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Research Article

Oncology

S-phase kinase-associated protein 2 (SKP2) is a component of the E3 ubiquitin ligase SKP1-Cul1-Fbox complex. Overexpression of SKP2 results in cell cycle dysregulation and carcinogenesis; however, the genetic lesions that cause this upregulation are poorly understood. We recently demonstrated that forkhead box P3 (FOXP3) is an X-linked breast cancer suppressor and an important repressor of the oncogene *ERBB2/HER2*. Since *FOXP3* suppresses tumor growth regardless of whether the tumors overexpress *ERBB2/HER2*, additional FOXP3 targets may be involved in its tumor suppressor activity. Here, we show that mammary carcinomas from mice heterozygous for a *Foxp3* mutation exhibited increased *Skp2* expression. Ectopic expression of *FOXP3* in mouse mammary cancer cells repressed *SKP2* expression with a corresponding increase in p27 and polyploidy. Conversely, siRNA silencing of the *FOXP3* gene in human mammary epithelial cells increased *SKP2* expression. We also show that *Foxp3* directly interacted with and repressed the *Skp2* promoter. Moreover, the analysis of over 200 primary breast cancer samples revealed an inverse correlation between FOXP3 and SKP2 levels. Finally, we demonstrated that downregulation of SKP2 was critical for FOXP3-mediated growth inhibition in breast cancer cells that do not overexpress ERBB2/HER2. Our data provide genetic, biochemical, and functional evidence that FOXP3 is a novel transcriptional repressor for the oncogene SKP2.

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FOXP3 is a novel transcriptional repressor for the breast cancer oncogene SKP2

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S-phase kinase-associated protein 2 (SKP2) is a component of the E3 ubiquitin ligase SKP1-Cul1-Fbox complex. Overexpression of SKP2 results in cell cycle dysregulation and carcinogenesis; however, the genetic lesions that cause this upregulation are poorly understood. We recently demonstrated that forkhead box P3 (FOXP3) is an X-linked breast cancer suppressor and an important repressor of the oncogene ERBB2/HER2. Since FOXP3 suppresses tumor growth regardless of whether the tumors overexpress ERBB2/HER2, additional FOXP3 targets may be involved in its tumor suppressor activity. Here, we show that mammary carcinomas from mice heterozygous for a *Foxp3* mutation exhibited increased *Skp2* expression. Ectopic expression of FOXP3 in mouse mammary cancer cells repressed SKP2 expression with a corresponding increase in p27 and polyploidy. Conversely, siRNA silencing of the FOXP3 gene in human mammary epithelial cells increased SKP2 expression. We also show that *Foxp3* directly interacted with and repressed the *Skp2* promoter. Moreover, the analysis of over 200 primary breast cancer samples revealed an inverse correlation between FOXP3 and SKP2 levels. Finally, we demonstrated that downregulation of SKP2 was critical for FOXP3-mediated growth inhibition in breast cancer cells that do not overexpress ERBB2/HER2. Our data provide genetic, biochemical, and functional evidence that FOXP3 is a novel transcriptional repressor for the oncogene SKP2.

Introduction

Cancer pathogenesis involves both the inactivation of tumor suppressor genes and the activation of oncogenes (1, 2). One of the most fascinating aspects of cancer biology is the interaction between cancer suppressor genes and oncogenes. Most of these interactions are at posttranslational levels. For instance, proteins encoded by tumor suppressor genes can inactivate oncogenes. One of the most clearly studied cases is tumor suppressor Rb, which inhibits the E2F family members of oncogenes (1, 2). Conversely, oncogenes can overcome the tumor suppressor proteins. For example, S-phase kinase-associated protein 2 (SKP2) causes the degradation of tumor suppressor FOXO (3) as well as CDK inhibitors, such as p27 (4, 5). It is less clear whether such antagonism exists at the transcriptional level. However, a recent study suggests that FOXO may repress the expression of *cyclin D* (6), a well-known oncogene. Likewise, we have recently demonstrated that forkhead box P3 (FOXP3), an X-linked tumor suppressor, represses transcription of ERBB2/HER2 oncogene (7).

The high-level expression of SKP2 was reported in a significant proportion of cancers (5). SKP2 is a component of the E3 ubiquitin ligase SCF with specificity for CDK inhibitor p27. However, under physiological conditions, Skp2 expression is maximal at the S and G2 phases and appears to primarily mediate p27 degradation at the G2 (8, 9) but not G0 and G1 phases (10). As such, Skp2 was found to be essential for progression into mitosis in cell cycles (4, 9, 11). Targeted mutation of *Skp2* causes delayed animal growth and cellular polyploidy, which were prevented by an additional

deletion of *p27*^{-/-} mice (8, 9). The increased expression of SKP2 has been shown in nearly 50% of breast cancers, especially among those with early onset and poor prognosis (12–15). The oncogenic effect of Skp2 overexpression is substantiated by cancer development in *Skp2* transgenic mice (16, 17). However, genetic lesions responsible for SKP2 overexpression in cancers remain largely undefined although gene amplification of SKP2 has recently been shown in non-small cell lung carcinomas (18). The transcriptional regulation of SKP2 has also been studied recently (19, 20), which paves the way to study whether aberrant genetic alterations on some specific transcriptional regulators of SKP2 could cause overexpression of this oncogene in cancers.

FOXP3 is a new member of the Forkhead/winged helix family of the transcription factors and was identified by position cloning as the causative mutation for lethal autoimmune diseases (21–24). In the T cell lineage, mutation of the *Foxp3* gene resulted in ablation of the suppressor function of Tregs although it is still debatable whether such mutation ablates the Treg lineages (25), as was originally proposed (26, 27). In addition, we reported that *Foxp3* mutation in nonhematopoietic cells, most likely the thymic epithelial cells, is necessary and sufficient to cause abnormal thymopoiesis (28). In support of an epithelial intrinsic function of *Foxp3*, we have recently observed that a germline mutation of *Foxp3* resulted in a high rate of spontaneous breast cancer and increased susceptibility to carcinogens in the mouse (7). In addition, widespread deletion and somatic mutations of FOXP3 have also been observed in human breast cancer samples. While we have identified FOXP3 as a transcriptional repressor of the HER2/ERBB2 oncogene (7), FOXP3 also suppressed growth and induced the cell death of MCF-7, a breast cancer line without HER2/ERBB2 overexpression (7). Therefore, it is likely that *Foxp3* may affect other pathways involved in breast cancer. Given the role of Skp2 in breast cancer (12–15), we determined whether *Foxp3* may regulate its expression

Nonstandard abbreviations used: ChIP, chromatin immunoprecipitation; FOXP3, forkhead box P3; HMEC, human mammary epithelial cell; SKP2, S-phase kinase-associated protein 2.

