The Regulatory T Cell–Associated Transcription Factor FoxP3 Is Expressed by Tumor Cells

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Abstract

FoxP3 is a member of the forkhead family of transcription factors critically involved in the development and function of CD25$^+$ regulatory T cells (Treg). Until recently, FoxP3 expression was thought to be restricted to the T-cell lineage. However, using immunohistochemistry and flow cytometric analysis of human melanoma tissue, we detected FoxP3 expression not only in the tumor infiltrating Treg but also in the melanoma cells themselves. FoxP3 is also widely expressed by established human melanoma cell lines (as determined by flow cytometry, PCR, and Western blot), as well as cell lines derived from other solid tumors. Normal B cells do not express FoxP3; however, expression could be induced after transformation with EBV *in vitro* and *in vivo*, suggesting that malignant transformation of healthy cells can induce FoxP3. In addition, a *FOXP3* mRNA variant lacking exons 3 and 4 was identified in tumor cell lines but was absent from Treg. Interestingly, this alternative splicing event introduces a translation frame-shift that is predicted to encode a novel protein. Together, our results show that FoxP3, a key regulator of immune suppression, is not only expressed by Treg but also by melanoma cells, EBV-transformed B cells, and a wide variety of tumor cell lines. [Cancer Res 2008;68(8):3001–9]

Introduction

The immune system is subject to many levels of control, and distinct populations of T cells with "regulatory" (or suppressor) function make a major contribution to such regulation (1, 2). The best understood is the population of regulatory T cells (known as Treg), which are characterized by constitutive expression of CD25 and the transcription factor FoxP3 (3, 4). Despite an essential role in preventing autoimmunity, however, Treg may also have a negative effect on health by down-regulating beneficial immune responses, such as those mounted against tumors. Numerous animal studies have shown that removing or inhibiting Treg dramatically improves tumor clearance and survival (5, 6). Furthermore, several reports have documented the presence of Treg within human tumor tissue, the frequency of which may negatively correlate with survival (5–9). Thus, Treg may play a major role in preventing the development of effective antitumor immunity.

FoxP3 is a member of the forkhead family of transcription factors and, at least in mice, seems to act as a "master switch" for the development and function of Treg (4, 10). Mice lacking functional expression of FoxP3 completely lack Treg and develop severe autoimmunity. Moreover, ectopic expression of FoxP3 in conventional murine T cells endows them with the full phenotype and function of Treg. In humans, there is also a strong association between FoxP3 expression and the Treg phenotype, although the relationship is more complex than in mice (10). For example, expression of FoxP3 can be transiently induced in human non-Treg cells by activation through the T-cell receptor (11–13). Nevertheless, FoxP3 does seem to play a critical role in human Treg activity, as mutations in *FOXP3* are associated with development of the multiorgan autoimmune disorder immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (14), in which Treg from affected patients have greatly reduced suppressive activity (15). Furthermore, ectopic expression of FoxP3 allows conventional human T cells to acquire many characteristics of Treg, although some studies have shown that their suppressive activity is inferior to that of *bona fide* Treg (16, 17).

Until recently, FoxP3 expression has been thought to be restricted to the T-cell lineage. In the present study, however, we provide several lines of evidence that FoxP3 is also expressed by tumor cells. Given the central contribution of FoxP3 to Treg function, the expression of FoxP3 by tumor cells may represent a novel mechanism by which cancers suppress the immune system to escape destruction.

Materials and Methods

Cells, tissue, and media. Complete medium (RF-10) consisted of RPMI supplemented with 2 mmol/L Glutamax, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10 mmol/L HEPES (Invitrogen) and 10% FCS (Thermo Trace). Melanoma cell lines (LM-Mel series) and glioma cell lines (LM-G-2 and LM-G-4) were generated by culture of surgically excised tissue after mechanical and/or enzymatic disruption. Additional melanoma cell lines (SK-Mel-14 and SK-Mel-28), as well as renal cell carcinoma lines (SKRC-01, SKRC-09, and SKRC-017), were obtained from the Memorial Sloan-Kettering Cancer Center. The following additional cell lines were obtained from American Type Culture Collection: A172 and U-87MG (glioma); DU 145, PC-3, and LN-CaP (prostate cancer); A549, CaLu-6, and NCI-H460 (lung cancer); HCT116, caco-2, and SW480 (colorectal cancer); HT-1376, HT-1197, and HT-5637 (bladder cancer); MCF7, MDA-MB-231, and MDA-MD-468 (breast cancer). All tumor cell lines, as well as primary fibroblast cultures (derived from normal human dermis or foreskin), were maintained in RF-10 and passaged using trypsin/EDTA (Invitrogen) or 2 mmol/L EDTA in PBS. Normal epidermal melanocyte cultures were

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

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obtained from Lonza Biosciences and cultured according to the manufacturer's recommendations. Melanoma tissue collected after surgical resection was formalin-fixed and embedded in paraffin for immunohistochemistry or used to generate single-cell suspensions for flow cytometry by mechanical disruption. These cells were cryopreserved in FCS + 10% DMSO until required. Collection of tissue for these studies was approved by the Austin Health Human Research Ethics Committee, and all patients gave informed consent. Peripheral blood mononuclear cells (PBMC) from buffy coats of healthy donors (obtained from the Australian Red Cross Blood Service) were prepared by Ficoll-Paque density gradient centrifugation (Amersham Biosciences). Natural killer (NK) cells, monocytes, and $CD4^+$ T cells were purified using the NK cell isolation kit or CD14 or CD4 microbeads, respectively, according to the manufacturer's recommendations (Miltenyi Biotec). The Treg cell line was generated after purification of $CD25^{hi}$ T cells from disaggregated melanoma tissue using CD3 microbeads (Miltenyi) followed by CD25 Dynabeads (Invitrogen). Treg cells were expanded using 1 μg/mL phytohemagglutinin and feeder cells in RF-10 containing 150 IU/mL interleukin 2 (IL-2).

Immunohistochemistry. Immunohistochemistry was performed on formalin-fixed paraffin sections after EDTA buffer (pH 8.0) retrieval, as described (18). Rabbit polyclonal anti-FoxP3 antibody was from Abcam, and monoclonal antibody (mAb) to Melan-A (clone A103) was produced at the Biological Production Facility, Ludwig Institute for Cancer Research.

