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This report is the first annual report on the grant “FoxP3 as a missing link between breast cancer and inflammation”. Given the importance of both cancer-cell intrinsic (cancer gene) and extrinsic (inflammation) pathways, it is especially intriguing and appropriate to investigate the effect of genetic alteration in genes that have both cancer cell-intrinsic and extrinsic functions, as they may play a unique—and as yet unrecognized—role in the pathogenesis of breast cancer. The FoxP3 gene is unique in this regard, in that its alteration can affect both cancer cell-intrinsic function and inflammation. Our proposed work will address the role for inflammation associated with adaptive and innate immune response in the pathogenesis of mammary cancer in female mice with a FoxP3 mutation. Despite a setback caused by an outbreak of mouse hepatitis virus that lead to termination of the mouse colonies specifically generated for the proposed studies, we made an important observation that targeted mutation of FoxP3 genes in the epithelial cells, resulted in expression of IL-1α and IL1β, key regulators of inflammation. More importantly, expression of IL1b associated with strong inflammation.
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(4) Introduction

This is the First Report on the grant “FoxP3 as a missing link between breast cancer and inflammation”. Apart from the breast cancer cell-intrinsic factors, an important issue is whether inflammation itself is part of the pathogenesis leading to the development and progression of breast cancer. We have hypothesized that FOXP3 mutation will increase inflammatory response in breast cancer. We proposed to test this hypothesis with studies in three specific aims.

Specific aim I. Contribution of innate inflammation to pathogenesis of breast cancer. We have produced Rag2−/−FoxP3sf/sf and FoxP3+/+Rag2−/− mice for cancer incidence. Since the Rag2−/− mice have no adaptive immune system, this model will allow us to determine whether in the absence of adaptive immunity, innate immune system can co-operate with an epithelial cell-intrinsic defect to cause development of mammary tumors in the mice. If so, we will take a genetic and a pharmacological approaches to abrogate the mast cells in order to identify their potential contribution to tumorigenesis.

Specific aim II. What is the significance of Treg-mediated suppression of innate immunity in pathogenesis of mammary tumor? We will adoptively transfer Treg into the Rag2−/−FoxP3sf/sf mice and determine whether it will suppress innate inflammation responsible for the development of breast cancer.

Specific aim III. Define in detail the impact of T-lineage-specific and epithelial cell specific deletion of the FoxP3 gene on the rate of breast cancer in a susceptible background. We will use the mice floxed FoxP3 locus to generate mice with T-lineage-specific or epithelial specific ablation of the FoxP3 gene and monitor the rate and types of breast cancer. The impact of such depletion of T cell activation and functional differentiation at the tumor sites will be determined using intracellular cytokine staining. Should polarized T cells be observed at the tumor sites, the function of the cytokine will be tested by neutralizing antibodies.

Essentially all aims depend on the tumorigenesis of the Rag2−/−Sf/sf mice and observe tumorigenesis in the mice for at least 14 months. Unfortunately, there were a major outbreak of murine hepatitis virus (MHV) infection in the facility. MHV is a mouse pathogen that cause significant alteration in inflammatory response. Because of their immune deficiency, all of the mice generated for the studies are infected, and therefore must be euthanasized in the last year. We are therefore forced to modify research strategy. On one hand, we started regeneration of the mice. At the same time, we have made some very interesting observation on a potential novel mechanism by which FOXP3 regulate inflammatory response. As detailed below.
(5) Body of Annual Report

Statement of work

The proposed studies consist of 4 main tasks.

**In task 1**, we will compare the incidence of tumors in BALB/cRag2⁻/⁻ FoxP3⁺/⁺, BALB/cRag2⁻/⁻ FoxP3⁻/⁻ mice. The mice will be observed for both spontaneous cancer and carcinogen DMBA-induced mammary tumor, using the regiment depicted in Fig. 2. The tumor incidence will be determined first by palpation and confirmed by H&E. Only those samples with confirmed malignancy in mammary tissue will be counted into cancer incidence. We have produced mice of designed genotype and are in the process of expanding the colonies. This will ensure that all the mice can be observed over 24 months.

This task is essentially finished. We have observed some breast cancer in the BALB/cRag2⁻/⁻ FoxP3⁻/⁻ mice. No tumors were observed in the BALB/cRag2⁻/⁻ FoxP3⁺/⁺ mice. The tumors were identified by H&E studies.

**In task 2**, we will determine the contribution of mast cells by two approaches. First, we will produce chimera mice using hematopoietic stem cells from new liver of new borne Rag2⁻/⁻ Kit⁺/⁺ or Kit⁻/⁻ mutant. The recipient mice Rag2⁻/⁻ FoxP3⁻/⁻ mice and will be irradiated for 500 Rad before receiving 10⁶ mononuclear cells from neonate livers. The donor mice will be produced by two steps of breeding. First the Kit⁺/⁺ mice will be crossed will Rag2⁻/⁻ mice to generate either Kit⁺/⁺ Rag2⁻/⁻ F1 mice. The F1 mice will be crossed to generate F2 neonate mice. PCR based genotyping of neonate liver cells will be used to identifying two types of HSC, Rag2⁻/⁻ Kit⁺/⁺ and Rag2⁻/⁻ Kit⁻/−. The liver HSC will be injected intravenously, and the chimera mice will be observed for the incidence of cancer.

This study was not carried out because we have to remove all MHV infected mice in the colony.

**In task 3**, we will adoptively transfer 10⁶ FoxP3-GFP⁺ cells from BALB/cxB6 FoxP3⁺/⁺ mice into BALB/c Rag2⁻/⁻ FoxP3⁻/⁻ mice. In order to isolate sufficient number of Treg, we will first purify CD4 T cells by negative selection, and then purify GFP⁺ cells by FACS sorting. We plan to use 20 recipient mice as recipients, and the other 20 mice will be left untreated. Tumor incidence will be compared.

This study was not carried out because we have to remove all MHV infected mice in the colony.

**In task 4**, we will produce lineage-specific knock out of the FoxP3 gene in either T cell-lineage or the epithelial cell lineage. We have backcrossed the FoxP3floxed allele into the BALB/c background for six generations, and are in the process of producing the BALB/cFoxP3floxed mice. These founders will be crossed with the WSP-Cre transgenic mice to produce FoxP3floxed/WASP-Cre⁺ mice, which will then be crossed with the BALB/cFoxP3floxed mice to produce the BALB/c WSP-Cre⁺FoxP3floxed and WSP-Cre FoxP3floxed mice, to be used for tumorigenicity studies, as detailed in task 1.

Similar breeding schedules have been planned for the BALB/c LCK-Cre⁺ FoxP3floxed mice.
This study was not carried out because we have to remove all MHV infected mice in the colony.

**Unexpected discoveries**

**FOXP3 is a transcriptional repressor of IL1α/β expression**

The key cytokines for inflammatory response is interleukin 1, which comes in two forms encoded by different genes, *IL1a* and *IL1b*. These genes are normally expressed in macrophages. However, in our analysis of cells with FOXP3 silencing, we observed a major elevation of *IL1A* and *IL1A*, as demonstrated in Fig. 1.

![MCF10A affi chip data](image)

**Fig. 1, Gene silencing of FOXP3 by shRNA increases transcripts of IL1A and IL1B. Data shown were relative levels, using control unsilenced cells as 1.0.**

Although IL1α protein can induce inflammation without further processing, IL1β is expressed as a precursor, which require further processing, as illustrated in Fig. 2.

![Diagram of molecules involved in IL1β processing](image)

**Fig. 2, Diagram of molecules involved in IL1β processing.**
We therefore analyzed gene array data to determine if these components are expressed in breast epithelial cells, and if so, whether they are regulated by FOXP3. As shown in Fig. 3, at least two genes are expressed at significant levels. More importantly, their expressions are increased when FOXP3 is silenced.

