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The Novel ETS Factor TEL2 Cooperates with Myc in B Lymphomagenesis†

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The human ETS family gene *TEL2*/*ETV7* is highly homologous to *TEL1*/*ETV6*, a frequent target of chromosome translocations in human leukemia and specific solid tumors. Here we report that TEL2 augments the proliferation and survival of normal mouse B cells and dramatically accelerates lymphoma development in Eμ-*Myc* transgenic mice. Nonetheless, inactivation of the p53 pathway was a hallmark of all TEL2/Eμ-*Myc* lymphomas, indicating that TEL2 expression alone is insufficient to bypass this apoptotic checkpoint. Although *TEL2* is infrequently up-regulated in human sporadic Burkitt's lymphoma, analysis of pediatric B-cell acute lymphocytic leukemia (B-ALL) samples showed increased coexpression of *TEL2* and *MYC* and/or *MYCN* in over one-third of B-ALL patients. Therefore, TEL2 and MYC also appear to cooperate in provoking a cadre of human B-cell malignancies.

Recurrent chromosome translocations are a hallmark of many human leukemias and lymphomas and either generate chimeric genes encoding proteins with altered functions or lead to the overexpression of oncogenes by their juxtaposition to potent enhancers, a scenario frequently observed in lymphoid malignancies (31).

ETS (for E26 transformation specific) transcription factors play important roles in hematopoietic development as well as in oncogenesis (10, 27). For example, *TEL1* (for translocation ETS leukemia, also known as *ETV6*) is required for normal yolk sac angiogenesis and adult bone marrow hematopoiesis (46, 47) and is a frequent target of aberrations involving the short arm of chromosome 12 in various hematopoietic malignancies (13), as well as in some solid tumors (44).

Recently we identified a close homologue of *TEL1* coined *TEL2*, located on the short arm of chromosome 6p21, a position implicated in a variety of cancers (25). *TEL2* has highest identity to TEL1 in the pointed (PNT) protein-protein interaction domain (62.5%) and the ETS DNA binding domain (85.4%) (30). TEL2 and TEL1 are most divergent in their central regions, which in TEL1 directs transcriptional repression by binding to corepressors and HDAC3 (7, 22) and in TEL2 contains a putative PEST sequence (residues 138 to 155) (15) that might direct its rapid turnover. Furthermore, unlike TEL1, which is ubiquitously expressed, *TEL2* expression is generally restricted to hematopoietic tissues (30).

As monomers, TEL2 and TEL1 can compete for the same recognition element and function as transcriptional repressors (19, 29), but they can also form heterodimers via their PNT domains (30). However, TEL2 and TEL1 play quite different biological roles. For example, while TEL1 inhibits Ras-induced colony formation (45), TEL2 augments it (19). Furthermore, TEL2 but not TEL1 is down-regulated during monocytic differentiation, and enforced TEL2 expression can block this differentiation program (19). Finally, TEL2 is expressed in many human tumor cell lines (www.ncbi.nlm.nih.gov/CGAP) and appears overexpressed in some human leukemia samples (19).

A tumor suppressor role for TEL1 is suggested by its loss during disease progression of TEL-RUNX1-expressing childhood pre-B-cell acute lymphocytic leukemia (B-ALL) (13, 33, 34). TEL-2's biological effects, and its ability to form heterodimers with TEL1, suggest that TEL2 may antagonize TEL1 functions and thus act as an oncogene. Here we report that TEL2 indeed is an oncogene that cooperates with Myc in lymphoma development and that *TEL2* and *MYC* expression levels are coordinately elevated in a subset of pediatric B-ALL patients. Therefore, TEL2 and MYC also appear to cooperate to promote human B-cell lymphomagenesis.

MATERIALS AND METHODS

Bone marrow transplantation and retroviral transduction of progenitor BMCs (Lin$^-$ BMCs). We injected 3-month-old preleukemic C57BL/6 Eμ-*Myc* transgenic mice (1) with 150 mg of 5-fluorouracil (Sigma Chemical, St. Louis, Mo.)/g of body weight. Bone marrow cells (BMCs) were isolated 48 to 72 h later by flushing femurs and tibias with Iscove's medium and 2% fetal bovine serum. Nonnucleated cells were lysed with Gey's solution (150 mM NH_4Cl, 10 mM $KHCO_3$) for 10 min at room temperature. BMCs not expressing lineage markers (Lin$^-$) were selected after incubation with biotinylated antibodies against Gr-1 (01212D; PharMingen, San Jose, Calif.), B220 (PharMingen 01122D), CD5 (PharMingen 01032D), and TER119 (PharMingen 09092D) and passing them over a column with streptavidin-coated beads (Dynabeads M-280 streptavidin; 112.16; Dynal, Brown Deer, Wis.). Unbound BMCs were spun down and resuspended in Iscove's medium and 20% fetal bovine serum (HyClone, South Logan, Utah) supplemented with 20 ng of mouse interleukin-3 (IL-3)/ml, 50 ng of human IL-6/ml, 50 ng of mouse IL-7 (Preprotech, London, United Kingdom)/ml, and 50 ng of stem cell factor (R&D Systems, Minneapolis, Minn.)/ml at a density of 2×10^6 cells per ml. After 48 h, BMCs were transduced with conditioned

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medium containing 1×10^6 to 2×10^6 viral particles of MSCV-TEL2-IRES-GFP/ml (19) or MSCV-IRES-GFP vector/ml (28) for two consecutive days in the presence of the same growth factors on RetroNectin-coated plates (Takara, Otsu, Japan). Flow cytometric analysis was performed on a FACSCalibur (Becton Dickinson, Franklin Lakes, N.J.). C57BL/6/129svJ mixed-background mice were lethally irradiated (850 cGy), and 10^6 transduced bone marrow cells were injected into the tail vein 24 h after irradiation. Prior to transplantation, we determined the percentage of green fluorescent protein-positive (GFP^+) cells in the transduced BMCs by fluorescence-activated cell sorter (FACS) analysis.

For secondary transplants, BMCs of diseased $BMTMYC^{TEL2}$ mice were collected as described above, and 10^6 cells were transplanted into sublethally irradiated (450 cGy) C57BL/6/129svJ mixed-background mice.

GFP expression in peripheral blood of transplanted mice. $BMTMyc^{TEL2}$ and $BMTMyc^{vector}$ mice were bled monthly by orbital sinus puncture until the time of euthanasia (2.5 to 4 months posttransplantation). Blood (20 μl) was collected in 1 ml of phosphate-buffered saline for FACS analysis of erythrocytes and platelets. After lysis of erythrocytes in Gey's solution, the percentage of GFP^+ leukocytes was determined. Dead cells were omitted from the analysis after staining with propidium iodide (PI; 0.018 mg/ml) and electronic gating to select PI-negative cells. The number of GFP^+ lymphocytes in the peripheral blood was determined by measuring the percentage of GFP^+ cells in the area of forward and sideward scatter of the FACS plot coinciding with that of lymphocytes, multiplied by the total number of white blood cells as determined by differential counts using a Hemavet 3700 (Drew Scientific, Cumbria, United Kingdom).