Flow cytometry and cell sorting. Fluorochrome-conjugated antibodies to human CD4 (clone UCHT2), CD19 (clone HIB19), CD31 (clone WM59), CD90 (clone 5E10), and isotype-matched control antibodies were from BD Biosciences. Anti-CD3 (clone S4.1) was from Invitrogen, anti-MCSP (clone EP-1) was from Miltenyi, and anti-FoxP3 (clone 236A/E7) was from eBioscience. FoxP3 staining was performed (after staining of surface antigens) using the FoxP3 buffer set from eBioscience. Flow cytometric analysis was performed on a FACSCalibur or FACSCanto II instrument (BD Biosciences), and data was analyzed using FlowJo (TreeStar Inc.). Mean fluorescence intensity (MFI) ratios were calculated by dividing the geometric mean channel fluorescence value of FoxP3 stained cells by that of isotype-matched control stained cells. Cell sorting was performed on a FACSAria (BD Biosciences).

EBV-mediated transformation of B cells. Supernatant was collected after 5-d culture of the lymphoblastoid marmoset cell line B95-8 latently infected with EBV. Virus-containing supernatant was 0.45 μm–filtered and diluted 1:10 with fresh RF-10 containing 1 μg/mL cyclosporine (Novartis). PBMC (2×10^6 cells/mL) were cultured in this medium for 30 d, changing half of the medium weekly with fresh RF-10 containing cyclosporine.

Mitogenic activation of B cells using *Staphylococcus aureus* Cowan I + IL-2. PBMC were incubated with 2 μmol/L carboxyfluoroscein diacetate (CFSE) in PBS + 0.1% FCS at 37°C for 7 min then washed twice in RF-10 and resuspended to 1×10^6 cells/mL in RF-10 + 100 units/mL IL-2. *Staphylococcus aureus* Cowan I (SAC; Pansorbin, Merck) was added to half the cells to a final concentration of 0.01%. Cultures with or without SAC were incubated for 4 d in a 24-well plate (2 mL/well) and then analyzed by flow cytometry.

RNA isolation and complimentary DNA synthesis. Total RNA was extracted using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions and immediately stored at −80°C. Complimentary DNA (cDNA) was synthesized from 1 μg total RNA (or 200 ng RNA in the case of Treg cells) in a 20-μL reaction for 60 min at 42°C. The reaction consisted of 1 μg random hexamer primers (Promega), 4 mmol/L $MgCl_2$, 1 mmol/L deoxynucleoside triphosphates (Applied Biosystems), 40 units of RNase inhibitor (Promega), and 10 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen).

PCR, gel electrophoresis, and DNA sequencing. In each 25 μL reaction, 1 μL of cDNA was used together with final concentrations of 2 mmol/L $MgCl_2$, 0.2 mmol/L deoxynucleoside triphosphates (Applied Biosystems), 0.625 units of Amplitaq Gold DNA polymerase (Applied Biosystems), and 2 ng of primers (Sigma-Genosys). Primer sequences were as follows:

FOXP3 (forward primer) 5′-GCCCTTGGACAAGGACCCGATG-3′
FOXP3 (reverse primer) 5′-CATTTGCCAGCAGTGGGTAGGA-3′
CYCLOPHILIN-A (*CYP-A*; forward primer) 5′-GTCAGCAATGGTGATCTTCTT-3′
CYP-A (reverse primer) 5′-GCAGAAAATTTTCGTGCTCTG-3′.

PCR involved 35 cycles (or 40 cycles, where stated) at 94°C for 1 min, 66°C for 1 min, 72°C for 1 min, and a final primer extension at 72°C for 10 min. PCR products were resolved using 1% agarose gel electrophoresis and documented using Safe Imager. Where required for sequencing, PCR products were cut from agarose gels and purified with Qiaquick gel extraction kits (Qiagen). Purified DNA was sequenced at the Micromon DNA Sequencing Facility (Monash University).

Western blot. Whole-cell lysates were prepared by sonication in SDS lysis buffer containing protease inhibitors. Samples were heated, and 20 μL (4×10^5 cell equivalents, except for $CD4^+$ T cells: 1×10^6 cell equivalents, and Treg cell line: 2×10^5 cell equivalents) were subjected to SDS-PAGE using NuPAGE 4% to 12% Bis-Tris gels under reducing conditions. Resolved proteins were transferred onto 8 × 7.5 cm membrane (Milipore) in Transblot semidry buffer (Bio-Rad Laboratories). The membranes were blocked with 5% nonfat dry milk in 1× TBS, then probed with 2 μg/mL anti-FoxP3 (clone PCH101; eBioscience) overnight at 4°C, followed by a horseradish peroxidase–conjugated goat anti-rat IgG (Chemicon) for 1 h at room temperature. Membranes were treated with ECL plus Western blotting detection kit (Amersham), and bands were detected using STORM 840 v2005 with ImageQuant software. Subsequently, membranes were washed in TBS-T and reprobed with rabbit antibody to β-actin (Cell Signaling) as described above.

Results

FoxP3 is expressed by both lymphocytes and tumor cells in metastatic melanoma tissue. With the aim of characterizing the frequency and localization of Treg in melanoma, we performed immunohistochemistry on metastatic melanoma tissue sections using a polyclonal anti-FoxP3 antibody. Surprisingly, in addition to the expected staining of cells with lymphocyte morphology (small, round, dense nucleus, and little cytoplasm), staining was also occasionally apparent in cells which had several morphologic features of tumor cells, such as a large, irregular nucleus and abundant cytoplasm (Fig. 1*A, left*). To definitively identify these $FoxP3^+$ cells as melanoma cells, two-color immunohistochemistry was performed using antibodies to both FoxP3 and Melan-A, a specific marker of melanocytic differentiation (19). As shown in Fig. 1*A* (*right*), $FoxP3^+$ cells were readily identified within Melan-A^+ tumor cell islands, whereas infiltrating Treg were located outside of these islands and lacked staining for Melan-A.