**Affymetrix Gene Chip**

![Graph of gene expression](image)

- **NOD1**: mRNA expression x1.5 after FOXP3 knock down.
- **NLRX1**: mRNA expression x2.0 after FOXP3 knock down.
- Others: no change, or, not expressed.

**Fig. 3.** FoxP3 silencing increase expression of NOD1 (NLRP1) and NLRX1.

To determine whether IL1b is processed and functional in epithelial tissues, we analyzed whether mammary tumors with FOXP3 mutation have mature IL1B protein, and if so, whether their expression correlates with inflammatory response. As shown in Fig. 4, mammary tumor from FoxP3 mutant mice show high levels of mature IL1 in epithelial cells. Correspondingly, large inflammatory infiltrates are found.
Fig. 4. Expression of mature IL1b in normal and tumor cells of a Foxp3 mutant mice. Presence of IL1b in normal epithelial cells suggest that inflammation can occur prior to tumorigenesis and argue for a role in tumor initiation.

(6) Key Research Accomplishments
Our conclusion that oncogenic mutation directly activates expression of IL1 suggest a novel mechanism of inflammatory response in breast cancer tissue.

(7) Reportable Outcomes:

Manuscripts:


(8) Conclusions and future plan:
Despite of the set back related to MHV infection, we were able to carry out some work as planned, while making a novel observation which have important implication in mechanism of inflammation in breast cancer. In the next funding period, we hope to complete regeneration of mice and therefore proceed with the proposed work as planned. Furthermore, we hope to pursue the new observation to its completion.

(9) References:
None.
FOXP3 Up-regulates p21 Expression by Site-Specific Inhibition of Histone Deacetylase 2/Histone Deacetylase 4 Association to the Locus

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Abstract

p21 loss has been implicated in conferring oncogenic activity to known tumor suppressor gene KLF4 and cancer drug tamoxifen. Regulators of p21, therefore, play critical roles in tumorigenesis. Here, we report that X-linked tumor suppressor FOXP3 is essential for p21 expression in normal epithelia and that lack of FOXP3 is associated with p21 down-regulation in breast cancer samples. A specific FOXP3 binding site in the intron 1 is essential for p21 induction by FOXP3. FOXP3 specifically inhibited binding of histone deacetylase 2 (HDAC2) and HDAC4 to the site and increased local histone H3 acetylation. Short hairpin RNA silencing of either HDAC2 or HDAC4 is sufficient to induce p21 expression. Our data provides a novel mechanism for transcription activation by FOXP3 and a genetic mechanism for lack of p21 in a large proportion of breast cancer. [Cancer Res 2009;69(6):2252–9]

Introduction

As a universal cyclin-dependent kinase inhibitor, p21 plays an important role in preventing cell cycle progression by acting at G1 checkpoint (1–4). p21 is down-regulated in many types of cancers, including breast cancer (5–7). Absence of p21 has been shown to confer oncogenic properties to KLF4 (8). Moreover, p21 loss is causatively related to the tamoxifen-stimulated growth of breast cancer (9). Surprisingly, p21 mutation is rarely observed in cancer (10). Instead, p21 has emerged as a major downstream target of tumor suppressor genes, including p53 (1, 11, 12), BRCA1 (13), CHK2 (14), KLF4 (15, 16), and KLF6 (17). Although p53-mediated regulation has been established as a classic example, the lack of correlation between p53 protein levels (usually used as an indication of p53 mutation) and the down-regulation of p21 would argue strongly that p53 mutation is perhaps not the major underlying cause for p21 loss in breast cancer (5–7). Likewise, although it has been shown that BRCA1-mediated (13) and Chk2-mediated (14) tumor cell cycle arrest and senescence require p21 function, mutations of these two genes had not been established as the genetic cause for the lack of p21 in the tumors. On the other hand, epigenetic factors have been suggested as possible mechanisms of p21 silencing in breast cancer cells (18–21).

We reported recently that heterozygous FOXP3 mutation leads to spontaneous development of mammary tumors (22). The significance of FOXP3 mutation in human is shown by both widespread somatic mutation and deletion of the gene in human breast cancer samples (22). Ectopic expression of the FOXP3 gene caused profound growth inhibition for breast cancer cell lines both in vivo and in vitro. Because Foxp3 is a transcription factor, it is important to identify critical targets of Foxp3 that are responsible for the tumor suppressor activity of Foxp3. In this context, we have reported that Foxp3 is a repressor for the HER-2/ErbB2 and Skp2 oncogenes (22, 23). Alternatively, it is possible that Foxp3 may activate additional tumor suppressor genes. To test this hypothesis, we used a gene array analysis to identify genes affected by Foxp3. We uncovered several tumor suppressor genes that were induced >2-fold after induction of FOXP3. We focused on p21, as it is the most highly induced tumor suppressor and because of its unique role in breast cancer biology. Here, we report that FOXP3 is a potent inducer of p21 in both normal epithelial cells and malignant breast cancer cell lines. Our data provide a novel mechanism for FOXP3-mediated activation of tumor suppressor gene.

Materials and Methods

Mice. Rag2−/− Foxp3−/− and Rag2−/− Foxp3sf/sf BALB/c mice have been described previously (24). Two-month-old virgin mice were used to analyze the effect of Foxp3 mutation on p21 expression and hyperplasia of mammary epithelia. All animal experiments were conducted in accordance with accepted standards of animal care and approved by the Institutional Animal Care and Use Committee of University of Michigan.

Cell culture. Breast cancer cell line MCF-7 and immortalized mammary epithelial cell line MCF-10A were purchased from the American Type Culture Collection. The HO15.19 cell line, which is the c-Myc null derivative of TGR-1 (25, 26), was a kind gift from Dr. John M. Sedivy of Brown University. A previously established tet-off FOXP3 expression system in the MCF-7 cells was also used (22, 23).

Microarray analysis of FOXP3-regulated genes. The FOXP3 tet-off MCF-7 cells (22, 23) were seeded in six-well plates and cultured with (20 μg/ml) and without doxycyclin in the culture media. After 48 h of incubation, cells were washed with ice-cold PBS twice and RNA extraction was performed with RNeasy Mini kit (Qiagen) according to the manufacturer’s protocol. Contaminated genomic DNA was eliminated with DNase I (Invitrogen) according to the manufacturer’s protocol. We conducted mRNA microarray analyses using HG-U133 Plus 2.0 (Affymetrix) according to the manufacturer’s protocol. We used the most current version of ENTREZ gene-based CDFs, as of July 2008, that has been maintained at University of Michigan for accurate analysis (27). dChip software (University of California at Los Angeles Clinical Microarray Core) was used to make a heat map of microarray profile according to the instruction of the software. Gene expression profiles of FOXP3 tet-off cells cultured with and without

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doxycyclin were compared. Differences of mRNA expression levels between FOXP3* and FOXP3* cells were calculated by Student's t test.

**FOXp3, p21, and histone deacetylase silencing.** Two FOXP3 short hairpin RNA (shRNA) constructs are FOXP3-993 shRNA and FOXP3-1355 shRNA (Genbank accession no. NM_014009). Oligonucleotides encoding small interfering RNA (siRNA) directed against FOXP3 are 5'-GCTTCA-CTGTTGGCATCATTCC-3' for FOXP3-993 shRNA [993–1013 nucleotides from transcription starting site (TSS)] and 5'-GAGCTTGGCAAGTGCTTTTG-3' for FOXP3-1355 shRNA (1355–1375 from TSS). The selected shRNA oligonucleotides were cloned into pSIREN-RetroQ vectors (Clontech) to generate siRNA according to manufacturer's protocol. The human p21 shRNA (CCGCTCTGCGCATAGAATTATT), human shHDAC2 (shHDAC2-1, CCGCCGCTATTTGGAATTA; shHDAC2-2, CGGCGAGA-TATTAAGCCTT), human shHDAC4 (shHDAC4-1, AGCGCAGCTTTGTATGAT; shHDAC4-2, AGACGGCAGCTTTGATTG), and control lentiviral vectors were purchased from Open Biosystems.