Mouse tissue collection. All animal procedures were carried out in accordance with the U.S. Public Health Service policy on the humane care and use of laboratory animals. Both $BMTMyc^{TEL2}$ and $BMTMyc^{vector}$ mice were euthanized 3 to 4 months after transplantation. For protein analyses, tissues were collected and snap-frozen in liquid nitrogen. For staining purposes, tissues were fixed in 10% paraformaldehyde overnight, prior to paraffin embedding, sequential sectioning, and histological staining.

Retroviruses. The retroviral vectors MSCV-TEL2-IRES-GFP, MSCV-TEL2DBDM-IRES-GFP, and MSCV-TEL2ΔPNT-IRES-GFP have been described elsewhere (19).

FACS analyses. To determine the lineage of cells in the bone marrow of diseased $BMTMyc^{TEL2}$ mice, the first few moribund animals were assessed for the following lineage markers: Mac1, Gr1, Thy 1.2, CD3, CD4, CD8, B220, immunoglobulin M (IgM), Sca1, c-Kit, and CD34. Because the mice clearly died of a B-lymphoid disease ($B220^+$, partly IgM^+), all subsequent animals were only checked for the lymphoid markers B220 and IgM. Single-cell suspensions of bone marrow were made in staining medium (SM; Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 20 mM sodium azide) containing 100 mg of gamma globulin (Sigma)/ml to block Fc receptors. After incubating for 30 min on ice, cells were washed and suspended in SM containing a titered excess of monoclonal antibodies (anti-sIgM [Southern Biotechnology Associates, Birmingham, Ala.] and anti-B220 [Pharmingen]) for 30 min on ice. Cells were then washed and resuspended in SM containing PI (0.018 mg/ml), filtered through 40-μm nylon mesh (Small Parts, Inc., Miami, Fla.), and analyzed using a BD Biosciences FACSCalibur flow cytometer. Dead cells were excluded from the analysis by electronic gating to select PI-negative cells.

Protein analyses. Protein extracts were prepared from mouse tissues, wild-type pro-B cells, or pro-B cells transduced with TEL2 retrovirus, using TRIzol reagent (Invitrogen, Carlsbad, Calif.) according to the manufacturer's instructions. The proteins were quantified using the bicinchoninic acid protein assay reagent (Pierce Chemical Co., Rockford, Ill.). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% gel) under reducing conditions and transferred to a polyvinylidine difluoride membrane (Millipore, Billerica, Mass.). Membranes were incubated with antibodies specific for p53 (Ab-7; Calbiochem, La Jolla, Calif.); p19 ARF (ab80; Abcam, Cambridge, United Kingdom); anti-actin (I-19 sc-1616; Santa Cruz); Bcl2 (554218; BD PharMingen); E2f1 (32-1400; Zymed Laboratories Inc., San Francisco, Calif.); c-Myc, β-catenin, and Mdm2 (Santa Cruz); and Bcl-X_L (Transduction Laboratories, Lexington, Ky.). The blots were developed by using the enhanced chemiluminescence kit (NEN-Perkin Elmer Life Sciences, Torrance, Calif.).

TEL2 antibody production. Because our original TEL2 antibody (30) had a low affinity, we raised a new antibody. A peptide corresponding to the 14 carboxy-terminal amino acids (DRIEFKDKRPEISP) of *TEL2* was synthesized, conjugated to keyhole limpet hemocyanin, and injected into New Zealand White rabbits (Rockland, Gilbertsville, Pa.). *TEL2*-specific antibodies were affinity purified on a CH-activated Sepharose 4B column (Sigma) with coupled DRIEF KDKRPEISP peptide.

In vitro culture of pro-B cells and retroviral infections. The BMCs of 4- to 6-week-old C57BL/6/129svJ mixed-background mice were harvested from the femurs and tibiae. After hypotonic lysis of the red blood cells in Gey's solution, the cells were plated on an S17 stromal layer (35) in RPMI 1640 medium supplemented with 10% fetal calf serum (HyClone), 55 μM 2-mercaptoethanol, 2 mM glutamine, penicillin (100 IU/ml), streptomycin (100 μg/ml), and murine IL-7 (50 ng/ml) to stimulate the outgrowth of B-cell progenitors. After 1 week of culture, the surviving cells were immunophenotyped by flow cytometry, and over 95% of cells were positive for the markers $B220^+$ and $CD43^+$ but negative for IgM, T-cell-specific or myeloid/macrophage-specific markers, consistent with a pro-B-cell phenotype (data not shown). The pro-B cells were infected twice daily for 48 h with retroviral vectors MSCV-IRES-GFP, MSCV-TEL2-IRES-GFP, MSCV-TEL2DBDM-IRES-GFP, and MSCV-TEL2ΔPNT-IRES-GFP in the presence of Polybrene (8 μg/ml) and IL-7. Infected GFP^+ cells were sorted by FACS and expanded in liquid culture in IL-7-containing growth medium. Greater than 98% of cells in the pro-B-cell–TEL2 cultures were GFP^+.

Cell cycle analysis and Annexin-V staining of cultured pro-B cells. Wild-type and TEL2-expressing pro-B cells were cultured on S17 stromal cells with IL-7. Apoptotic cells were identified by FACS after Annexin-V–fluorescein isothiocyanate staining, and PI staining was used to exclude dead cells. Cell cycle analysis was performed by FACS by quantitative staining of the DNA in cell nuclei through a rapid one-step protocol with PI. Briefly, the samples were prepared at a concentration of 10^6 cells/ml and washed in a hypotonic solution containing PI and 0.1% Triton X-100, resulting in bare nuclei that are quantitatively stained with PI. The nuclei were treated with DNase-free RNase and filtered through a 40-μm-diameter mesh to eliminate clumps of nuclei. Finally, the percentages of cells within G_0/G1, S, or G_2/M were determined by measuring the DNA content using flow cytometry.

Patient samples and real-time quantitative RT-PCR. With the approval of the St. Jude Children's Research Hospital Institutional Review Board, patient samples were obtained from Istanbul University, Institute for Experimental Medicine, Department of Genetics. Bone marrow samples were collected at the time of diagnosis from children with ALL or from healthy donors, with informed consent of their parents according to Turkish law. No identifiers were linked to any of these patient samples. Mononuclear cells were separated by Ficoll-Hypaque centrifugation. Total RNA was isolated by guanidium thiocyanate-phenol-chloroform extraction, and cDNA synthesis was performed using 1 μg of RNA and random hexamer primers. Real-time reverse transcription-PCR (RT-PCR) was performed using a 7900HT sequence detection system (ABI, Foster City, Calif.), TaqMan universal PCR master mix reagents, and a 1/10 volume of the cDNA. Human *TEL2* and *MYC* primers and probes were designed using Primer Express version 2.0 software (ABI). Amplification of 18S rRNA was performed in the same reaction tube as an internal control with an alternatively labeled probe (ABI). cDNA from total RNA of normal bone marrow (bone marrow pooled from eight male and eight female Caucasians; BD Bioscience) and four separate healthy donors was analyzed, and the average value of these samples was used as a control. Triplicate experiments were performed for each of the control and experimental samples.