We then generated cell suspensions from disaggregated melanoma tissue and analyzed them using six-color flow cytometry, using a hierarchical gating strategy to identify melanoma cells within the mixed population (Fig. 1*B*). T cells were excluded on the basis of forward and side scatter and then by gating out $CD3^+$ and $CD4^+$ events. Endothelial cells and tissue fibroblasts were excluded by gating out $CD31^+$ and $CD90^+$ events, respectively, and melanoma cells within the remaining population were identified by staining for MCSP (20). Fluorescence minus one isotype-matched negative controls were used to assess the level of background staining within this population and this pattern compared with staining with anti-FoxP3. Generally, the addition of anti-FoxP3 produced a shift of the entire $MCSP^+$ population compared with the control; the magnitude of this shift varied greatly, ranging from barely detectable to >1 log shift. In addition, many samples had a small subpopulation of $FoxP3^{bright}$ cells that clearly expressed much higher levels of FoxP3 than the bulk population. The examples shown in Fig. 1*B* illustrate these different patterns. For the seven samples analyzed, the mean proportion of $FoxP3^{bright}$ cells was

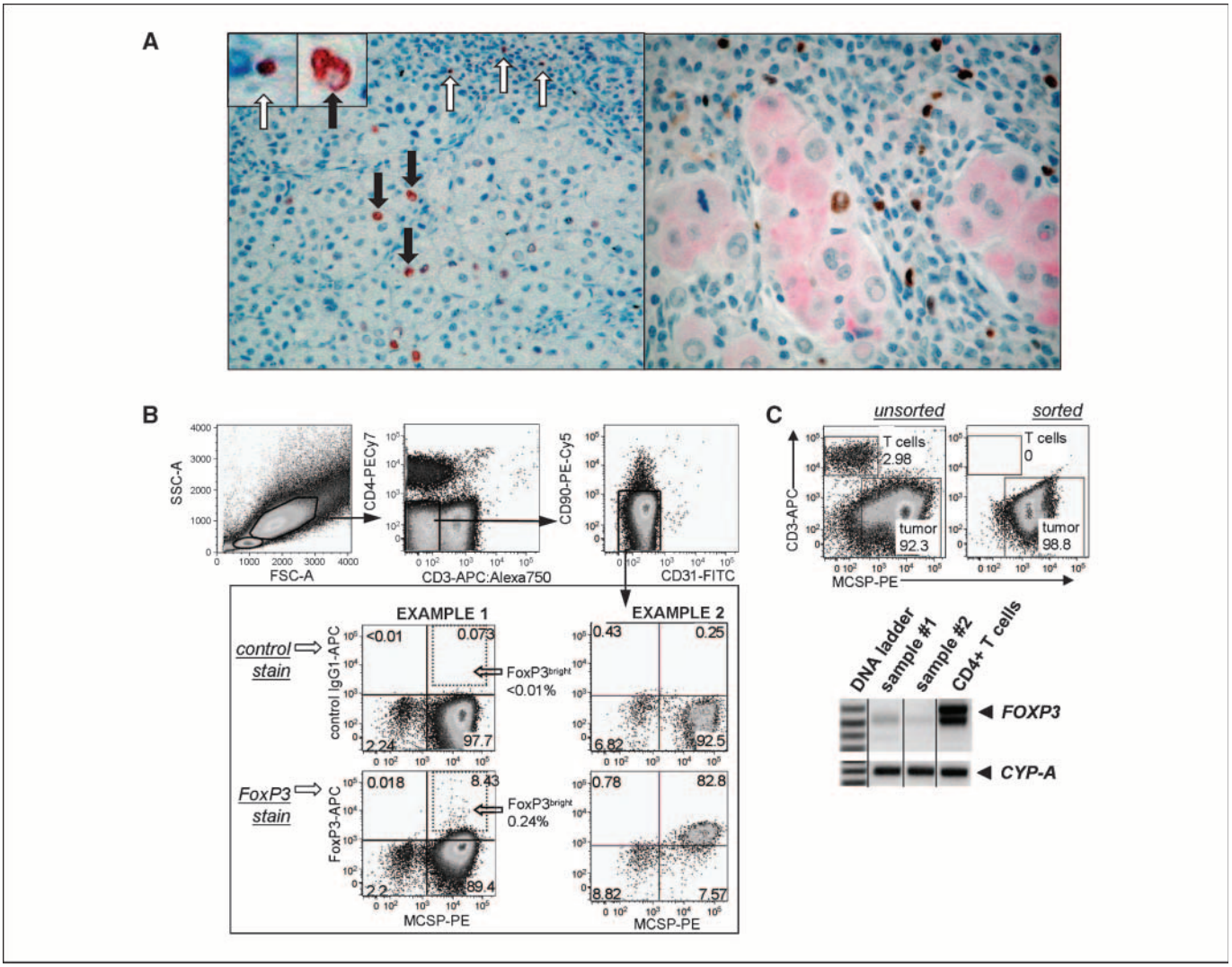

Figure 1. FoxP3 expression in metastatic melanoma tissue is not restricted to T cells. *A,* immunohistochemistry analysis of tissue sections. *Left,* FoxP3 staining was detected in cells with the morphology of lymphocytes (*open arrows*) and tumor cells (*solid arrows*) at 100× magnification. An enlarged (300%) view of each cell type is shown in the inset. *Right,* co-staining for FoxP3 (*brown*, nuclear) and Melan-A (*pink*, cytoplasmic) at 400× magnification. *B,* cell suspensions were generated from metastatic melanoma tumor tissue and stained for flow cytometry. *Top,* plots show the hierarchical gating strategy for identification of melanoma cells; *bottom,* plots show two different patterns of staining observed with anti-FoxP3, relative to isotype control. *C,* tumor cells were sorted from disaggregated tumor tissue, and expression of mRNA for *FOXP3* or *CYP-A* was assessed by RT-PCR; *vertical lines,* noncontiguous lanes that have been electronically juxtaposed. The original gels are presented in Supplementary Fig. S1. Peripheral blood $CD4^+$ T cells are shown for comparison. Expression of CD3 and MCSP was determined by flow cytometry after sorting to confirm that the *FOXP3* transcripts did not originate from Treg (sample 1).

0.1 + 0.09% (SD; range, 0–0.24%). This population likely corresponds to the scattered $FoxP3^+$ melanoma cells identified in immunohistochemistry analysis of tissue sections.

To confirm that melanoma cells expressed *FOXP3* transcripts, the disrupted tumor tissue was subject to high-stringency cell sorting to purify the melanoma cells (identified as $MCSP^+$ $CD3^-$ $CD4^-$ $CD31^-$ $CD90^-$), which were then analyzed by reverse transcription–PCR (RT-PCR; Fig. 1*C*). PCR using *FOXP3* primers produced a pattern of bands similar, but not identical, to that observed for $CD4^+$ Treg (see later), thus confirming the presence of *FOXP3* transcripts in sorted melanoma cells. Importantly, analysis of the sorted populations confirmed that they were >98% $MCSP^+$ and that the level of T-cell contamination was <0.005%. Thus, *FOXP3* transcripts can be amplified from pure, freshly isolated melanoma cells, providing further evidence that these cells can express FoxP3.