Conflict of interest: The authors have declared that no conflict of interest exists.

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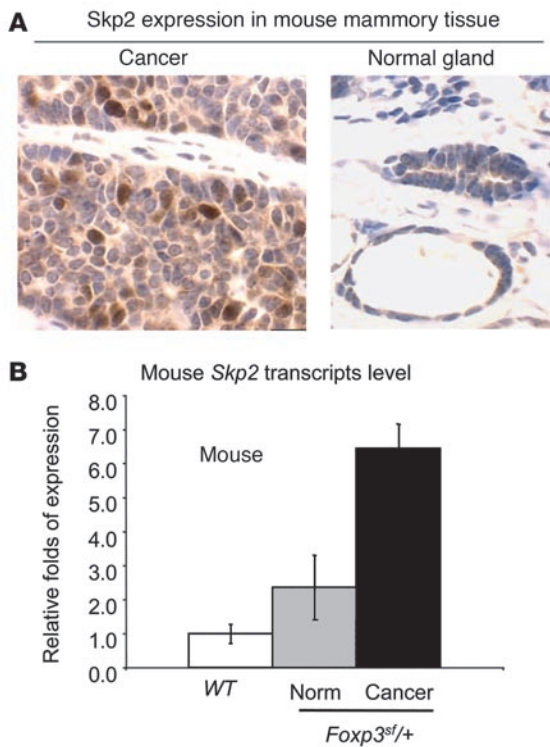


Figure 1

A naturally occurring mutation in the *Foxp3* locus results in increased *Skp2* expression. (A) Immunohistochemical staining of the *Skp2* protein in mammary cancers and an adjacent normal mammary gland in 1 *Foxp3*^{sl/+} mouse. Original magnification, ×40. (B) Relative levels of *Skp2* transcripts in normal mammary epithelium of WT and *Foxp3*^{sl/+} mice and the cancerous tissue in the *Foxp3*^{sl/+} mice, as revealed by real-time RT-PCR of LCM samples. The expression of *Skp2* was normalized against the internal control, the *Hprt* gene. Data shown are means ± SD of 3 samples in each group. Highly significant differences were observed between cancerous and normal tissue ($P < 0.001$, ANOVA test when either internal standards were used).

in normal and malignant breast epithelial cells. Here, we report that *Foxp3* is a transcriptional repressor of *Skp2*, a breast cancer oncogene. These data indicate that defect in *FOXP3* is a potential mechanism for *SKP2* upregulation in breast cancer and illustrate an intriguing interplay between the oncogenes and tumor suppressor genes in cell-cycle regulation and carcinogenesis.

Results

Inactivation of the Foxp3 locus resulted in increased Skp2 expression. We have reported that mice heterozygous for the *Foxp3* gene developed spontaneous mammary cancer at high rates (7). Moreover, while most of the *Foxp3* transcripts in the normal epithelial cells were from WT alleles, all of the transcripts from the cancerous tissues were transcribed from the mutant allele. Thus, in the cancer cells, the *Foxp3* locus is silenced. To determine whether *Foxp3* represses *Skp2* expression, we stained the normal and cancerous mammary tissues with anti-*Skp2* and anti-p27 antibodies. As shown in Figure 1A, *Skp2* was found to be highly expressed in cancer cells but not in normal epithelial cells from the same mouse. To quantify increases in *Skp2* transcripts, we isolated cells from frozen sections by laser microdissection and extracted mRNA for real-time RT-PCR analysis. We compared the expression of *Skp2* in normal mammary epithelial cells from either WT or *Foxp3*^{sl/+} mice as well as mammary cancer tissues from mutant mice. As shown in Figure 1B, in comparison with the WT epithelial cells, the heterozygous epithelial cells expressed 2-fold higher levels of *Skp2*, which suggests a *Foxp3* gene dose effect on the levels of *Skp2*. Moreover, in the cancerous tissue that has silenced the WT allele (7), expression of *Skp2* was substantially enhanced.

A potential caveat of this interpretation is that upregulation of *SKP2* may be due to cancer rather than to the silencing of the *Foxp3* locus. Although the WT mice had lower incidences and later onsets of mammary cancer than the heterozygous mice, cancer did

arise, both spontaneously and in response to carcinogen treatment (7). Thus, by comparing mouse mammary cancer tissues from WT and *Foxp3*^{sl/+} mice for expression of *Skp2*, one may be able to discern the contribution of *Foxp3* mutation versus the nonspecific effect of cancer growth. As shown in Table 1, 80% of the spontaneous cancers in the WT mice did not overexpress *Skp2*. In contrast, 71% of the spontaneous tumors from the *Foxp3*^{sl/+} mice did. A similar trend was observed in the carcinogen-induced mammary tumors. Thus, inactivation of the *Foxp3* locus is likely responsible for increased *Skp2* expression in the mammary tumors.

Foxp3 as a transcriptional repressor of Skp2. Since *Foxp3* is a transcription factor capable of repressing or promoting the expression of a large cohort of genes (29, 30), we evaluated whether *Skp2* can be a direct target of *Foxp3*. We first transfected a mouse mammary cancer line, TSA, with the V5-tagged *Foxp3* protein and generated a polyclonal *Foxp3*-V5, CL30, and 2 subclones, CL302 and CL305. Using real-time PCR analysis, we found that the CL302 and 305 have approximately 5-fold higher *Foxp3* transcript than the CL30 line. *Skp2* transcripts were found to decrease by around 10- to 20-fold in the *Foxp3*-V5 transfectant line or clones compared with the vector control. The extent of reduction correlated with the *Foxp3* transcript levels. In contrast, no changes in *p27* mRNA levels were detected (Figure 2A). Since *Skp2* regulates the degradation of *p27*, we also examined the levels of these 2 proteins in *Foxp3*-V5 transfectants. As shown in Figure 2B, *Foxp3* transfection dramatically reduced *Skp2*. Correspondingly, *p27* was significantly increased in the *Foxp3*-V5 transfectant. To determine whether the increase of *p27* was caused by more rapid degradation, we treated vector

Table 1

Inactivation of the *Foxp3* locus contributed to *Skp2* elevation in mouse mammary tumors

	Spontaneous tumor		Carcinogen-induced tumor ^A	
	WT (n = 5)	<i>Foxp3</i> ^{sl/+} (n = 7)	WT (n = 10)	<i>Foxp3</i> ^{sl/+} (n = 12)
Skp2				
(-)	4	2	7	4
(+)	1	5	3	8

Arabic numerals indicate the number of mice carrying mammary tumors. ^A7,12-Dimethylbenz[*a*]anthracene was used to induce mammary tumorigenesis in mice. Detailed protocol has been described previously (7). χ^2 test revealed that the difference in frequency of *SKP*⁺ cases between the tumors (including both spontaneous and chemically induced) from the *Foxp3*^{sl/+} mice and the WT littermates was significant ($P = 0.0152$).