RESULTS

TEL2 accelerates Myc-driven B-cell lymphomagenesis. We assessed if TEL2 was able to cooperate with c-Myc by using the Eμ-*Myc* transgenic mouse model (1) of human Burkitt's lymphoma (BL), which bears *MYC*/*Ig* translocations (3, 16). Myc overexpression in these mice forces the overproduction of cycling B-lineage cells (2) which are nontumorigenic due to their high rate of apoptosis (21). Ultimately, secondary changes in these tumors bypass this checkpoint and the mice succumb to a lethal B lymphoma by 4 to 6 months of age (11).

Following 5-fluorouracil treatment, bone marrow cells from preneoplastic Eμ-*Myc* mice were infected with MSCV-TEL2-IRES-GFP or control MSCV-IRES-GFP retroviruses (Fig. 1A) and then transplanted into 18 lethally irradiated syngeneic mice in three independent experiments. The course of disease was monitored by eye bleeds of mice transplanted with an equal number of BMCs that were transduced at equal efficiency (~50% GFP^+) (Fig. 1B). The average number of GFP^+ lymphocytes in the peripheral blood of mice transplanted with Eμ-*Myc* bone marrow expressing TEL2 ($BMTMyc^{TEL2}$) was

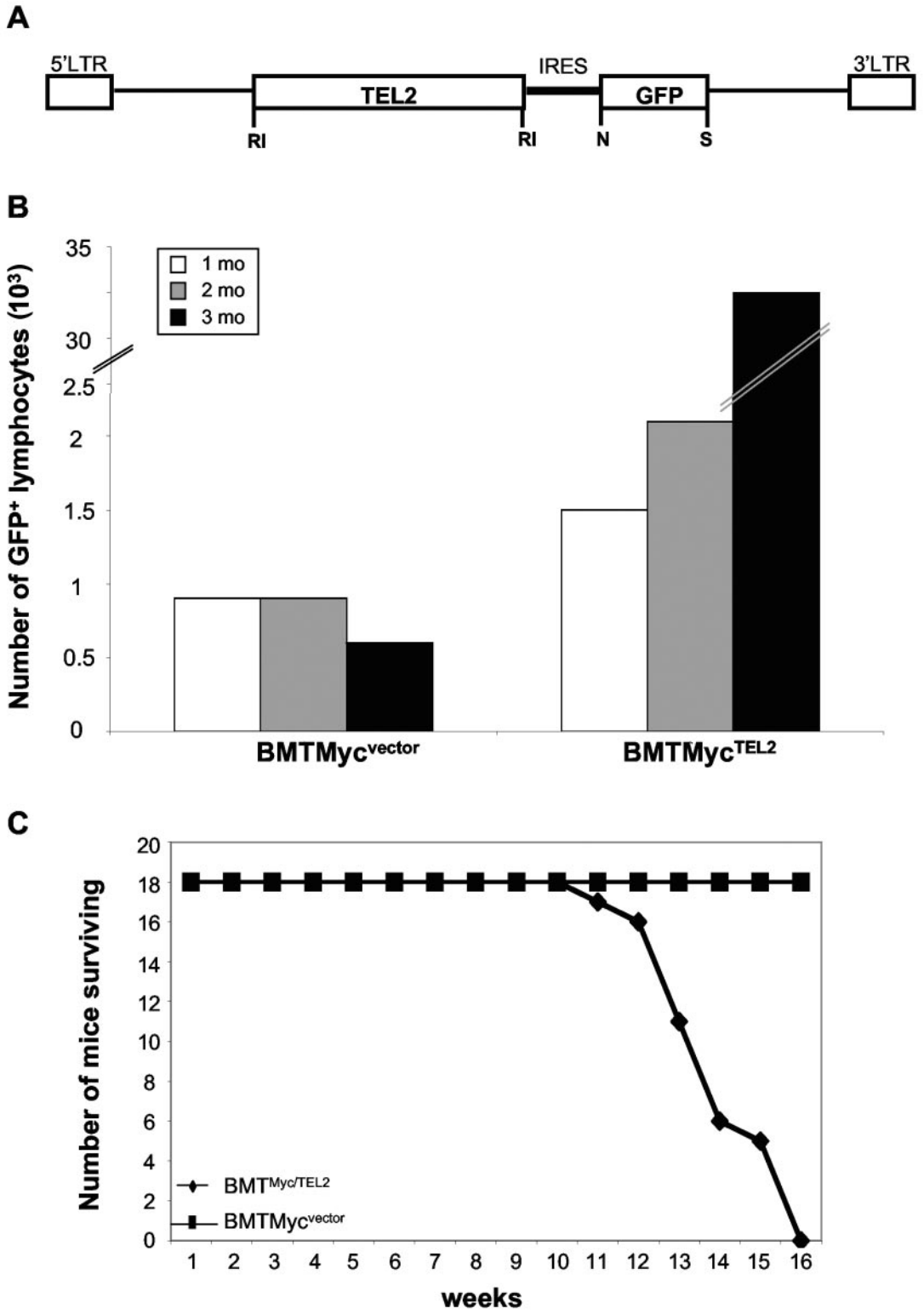

FIG. 1. c-*Myc* and *TEL2* cooperate in murine B lymphomagenesis. (A) Schematic of the MSCV-TEL2-IRES-GFP retroviral vector showing the *TEL2* cDNA followed by the *IRES* and *GFP* marker gene and the 5′ and 3′ long terminal repeats (LTR). RI, EcoRI; N, NotI; S, SalI. (B) Average numbers of GFP^+ lymphocytes in C57BL/6 mice transplanted with 10^6 Eμ-*Myc*/vector-transduced BMCs (48% GFP^+) or with 10^6 Eμ-*Myc*/TEL2-transduced BMCs (50% GFP^+). (C) Survival curve of C57BL/6 mice transplanted with 10^6 Eμ-*Myc*/vector-transduced BMCs or with 10^6 Eμ-*Myc*/TEL2-transduced BMCs. All $BMTMyc^{TEL2}$ mice died of B-cell lymphoma by 16 weeks posttransplantation. The cumulative data of three independent experiments are shown.

slightly augmented at 1 month ($1.5 \times 10^3/\mu l$) versus those receiving vector alone ($BMTMyc^{vector}$; $10^3/\mu l$) (Fig. 1B). In the following 2 months, the numbers of GFP^+ lymphocytes in peripheral blood decreased slightly in $BMTMyc^{vector}$ mice to $0.8 \times 10^3/\mu l$. Strikingly, the numbers of GFP^+ lymphocytes in the peripheral blood of $BMTMyc^{TEL2}$ mice almost doubled by the second month ($2.1 \times 10^3/\mu l$) and increased an additional 15-fold ($35.1 \times 10^3/\mu l$) by the third month. Therefore, TEL2 provides a profound growth advantage to Myc-expressing B lymphocytes.