**Western blot.** The anti-FOXp3 (Abcom, 1:1,000), anti-hFOXY (eBioscience, 1:100), anti-p21 (Cell Signaling, 1:1,000), and anti-β-actin (Sigma, 1:3,000) were used as primary antibodies. Anti-rabbit or mouse IgG horseradish peroxidase-linked secondary antibody at 1:3,000 to 1:5,000 dilutions (Cell Signaling) was used. To ensure equal loading of proteins, the membranes were stripped under the same conditions as described above. They were then incubated with enhanced chemiluminescence reagents (Amersham Biosciences) and exposed to X-ray film for 1 to 5 min.

**Chromatin immunoprecipitation.** Chromatin immunoprecipitation (ChIP) was carried out according to published procedure (28). Briefly, the FOXP3-transfected tet-off cells were sonicated and fixed with 1% paraformaldehyde. The anti-FOXp3, anti-acetyl-H3 (Cell Signaling), anti-histone deacetylase 1 (HDAC1), anti-HDAC2, anti-HDAC3, anti-HDAC4, anti-HDAC5, anti-HDAC7 (Cell Signaling), and anti-IgG (Santa Cruz Biotechnology) antibodies were used to pull down chromatin associated with FOXp3. The amounts of the specific DNA fragment were quantitated by real-time PCR and normalized against the genomic DNA preparation from the same cells. The ChIP real-time PCR primers are listed in Supplementary Table S3.

**Quantitative real-time PCR.** Relative quantities of mRNA expression were analyzed using real-time PCR (ABI Prism 7500 Sequence Detection System, Applied Biosystems). The SYBR (Applied Biosystems) green fluorescence dye was used in this study. The primer sequences are listed in Supplementary Table S3.

**Immunohistochemistry.** Immunohistochemistry was performed by the avidin-biotin complex method. Expression of FOXP3 in human breast cancer or normal tissue samples was determined using immunohistochemistry, as described (22, 23). The p21 mouse monoclonal antibody (Cell Signaling, 1:100) and biotin goat anti-mouse IgG (Santa Cruz, 1:200) were used as secondary antibodies. FOXP3 and p21 staining were scored double blind.

**Statistical analysis.** Data are shown as means ± SD. Statistical analysis was performed with Student's t test for means from two groups. ANOVA test was used for ANOVA between several groups. χ² test was used to compare the relationship between the expression of FOXp3 and p21.

**Results**

p21 is up-regulated after FOXp3 induction and contributes to its tumor suppressor activity. We used the MCF-7 cell lines engineered to express FOXp3 in the absence doxycyclin. The cells cultured in the presence or absence of doxycyclin for 48 hours were compared with five independent RNA isolates in each group by gene array analysis. A summary of the gene array data, depicting genes that are induced by >2-fold, is shown in Fig. 1A. The full data set is shown in Supplementary Tables S1 and S2, and the raw data are deposited to MIAExpress (accession no. E-MTAB-73).

Among the FOXp3-induced genes are several tumor suppressors, including p18, p21, LAT2, and ARHGAPS (Fig. 1A). We have chosen

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**Figure 1.** Identification of p21 as a FOXp3-induced tumor suppressor gene. A, gene expression profiles in a panel of five flasks of FOXp3* cells and five flasks of FOXp3* cells. Expression values of each row were normalized to the average expression value of each gene among the FOXp3* cells. Color scales of gene expression levels are indicated at the bottom of the figure. Known tumor suppressor genes are indicated at the left side of the heat map. B and C, confirmation of p21 up-regulation by FOXp3. After removing doxycyclin from the medium, the cells were collected at 0, 24, 36, 48, 72, and 96 h and the FOXp3 expression was measured by real-time PCR (B) and Western blotting (C). B, the mRNA levels of p21 were measured by real-time PCR for the pBI-GFP vector control cells and pBI-FOXp3/GFP cells. The mean of the 0 h is artifically defined as 1.0. Columns, mean of three independent experiments; bars, SD. C, the protein levels of FOXp3 and p21 were detected in the GFP control and FOXp3/GFP cells by Western blotting without doxycyclin from 0 to 5 d.
p21 as the prototype to study the mechanism by which FOXP3 activates tumor suppressors as the relevance of defective p21 in breast cancer is well established. In addition, real-time PCR showed induction of p18 is <2-fold (data not shown). We first used real-time PCR and Western blot to confirm the induction of p21 after the inducible expression of FOXP3. As shown in Fig. 1B, p21 transcripts were induced by 7-fold in the MCF-7-pBI-FOXP3/green fluorescent protein (GFP) cell line after removal of doxycyclin, but not the MCF-7-pBI-GFP/control cell line under the same culture condition. Western blot analysis confirmed that accumulation of p21 protein followed that of FOXP3 (Fig. 1C). To determine whether induction of p21 contributed to tumor suppression, we transfected the MCF-7 cell lines with either control vector or p21 shRNA. The transfectants were cultured in the absence of doxycyclin for 10 days and were stained by crystal violets. As shown in Fig. 2B, p21 shRNA specifically increased the number of colonies in the cell line that expressed FOXP3, but barely so for those that expressed GFP. Microscopically, the sizes of colonies were usually larger in the shRNA group, even for those that expressed GFP only, consistent with the notion that endogenous p21 protein can inhibit cell growth. Western blot analysis of the colonies indicated that p21 suppression activity of the FOXP3 gene.

FOXP3 maintains p21 levels in normal mammary epithelial cells. An important issue is whether FOXP3 expression contributes to expression of p21 in normal mammary epithelial cells. As shown in Fig. 2C, the FOXP3 protein can be identified by Western blot in immortalized human mammary epithelial cell line MCF10A. MCF10A was transfected with either control vector or FOXP3 shRNAs. The untransfected cells were removed by selection with puromycin. At 2 wk after transfection, the protein levels were determined by Western blotting, using specific anti-FOXP3, anti-p21, and β-actin antibodies as loading control. The mRNA levels of the FOXP3 and p21 transcripts were quantitated by real-time PCR. The RNA inputs were normalized against the housekeeping gene GAPDH. The vector control was defined as 1.0. Columns, mean of triplicates and representative of three independent experiments; bars, SD.

Figure 2. p21 induction is an underlying mechanism for the tumor suppressor activity of FOXP3. A, MCF-7 cells with inducible expression of either FOXP3 (1 and 2) or GFP (3 and 4) were supertransfected with either vector control (1 and 3) or p21 shRNA (2 and 4). After removing untransfected cells by drug selection, the cultures were maintained in doxycyclin-free conditions for 10 d. Photographs of viable (2–4) and apoptotic MCF-7 cells (1). Magnification, 100×. B, the colony number per 60-mm² plate. Columns, mean of triplicates and representative of three independent experiments; bars, SD. C and D, silencing of FOXP3 resulted in down-regulation of p21 protein (C) and p21 mRNA (D) in human mammary epithelial cell line MCF10A. MCF10A was transfected with either control vector or FOXP3 shRNAs. The untransfected cells were removed by selection with puromycin. At 2 wk after transfection, the protein levels were determined by Western blotting, using specific anti-FOXP3, anti-p21, and β-actin antibodies as loading control. The mRNA levels of the FOXP3 and p21 transcripts were quantitated by real-time PCR. The RNA inputs were normalized against the housekeeping gene GAPDH. The vector control was defined as 1.0. Columns, mean of triplicates and representative of three independent experiments; bars, SD.