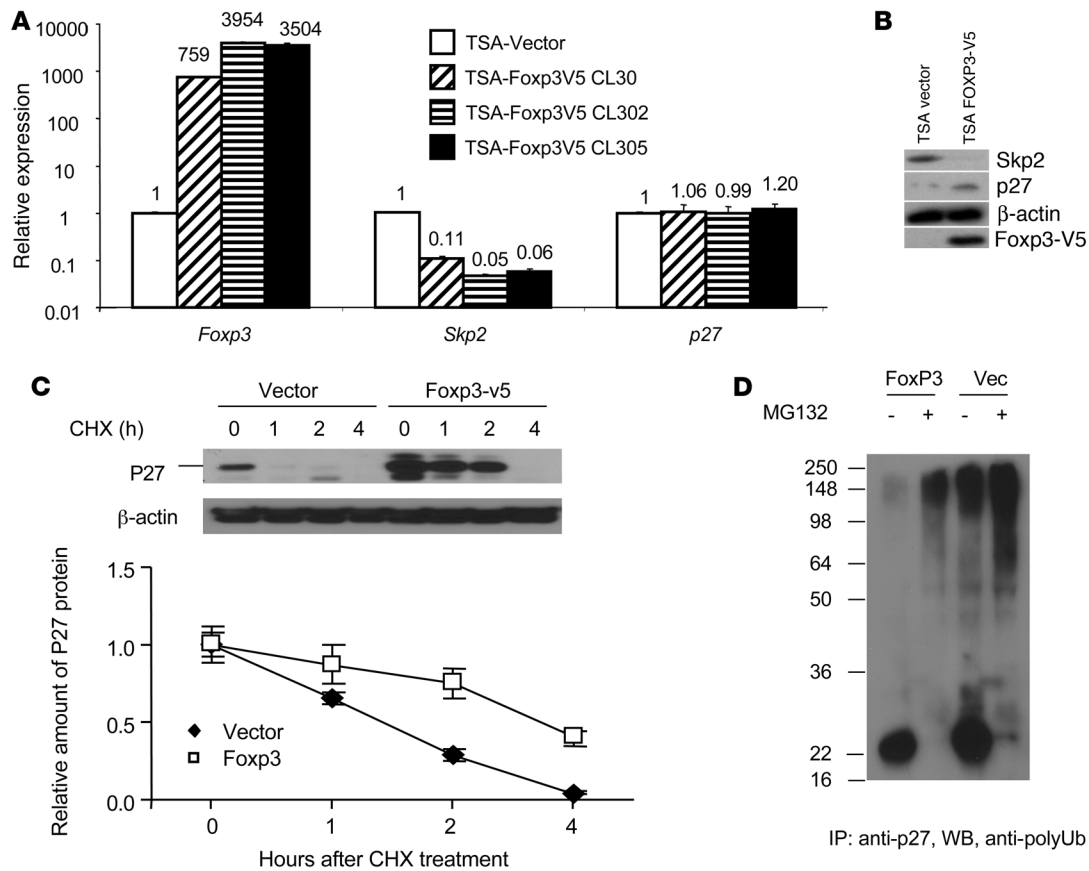


Figure 2

Foxp3 represses *Skp2* transcription. (A) Transfection of *Foxp3*-V5 into TSA cells repressed expression of the *Skp2* gene. The mRNA levels of *Foxp3*, *Skp2*, and *p27* were measured by real-time PCR for the vector control cells, polyclonal *Foxp3*-V5 transfectants (CL30), and 2 stable *Foxp3*-V5 transfectant clones, CL302 and CL305. Data shown are relative amounts of transcripts after normalizing against the amounts of total RNA based on the levels of *Hprt* mRNA. The means of the vector group are artificially defined as 1.0. Data shown are means \pm SD of 3 independent experiments. (B) *Foxp3* reduces *Skp2* with a corresponding increase in *p27*. Lysates of *Foxp3*-V5 or vector-transfected TSA cell lines were analyzed by Western blot using SKP2, *p27*, β -actin, and anti-V5 (which recognize V5-tagged *Foxp3*) antibodies. (C) *Foxp3* increased stability of *p27*. Vector or *Foxp3*-V5 transfectants were treated with cycloheximide (CHX, 100 μ M) for the indicated intervals. Cells were collected and *p27* protein levels were detected by Western blot. The upper panel shows representative experiments while the lower panel shows the decay of *p27*, using time 0 as 1.0. Protein loading equivalence was assessed by the expression of β -actin. The relative intensity of bands was measured relative to their respective β -actin bands using BandScan software (version 4.3; Glyco). (D) Polyubiquitination of the *p27* in vector or *Foxp3* transfectants. Vector or *Foxp3*-V5-transfected TSA cell lines were either left untreated or treated with proteasome inhibitor MG132 (40 μ M) for 3 hours. Equal aliquot of the cellular lysates were immunoprecipitated with anti-*p27* antibodies. The precipitates were separated by SDS-PAGE and transferred into nitrocellulose membrane, which were then autoclaved in water for 30 minutes and blotted with HRP-conjugated anti-polyubiquitin antibody Ub P4D1 (33).

or *Foxp3*-V5-transfected TSA cells with cycloheximide and measured the levels of *p27* at 0, 1, 2, and 4 hours after treatment by Western blot. As shown in Figure 2C, *p27* was degraded at a much faster rate in the vector-transfected TSA cells. Consistent with this notion, we observed reduced ubiquitination of *p27* in the *Foxp3*-transfected cells (Figure 2D).

To further confirm that the downregulation of *Skp2* by *Foxp3* occurred at the transcription level, we cloned the 2.0-kb region upstream of the murine *Skp2* gene into the luciferase reporter vector pGL2 and tested the effects of *Foxp3* of this promoter's activity by luciferase assay. As shown in Figure 3A, *Foxp3* substantially repressed the promoter activity of the *Skp2* gene.