With time $BMTMyc^{TEL2}$ mice became progressively moribund, had obvious signs of lympholeukemia (white blood cell count of 1×10^5 to $2 \times 10^5/\mu l$), and by 4 months all $BMTMyc^{TEL2}$ mice died of aggressive lymphoma (Fig. 1C). By contrast, $BMTMyc^{vector}$ mice survived 6 to 7 months posttransplant. Disease arising in $BMTMyc^{TEL2}$ mice was characterized by large numbers of malignant $B220^+$ lymphoblasts in the peripheral blood (Fig. 2A) and bone marrow (data not shown) that expressed GFP^+ (Fig. 2B, left panel), and a small percentage (2 to 15%) also expressed surface IgM (Fig. 2B, right panel). All diseased $BMTMyc^{TEL2}$ mice displayed splenomegaly and lymphadenopathy and had diffuse GFP^+ lymphoblastic infiltrates of the liver, lung, stomach, kidney, brain, and lymph nodes (Fig. 2C to F and data not shown). Therefore, $BMTMyc^{TEL2}$ mice develop disseminating B-cell lymphoma identical to that manifested in Eμ-*Myc* transgenic mice but with a much-reduced latency period.

Lymphomas arising in Eμ-*Myc* mice are transplantable and produce IL-7-independent cell lines when put into culture (21). Indeed, cells from the peripheral blood or spleens of diseased $BMTMyc^{TEL2}$ mice efficiently produced IL-7-independent cell lines. Further, injection of 10^6 bone marrow cells of two diseased $BMTMyc^{TEL2}$ mice into sublethally (450 cGy) irradiated syngeneic secondary recipients led to death of all recipients within 4 to 5 weeks posttransplant, whereas mice transplanted with $BMTMyc^{vector}$ bone marrow (from the same primary transplant) lacked any signs of disease within 8 months following transplant (data not shown). Finally, lymphomas arising in the secondary $BMTMyc^{TEL2}$ recipients were phenotypically identical to those arising in primary recipients (data not shown). Therefore, TEL2 and Myc cooperate to fully transform B cells.

To confirm that TEL2 transcriptional activity was required for cooperation with Myc, we also evaluated the activity of a TEL2–DNA-binding domain mutant (DBDM) that fails to bind DNA (19). Bone marrow derived from precancerous Eμ-*Myc* mice was infected with MSCV-TEL2-DBDM-IRES-GFP or MSCV-TEL2-IRES-GFP retroviruses, and these cells expressed comparable levels of TEL2-DBDM and TEL2 proteins (data not shown). Lethally irradiated mice were then transplanted and observed for disease. All $BMTMyc^{TEL2}$ mice developed lympholeukemia within 4 months, whereas the $BMTMyc^{TEL2\text{-}DBDM}$ mice, like $BMTMyc^{vector}$ recipients, lacked signs of disease until 6 to 7 months posttransplant. Therefore, TEL2 must bind to its target genes to cooperate with Myc in lymphomagenesis.

p53 is mutated in Eμ-*Myc*/TEL2 tumors. Genes that cooperate with Myc in the Eμ-*Myc* lymphoma model often disable Myc's apoptotic response (11, 12, 38–40). The emergence of lymphoma in Eμ-*Myc* transgenic mice is associated with inactivation of the p53 tumor suppressor pathway in most tumors (11) through missense point mutations in p53 that create dominant-negative forms of the protein (~25% of lymphomas), biallelic deletion of *Arf* (~25%), and/or overexpression of Mdm2 (~50%) (11). Immunoblotting using an anti-TEL2 antibody confirmed that all Eμ-*Myc*/TEL2 tumors expressed high levels of TEL2 protein (Fig. 3A). Strikingly, supraphysiological levels of p53, a hallmark of p53 mutations (11), were evident in 12 out of 15 lymphomas arising in $BMTMyc^{TEL2}$ mice (Fig. 3A, lanes 1 to 15), compared to absent or low levels of p53 expressed in FACS-analyzed $B220^+$ normal spleen cells and in $BMTMyc^{vector}$ spleen cells (Fig. 3A, lanes B220, Eμ-Myc T, and $BMTMyc^{vector}$). p53 negatively regulates the transcription of *Arf* (42), and thus another hallmark of tumors bearing p53 mutations is a dramatic up-regulation of Arf protein levels (11). Indeed, moderate to high levels of $p19^{Arf}$ protein were detected in all but one lymphoma of Eμ-*Myc*/TEL2 BMT mice (Fig. 3A). Thus, there is a marked bias towards p53 mutations in tumors of Eμ-*Myc*/TEL2 BMT mice.

TEL2 suppresses Myc-induced apoptosis, augments B-cell proliferation, and provokes B-cell immortalization. The ability of TEL2 to accelerate disease in Eμ-*Myc* transgenic mice suggested that TEL2 should impair Myc-induced apoptosis and/or accelerate B-cell growth. To assess the effects of TEL2 on Myc-induced apoptosis, Lin^- BMCs from Eμ-*Myc* mice were transduced with MSCV-TEL2-IRES-GFP or MSCV-IRES-GFP retroviruses and cultured in vitro for 5 days, and GFP-sorted cells were assessed for their apoptotic index (Fig. 4A). Levels of c-Myc were greatly elevated in Eμ-*Myc* cultures versus wild-type bone marrow (Fig. 4B), and the apoptotic indices of Eμ-*Myc*- and Eμ-*Myc* vector-transduced cells were high (around 30%) (Fig. 4A). Notably, expression of TEL2 reduced the apoptotic index of Eμ-*Myc* cells about twofold (15%) (Fig. 4A). Therefore, TEL2 suppresses Myc-induced apoptosis.