Figure 3. FoxP3 mutation in benign mammary epithelia increased p21 transcripts and increased proliferation of epithelial cells. A, FoxP3 mutation increased p21 transcripts. Mammary epithelia from virgin Rag2 FoxP3sf sf and Rag2 FoxP3sf sf BALB/c mice were isolated by microdissection. The p21 and FoxP3 transcripts were measured by real-time PCR. Columns, mean of percentage of housekeeping gene Hprt; bars, SE. Three independent mice were used in each group. B, increased proliferation of the mammary epithelial cells as revealed by Ki67 staining. Representative fields from each group. The means and SDs from groups of three mice are shown in the insert (P < 0.05). C, H&E staining of 2-month-old virgin Rag2 FoxP3sf sf and Rag2 FoxP3sf sf BALB/c mice. Representative of three mice per group.
transcripts. As shown in Fig. 2D, FOXP3 silencing caused a 5-fold to 10-fold reduction of the p21 transcripts, which revealed a critical role for FOXP3 in maintaining p21 expression in mammary epithelial cells. A similar effect was observed when FOXP3 was silenced in the early passage of primary human mammary epithelial culture (Supplementary Fig. S1).

To test the role of FoxP3 in p21 expression in vivo, we microdissected mammary epithelium from 2-month-old Rag2<sup>−/−</sup>, FoxP3<sup>sf/sf</sup> and Rag2<sup>−/−</sup> FoxP3<sup>−/−</sup> mice. The amounts of p21 transcripts were determined by real-time PCR. As shown in Fig. 3A, the FoxP3 mutation caused ~6-fold reduction in p21 transcripts. Perhaps due to nonmediated decay caused by frameshift mutation, mammary epithelia from FoxP3 mutant mice lacked FoxP3 transcripts. Correspondingly, dramatically increased numbers of breast epithelial cells in the Rag2<sup>−/−</sup> FoxP3<sup>sf/sf</sup> mice have entered the cell cycle, as judged by Ki67 staining (Fig. 3B). H&E staining of the mammary tissue indicated extensive ductal hyperplasia in the Rag2<sup>−/−</sup> FoxP3<sup>sf/sf</sup> mice (Fig. 3C). These data showed that the FoxP3 mutation leads to reduced p21 expression and increased proliferation of normal epithelium in vivo. Because the young mice had yet to develop mammary tumor at this age, down-regulation of p21 is not due to the secondary effect of malignant transformation.

**Correlation between expressions of FOXP3 and p21 in human breast cancer.** The majority of breast cancer samples lack p21 and FOXP3 expression (5–7, 22, 23). An important issue is, therefore, whether the expression of the two genes is interrelated among human breast cancer samples. To address this issue, we analyzed 62 cases of breast cancer samples in TMA for expression of FOXP3. As shown in Fig. 4, among the FOXP3<sup>−</sup> samples, 66% are also p21<sup>−</sup>. In contrast, only 30% of the FOXP3<sup>+</sup> samples expressed p21. The strong correlation between FOXP3 and p21 expression suggests that FOXP3 down-regulation may be an important factor for the lack of p21 among breast cancer tissue.

**Specific binding of FOXP3 to the p21 locus is essential for activation of p21.** Two p21 mRNA isoforms (1, NM_078467 and 2, NM_000389) have been reported with different exon 1–exon 2 junctions. To properly align the genomic structure of the locus, we sequenced the transcripts, which revealed a critical 5′ to TSS, with the maximal activity requiring −340 and +365 bp at 5′ and 3′, respectively. Further extension at 3′ significantly reduced the p21 induction (Fig. 5B). We, therefore, used the optimal reporter to confirm the function of the forhead binding site at the 0.2 kb at 3′ of TSS. As shown in Fig. 5C, whereas wild-type (WT) reporter is induced by FOXP3 expression, mutation of the forhead binding site abrogated induction. These data showed that the specific cis-element is essential for FOXP3-mediated activation of p21 locus.

It has been shown that c-Myc can target the p21 promoter and inhibit its expression (33–35). To determine whether the FOXP3 gene regulates p21 directly, we measured the effects of FOXP3 on the p21 promoter activity in the c-Myc knockout cell line. As shown in Fig. 5D, the promoter activity of p21 was significantly induced by FOXP3 in c-Myc knockout cells. The relatively low induction, compared with HEK 293 cells, is likely due to the drastically reduced transfection efficiency of the Myc-deficient cell line.

**Localized chromatinf modification as a mechanism for FOXP3-induced expression of p21.** Recent studies showed that FOXP3-mediated induction of gene expression is associated with histone acetylation (36). We, therefore, used anti–acetyl-H3 antibodies to monitor local chromatin changes associated with FOXP3 binding. As shown in Fig. 6A and B, in cells expressing FOXP3, H3 acetylation in the −0.2 kb site of p21 was increased by 2-fold. The increase in the neighboring areas mirrored what was observed with FOXP3 binding. These data showed that FOXP3 enhances the H3 acetylation of p21, especially at the 0.2 kb region. We carried out

![Figure 4](Image)

**Figure 4.** A positive correlation between FOXP3 and p21 expression in human breast cancer. Tissue microarray samples were stained with either anti-FOXP3 antibody or anti-p21 antibody and scored by a double-blind fashion. Samples with nuclear staining by the anti-FOXP3 antibody were scored as positive. Samples with >10% p21 staining in nuclear and/or cytosolic were scored as positive. Magnification, 600×. Bottom, summary data from 62 independent cases. The P value of the χ² test is listed.

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4 Our unpublished observation.
ChIP analysis using antibodies specific for HDAC1 to HDAC7. The MCF-7 cells with or without FOXP3 induction were compared. As shown in Fig. 6B, a generalized reduction of HDAC association to the p21 locus was observed after FOXP3 induction. However, by far, the strongest effect was observed at the 0.2-kb site, wherein FOXP3 associates with the p21 locus. Moreover, although a reduction of HDAC1 to HDAC7 was observed after FOXP3 binding, the most significant reduction was observed on HDAC2 and HDAC4, as these two HDACs showed the strongest association before FOXP3 induction.

To determine whether HDAC2 and HDAC4 are involved in p21 up-regulation, we used shRNAs to modulate their expression. As shown in Fig. 6C, two independent shRNAs were specifically silencing the expression of either HDAC2 or HDAC4. Correspondingly, the levels of p21 transcripts were increased by 2-fold to 3-fold after the silence of either gene. Note that some of the p21 protein induced by HDAC shRNAs had a molecular weight of 15 kDa rather than 21 kDa. This is likely due to the cleavage of p21 associated with a general increase in histone acetylation, as reported by others (37). To test if HDAC2 and HDAC4 are necessary mediators of p21 induction by FOXP3, we transfected FOXP3 into MCF-7 cells, in which HDAC2 and HDAC4 are silenced, and compared the levels of p21 in either FOXP3-transfected or vector control–transfected cells. As shown in Fig. 6C, FOXP3-mediated induction of p21 is abrogated in the HDAC4-silenced cell lines. These data further support the notion that FOXP3-mediated induction of p21 is

Figure 5. FOXP3 as a transcription activator for p21. A, ChIP. Top, a diagram of the p21 gene, including the promoter and exon 1 to 3 (NM_000389). Black asterisks, forkhead binding motifs; black columns, regions surveyed by real-time PCR. Middle, amount of DNA precipitated by either control IgG or anti-FOXP3 monoclonal antibody expressed as percentage of the total input genomic DNA. Columns, means of triplicates; bars, SD. This experiment has been repeated twice with similar results. Bottom, specificity of the ChIP assay, as shown by the requirements for both FOXP3 induction and anti-FOXP3 antibody. B, identification of the promoter region most responsive to FOXP3-mediated induction. The HEK293 cells were transfected with either vector control or FOXP3 (1 μg/well) in conjunction with the luciferase reporter driven by different 5’ promoter regions of the p21 gene (0.5 μg/well). pRL-TK was used as internal control. The luciferase activity from the cells transfected with the pGL2 basic vector was arbitrarily defined as 1.0. Columns, means of triplicates repeated at least thrice; bars, SD. C, site-directed mutagenesis of one candidate forkhead/HNF-3 binding motif in the p21 promoter abrogated the induction of the p21 transcription activity by FOXP3. The WT forkhead-binding motif TGTGTGC was mutated into CCCTATAA. The promoter activity was measured and normalized as detailed in B. Columns, means of triplicates; bars, SD. This experiment has been repeated twice with similar results. D, p21 transcription is directly induced by FOXP3 by a c-Myc–independent mechanism. Transfection of FOXP3 into c-Myc−/− cells increased p21 activity, as by a luciferase assay. Columns, means of triplicates; bars, SD. This experiment has been repeated thrice.
mediated by the disruption of HDAC2-mediated and HDAC4-mediated repression.