Analysis of the *Skp2* promoter revealed 4 potential binding sites within the 2 kb promoter region (Figure 3B). We carried out

chromatin immunoprecipitation (ChIP) to determine whether *Foxp3* binds to the promoter. The nuclear preparations from the *Foxp3*-transfected cells were fixed with paraformaldehyde. After sonication, the *Foxp3*-associated genomic DNA was immunoprecipitated and quantitated by real-time PCR. To avoid artifacts associated with differential amplification, we compared the quantity of precipitated DNA with the total input of genomic DNA, amplified by the same pairs of primers. In addition, the small amount of DNA precipitated by the IgG control was subtracted. As shown in Figure 3B, the primers corresponding to the -0.8-kb and -1.2-kb regions yielded significant amounts of product, which was equal to 5%–6% of input DNA. In contrast, those corresponding to either the -2.2- or +0.6-kb region yielded no specific signal.

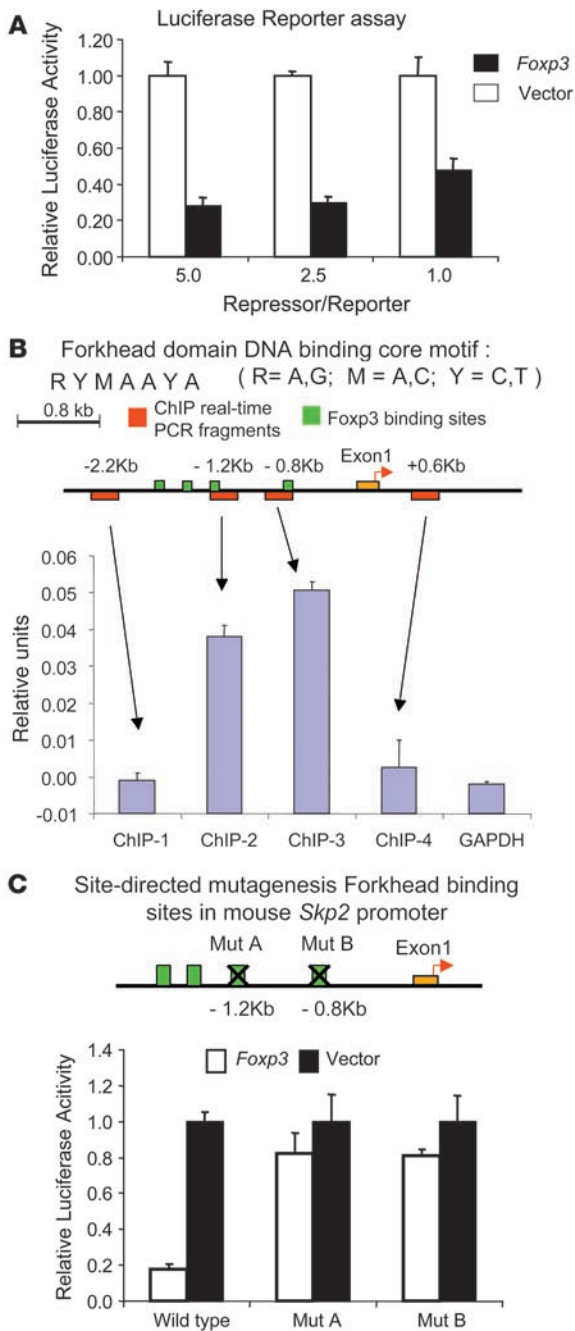


Figure 3

Foxp3 binding to *Skp2* is important for transcriptional repression. (A) *Foxp3* represses mouse *Skp2* promoter activity. Either *Foxp3* cDNA or empty vector was transiently cotransfected with reporter vector at different ratios illustrated in the figure. Cells were transfected with either vector control or *Foxp3* (1 µg/well) in conjunction with the luciferase reporter driven by 5' promoter regions of the *Skp2* gene (0.2 µg, 0.4 µg and 1.0 µg per well). Forty-eight hours later, the cell lysates were harvested and measured for luciferase activity. The luciferase activity from the cells transfected with the pGL2-basic vector was arbitrarily defined as 1.0. Data shown are means ± SD of triplicates and have been repeated at least 3 times. (B) The upper panel depicts the 5' region of the *Skp2* gene. The lower panel shows the amount of DNA precipitated by anti-V5 mAbs after subtracting a minute portion precipitated by IgG control. The data shown are a fraction of the total genomic DNA isolated from the same number of cells. (C) Deletion of 1 of the 2 *Foxp3*-binding sites in the *Skp2* promoter region prevented FOXP3-mediated suppression. The deleted sequences are mut A: ACTAAAC-CAATATTCTAAT and mut B: TAAAAATAAACCATC. The promoter activity was measured in the human breast cancer line T47D.

function. We chose a *Foxp3*-transfected TSA cell line with moderate levels of the *Foxp3*-V5 protein to test the effect of *Foxp3* expression (Figure 4A) on the cellular function of *Skp2* in order to avoid possible artifacts associated with overexpression. Real-time PCR revealed that the levels of *Foxp3* transcripts in the stable transfectants is about 4.5-fold that of the ex vivo mammary epithelial isolates after normalizing against *Ck19* transcripts (Figure 4A). Since not all mammary epithelial cells express *Foxp3*, the difference between the transfectants and physiological levels of normal cells is likely to be even smaller. As shown in Figure 4B, only slightly more than 50% of the transfectants had demonstrable levels of the *Foxp3*-V5 fusion protein. This allowed us to compare the DNA contents of the *Foxp3*^{hi} and *Foxp3*^{lo} subsets from the same culture in addition to comparing them with control vector transfectants. As shown in Figure 4B, less than 1% of the control vector-transfected cells had >4C DNA content, as expected. The same pattern was observed in the *Foxp3*^{lo} subset from the *Foxp3* transfectants. In contrast, about 25% of the *Foxp3*^{hi} cells had >4C DNA contents. To determine whether the polyploidy can be attributed to downregulation of *Skp2*, we ectopically expressed the *Skp2* cDNA in the *Foxp3*-V5 transfectants. As shown in Figure 4C, the ectopic expression of *Skp2* significantly reduced the percentage of cells with polyploidy. These data demonstrate that, by suppressing *Skp2* expression, *Foxp3* has a very significant impact on cell cycle progression.