To gain insights into TEL2's effects on B-cell growth and survival, wild-type B-cell progenitors, grown on S17 stromal cells with IL-7 (35), were transduced with MSCV-TEL2-IRES-GFP or MSCV-IRES-GFP retrovirus. Within 1 to 2 weeks of culture, 90% of cells were $B220^+$, $CD43^+$, IgM^- pro-B cells as judged by flow cytometry (data not shown). The proliferative rates of TEL2-transduced, vector-transduced, or nontransduced pro-B cells were then determined. Interestingly, TEL2-expressing pro-B cells grew three times faster than vector-transduced or nontransduced pro-B cells (Fig. 5A), and this was associated with an increased percentage of cells in $S/G_2/M$ phases of the cell cycle (44.5 versus 33% for wild-type pro-B cells); therefore, TEL2 accelerates cell cycle traverse. When the cells were deprived from the S17 feeder layer and IL-7, TEL2 alone was sufficient to override cell cycle arrest of pro-B cells short term (Fig. 5B). While approximately 85% of wild-type pro-B cells arrested in G_0 following a 24-h withdrawal of S17 stroma and IL-7, 39% of TEL2-expressing pro-B cells remained in the S/G2/M phase (Fig. 5B). However, after withdrawal for 72 h only 35% of the TEL2 pre-B cells survived and these were not cycling, since 96% of these cells were in the G_0/G1 phase of the cell cycle (data not shown).

Finally, the effects of TEL2 on B-cell growth required its protein-protein interaction and transcription functions, as B-cell progenitors transduced with retroviruses that expressed either TEL2ΔPNT, which lacks the pointed protein-protein interaction domain (19), or TEL2-DBDM were comparable to

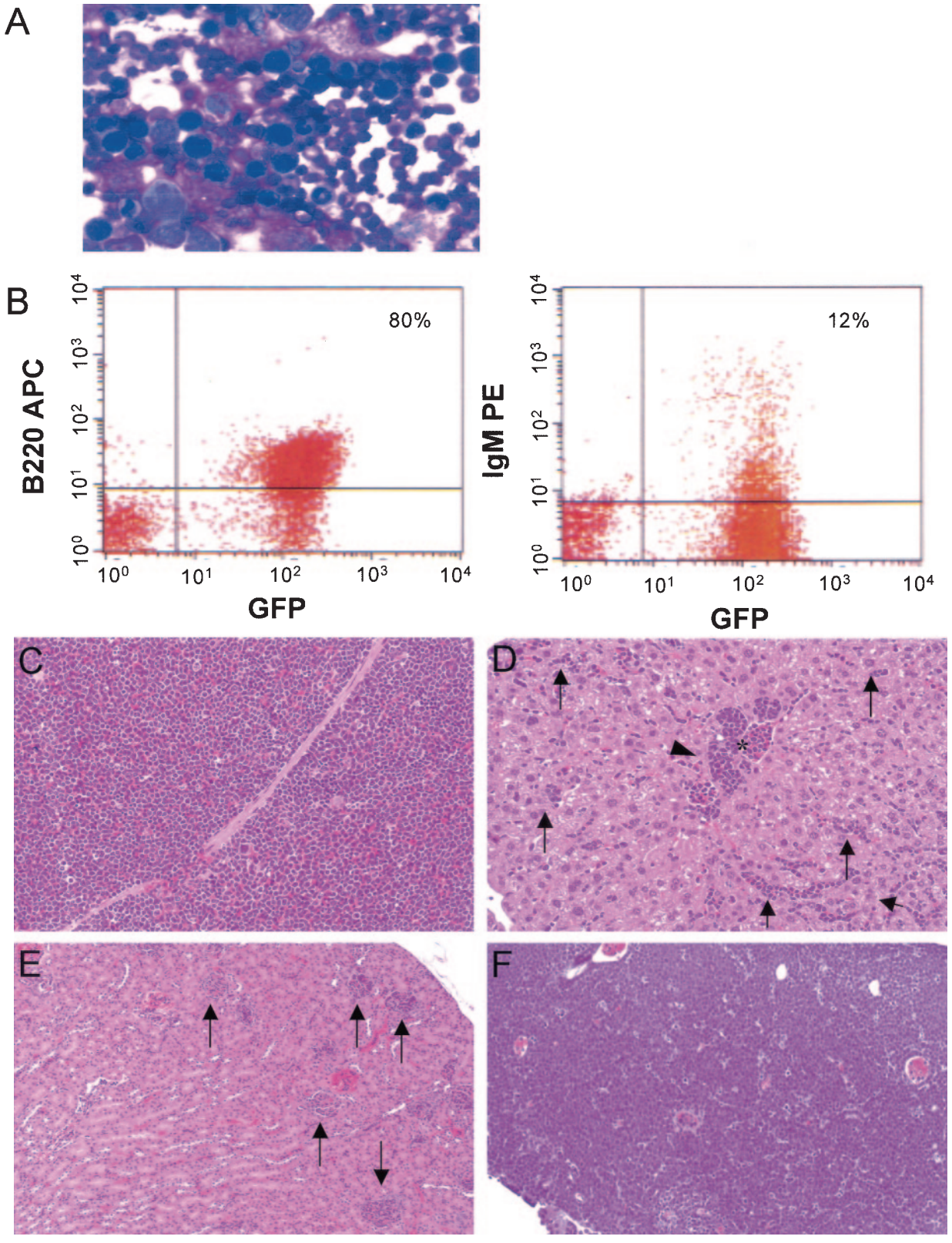

FIG. 2. Eμ-*Myc*/TEL2-transplanted mice develop B220$^+$ B-cell lymphoma. (A) Wright-Giemsa-stained peripheral blood smear showing cells with morphological features of lymphoblasts in diseased animals transplanted with Eμ-*Myc*/TEL2 bone marrow. Magnification, ×400. (B) Flow cytometric immunophenotyping of BMCs of a diseased BMTMyc^{TEL2} mouse. The lineage-specific B220 antibodies were labeled with allophycocyanin (APC), and the IgM antibodies were labeled with phycoerythrin (PE). Most of the cells (80%) were B220$^+$, and a small (10%) subpopulation was also IgM$^+$, which is typical for B-cell lymphomas occurring in Eμ-*Myc* mice. Diseased BMTMyc^{TEL2} mice were characterized by massive organ infiltration of lymphoblastic cells. Hematoxylin and eosin staining showed the following: an extensive presence of neoplastic cells in the spleen (C); neoplastic lymphocytes forming cuffs around vessels (arrowheads), expanded sinusoids (arrows), and distended pulmonary veins (*) in the liver (D); neoplastic cells in the glomerular capillaries (arrows) in the kidney (E); and extensive infiltration of lymphoblastic cells in the lymph nodes (F). Magnification, ×100 (lymph node) or ×200 (spleen, liver, and kidney).