**Discussion**

Mechanism of p21 regulation in normal and cancerous epithelial cells may hold the keys to the molecular mechanism of carcinogenesis. Here, we present several lines of evidence demonstrating a critical role of p21 as a downstream target of FOXP3, and its expression contributes to the FOXP3-mediated growth inhibition of a breast cancer cell line.

First, in confirming the cDNA microarray data, we showed that inducible expression of FOXP3 induced the p21 transcripts and protein in breast cancer cell line MCF-7. The induction is mediated by transcription regulation, as it is reflected in luciferase assay. ChIP analysis revealed that a specific site at 0.2 kb downstream of FOXP3 specifically increased acetylation of histone H3 associated with the FOXP3 binding site by inhibiting association of HDAC 2 and 4. FOXP3 increases acetylation of histone H3 associated with the FOXP3 binding site. The increased acetylation of H3 is due to FOXP3-mediated inhibition association of HDAC2 and HDAC4 to the site. The MCF-7 cells with inducible FOXP3 expression were cultured with (-FOXP3) or without (+FOXP3) doxycyclin for 4 d and subjected to ChIP analysis using acetyl-H3 (A) and HDAC1, HDAC2, HDAC3, HDAC4, HDAC5, and HDAC7 (B) antibody or control IgG. Precipitated genomic DNA was probed for the promoter/enhancer regions of the p21 locus by real-time PCR. The amounts of DNA precipitated were expressed as percentage of the total input genomic DNA. Columns, means of triplicates; bars, SD. Representative of three separate experiments.

C, shRNA silencing of either HDAC2 or HDAC4 is sufficient to induce p21 expression. Data in bar graphs are from real-time PCR quantitation of shRNA efficacy and the effect of shRNA silencing using the means of the vector group as 1.0. Bottom, Western blotting. Note that some p21 proteins in HDAC4 or HDAC2 shRNA groups are degraded products with a molecular weight of 15 kDa whereas those in the control group consisted of mostly intact p21. These experiments have been repeated four times with similar results. D, silencing HDAC4 abrogates induction of p21 by FOXP3. Control or HDAC2/HDAC4-silenced MCF-7 cells were transfected with FOXP3 cDNA. After removal of nontransfected cells by blasticidin, the levels of p21 transcripts were determined by real-time PCR. Data have been repeated twice. E, diagram of a proposed mechanism of FOXP3-mediated gene activation. FOXP3 removes association of HDAC2 and HDAC4 and, thereby, increases H3 acetylation and gene activation.
TSS is necessary for FOXP3-mediated induction by FOXP3. Moreover, the induction is not an artifact of FOXP3 overexpression, as shRNA silencing of the FOXP3 gene leads to a dramatic reduction of p21 in primary mammary epithelial cells.

Second, to determine whether induction of p21 contributes to growth inhibition of the tumor cell line, we tested whether blunting p21 induction by shRNA abrogate growth inhibition by FOXP3. Our data showed the significant, albeit incomplete, rescue of FOXP3-mediated growth inhibition. The significant rescue shows an important role of p21 induction in FOXP3-mediated growth inhibition in MCF-7 cell line. Other recent studies indicate that FOXP3 also inhibits growth by repressing the expression of HER-2 and SKP2 (22, 23). Thus, depending on tumor cell lines used, FOXP3-mediated inhibition of oncogenes and induction of tumor suppressor may work either independently or in concert to cause growth inhibition of breast cancer cell line.

Third, our analysis of 62 cases of breast cancer samples showed a significant correlation between expression of FOXP3 and p21. Nevertheless, not unlike other tumor suppressor targets, there was no 1:1 correlation between expression of FOXP3 and p21. For instance, ~30% of cases that stained positive for FOXP3 still lack detectable p21. This is, in part, due to the fact that nearly one third of breast cancer samples show somatic missense mutation of FOXP3 (22, 23). Conversely, nearly one third of the FOXP3 negative tumor cells still express p21. This can be due to either the false-negative staining of FOXP3, perhaps relating to the quality of clinical samples, when viewed in the context of the data in mice can be explained, the positive association between p21 and FOXP3 in instance, no 1:1 correlation between expression of FOXP3 and p21. For nevertheless, not unlike other tumor suppressor targets, there was suppressor may work either independently or in concert to cause growth inhibition of breast cancer cell line.

Finally, whereas a number of studies have addressed the mechanism of FOXP3-mediated gene repression, the mechanism by which FOXP3 directly induces gene expression remained largely obscure. A recent report showed association between FOXP3-induced gene activation and histone acetylation (36), although the mechanism and significance of such acetylation have not been addressed. Our data showed that FOXP3 binding to a specific site in intron 1 of p21 increased histone H3 acetylation by reducing binding of HDAC4 and HDAC2 to the same site (Fig. 6D). Gene silencing with shRNA confirmed the significance of these two HDACs in p21 expression, although FOXP3 did not repress expression of either HDAC2 or HDAC4 (Supplementary Fig. S4). Therefore, our data provide a novel mechanism for FOXP3-mediated transcription activation. Because FOXP3 has been shown to recruit histone acetyl transferases (38), it is of interest to investigate whether this interaction contributes, either directly or indirectly, to increased H3 acetylation in the p21 locus.

Taken together, our data showed p21 as a downstream target for FOXP3, the first X-linked tumor suppressor in breast cancer. Because p21 serves as an important target for all major tumor suppressor genes of breast cancer and because irreversible genetic lesion to p21 is relatively rare, it might be possible to reactivate p21 in cancer by inducing FOXP3. Whereas p21 induction can be achieved by a general silencing of HDAC2 and HDAC4, the induced p21 are rapidly degraded, presumably due to the simultaneous induction of other proteins involved in p21 cleavage (37). On the other hand, our data showed that p21 induced by FOXP3 remained intact and mediates tumor suppression. Therefore, reactivating FOXP3 may prove to be a more relevant approach.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

An Epigenetic Mechanism of p21 Induction by FOXP3

Activating Transcription Factor 2 and c-Jun–Mediated Induction of FoxP3 for Experimental Therapy of Mammary Tumor in the Mouse

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Abstract

FOX P3 is inactivated in breast cancer cells by a number of mechanisms, including somatic mutations, deletion, and epigenetic silencing. Because the mutation and deletion are usually heterozygous in the cancer samples, it is of interest to determine whether the gene can be induced for the purpose of cancer therapy. Here, we report that anisomycin, a potent activator of activating transcription factor (ATF) 2, and c-Jun-NH2-kinase, induces expression of FoxP3 in both normal and malignant mammary epithelial cells. The induction is mediated by ATF2 and c-Jun. Targeted mutation of ATF2 abrogates both constitutive and inducible expression of FoxP3 in normal epithelial cells. Both ATF2 and c-Jun interact with a novel enhancer in the intron 1 of the FoxP3 locus. Moreover, shRNA silencing of ATF2 and FoxP3 reveals an important role of ATF2-FoxP3 pathway in the anisomycin-induced apoptosis of breast cancer cells. A low dose of anisomycin was also remarkably effective in treating established mammary tumor in the mice. Our data showed that FoxP3 can be reactivated for cancer therapy. [Cancer Res 2009;69(14):5954–60]

Introduction

The overwhelming majority of tumor suppressor genes are autosomal, and their inactivations involve two genetic events (1–4). The best defined two hits of the tumor suppressors in cancer cells are usually irreversible, such as deletion or mutations (3, 4). More recently, however, it has become increasingly clear that epigenetic inactivation of tumor suppressors plays a critical role in inactivating tumor suppressor genes (5). On the other hand, due to X-inactivation, the X-linked tumor suppressor genes are operatively hemizygous, and can be inactivated by a single hit (6). This notion has been substantiated by the recent identification of two tumor suppressor genes, FoxP3 for breast cancer (7) and WTX for Wilms’ tumor (8). Because the majority of mutations and/or deletions associated with X-linked tumor suppressor genes are heterozygous (7, 8), most cancer cells have a wild-type (WT) allele that has not been irreversibly inactivated (7).