FoxP3 is widely expressed by melanoma cell lines. Further confirmation of FoxP3 expression in melanoma cells was obtained by analyzing a panel of established melanoma cell lines by flow cytometry (Fig. 2). The cell line shown in Fig. 2*A* (SK-Mel-14) was uniformly positive for FoxP3, although the intensity of staining was not as high as $CD4^+$ $CD25^+$ T cells. Staining of freshly isolated NK cells and monocytes was negative, as expected. Staining for FoxP3 on additional melanoma cell lines is summarized in Fig. 2*B*. Staining with anti-FoxP3 resulted in a shift in the fluorescence of the entire population compared with the isotype control; to quantify the intensity of this shift, the MFI was determined after staining with anti-FoxP3 or isotype control and a ratio of the two values were calculated (this calculation was necessary to normalize the different levels of background fluorescence). The same calculation was also performed for a range of negative control cells to establish the background MFI ratio (indicated by a dotted line).

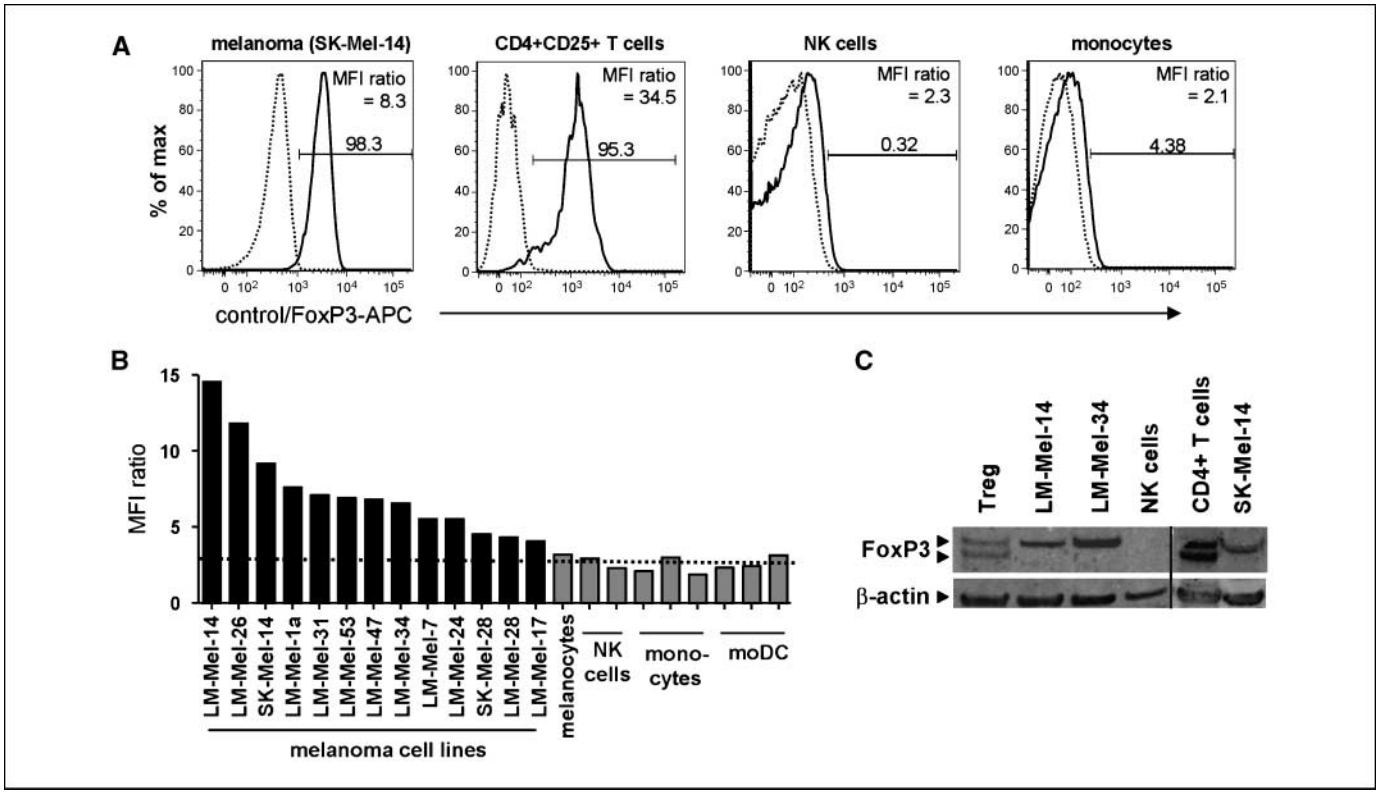

Figure 2. FoxP3 is widely expressed in melanoma cell lines. *A* and *B,* cells were stained using anti-FoxP3 mAb (*solid line*) or isotype-matched negative control (*broken line*) and analyzed by flow cytometry. In *A*, one representative melanoma cell line is shown together with peripheral blood $CD4^+$ $CD25^+$ Treg cells, NK cells, and monocytes for comparison. In *B*, results for a panel of melanoma cell lines are shown (*black columns*), together with a range of negative controls (peripheral blood NK cells/monocytes and 7-d cultured monocyte-derived DCs; *gray columns*). For each sample, the MFI was determined after staining with anti-FoxP3 and isotype control, and the ratio was calculated. *C,* Western blot analysis of lysates prepared from three melanoma cell lines, freshly isolated peripheral blood NK cells or $CD4^+$ T cells, and a short-term expanded Treg cell line. The vertical line delineates two blots performed on different days (full-length original blots are presented in Supplementary Fig. S1).

The majority of melanoma cell lines had MFI ratios well above this background level, although the intensity of FoxP3 expression varied considerably and a small proportion of cell lines had ratios only slightly above background. In contrast, cultured normal epidermal melanocytes did not express detectable FoxP3. Furthermore, as shown in Fig. 2*C*, FoxP3 protein could be detected in melanoma cell lines by Western blotting. Thus, FoxP3 is widely expressed in melanoma cell lines (but not normal melanocytes); however, the intensity of expression is variable and a small number of melanoma cell lines express only borderline levels of FoxP3.

The FOXP3 gene is expressed in melanoma cells as three mRNA variants, one of which is predicted to encode a novel protein. To confirm *FOXP3* gene expression in melanoma cells, RNA was extracted from a panel of melanoma cell lines and used to generate cDNA by reverse transcription, which was then subject to PCR using primers specific for *FOXP3* or the housekeeping gene *CYP-A*. PCR products could be readily detected after amplification using *FOXP3*-specific primers (35 cycles), confirming that melanoma cell lines express *FOXP3* mRNA (Fig. 3*A*).