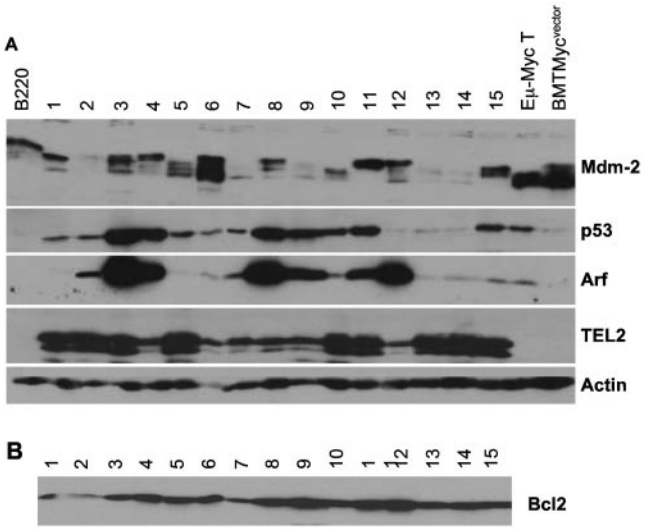

FIG. 3. Eμ-*Myc*/TEL2 tumors display a marked bias towards p53 mutations. (A) Western blots of spleen cell lysates from diseased $BMTMyc^{TEL2}$ mice (lanes 1 to 15) were incubated with Mdm2, p53, Arf, TEL2, and actin antibodies, indicated at the right of the panels. Controls were a 100-μg aliquot of lysate of sorted B220 normal spleen cells (B220), of a Eμ-*Myc* tumor cell line bearing a p53 mutation, and of a spleen of a healthy $BMTMyc^{vector}$-transplanted mouse euthanized 3 months after transplantation ($BMTMyc^{vector}$). All but three infiltrated spleen samples overexpressed mutant p53 (lanes 12 to 14). All but one lymphoma (lane 1) expressed moderate to high levels of Arf. As expected, all Eμ-*Myc*/TEL2 tumors expressed TEL2. Actin is shown as a loading control. (B) Western blot of the same filter with Bcl-2 antibody. All Eμ-*Myc*/TEL2 BMT tumors expressed elevated levels of Bcl-2, which is expressed at very low levels in sorted $B220^+$, IgM^- Eμ-*Myc* spleen cells (12; see also Fig. 6 of reference 12).

vector-only-transduced cell cultures in their growth properties (data not shown).

To determine the effects of TEL2 on pro-B-cell survival, we performed Annexin-V assays in the presence or absence of S17 stroma and IL-7. Notably, in the presence of S17 stroma and IL-7, TEL2-expressing pro-B cells showed a threefold lower apoptotic index than wild-type or vector-transduced pro-B cells (Fig. 5C). TEL2 also provided a remarkable short-term survival advantage to pro-B cells when they were deprived of IL-7 and S17 stroma contacts (Fig. 5C). Therefore, TEL2's effects on promoting survival may also contribute to the increased proliferative rates of TEL2-expressing B cells.

These findings suggested that TEL2 targets both cell cycle and apoptotic regulators. We therefore canvassed likely culprits in these responses, including the cell cycle regulators E2f1, c-Myc, $p27^{KIP1}$, and $p16^{ink4a}$ and the apoptotic regulators Bcl-2 and Bcl-X_L. By 1 month of culture, there were obvious increases in the levels of E2f1 and c-Myc in TEL2-expressing versus control pro-B-cell cultures (Fig. 5D), but there was essentially no change in the levels of $p27^{KIP1}$ (data not shown). Further, although there were no differences in Bcl-X_L expression (data not shown), there was a marked up-regulation of Bcl-2 levels in TEL2-expressing pro-B cells (Fig. 5D). Myc suppresses Bcl-2 and Bcl-X_L expression in B cells, and bypass of this pathway also contributes to Myc-induced lymphomagenesis (12). Therefore, TEL2 may impair Myc-induced apoptosis through its ability to up-regulate Bcl-2. Indeed, immunoblotting confirmed that malignant spleen cells of Eμ-*Myc*/TEL2 BMT mice expressed high levels of Bcl-2 (Fig. 4B), while precancerous $B220^+$, IgM^- Eμ-*Myc* spleen cells express very low levels of Bcl2 (12).

Strikingly, unlike wild-type- or vector-only-transduced cultures that slowed in their growth rates and senesced by 1 to 2 months, TEL2-expressing pro-B cells grew indefinitely without undergoing replicative crisis. Therefore, TEL2 behaves as an immortalizing oncogene in pro-B cells. Immortalization of mouse cells usually involves missense mutations of p53 or deletion or silencing of *Arf* or *p16^Ink4a^* (18, 32, 48, 49). We therefore assessed the status of p53, Arf, and $p16^{Ink4a}$ of TEL2-expressing pro-B cells with time in culture. Immortalization via p53 mutations provokes profound increases in p53 protein and in $p19^{Arf}$, due to the loss of the p53-to-Arf feedback loop (18, 32, 48). However, p53 protein levels remained very low in TEL2-expressing cultures. A modest increase in $p19^{Arf}$ protein was noted, but by 4 months Arf expression was entirely silenced (Fig. 5D), consistent with studies showing that loss of *Arf* alone is sufficient to immortalize mouse pre-B cells

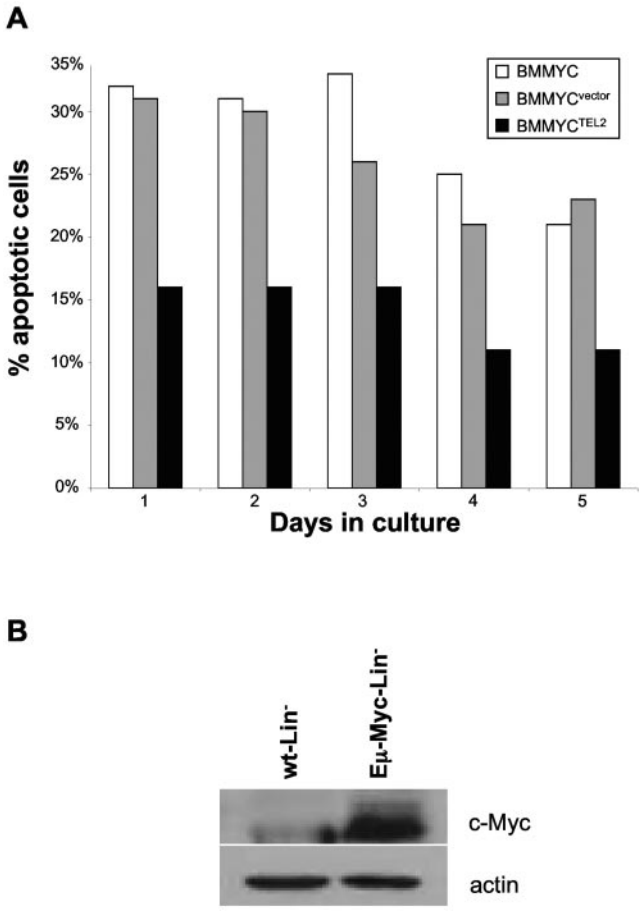

FIG. 4. TEL2 inhibits Myc-induced apoptosis. (A) Eμ-*Myc* BMCs transduced with MSCV-TEL2-IRES-GFP or MSCV-IRES-GFP viruses were cultured in vitro for 5 days. At each day the percentage of cells expressing Annexin-V was determined using flow cytometry. (B) Immunoblot showing c-Myc expression in wild-type Lin^--selected and Eμ-*Myc* Lin^--selected cells to confirm c-Myc overexpression in Eμ-*Myc* cells. Immunoblotting against actin was used as a loading control.