Materials and Methods

Antibodies and reagents. Anti–ATF-2 (20F1), phospho-ATF2-(Thr71), and activated caspase-3 were purchased from Cell Signaling, Inc. Other vendors are as follows: anti-Foxp3 (eBioscience; #14-5779-82); and anti–β-actin (1-19), c-Jun (NX), and phosphor-c-Jun (KM-1; Santa Cruz Biotechnology, Inc.), Chemicals SP600125, SB203580, and PD98059 were purchased from CalBiochem, Inc., whereas anisomycin was purchased from Sigma, Inc.

Experimental animals. Mice heterozygous for Atf2 null mutation (Atf2tm1Glm/Atf2tm1Glm; ref. 11) were revived from the frozen embryo bank in The Jackson Laboratory. Heterozygous mice were crossed to produce Atf2−/− and Atf2+/- littermates. BALB/c mice were purchased from Charles River through a National Cancer Institute Subcontract. All studies involving animal has been approved by University Committee on Use and Care of Animals at University of Michigan.

Preparation of mammary epithelial cell culture. Mouse mammary fat pads were removed from 6- to 8-wk-old virgin female mice and minced into small pieces. After collagenase digestion at 37°C in a shaking incubator in DMEM supplemented with 5% FCS, cells were sieved through a 70-μm cell strainer (BD Falcon) to obtain a single-cell suspension. The cells were cultured in DMEM supplemented with 10% fetal bovine serum and 10 ng/ml epithelial growth factor. At day 3 of culture, fibroblast cells were removed by a short digestion with 0.05% trypsin-EDTA as less adherent cells.

Measurement of FOX P3 transcripts. Total cDNA were prepared from breast cancer cell lines or epithelial cultures. The levels of FOX P3 mRNA were measured by reverse transcription-PCR (RT-PCR) under two conditions. Full-length encoding regions were analyzed by aagarose

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Anisomycin rapidly induces FoxP3 expression in mouse and human breast cancer cell lines. A, induction of FoxP3 transcripts by anisomycin (0.1 μg/mL). Top, kinetics of FoxP3 induction in mouse mammary tumor cell line TSA, as measured by RT-PCR for cDNA covering the entire coding region. Bottom, the induction of FoxP3 in TSA by real-time PCR. Columns, means of three independent experiments; bars, SD. The transcript level of 0 h is artificially defined as 1.0. B, induction of FoxP3 by anisomycin (0.01, 0.05, 0.1, 0.25, 0.5, 1.0 μg/mL), but not by PMA (0.01, 0.1, 0.5, 1.0, 2.5 μg/mL), in mouse mammary tumor cell line 4T1 or human breast cancer cell line MCF-7 by RT-PCR, using primers spanning from start codon to stop codon. C, anisomycin induces FoxP3 protein, as revealed by Western blot. The specificity of analysis and dependence of FoxP3 transcript is confirmed by FoxP3 shRNA. All data are representative of at least three independent experiments.

**Figure 1.**

**Transcriptional Activation of FoxP3**

**ShRNA lentiviral vector.** The lentivirus-based shRNA expressing vectors were created by introducing the murine U6 RNA polymerase III promoter and a murine phosphoglycerate kinase promoter (pGK)–driven enhanced green fluorescent protein expression cassette into a vector of pLent6/V5-D-TOPO backbone without cytomegalovirus promoter. Hairpin shRNA sequence of FoxP3, JNK1, JNK2, and Akt2 (FoxP3: 5′-aagccatggcaaaacatgctattcctt-3′; FOXP3: 5′-gcaacgggaactctactgaa-3′; JNK1: 5′-gcaaggaaggtggcaattcctt-3′ and 5′-agacgtaaagacagcc-3′; Akt2: 5′-ctccgctttttagaac-3′ and 5′-agacgtaaagacagcc-3′) were cloned into the lentiviral shRNA expressing vectors by restriction sites of ApaI and EcoRI.

**Electrophoresis mobility shift assay.** Anisomycin were added to 4T1 cells in conjunction with either vehicle control or kinase inhibitor SP600125 at a dose of 2 μg/mL for 2 h before cells lysed. The nuclear extracts were mixed with either WT or mutant probes in the presence of either control anti-c-Jun (Santa Cruz; sc-45X) or anti-ATF2 (Cell Signaling, #9226) antibodies, as indicated and analyzed by electrophoresis, as described (12).

**Western blot.** Protein samples for Western blot were prepared by lysing cultured cells in SDS sample buffer, resolved on 10% SDS-PAGE, and electroblotted onto nitrocellulose membranes. Membranes with transferred proteins were incubated with primary antibody followed by incubation with horseradish peroxidase conjugated to the secondary antibody. Chemiluminescence reaction using the enhanced chemiluminescence kit (Amersham Biosciences) was detected by film.

Chromatin immunoprecipitation (ChiP) was carried out according to a published procedure (13). Briefly, the vehicle or 2-h anisomycin-treated 4T1 cells were sonicated and fixed with 1% paraformaldehyde. The anti–phospho-c-Jun or anti–phosphor-ATF2 antibodies or control rabbit IgG were used to pull down chromatin associated with these proteins. The amounts of the specific DNA fragments were quantitated by real-time PCR and normalized against the genomic DNA preparation from the same cells.

**Immunofluorescence staining.** TSA cells were treated or left untreated with vehicle DMSO or 50 ng/mL of anisomycin for 24 h. After that, the cells were fixed with methanol, permeabilized with 0.3% Triton-X100, and stained with rabbit antibody against cleaved Caspase 3 (Cell signaling, #9661s) overnight. The stained cells were then visualized with Cy3-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratory).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay has been described in details (14).

DNA contents Anisomycin-treated or control cells were stained by Propidium Iodide using “PI/RNase Staining Buffer” from BD Biosciences according to the manufacturer’s manual.

**Results**

**Induction of FoxP3 by anisomycin.** We have recently shown that the expression of FoxP3 cDNA leads to rapid cell death of breast cancer cell lines (7, 15). These results raised the possibility that the induction of FoxP3 may represent a novel therapeutic approach for the treatment of breast cancer. When we screened for drugs that induce FoxP3 expression in mammary tumor cell lines, we observed a rapid induction of FoxP3 mRNA by anisomycin. As shown in Fig. 1A, significant levels of the FoxP3 transcripts were induced in a mouse mammary tumor cell line, TSA, as early as 4 hours after anisomycin treatment. Similar effects were observed in another mouse mammary tumor cell line 4T1 and human breast cancer cell line MCF-7 (Fig. 1B), which we showed to harbor the WT FOXP3 gene (7). In contrast, treatment of phorbol 12-myristate 13-acetate (PMA) did not result in any induction of FoxP3 (Fig. 1B). Because higher doses of anisomycin can inhibit translation, we tested induction of the FoxP3 protein by Western blot. As shown in Fig. 1C, low doses of anisomycin induced high levels of FoxP3 protein, which indicated that activation of FoxP3 locus can be achieved at doses that did not prevent translation of the FoxP3 protein. Importantly, the induction of FoxP3 protein can be prevented by FoxP3 shRNA. These data not only proved the specificity of Western blot but also confirmed that the accumulation of FoxP3 protein depends on induction of FoxP3 mRNA.