Previous reports have shown that, in Treg, *FOXP3* mRNA is expressed as two variants: full-length and an alternatively spliced version lacking exon 3 (Δ3; although note that these reports refer to exon 3 as exon 2 because the true exon 1 is not considered, presumably because it is within the 5′ untranslated region; refs. 10, 17, 21). Using our primers, these two products would be expected to result in band sizes of 608 and 503 bp, respectively. For melanoma cells, however, an additional band (~100 bp smaller than the Δ3 product) was frequently detected, which was not apparent in Treg. To better visualize this band, the PCR was repeated for selected cell lines at 40 cycles instead of 35 cycles (Fig. 3*B*). This confirmed the presence of a third PCR product in melanoma cells, which was absent from both freshly purified $CD4^+$ T cells and a Treg-cell line expanded in culture for several weeks. Sequencing of one melanoma cell line (LM-Mel-34) revealed that this product was lacking both exons 3 and 4 and therefore corresponds to a novel *FOXP3* mRNA variant (Δ3,4; Fig. 3*C*). Sequencing of the upper (608 bp) band confirmed that it was identical to the published sequence for full-length *FOXP3*.

The previously described Δ3 splice variant contains an in-frame excision of exon 3 (105 bp), which is predicted to result in removal of part of the repressor domain of the normal FoxP3 protein without affecting sequences downstream of the third exon (10, 17). In contrast, the novel Δ3,4 isoform contains a 244 bp excision, which is expected to result in a translation frame-shift. The predicted amino acid sequence is shown in Fig. 3*D*; this sequence is identical to FoxP3 in the NH_2 terminal portion encoded by exon 2, but this region is followed by a 101–amino acid sequence with no significant homology to FoxP3 (or any other sequence in the public databases) and a premature stop codon, resulting in a protein that is approximately half the size of FoxP3.

FoxP3 is also expressed in cell lines derived from other types of solid tumor. Given that FoxP3 was widely expressed in melanoma cell lines, we were interested to determine if cell lines derived from other types of solid tumor also expressed FoxP3.

Accordingly, an extensive panel of tumor cell lines was examined for FoxP3 expression by flow cytometry; this panel comprised three to four cell lines, each from colon cancer, lung cancer, breast cancer, prostate cancer, glioblastoma, renal cell carcinoma, and bladder cancer (Fig. 4*A*). As per the melanoma cell lines, the intensity of FoxP3 expression was determined relative to isotype control staining and calculated as an MFI ratio. The gray region indicates the mean background MFI ratio as determined in Fig. 2*B*.

This analysis revealed that at least one cell line from each type of tumor expressed readily detectable levels of FoxP3. Some, such as prostate cancer and colon cancer, were uniformly positive, whereas others, such as breast cancer, seemed to express FoxP3 less frequently. However, given the small sample size for each type of

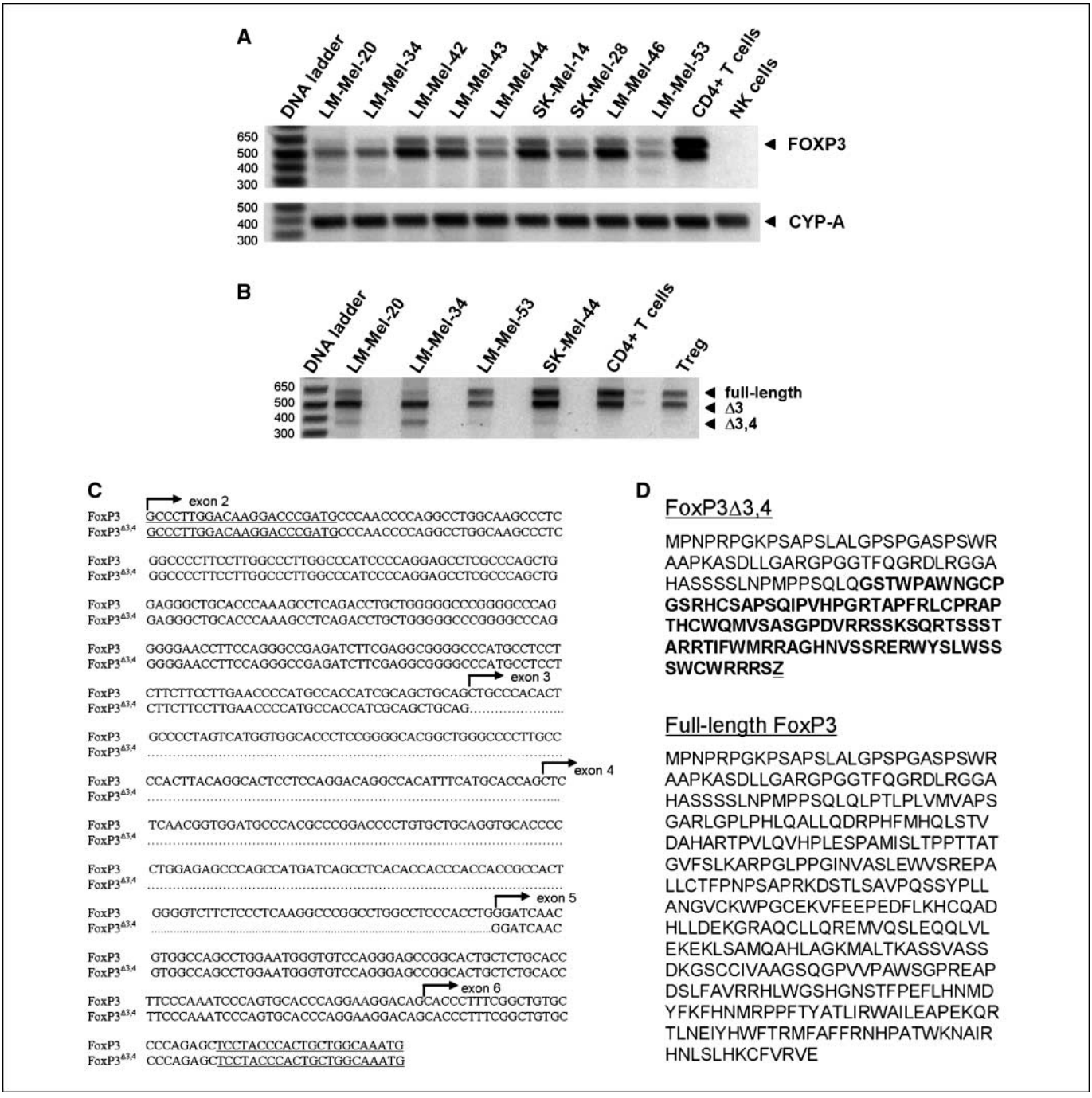

Figure 3. *FOXP3* mRNA is expressed as three distinct isoforms in melanoma cells. *A,* RNA was extracted from cells and reverse transcribed, and the cDNA was amplified by PCR (35 cycles) using primers specific for *FOXP3* and *CYP-A*. Representative melanoma cell lines and peripheral blood $CD4^+$ T cells and NK cells. Gels have been cropped for clarity; full-length gels are presented in Supplementary Fig. S1. *B, FOXP3* PCR was repeated at 40 cycles to better visualize the third PCR product. Four melanoma cell lines, $CD4^+$ T cells, and a short-term expanded Treg cell line. *C,* sequence of the upper (608 bp) and lower (364 bp; Δ3,4) *FOXP3* PCR products. Primer sites are underlined. *D,* predicted amino acid sequence of the Δ3,4 isoform compared with the published protein sequence for full-length FoxP3. For the Δ3,4 isoform, the sequence in bold type differs from the wild-type protein, and the premature stop codon is underlined.