Foxp3 and *SKP2* expression in normal and malignant human breast epithelial cells. A critical issue is whether *Foxp3* expression regulates *SKP2* in human breast epithelial cells. To substantiate that inactivation of *FOXP3* is a primary event leading to overexpression of *SKP2*, we transduced the early passage of normal human mammary epithelial cells (HMECs) with lentiviral vector encoding siRNA specific for *FOXP3* or control lentiviral vector. The untransduced cells were eliminated by blasticidin. As shown in Figure 5A, the *FOXP3* siRNA transduction caused a more than 100-fold reduction in the *FOXP3* transcript. Corresponding to this, a 4-fold increase of the *SKP2* transcripts was observed (Figure 5B). These data demonstrate that in HMECs, *FOXP3* is an important regulator for the *SKP2* gene.

To identify *FOXP3* targets in malignant breast epithelial cells, we produced cell lines with the inducible expression of *FOXP3* from MCF-7, a human mammary cancer cell line that does not overexpress the *HER2* oncogene, as diagrammed in Figure 6, upper

To determine the significance of the interaction, we determined whether deletion of either binding site disrupted the repression of promoter activity by *Foxp3*. As shown in Figure 3C, while the WT promoter was repressed by *Foxp3*, deletion of either site eliminated the repression. Thus, data presented in this section demonstrate that the binding of *Foxp3* to specific sites in the *Skp2* promoter is essential for *Foxp3* repression of *Skp2* expression.

Foxp3 expression caused polyploidy of breast cancer cell lines. One of the clearly defined functions of *Skp2* is to regulate the p27 levels at the G2/M phase in the cell cycle (4, 9). As a manifestation of defective G2/M transition, liver cells in the *Skp2*-deficient mice showed drastically increased polyploidy (8, 9). Therefore, the percentage of cells with polyploidy can be used as a valuable parameter for *Skp2*

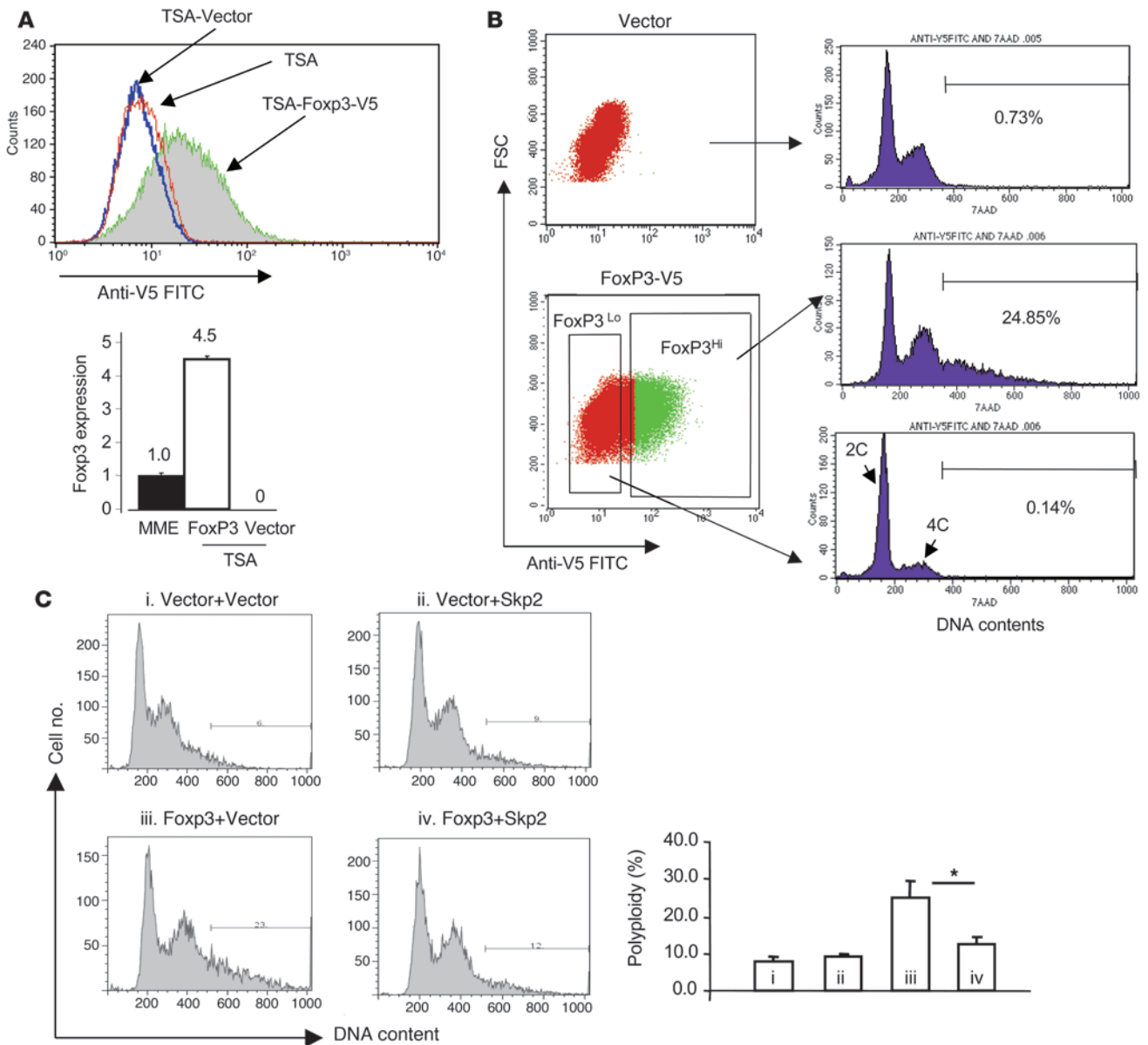


Figure 4

Polyplody of the Foxp3^{hi} but not the Foxp3^{lo} mouse mammary cancer cells. **(A)** Modest Foxp3-V5 expression in the Foxp3 transfectant compared with either untransfected parental cells or control vector-transfected cells. Data shown in the upper panel are histograms depicting Foxp3-V5 protein levels, while those in the lower panel show levels of *FoxP3* mRNA in Foxp3-V5- or vector-transfected TSA or LCM captured mouse mammary epithelial cells (MME), as measured by quantitative real-time PCR. **(B)** Foxp3 expression caused polyplody. Left panel shows the level of the Foxp3-V5 fusion protein in vector control (top) or Foxp3-transfected TSA cells (bottom) while the right panel shows the DNA contents of vector control (top), Foxp3^{hi} (middle), and Foxp3^{lo} cells (bottom). **(C)** Ectopic expression of Skp2 alleviated polyplody induced by Foxp3. Vector (i, ii) or Foxp3 (iii, iv) transfectants of TSA were transfected with either vector control or Skp2. After removing untransfected cells with blasticidin, the transfectants were fixed with Cytofix/Cytoperm buffer (BD) and tested with DNA contents using 7-AAD. Representative profiles are shown in the left and middle panels while summary data from 3 independent experiments are shown in the right panel. **P* < 0.05.