**Involvement of ATF2, c-Jun-NH2-kinase in FoxP3 induction.** As a first step to identify the mechanism by which anisomycin induced FoxP3, we treated mammary tumor cell line with anisomycin in conjunction with inhibitors of overlapping specificity, including ATF-2/c-Jun-NH2-kinase (JNK) inhibitor SP10096 (SP), p38α inhibitor SB203580 (SB), and p41/42 MAP kinase inhibitor PD98059 (PD). As shown in Fig. 2A, SP completely prevented the induction of FoxP3. On the other hand, SB and PD had little effect. These data raised the possibility that ATF-2 and JNK pathways may be involved in the induction of FoxP3 by anisomycin.

To test this hypothesis, we generated lentiviral vectors expressing shRNA for JNK1/2 or ATF2. The efficacy of shRNA
silencing and the effect of the silencing on anisomycin-mediated induction of FoxP3 is shown in Fig. 2B. These data showed that silencing either JNK or ATF2 resulted in abrogation of the induction of the FoxP3 transcripts and protein by anisomycin. These data provide important genetic evidence for the involvement of JNK and ATF2 in anisomycin-induced FoxP3 expression.

Interestingly, a recent study showed that mice with heterozygous deletion of the ATF2 gene developed spontaneous mammary tumors (10). Because FoxP3 heterozygous mutants have the same phenotype, it is intriguing that ATF2 may be responsible for constitutive and/or inducible expressions of FoxP3 in mammary epithelial cells. To address this issue, we obtained ATF2−− mice from the frozen embryo bank of The Jackson Laboratory. The ATF2+/− and the ATF2−− mice were obtained by F1 cross. A previous report indicated that the only a small fraction of the ATF2−− mice survive to adulthood (11). We obtained two ATF2−− females, from which we obtained two independent primary mammary epithelial cell cultures (Fig. 2D). The epithelial origin of the cultures was shown by the expression of CK19. Because T cells are the major source of FoxP3 transcripts in vivo, we also confirmed that the primary culture has no T-cell contamination.

**Figure 2** A critical role of ATF2 and JNK1/2 in FoxP3 induction by anisomycin. A, inhibitor of JNK and ATF-2 prevented induction of FoxP3. 4T1 cells were stimulated with anisomycin (0.1 μg/mL) in presence or absence of inhibitors (2 μg/mL) for ATF-2 and JNK SP, SB, and PD. The induction of FoxP3 was measured by RT-PCR 16 h after induction. B, effect of shRNA silencing of ATF2 and JNK1/2 on anisomycin-induced FoxP3 expression. Top, efficacy of shRNA using lentiviral vector control or that with given shRNA, as measured by Western blot. Levels of β-actin are used as loading controls. Middle, RT-PCR results as determined by electrophoresis. Two independent shRNA (called 1 and 2) were used. 4T1 cells treated with either vehicle control (−) or 0.1 μg/mL of anisomycin for 16 h. Bottom, real-time PCR results. The FoxP3 mRNA levels were determined by RT-PCR as detailed in Fig. 1 legends. C, shRNA for ATF2 prevented induction of FoxP3 protein, as measured by Western blot. D, targeted mutation of ATF2 abrogated both constitutive and inducible expression of the FoxP3 gene in mouse mammary epithelial culture. Top and middle left, morphology of epithelial culture. Note higher cellular density of the ATF2−− epithelial culture. Top right, the primary cell culture expressed epithelial cell marker (CK19) and had no T-cell contamination as judged by the lack of CD3 transcript. RT, reverse transcriptase. Middle right, constitutive and inducible expression of FoxP3 in ATF2++ but not ATF2−− cultures. Bottom, real-time PCR quantitation of FoxP3 induction. Columns, means of three independent experiments; bars, SD. All data have been repeated at least thrice.
contamination by the lack of CD3 transcripts (Fig. 2D). WT
epithelial cultures expressed significant amounts of Foxp3
transcripts, which were further induced by the treatment of
anisomycin. ATF2−/− cells had no detectable Foxp3
transcripts and did not express Foxp3 after stimulation by anisomycin.
These data revealed an essential role for ATF2 in both
constitutive and inducible expressions of Foxp3 in normal
epithelial cells. On the other hand, the thymocytes from the
ATF2−/− mice had normal number of CD4+FoxP3+ T cells (data
not shown). Therefore, the function of ATF2 in Foxp3
expression seems to be epithelia specific.

Identification of the Foxp3 enhancer associated with ATF2 and c-Jun.
To study the mechanism of ATF2/JNK-mediated induction of Foxp3, we carried out ChIP to identify an
anisomycin-inducible binding site of the Foxp3 locus. To identify specific ATF2 binding sites, we treated the 4T1 cell line with or
without anisomycin and carried out ChIP with either control IgG or
anti–phospho-ATF2 antibodies. Because JNK regulate transcription by
phosphorylation of c-Jun (16), we used anti–phospho-c-Jun antibodies in the ChIP. To identify the Foxp3 sequence associated
with p-ATF2 and p-c-Jun, we first analyzed the 5′ sequence of the
Foxp3 gene and identified 14 potential AP1 and cAMP-responsive
element binding protein sites. PCR primers were designed across
the 10.4 kb regions, and the amounts of each PCR product were
normalized against that amplified from the input DNA. The
quantitative real-time PCR results were shown in Fig. 3A, whereas
the PCR products from two major peaks were shown in Fig. 3B.
These data reveal two potential sites for ATF2/cJun interaction.
The first is hereby called P2, which is 4.8 kb 5′ of exon 1. The
second and the stronger binding site P10 is 4.2 kb 3′ of exon 1.
Importantly, whereas the P2 ATF2/cJun association is not
inducible by anisomycin, the P10 binding is enhanced by >2-
fold by anisomycin. Moreover, comparison of mouse and human
Foxp3 sequence revealed that the P10, but not the P2 site, is
highly conserved (Supplementary Fig. S1). Therefore, we focused
on the potential significance of P10 as the site for p-ATF2 and p-
cJun interaction.

Sequencing comparison identified a typical AP1 site within
the P10 (Supplementary Fig. S1). To directly show interactions of
ATF2 and c-Jun to the Foxp3 promoter, we radiolabeled an

Figure 3. Identification of cis-elements that interact with ATF2/c-Jun heterodimer. A to C, ChIP scanning of potential c-Jun and ATF2 binding sites. The 4T1 cells were treated with either vehicle control or anisomycin for 2 h. The chromatin was cross-linked with paraformaldehyde and precipitated with either control IgG, anti–phospho-ATF2, or anti–phospho-c-Jun. The amounts of DNA were quantified by PCR. A, quantitative analysis of the ATF2 and c-Jun binding sites in the Foxp3 locus. Top, the genomic structure of the 5′ of the Foxp3 locus. The DNA quantitation was shown in the bar graph (bottom). Data shown are means of triplicate samples and have been repeated thrice. B, PCR amplification of Foxp3 sequences P2 and P10 in ChIPs from anisomycin-treated and untreated cells. Note that although the binding of P10 to ATF2 and c-Jun are inducible, that of P2 was largely unaffected by anisomycin. C, the ATF2 and cJun bound to the AP1 site in P10. The sequence of WT probe (P10) is agatgcacgtcaccacatgcc (bold letters for core AP1 sequence), that for P10-Mt1 is agatgcacgtcggcccacatgcc (bold letters indicate mutations), whereas that for P10-Mt2 is agatgcacgtcgccacatgcc. The data have been repeated thrice.
oligonucleotide probe containing conserved AP1 site as well as two control oligos with mutations in the AP1 site and tested their binding to nuclear extracts. As shown in Fig. 3C, the nuclear extracts from anisomycin-treated, but not those from the untreated, 4T1 cells showed strong interaction with the WT P10 probe. The specificity was confirmed as mutations in the AP1 site significantly reduced the binding. Furthermore, the involvement of ATF2 and c-Jun was shown as their specific antibodies abolished the binding of nuclear extracts to WT probe. Thus, both ChIP and electrophoresis mobility-shift assay identify a specific activator protein (AP1) site with 4.2 kb 3’ of the TSS, which binds to both p-ATF2 and p-cJun by anisomycin-inducible fashion.