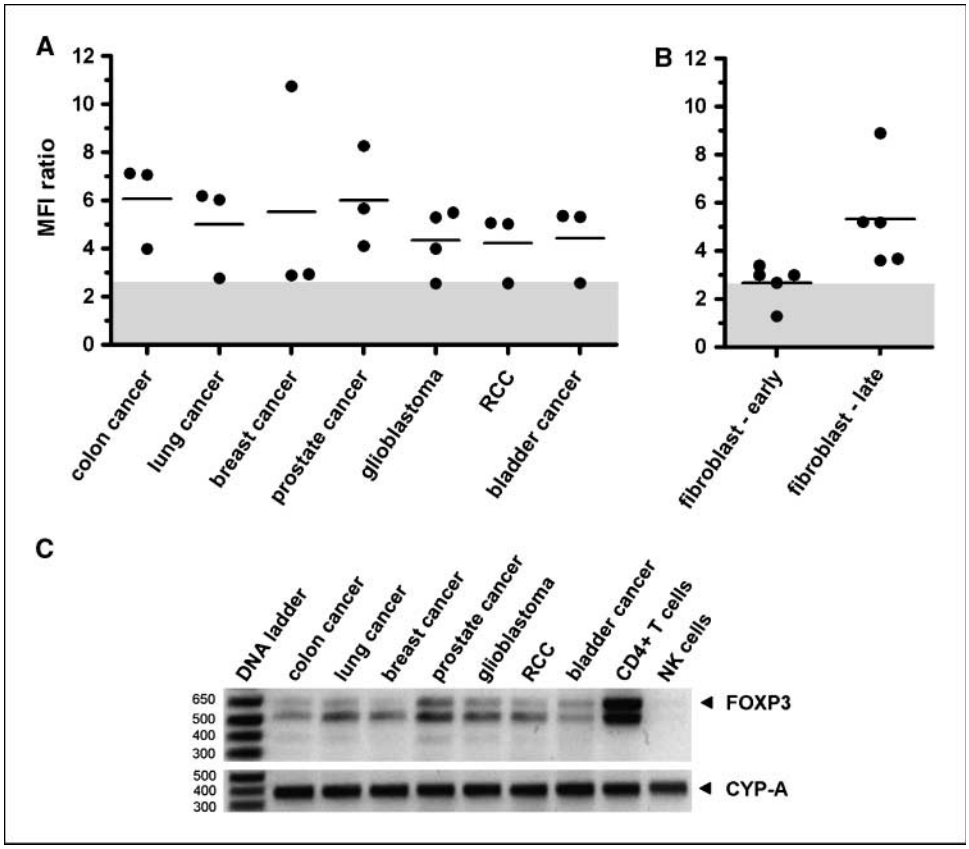

Figure 4. Expression of FoxP3 in cell lines derived from other types of solid tumor or normal fibroblasts. *A* and *B,* tumor cell lines and normal fibroblast cultures (early and late passage) were analyzed by flow cytometry. The MFI was determined after staining with anti-FoxP3 and isotype control, and the ratio was calculated. *Gray region,* background level as determined in Fig. 2*B*. *C,* RNA extracted from 1×10^6 cells was reverse transcribed, and the cDNA was amplified by PCR (35 cycles) using primers specific for *FOXP3* and *CYP-A*. Representative cell lines derived from the various types of cancer [colon cancer (CaCo2), lung cancer (NCI-H460), breast cancer (MDA-MB-468), prostate cancer (PC3), glioblastoma (A172), renal cell carcinoma (SKRC09), and bladder cancer (5637)] and $CD4^+$ T cells and NK cells purified from peripheral blood. Gels have been cropped for clarity; full-length gels are presented in Supplementary Fig. S1.

cancer, these differences cannot be considered conclusive. Early passage cultures of normal fibroblasts lacked convincing staining for FoxP3, suggesting that the expression of FoxP3 is restricted to malignant cell lines. Interestingly, however, extended culture of fibroblasts did eventually result in induction of FoxP3 expression (Fig. 4*B*). The mechanism responsible for this observation is unclear at present, although it is possible that FoxP3 is induced in normal tissue cells as they approach replicative senescence.

To confirm expression of *FOXP3* transcripts, one cell line from each type of cancer was selected on the basis of unequivocal expression of FoxP3 protein by flow cytometry and analyzed by RT-PCR. As shown in Fig. 4*C*, each cell line tested positive for *FOXP3*, and several cell lines also expressed the Δ3,4 isoform described in Fig. 3. Thus, expression of FoxP3 in tumor cell lines is not restricted to melanoma.

FoxP3 expression is induced during EBV-mediated B-cell transformation *in vitro* and *in vivo*. Infection of B cells with EBV induces transformation and can serve as an *in vitro* model for B-cell lymphoma formation. We used this model to investigate whether transformation is associated with *de novo* induction of FoxP3 expression. PBMC from four healthy donors were infected with EBV *in vitro* and cultured in the presence of cyclosporine to block the expansion of T cells (Fig. 5*A–C*). Before culture, $CD19^+$ B cells did not express detectable FoxP3, as expected. After a 30-day culture, the small B cells still lacked FoxP3 expression whereas the transformed B-cell blasts had clearly up-regulated FoxP3 expression. A time course analysis revealed that blasts were first detectable at around day 15 of culture, by which time they had already begun to up-regulate FoxP3. With further culture, the level of FoxP3 expression progressively increased until the last day of analysis (day 30). In contrast, expression of FoxP3 in the small B cells remained low to undetectable throughout the culture period, arguing against a nonspecific induction of FoxP3 in cultured B cells. Expression of *FOXP3* mRNA was confirmed for one of these cultures by RT-PCR (not shown).