panel. We analyzed the expression of *SKP2* at different time points after the cells were cultured in the absence of doxycycline, which induced the expression of FOXP3 (7). The levels of *SKP2* were quantitated by real-time PCR and were compared with control cell lines expressing GFP but not FOXP3 under the same conditions. The relative levels of the *SKP2* transcripts of the control cell lines and the FOXP3-expressing cells at different times are presented in

Figure 6, lower panel. Using the levels of uninduced cells as references, we observed nearly a 4-fold reduction of *SKP2* mRNA within 24 hours of removing doxycycline in the FOXP3 transfectants. By 48 hours, more than an 8-fold reduction was observed. No reduction of *SKP2* transcript was observed in control cell lines cultured under the same conditions. These data demonstrate a rapid repression of the *SKP2* transcripts following FOXP3 induction.

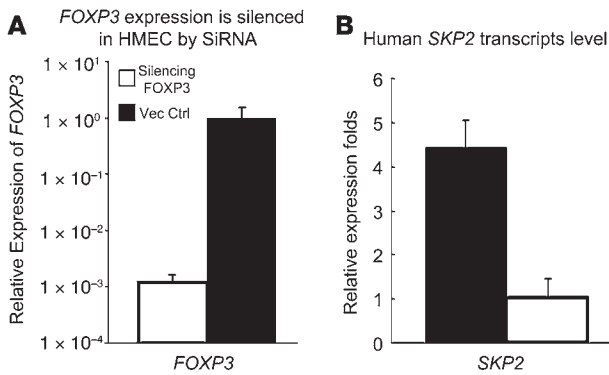


Figure 5

Silencing of *FOXP3* resulted in upregulation of *SKP2* in primary HMEC. *FOXP3* was silenced in HMEC by using siRNA. *FOXP3* and *SKP2* transcripts were quantified by real-time PCR. The RNA inputs were normalized against housekeeping gene *GAPDH*. Data shown are means ± SEM of relative levels of *FOXP3* and represent 3 independent experiments ($P < 0.01$, Student's *t* test). In **A**, the vector control was defined as 1.0; in **B**, silenced cells were defined as 1.0. Data shown are means ± SD of triplicates and represent 3 independent experiments.

We have reported that the *FOXP3* locus is frequently inactivated in the majority of, although not all, mammary cancer tissues in humans. On the other hand, *SKP2* is overexpressed in nearly 50% of the breast cancer samples (12–15). If a loss of *FOXP3* contributes to *SKP2* expression, one may expect an increased rate of the *SKP2*⁺ samples among the *FOXP3*⁻ tumors. To address this issue, we independently stained and scored in a double-blinded fashion 206 cases of breast cancer samples in tissue microarray for their expression of *SKP2* and *FOXP3*. As shown in Figure 7, among the *FOXP3*⁺ samples, less than 30% of the cells expressed *SKP2*. In contrast, more than 56% of the *FOXP3*⁻ samples showed *SKP2* overexpression. Statistical analysis revealed that the difference was highly significant ($P = 0.0016$).

cancer, especially breast cancer (5). Overexpression of *SKP2* is associated with a poor prognosis in breast cancer and is prevalent in cancer that develops in young women (15). While overexpression of *Skp2* has been reported in multiple lineages of cancer and may confer resistance to antisteroid therapy (5, 15), the mechanisms for its overexpression have not been well understood. Here, we demonstrate that *Foxp3*, which we have recently demonstrated to be the first X-linked breast cancer suppressor gene, to our knowledge, is, we believe, a novel transacting regulator for *Skp2* expression. Our conclusion is based on 4 lines of evidence.

The ectopic expression of SKP2 bypass FOXP3-mediated growth inhibition for a HER2^{lo} breast cancer cell line. Our previous studies demonstrated that *FOXP3* can suppress the growth of both *ERBB2*^{hi} and *ERBB2*^{lo} tumor cell lines. While the repression of *ERBB2*^{hi} tumor cell line TSA can be rescued by the ectopic expression of *ERBB2*, the target responsible for growth inhibition of the *ERBB2*^{lo} tumor cells remained to be identified. To determine the relevance of *SKP2* repression in growth inhibition by *FOXP3*, we ectopically expressed either vector or *SKP2* into the MCF-7 cell line with TetOff inducible expression of *FOXP3*. The impact of the *SKP2* expression was visualized by colony formation following TetOff induction of *FOXP3*. As shown in Figure 8, in the vector-transfected group, TetOff induction of *FOXP3* wiped out all MCF-7 colonies, as expected. Remarkably, ectopic expression of *SKP2* resulted in almost complete restoration of the colonies (Figure 8, lower panel) although the colony size is still somewhat less than in the culture without *FOXP3* induction (Figure 8, upper and middle panels). These results demonstrate a critical role of *SKP2* downregulation in the *ERBB2*^{lo} breast cancer cell line.

First, inactivation of the *Foxp3* locus resulted in the elevation of *Skp2* expression. Mammary epithelial cells from mice heterozygous for *Foxp3* mutation had 2-fold higher levels of *Skp2*. Since *Foxp3* is an X-linked gene and is dose compensated (27), the elevation was likely due to *Skp2* overexpression among the cells