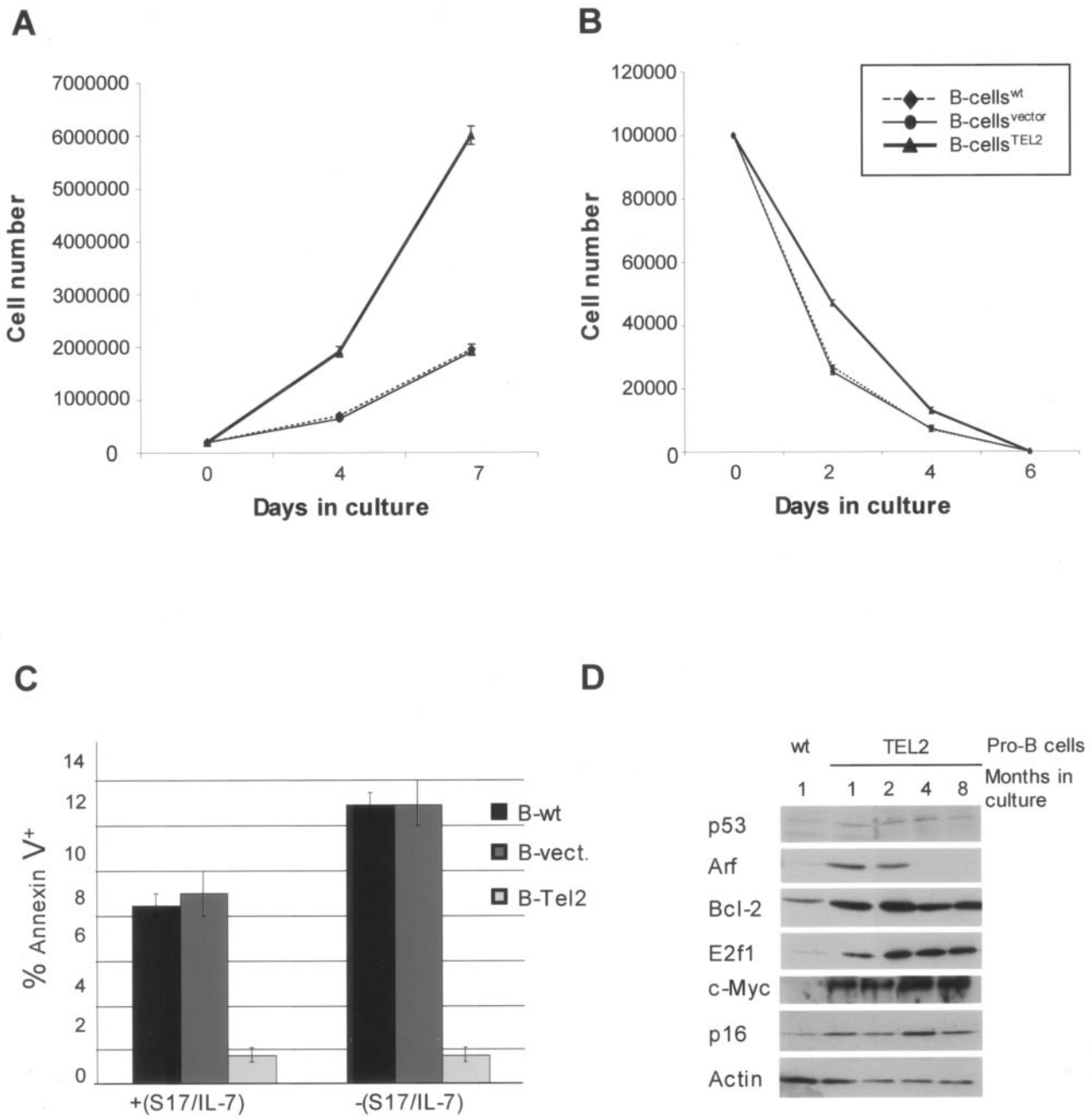

FIG. 5. TEL2 accelerates pro-B-cell proliferation and promotes pro-B-cell survival and immortalization. (A) Primary pro-B cells were transduced with MSCV-TEL2-IRES-GFP or MSCV-IRES-GFP. Sorted GFP^+ cells (10^5) were plated onto an S17 stromal layer with IL-7 and counted after trypan blue staining to exclude dead cells from the total counts. The growth curve shows the number of cells in the different cultures at the indicated days during 1 week of culture. The average of three experiments is shown. Bars indicate the standard deviation. (B) Cells (10^5) were plated without S17 stromal cells and IL-7. The number of live cells at different days in the cultures was determined by counting trypan blue-negative cells at the indicated days during 1 week of culture. The average of three experiments is shown. Bars indicate the standard deviation. (C) Apoptotic indices (Annexin-V^+) of wild-type pro-B cells, vector-only-expressing pro-B cells, and TEL2-expressing pro-B cells were compared after 3 weeks in culture on S17 feeder cells and IL-7 (left) by using flow cytometry. After these 3 weeks of culture, 10^7 cells were taken and deprived of S17 and IL-7 for 24 h and the apoptotic index was determined (right). TEL2-expressing pro-B cells are less sensitive to growth factor deprivation initially, most likely due to their increased Bcl-2 expression. The average of three experiments is shown. Bars indicate the standard deviation. (D) TEL2-expressing pro-B cells were cultured for 8 months. At 1, 2, 4, and 8 months, 10^7 cells were removed from the culture, lysed, and assessed for their expression of p53, Arf, Bcl-2, E2f1, c-Myc, and $p16^{ink4a}$. Their expression levels were compared to levels in wild-type pro-B cells and cultured for 1 month, after which time they senesced. TEL2-expressing pro-B cells expressed high levels of Bcl-2, E2f1, and c-Myc, which would promote their growth and survival. As a result of this proliferative stress, TEL2-expressing pro-B cells show a slightly increased expression of p53 and a considerable increase in Arf expression during the first 2 months of culture. Thereafter, Arf expression was lost, indicating the emergence of immortalized cells. Also note that Arf expression was selectively silenced as $p16^{Ink4a}$ expression increased with time in culture.