To test whether the P10 sequence was a functional FoxP3 enhancer, we generated a series of constructs consisting of the basal promoter and putative enhancer elements. As shown in Supplementary Fig. S2, a 265 bp sequence 5’ of TSS of the FoxP3 locus plus 50 bp down-stream of TSS is sufficient to convey a significant basal promoter activity. This fragment is therefore chosen to measure the enhancer activity. As shown in Fig. 4, addition of three copies of P2 fragment increased the promoter activity by ~2-fold, which suggests that P2 is at best a weak enhancer. Inclusion of three copies of P10 sequences, however, increased the Foxp3 promoter activity by 10-fold. Surprisingly, this seems unidirectional as the inversion of the P10 fragment eliminated its enhancer activity. Moreover, the involvement of AP1 site in P10 was confirmed as a mutation of the AP1 site significantly reduced the enhance activity. Moreover, addition of P2 to P10 failed to further enhance the promoter activity. Taken together, our data showed that anisomycin induced ATF2/c-Jun interaction with a specific enhancer within the intron 1 of the FoxP3 gene.

A critical role for ATF2-FoxP3 pathway in anisomycin-induced apoptosis and the therapy of breast cancer. Our recent studies have shown that the induced expression of FoxP3 caused apoptosis of breast cancer cell lines (7, 11, 15). To determine whether anisomycin treatment caused apoptosis of breast cancer cells, we measured the cytotoxic effect of anisomycin on several of breast cancer cell lines, by MTT assay. As shown in Fig. 5A, both mouse (TSA) and human breast cancer cell lines (BT474, MCF-7) were highly susceptible to anisomycin, with an IC50 between 50 and 100 ng/mL. The reduced viability is due to apoptosis as revealed by the increased expression of active caspase 3 in TSA cells (Fig. 5B, top) with less than 2C DNA contents (Fig. 5B, bottom). Given the critical role for ATF2 in FoxP3 induction, we tested the contribution of ATF2 to anisomycin-induced cell death by comparing the dose response to anisomycin in cells transfected with either vector alone or those with ATF2 shRNA. As shown in Fig. 5C and D, ATF2 and FoxP3 shRNAs increased resistance to anisomycin by nearly 3-fold. These data show a critical role for the ATF2-FoxP3 pathway in anisomycin-induced cell death of breast cancer cells.

To test whether induction of FoxP3 by ATF2-FoxP3 pathway can be explored for breast cancer therapy, we injected the TSA cell line into the mammary pad. Seventeen days later, when the cancer cells established locally, the mice were treated with either vehicle control or anisomycin. As shown in Fig. 6, the growth of the TSA tumor cells in syngeneic mammary pad is abrogated by anisomycin. These data show the potential of ATF2-FoxP3 pathway in the therapeutic development for breast cancer.

Discussion

FOX3P is an X-linked gene that is subject to X-inactivation (7, 17). Our inquiry into the high incidence of spontaneous mammary tumors in mice heterozygous for the Scuffy mutation led to the identification of it as the first X-linked tumor suppressor for
breast cancer in mouse and in woman (7). FOXP3 acts as a transcriptional repressor of oncogenes such as ErbB2 and SKP2 (7, 15). Moreover, ectopic expression of FOXP3 cause an apoptosis of breast cancer cells (7). These data showed that the induction of FOXP3 in the tumor cells may prove valuable for the treatment of breast cancer.

Because the one WT allele was not irreversibly inactivated in the overwhelming majority of breast cancer samples analyzed (7), it is theoretically possible to reactivate the expression of FOXP3 locus for the treatment of breast cancer. The data presented herein showed such reactivation by anisomycin.

We observed that anisomycin, a drug commonly used to activate MAP kinases, rapidly induced FOXP3 expression in multiple breast cancer cell lines tested. Using shRNA specific for Jnk and Atf2, we showed that the Jnk and Atf2 genes are required for the induction of FOXP3 expression. Moreover, biochemical analysis allowed us to identify critical cis-element involved in the induction of FOXP3 and that this cis-element interacts with c-Jun and ATF2 to cause the activation of the FoxP3 gene. It is worth considering whether the effect of anisomycin can be related to reactivation of X-inactivated FoxP3, given recent reports of chromatin modification-dependence of c-Jun–induced transcriptions (18, 19).

Using mammary epithelial culture isolated from WT and ATF2+/− mice, we showed that the targeted mutation of Atf2 not only reduced the basal levels of FoxP3 transcripts in the mammary epithelial cells, but also eliminated its induction by anisomycin. Therefore, ATF2 plays an essential role in both constitutive and inducible expression of FoxP3. It is of great interest to note that mice heterozygous for Atf2-null allele spontaneously developed mammary tumors (10). The similarity in tumor onset suggests that the lack of FoxP3 expression may be an underlying cause for the spontaneous mammary tumors, although the Atf2+/− mice available to us is in 129/SV background, which is not suitable to address this issue.

FOXP3 is expressed in both T cells and epithelial cells (7, 20, 21). Because the majority of the Atf2−/− mice die shortly after birth (11), a systemic analysis of the effect on expression of FOXP3 in the T-cell lineage remained to be determined. However, our preliminary analysis suggested that Atf2 is not essential for FOXP3 expression in the thymocytes (data not shown). Therefore, ATF2 may play different roles in different lineages. Consistent with this notion, both cis-element and the trans-activating factors identified here differ from what were reported in FoxP3 induction in T cells (22–25).
Independent experiments. (Figure 6) Anisomycin conferred significant therapeutic effect for established mammary tumor in mice. TSA cells (5 × 10^5) were injected into mammary fat pads of syngeneic BALB/c mice. After 17 d of injection, the mice with palpable tumors were randomly grouped and treated with anisomycin or vehicle control i.p., every 2 d for 8 times, at a dose of 0.5 mg per mouse. The dose used did not give obvious side effects and was ~1/20 of LD50 in the mice. The tumor diameters were derived from the average of largest diameters in two dimensions. The tumor volumes defined as 0.75πr^3, where r is radius. A, the growth kinetics is shown; B, photographs of tumors before, and at the end of treatments were shown at the bottom. Similar therapeutic effects have been observed in three independent experiments.

Finally, an important but unresolved issue is how to reactivate tumor suppressor function in the tumor cells. Classic tumor suppressors are inactivated by two irreversible hits (2). Therefore, despite an elegant recent approach (9), restoring the function of classic tumor suppressors remains a major challenge for cancer therapy. On the other hand, recent data from our group and that of another indicated that X-linked tumor suppressor genes are subject to X-inactivation (7, 8). Because X-inactivated genes are not subject to selection during tumor growth and because deletion and mutation found in the majority of the cases are heterozygous (7, 8), it may be possible to reactivate FOXP3 for the treatment of breast cancer. In this regard, we have shown that anisomycin cause apoptosis in an ATF2- and FOXP3-dependent manner. Moreover, the doses used here do not interfere with protein translation and cause no obvious side effect, yet the drug causes dramatic inhibition of growth of established mammary tumors in syngeneic hosts. Although more work is needed to evaluate the potential for anisomycin for breast cancer treatment, our data suggest that one may be able to reactivate X-linked tumor suppressor genes for cancer treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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