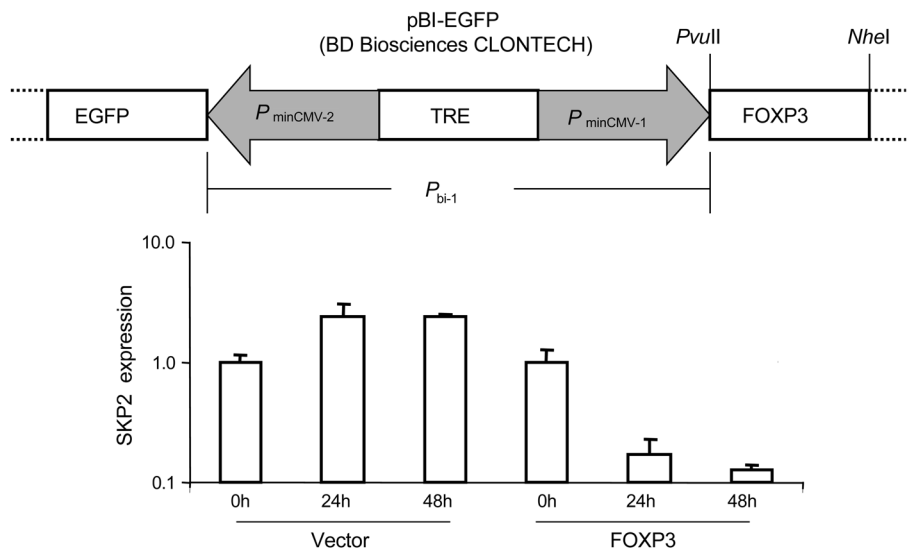
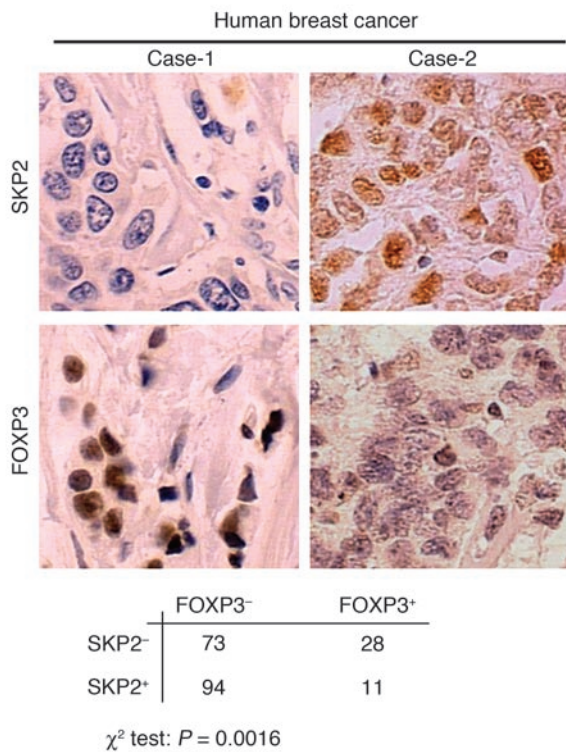


Figure 6

Inducible *FOXP3* expression in TetOff breast cancer cells rapidly downregulated *SKP2* expression. Upper panel shows a diagram depicting the promoter structure in pBI-EGFP vector. The bidirectional promoter P_{bi-1} was responsive to the tTA regulatory proteins in the TetOff system. P_{bi-1} contains the tetracycline-responsive element (TRE) that is between 2 identical minimal CMV promoters (P_{minCMV}). EGFP is at one side while human *FOXP3* cDNA was inserted at the *PvuII*/*NheI* sites in the opposite direction. A control vector with GFP but not *FOXP3* cDNA was also used. Lower panel presents kinetics of *SKP2* repression by *FOXP3*. *FOXP3* was induced by the withdrawal of doxycycline in medium. The transcript levels were first normalized for RNA input by using *GAPDH* and then compared with those observed in the control cell lines, defined as 1.0.

Discussion

As a component of the SCF E3 complex, *SKP2* has emerged as an important oncogenic protein for several major types of



that expressed the mutant allele. More importantly, the spontaneous mammary cancer that developed in the *Foxp3* mutant mice, which had completely inactivated the *Foxp3* locus, showed drastically higher levels of *Skp2* transcripts. This is further supported by the fact that mammary cancers observed in WT BALB/c mice showed much less *Skp2* overexpression.

Second, transfection of *Foxp3* into mammary cancer cell lines repressed the levels of *Skp2* mRNA. Using an *Skp2* promoter-driven luciferase reporter, we demonstrated that *Foxp3* significantly repressed the promoter activity. Moreover, ChIP assay demonstrated that Foxp3 binds to specific regions of *Skp2*. The significance of the binding sites was demonstrated by the fact that deletion of either site ablated the repression.

Third, transfection of *Foxp3* resulted in an increase of p27, the major target of *Skp2*. The increase of p27 was likely restricted to the G2/M phase, as a high proportion of *Foxp3*-expressing cells showed polyploidy, which was also observed in the hepatocytes of

Figure 8

Ectopic expression of *Skp2* substantially alleviates growth inhibition of MCF-7 cell lines by FOXP3. MCF-7 cell lines with inducible expression of either GFP (iii, iv) or FOXP3 (i, ii) were supertransfected with either vector control (i, iii) or SKP2 (ii, iv). After removing untransfected cells by drug selection, the cultures were maintained in tetracycline-free conditions for 2 weeks. Upper and middle panels show photographs of viable (ii, iii, iv) or apoptotic MCF-7 cells. Original magnification, $\times 10$. The lower panel shows colony numbers per 100-mm² plate. At the end of 2 weeks of culture, the dead cells were removed and the plates were stained with violet crystal. The visible colonies were counted. Data shown are means of SD of triplicates and are representative of 3 independent experiments. $**P < 0.001$ when group I and II were compared by Student's *t* test.

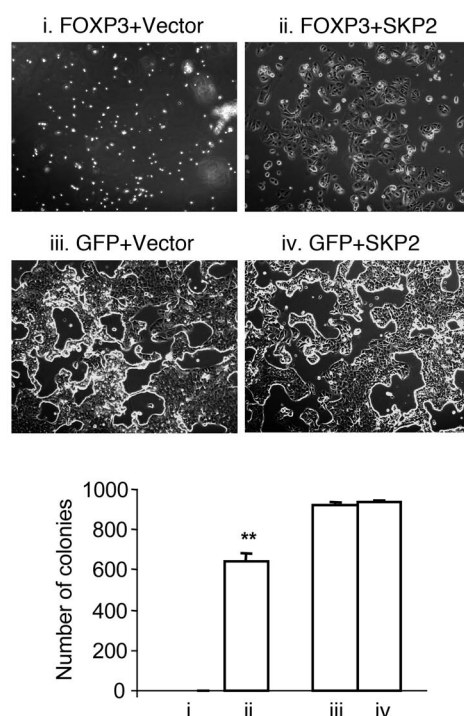
Figure 7

Significant reduction in the rate of SKP2 upregulation in the FOXP3⁺ breast cancer samples. Tissue microarray samples of human breast cancer were stained with either anti-FOXP3 antibody or anti-SKP2 antibody. The samples were scored in a double-blind fashion. The top panels show staining patterns of either FOXP3 or SKP2 in 2 representative cases. Original magnification, $\times 60$. Summary data from 206 independent cases are presented in the lower panel. The P values of the χ^2 tests are listed.

the *Skp2*-deficient mice (8, 9). These results indicate that *Foxp3* serves as an important regulator for cell cycle progression.