Several reports have shown that proliferating T cells can up-regulate FoxP3 expression after activation through the TCR (11–13). It was therefore important to rule out the possibility that FoxP3 induction in B cells was similarly simply due to the cells becoming activated and proliferating. To control for this possibility, PBMC were labeled with CFSE and cultured with whole, fixed SAC cells and IL-2. This combination has potent B-cell mitogenic activity due to the presence of protein A on the bacterial surface which can cross-link the B-cell receptor. After a 4-day culture, a blast population was readily detectable (Fig. 5*D*). Within this population, B cells ($CD19^+$ $CD3^-$) had clearly undergone several rounds of cell division, as assessed by CFSE dye dilution. Nevertheless, FoxP3 expression in these dividing B cells remained undetectable (although the level of autofluorescence increased, as expected for activated cells). As expected, PBMC cultured in the absence of SAC did not generate a blast population, the B cells did not divide, and they also lacked FoxP3 expression. These results suggest that activation and initiation of cell division is not sufficient to up-regulate FoxP3 expression in B cells. Therefore, the induction of FoxP3 expression after EBV transformation must be due to some aspect specific to the transformation process.

Posttransplant lymphoproliferative disorder (PTLD) is a complication of solid organ and bone marrow transplantation in which B-cell lymphomas develop after EBV-induced transformation of B cells (22). Given that transformation of B cells *in vitro* with EBV

seemed to induce FoxP3 expression, we hypothesized that B-cell lymphomas from PTLD patients would also express FoxP3. To test this, cryopreserved cell samples from a patient who developed PTLD after a heart transplant were assessed for FoxP3 expression (Fig. 5*E*). Normal $CD19^+$ B cells within the patient's PBMC did not express FoxP3, as expected. However, when disaggregated lymphoma tissue from the same patient was analyzed, a distinct population of $FoxP3^+$ $CD19^+$ B cells could be detected. These were clearly not Treg, as $CD4^+$ T cells were gated out of the analysis. Interestingly, the $FoxP3^+$ B cells expressed a lower level of CD19 than the $FoxP3^-$ B cells, although the significance of this observation is unclear at present.

Discussion

Using a wide variety of approaches, the present study shows that the FoxP3 transcription factor is expressed by melanoma cells, virally transformed B cells, and cell lines derived from a variety of solid tumors. This observation is particularly interesting, as it was previously believed that FoxP3 expression was strictly limited to T cells (23–25). During the preparation of this manuscript, however, two reports were published, describing the expression of FoxP3 outside the T-cell lineage. First, Zuo et al. showed that FoxP3 is expressed in normal breast epithelium but down-regulated in breast cancer due to reduced levels of *FOXP3* transcription or

