metastasis in vivo, we intravenously injected MDA-MB-231 wild-type (WT) cells or MDA-MB-231 cells stably expressing FOXP3 into NSG mice. At 7 weeks after injection, significant reductions in the number and size of lung and liver metastases were observed in the mice injected with FOXP3-overexpressing cells (Fig. 6C and D). Intravenous injection of miR-146a/b inhibitors beginning 3 weeks after implantation of FOXP3-overexpressing MDA-MB-231 cells (Fig. 6E) increased the size of lung and liver metastases substantially, but the number of metastases did not change (Fig. 6F and G). Significant increases of IRAK1 and TRAF6 transcripts in the tumors were also detected after treatment with miR-146a/b inhibitors (Supplementary Fig. S8).

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

We have demonstrated that nuclear FOXP3 is expressed in normal breast epithelial cells but is lost in 70% to 80% of breast cancer cells in human breast cancer samples (18). In cancer cells that do express FOXP3, it localizes predominately to the cytoplasm. In contrast, FOXP3 localizes predominately to the nuclei of normal cells (16–18, 20). The cytoplasmic localization is associated with the loss of tumor inhibition (32). Thus, we used IHC analysis with a specific human FOXP3 antibody to obtain two breast cancer sample groups, one group of eight nuclear FOXP3+ samples and one group of 12 FOXP3− samples (Fig. 7A). Expression levels of miR-146a/b are significantly higher in microdissected cells from nuclear FOXP3+ samples (approximately 2.3- and 2.2-fold, respectively) than in those from FOXP3− samples (Fig. 7B). Conversely, mRNA expression of IRAK1 and TRAF6 was significantly lower in microdissected nuclear FOXP3+ cancer cells (approximately 2.0- and 1.7-fold, respectively) than in FOXP3− cancer cells (Fig. 7C). Therefore, FOXP3 defects are likely a major
miR-146a/b in human breast cancer BT549 cells (33) and leukemia U937 cells (35), respectively, these miRs were not identified as potential FOXP3 targets in our assays.

Previous studies reported data consistent with the existence of an miR-146–NF-κB negative feedback regulation loop (2, 3). Our in vitro and in vivo analyses suggest that FOXP3-mediated induction of miR-146a/b results in downregulation of IRAK1 and TRAF6 and subsequently inhibits NF-κB activation, leading to tumor suppression in breast cancer cells. Furthermore, we identified two forkhead-binding motifs in the proximal promoter region of miR-146a that are required for transcriptional regulation of miR-146a by FOXP3. Interestingly, the forkhead-binding motifs are adjacent to NF-κB–binding sites (2), and our data show that FOXP3 interacts synergistically with NF-κB to induce transcriptional activity of miR-146a, even though FOXP3 and NF-κB have opposing functions in tumorigenesis. Recent studies found that FOXP3 and NF-κB colocalize in the nuclei of gastric cancer cells after TNFα stimulation, and FOXP3 interacts with NF-κB and represses its activation, suggesting FOXP3 as a negative determinant of miR-146a/b levels and their target signaling in human breast cancer.

**Discussion**

FOXP3-regulated miRs and their regulatory effects remain largely unexamined in Tregs and cancer cells. Here, our preliminary examination of potential FOXP3-targeted miRs in breast cancer cells identified a functional FOXP3–miR-146–NF-κB axis in breast epithelial cells. Specifically, miR-146a/b contributes at least in part to FOXP3-mediated suppression of tumor growth in breast cancer cells, although FOXP3 has an miR-146a/b–independent tumor-suppressive role during tumor metastasis. MiR146a/b is both substantially upregulated after FOXP3 induction in breast epithelial cells. However, our results indicate that only miR-146a is targeted directly by FOXP3 in MCF7 cells. Although miR-155 was reported to be induced by FOXP3 in Tregs and cancer cells (33, 34) and miR-7 and miR-183 were identified as potential FOXP3 targets in human breast cancer.
regulator of NF-xB activation (36, 37). Our data also validated a direct interaction between FOXP3 and NF-xB in MCF7 cells after stimulation with TNFα. The attenuation of this interaction by miR-146a/b mimics suggests a mechanism by which FOXP3 regulates the miR-146–NF-xB negative feedback loop in breast epithelial and cancer cells. Although miR-146b was also induced by FOXP3, we did not identify any significant forkhead-binding motifs in the potential promoter region of this miR. However, it remains possible that miR-146b is indirectly regulated by FOXP3 through an as yet undiscovered mechanism. Interestingly, direct targeting of miR-146b by STAT3 can induce miR-146b to inhibit NF-xB activation in breast cancer cells (38). Although STAT3 is not a FOXP3-target gene (19), FOXP3 acts as a cotranscriptional factor with STAT3 in tumor-induced Tregs (39), perhaps suggesting a FOXP3/STAT3–miR-146b–NF-xB axis in breast cancer cells.