Last, we have demonstrated that the *Foxp3*-mediated repression of SKP2 occurred in normal and malignant breast epithelial cells in humans. Thus, silencing the *FOXP3* gene by siRNA in primary mammary epithelial cells resulted in a significant increase of the *SKP2* transcript, while the induced expression of FOXP3 in breast cancer cell lines caused a time-dependent repression of *SKP2* expression. More importantly, our analysis of 206 clinical samples demonstrated a substantially reduced rate of SKP2 overexpression among the FOXP3⁺ breast cancer samples. These data indicate that FOXP3-mediated repression likely serves as an important guardian for overexpression of SKP2 in vivo. It should be noted that, as is often observed among clinical samples, there is not a 1:1 correlation between FOXP3 expression and the absence of SKP2 in breast cancer. Likewise, not all FOXP3⁻ cancers overexpress SKP2. Thus, in addition to FOXP3, other factors also contribute to SKP2 expression. It is worth noting that in non-small cell lung carcinomas, overexpression of *Skp2* is associated with the amplification of the *Skp2* gene (18). Additional studies are needed to determine whether FOXP3 can attenuate SKP2 expression in the case of gene amplification, as we have demonstrated in the HER2 gene amplification (7).

Taken together, genetic, biochemical, and functional analysis provides strong evidence that *Foxp3* is a transcriptional repressor of





the *SKP2* oncogene. Identification of a functionally important non-Her2 target explains why Foxp3 also represses the growth of MCF-7, a breast cancer cell line without HER2/ERBB2 overexpression.

Methods

Cell lines, experimental animals, and human tissue microarrays. HMECs (HMEC-1; CC-2551) and mammary epithelial cell growth medium (MEGM; CC-3051) were purchased from Cambrex Clonetics. TSA is an aggressive and poorly immunogenic cell line established from the first in vivo transplant of a moderately differentiated mammary adenocarcinoma that arose spontaneously in a BALB/c mouse (31). Breast cancer cell line MCF-7 was purchased from ATCC.

The female mice heterozygous for the *Scurfy* mutations (*Foxp3^{fl/+}*) were backcrossed to the BALB/c background for at least 12 generations at the University of North Carolina (Chapel Hill, North Carolina, USA). The animals were housed at the University Laboratory Animal Facility at The Ohio State University and the University of Michigan under specific pathogen-free conditions. Spontaneous tumor and carcinogen-induced tumor incidence observation were reviewed and approved by The Ohio State University and University of Michigan Institutional Animal Care and Use Committees.

The normal and cancerous breast tissues were obtained from The Ohio State University Department of Pathology Tissue Bank, Zymed Laboratories Inc., and US Biomax Inc.

Antibodies. Affinity purified rabbit anti-Foxp3 antibodies, specific for the FOXP3 N terminus peptide starting at position 25 ([C]LLGTRGS GGPFQGRDLRSGAH), have been described (28) as well as antibodies for immunohistochemical staining and Western blotting (p27 [M-197, 1:500, Santa Cruz Biotechnology Inc.], SKP2 [H-435, 1:100, Santa Cruz Biotechnology Inc.]).

FOXP3-silencing lentiviral vector. The lentivirus-based siRNA-expressing vectors were created by introducing the murine U6 RNA polymerase III promoter and a murine phosphoglycerate kinase promoter-driven (pGK) EGFP expression cassette into a vector of pLenti6/V5-D-TOPO backbone without CMV promoter. A hairpin siRNA sequence of *FOXP3* (target sequence at the region of 1256 to 1274 nucleotides; 5'-GCAGCGGACACT-CAATGAG-3') was cloned into the lentiviral siRNA-expressing vectors by restriction sites of *ApaI* and *EcoRI*.

ChIP. ChIP was carried out according to a published procedure (32). In brief, the Foxp3-V5-transfected TSA cells were sonicated and fixed with 1% paraformaldehyde. The anti-V5 antibodies or control mouse IgG were used to pull down chromatin associated with Foxp3-V5. 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 following are the ChIP real-time PCR primers from upstream *Skp2*: ChIP-1 forward, TGTGATGGGCACACATACAG; reverse, TGTCTCT-

GGAAGCCTCAGC; ChIP-2 forward, CGAATCTTGCTCTCTCCACA; reverse, CATGCAAAATTCAGGTGTGC; ChIP-3 forward, GGACAGGCTGTGGATTGAGT; reverse, CCAAGAGGAGCGATGGTTTA; and ChIP-4 forward, TGCTGGGACTTTTCTCCACT; reverse, AGACACCCATGCCTGATAGC; *Skp2* forward, TTAGTCGGGAGAAGTTTCCAGGTG; *Skp2* reverse, AGTCACGTCTGGGTGCAGATTT.

Immunohistochemistry. Expression of Foxp3 and Skp2 in mouse and human breast cancer samples was determined using immunohistochemistry as described. The samples consisting of more than 5% cells with nuclear expression of Foxp3 were scored as positive. SKP2 scoring followed the previous description (12–15): those with more than 5% of either nuclear-positive or cytoplasm-positive staining accumulations were scored as Skp2⁺. FOXP3 and SKP2 staining were scored double blinded.

Colony formation assay. The colony formation assay was carried out as we have described previously (7).

Antibodies, immunoprecipitation, and Western blot. Antibodies specific for SKP2 (Santa Cruz Biotechnology Inc.) and p27 (Cell Signaling) were used for immunoprecipitation and/or Western blot. A special procedure was used to detect polyubiquitinated p27, according to what was reported by Ji et al. (33), using HRP-conjugated ubiquitin (Ub P4D1) (sc-8017, Santa Cruz Biotechnology Inc.).

Statistics. Comparisons between 2 groups were performed using unpaired 2-tailed Student's *t* test. ANOVA test was used for analysis of variance between several groups. χ^2 test was used for analysis. All values are presented as mean \pm SD. Differences between groups were considered statistically significant at *P* < 0.05.

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