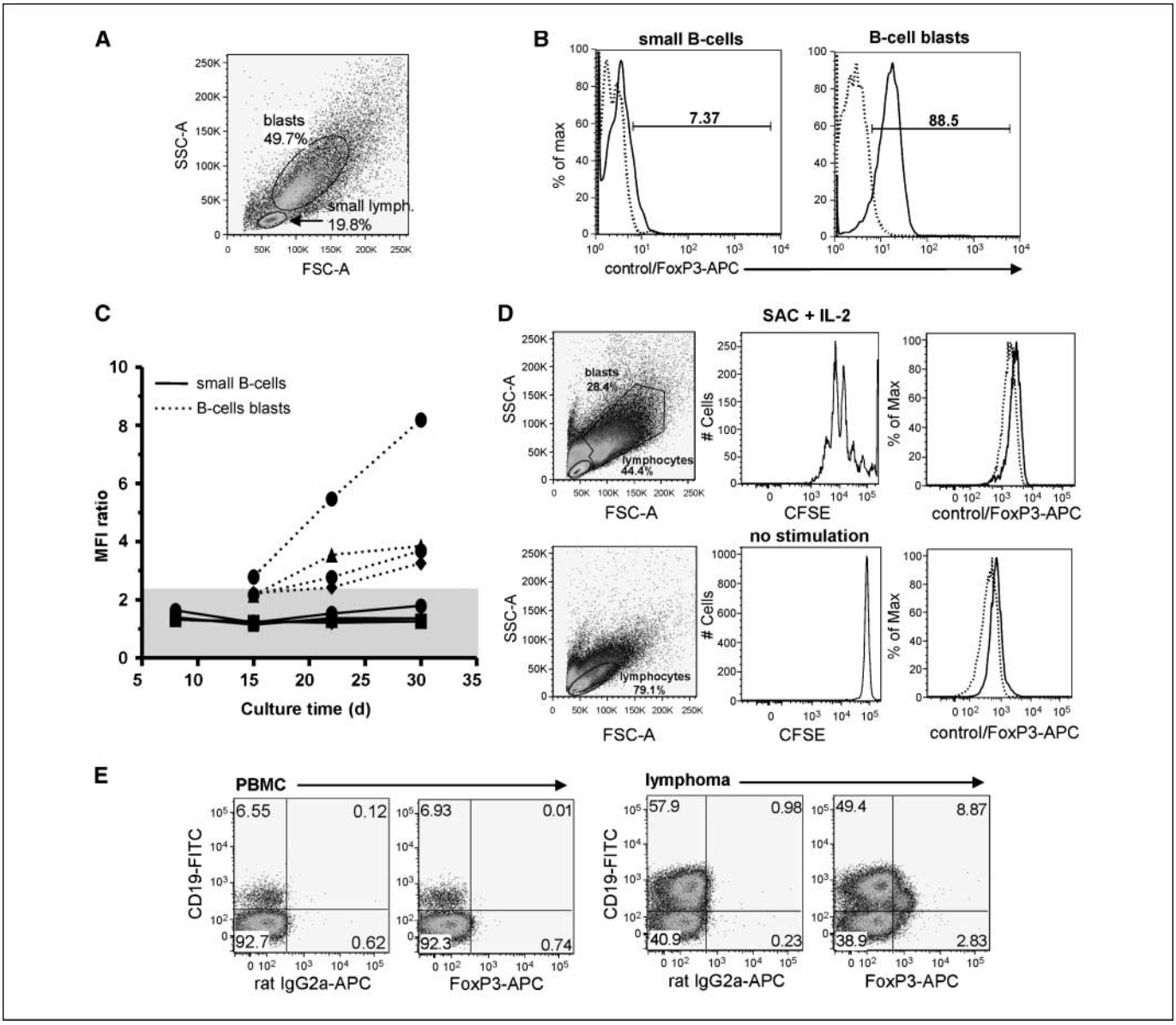

Figure 5. *De novo* induction of FoxP3 expression during EBV transformation of B cells *in vitro* and *in vivo*. *A-C,* PBMC from four healthy donors were infected with EBV *in vitro* and monitored for expression of FoxP3 by flow cytometry. After a 30-d culture, cells were gated into small lymphocyte and blast populations on the basis of forward and side scatter variables (*A*), and the $CD19^+$ B cells within each gate were assessed for FoxP3 expression (*solid line*) compared with staining with isotype matched control mAb (*broken line*; *B*). *C*, full time-course analysis of all four donors. The B-cell blast population is only displayed from day 15, the time point at which it was first clearly detectable. *D,* as a control, PBMC were labeled with CFSE and cultured for 4 d with SAC + 100 units/mL IL-2 (*top*) or IL-2 alone (*bottom*). After gating on blasts (SAC + IL-2) or total lymphocytes (IL-2 alone), B cells were identified as $CD19^+$ $CD3^-$ and gated to analyze the CFSE division profile (*middle*) or FoxP3 expression (*right*). *E,* analysis of PBMC and tumor cells from a patient with PTLD. Cells were stained for CD19 and either isotype control or anti-FoxP3 as indicated. Analysis is gated on $CD4^-$ lymphocytes.

deletion of the *FOXP3* locus (26). On the other hand, Hinz et al. provided evidence that FoxP3 is up-regulated by pancreatic carcinoma cells compared with normal pancreas (27), which is more in keeping with our results.

The function of FoxP3 in cancer cells may depend on the nature of the tumor. In breast cancer, loss of FoxP3 expression is thought to contribute to tumorigenesis by allowing enhanced expression of the *HER-2/ErbB2* oncogene, which plays a key role in breast cancer progression (26). Therefore, FoxP3 expression would be expected to be selected against, and in keeping with this concept, we found only one of three breast cancer cell lines to be FoxP3$^+$. In contrast, the *HER-2/ErbB2* oncogene is not reported to be involved in the pathogenesis of melanoma (28), and therefore, there would be no reason for FoxP3 expression to be selected against. In fact, given the established role for FoxP3 in directing Treg activity, up-regulated FoxP3 expression in melanoma cells may endow them with Treg-like activity and enable FoxP3$^+$ melanoma cells to resist immune attack. In support of this hypothesis, FoxP3-expressing melanoma cells could effectively suppress T-cell proliferation and, in some cases, were even more potent than Treg (data not shown). Further studies are required to directly show that FoxP3 is involved in this process for melanoma cells. However, FoxP3-expressing pancreatic carcinoma cell lines have also been shown to suppress T-cell proliferation, and knockdown of FoxP3 using RNA interference could effectively reduce the suppressive activity of these cells (27). It is therefore likely that FoxP3 will have an analogous function in other types of tumor cells, including melanoma.

Even in T cells, FoxP3 expression does not automatically lead to acquisition of full regulatory activity, as human T cells can transiently induce FoxP3 expression without acquisition of Treg function (11, 12). However, recent evidence suggests that FoxP3 expression needs to be stabilized by promoter demethylation for Treg activity to be induced, which does not occur after activation of conventional T cells (13, 29). In contrast, expression of FoxP3 by tumor cells was stable (as determined by repeat analyses of the same cell lines; not shown). This is in keeping with the observation that aberrant DNA methylation and demethylation patterns are common events in melanoma (30), suggesting that the FoxP3 promoter may become demethylated in melanoma cells, leading to stable FoxP3 expression and thus, potentially, acquisition of regulatory activity.

In addition to solid tumors, we have shown that B-cell lymphomas can also aberrantly express FoxP3. Transformation of normal B cells with the oncogenic virus EBV induced FoxP3 expression, and a subpopulation of B lymphoma cells from a PTLD patient were FoxP3$^+$. For several years, it has been recognized that adult T-cell leukemia/lymphoma is frequently associated with FoxP3 expression (31). The common interpretation of this observation is that these malignancies are derived from the Treg lineage. However, our observation of *de novo* FoxP3 induction in malignant B cells raises the possibility that a proportion of FoxP3$^+$ T-cell leukemias are in fact derived from non-Treg cells that have begun expressing FoxP3 during tumorigenesis.

Our data raise the possibility that FoxP3 may be a potential tumor antigen that could be targeted by vaccination. Although FoxP3 is a self antigen, tolerance is apparently very limited, as T-cell responses against FoxP3 could be readily induced after vaccination of mice (32). The surprising absence of functional tolerance to FoxP3 may be due to lack of expression in thymic epithelium (25), although an earlier study did detect thymic FoxP3 expression (33); whether or not FoxP3 is expressed in thymic epithelium is therefore currently unclear. A potential concern in targeting FoxP3 as a tumor antigen is the possibility that Treg would also be targeted for destruction, potentially leading to autoimmunity. Of note, however, is our observation that a number of tumor cell lines expressed a *FOXP3* mRNA isoform lacking exons 3 and 4, which was not detectable in Treg. This isoform is predicted to encode a novel protein, which is approximately half the size of FoxP3 and, due to a translation frame-shift, includes a 101–amino acid sequence that has very limited homology to *FOXP3*, and any other known protein sequence. Targeting this portion by vaccination would allow the generation of T cells that recognize tumor cells but not Treg, thereby eliminating the possibility of autoimmune complications. Interestingly, Zuo et al. also detected novel *FOXP3* mRNA variants in breast cancer cells, one of which seems to be identical to the Δ3,4 variant identified here in melanoma and a variety of other tumor cell lines. Therefore, alternative splicing of the *FOXP3* gene seems to be a common theme in cancer, and targeting this variant by vaccination could prove beneficial in a variety of malignancies.

This study shows that FoxP3 is expressed in melanoma cells both directly *ex vivo* and after culture *in vitro*. Thus, FoxP3 expression by melanoma cells is neither a culture artifact nor a transient phenomenon. We have also shown FoxP3 expression in B cells that have been transformed with EBV *in vitro* and *in vivo*, as well as a variety of other tumor cell lines, although further investigation is required to determine if each of these tumors also expresses FoxP3 *in vivo*. These observations suggest a reinterpretation of previous studies in which Treg prevalence in tumors was investigated by measuring *FOXP3* transcripts in the tumor tissue, as some of these transcripts may have originated from the tumor cells themselves and not from Treg. Clearly, FoxP3 expression and function can no longer be considered to be restricted to the T-cell lineage but may instead play a wider role in biology; for example, by endowing tumor cells with immune suppressive activity. These observations raise the possibility of enhancing tumor immunogenicity by knocking down FoxP3 expression (for example, using RNA interference) or, alternatively, by vaccinating against the FoxP3 Δ3,4 isoform.

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