Our data show that FOXP3-mediated inhibition of cell proliferation and tumor growth is partly blocked by miR-146a/b inhibitors, suggesting a contribution of miR-146a/b to FOXP3-
Figure 6. Effects of FOXP3 on tumor growth, migration, and metastasis. A, representative images of scratch assays with FOXP3-Tet-off MCF7 cells cultured with (FOXP3+) or without Dox (FOXP3−) for 24 hours and then transfected with 100 nmol/L scramble miR or miR-146a/b inhibitors (+). *P < 0.05, two-tailed t test. B, quantification of migratory cells in Transwell assays performed with FOXP3-Tet-off MCF7 cells cultured with (FOXP3+) or without Dox (FOXP3−) for 24 hours and then transfected with miR-146a/b inhibitors (P value by a two-tailed t test). Data are presented as the mean and SD of triplicate samples. All in vitro experiments were repeated three times. C and D, MDA-MB-231 cells stably expressing FOXP3 or a control vector were injected intravenously into immunodeficient NSG mice at 8 weeks of age. At 7 weeks after injection, the lungs (C) and livers (D) were removed (left top) and stained with H&E (left middle) and for the detection of FOXP3 (left bottom). Arrowheads, the FOXP3+ tumors. Dotted lines circumscribe metastatic lesions in livers (left middle panels in D). Scale bar, 1 mm. Right top graphs, quantification of gross metastatic nodules. Right bottom graphs, quantification of tumor burden (tumor area/tissue area × 100%). E, timeline of therapeutic delivery of miR-146a/b inhibitors. F, representative images of low-power magnification of H&E-stained sections used for analyses of size and distribution of metastatic tumors in the lung (left) and liver (right) from miR-146a/b−/− (left) and control mice (right). G, quantification of gross tumor nodules and burden of the lungs (left) and livers (right) from miR-146a/b−/− (left) and control mice (right). Data are shown graphically with black circles representing the number of tumor nodules from each mouse and columns representing the median and interquartile range of tumor burden. Horizontal lines represent the average or median value. P values were determined by two-tailed t-test or Mann–Whitney test. Wks, weeks; miR-146a/b−/−, miR-146a/b inhibitors. All in vivo experiments were repeated two times.

triggered tumor suppression. Although a previous study suggested that ectopic miR-146a/b downregulates expression of EGFR in MDA-MB-231 cells, inhibiting invasion and migration in vitro and suppressing experimental lung metastasis in vivo (4), our results do not support a similar role for FOXP3-induced miR-146a/b in MCF7 cells. Although FOXP3 also inhibited cell migration and tumor metastasis, this inhibition was not disturbed by miR-146a/b inhibitors in breast cancer cells. Interestingly, when compared with the expression levels of miR-146a/b in MCF10A normal breast epithelial cells, the expression levels are low in less invasive breast cancer cell lines such as MCF7 and T47D and high in more invasive breast cancer cell lines such
as MDA-MB-231 (4, 5, 31), suggesting that miR-146a/b functions may be lost in tumor development but not in tumor metastasis. In contrast, the expression levels of CXCR4 and MMP9, which promote tumor metastasis, were dramatically reduced by FOXP3 in MCF7 cells (40, 41). CXCR4 has been reported as a potential target of miR-146a (11), but miR-146a did not contribute to the FOXP3-induced regulation of CXCR4 in MCF7 cells. Thus, our future studies will examine whether CXCR4 and MMP9 bypass miR-146a to contribute to FOXP3-mediated suppression of tumor metastasis.

This study also reveals the involvement of the FOXP3–miR-146–NF-κB axis in apoptotic signaling in breast cancer cells. MiR-146a/b, through repression of IRAK1 and TRAF6, reduces NF-κB activation (2, 3), which can inhibit apoptosis in cancer cells by inducing antiapoptotic factors such as BCL2 and BCL2L1 (42–44). Interestingly, BCL2L1 expression was reduced by FOXP3 through miR-146a/b, but BCL2 expression was not. BCL2L1 is a well-characterized apoptotic inhibitor that can promote evasion of autophagic cell death (45, 46). Notably, BCL2L1 is likely to be regulated by p65, whereas BCL2 is not (42, 47). In fact, our data show that nuclear p65 but not total p65 is reduced after FOXP3–miR-146 induction in breast cancer cells, suggesting that the FOXP3–miR-146 axis inhibits NF-κB activation through specific repression of p65 nuclear translocation, which may also contribute to FOXP3–miR-146-induced apoptosis. Likewise, NF-κB–targeting genes TRAF1/2, which can inhibit apoptosis (43), were also inhibited by FOXP3 through miR-146a/b in breast epithelial cells. Thus, the identification of FOXP3–miR-146–NF-κB axis–induced apoptosis advances our understanding of FOXP3-mediated tumor suppression.

In summary, miR-146a/b functions in FOXP3-mediated tumor suppression, especially in regulating tumor growth and apoptosis (Supplementary Fig. S9). Identification of the FOXP3–miR-146–NF-κB axis in tumorigenesis reveals new therapeutic targets for cancers with FOXP3 defects.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: R. Liu, C. Liu, Y. Liu, L. Wang
Development of methodology: R. Liu, C. Liu, W.-H. Yang, X. Liu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Liu, L. Wang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Liu, C. Liu, D. Chen, X. Liu, C.-G. Liu, C.M. Dugas, L. Wang
Writing, review, and/or revision of the manuscript: R. Liu, Y. Liu, L. Wang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): W.-H. Yang, F. Tang, P. Zheng
Study supervision: R. Liu, L. Wang

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Figure 7. Effects of FOXP3 defects on miR-146a/b, IRAK1, and TRAF6 mRNA levels in human breast cancer samples. A, representative IHC analysis with a specific antibody against human FOXP3 used to identify nuclear FOXP3<sup>+</sup> and FOXP3<sup>+</sup> primary breast cancer samples. Red arrows, Tregs with FOXP3 staining. B, quantification of miR-146a/b expression in microdissected nuclear FOXP3<sup>+</sup> cancer cells and FOXP3<sup>+</sup> cancer cells. C, quantification of IRAK1 and TRAF6 mRNA expression in microdissected nuclear FOXP3<sup>+</sup> cancer cells and FOXP3<sup>+</sup> cancer cells. All P values were calculated by two-tailed t test. All experiments were repeated two times.
References


9. Bhaumik D, Scott GK, Schokrpur S, Patil CK, Campisi J, Benz CC. Expres...


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