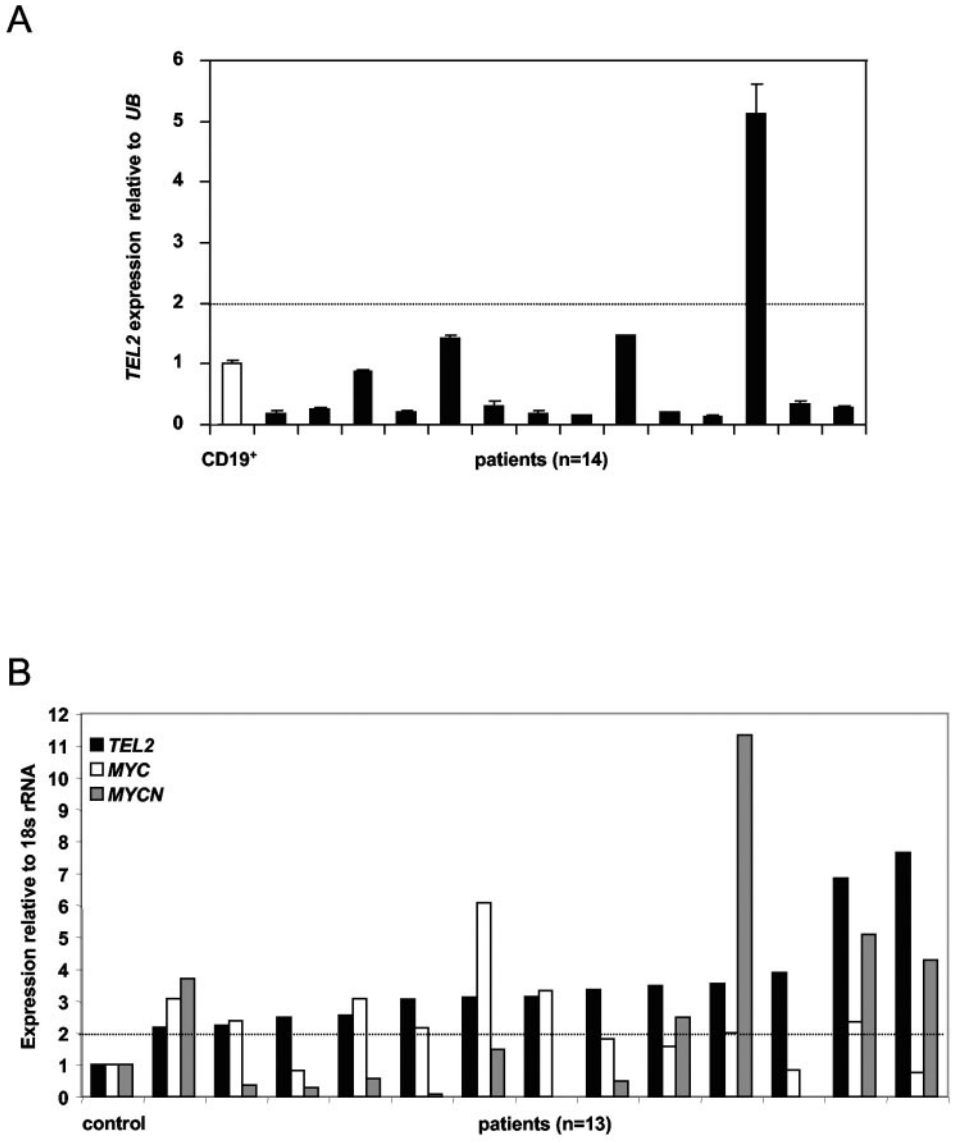

FIG. 6. Expression of *TEL2* in BL patients and of *TEL2*, *MYC*, and *MYCN* in pediatric B-ALL patients. (A) *TEL2* mRNA levels in 14 BL samples was determined by quantitative RT-PCR and compared with the level of *TEL2* mRNA in $CD19^+$ FACS-analyzed peripheral blood B cells of a normal donor. The relative levels of *TEL2* mRNA in the different samples were determined after normalization for the amount of *UB* mRNA. *TEL2* mRNA levels in $CD19^+$ FACS-analyzed peripheral blood B cells are twofold lower than in control bone marrow (data not shown). One BL sample showed a fivefold-elevated level of *TEL2* mRNA. The dotted line indicates the level of twofold overexpression. (B) *TEL2* and *MYC*/*MYCN* mRNA levels were determined in bone marrow aspirates of 23 B-ALL patients by quantitative RT-PCR and compared to the levels in normal bone marrow (control). The level of 18S rRNA was used as internal control. Data for the 13 patients (patients 11 to 23) (see also Table S1 in the supplemental material) with *TEL2* RNA levels twofold higher than that in control bone marrow are shown. The levels of *MYC*, *MYCN*, and *TEL2* mRNA of all 23 patients are shown in Fig. S1 in the supplemental material. The mRNA levels of *TEL2*, *MYC*, and *MYCN* in bone marrow were set at 1. The dotted line indicates twofold overexpression compared to normal bone marrow. Only patients 13 and 18 failed to show combined up-regulation of *TEL2* and *MYC*/*MYCN*, whereas the N-MYC level in patients 17 and 21 could not be determined due to insufficient RNA.

in culture (32). Loss of $p19^{Arf}$ protein likely occurred through selective silencing of *Arf* transcription, as with time TEL2-expressing cultures showed significant increases in $p16^{Ink4a}$ (Fig. 5D), similar to that noted in pre-B-cell lines established from *Arf*-null mice (32). Therefore, TEL2-induced immortalization involves loss of *Arf* but not $p16^{Ink4a}$.

***TEL2*, *MYC*, and *MYCN* are overexpressed in a subset of human B-ALL.** *TEL2* is not present in the mouse genome, but because TEL2 cooperated with Myc in lymphoma development in Eμ-*Myc* transgenic mice, we evaluated *TEL2* expression in human BL, which bears *MYC*/*Ig* translocations (3, 16). However, quantitative RT-PCR of 14 primary, sporadic BL samples established that *TEL2* was overexpressed in only one BL sample relative to its expression in normal peripheral blood $CD19^+$ B lymphocytes (Fig. 6A). Therefore, activation of *TEL2* appears infrequent in sporadic BL.

MYC is overexpressed in a large number of human tumors, including lymphoma and leukemia (37). Previously we determined, in a collection of 23 pediatric ALL samples heavily biased towards t(12;21) pre-B-ALL, that some ALL expressed

elevated levels of *TEL2* mRNA (19). To determine if the combination of elevated expression of *MYC* and *TEL2* might collaborate in other pediatric lymphomas or leukemias, we evaluated their expression in eight pediatric T-ALL (mostly FAB subtypes L1 and L2) (see Table S1 in the supplemental material) and 23 pediatric B-ALL (mostly FAB subtype L1 and L2) (Table S1) compared to that in normal bone marrow. All T-ALL expressed low levels of *TEL2* (data not shown). However, 13 out of 23 B-ALL expressed *TEL2* at levels two- to eightfold higher than those expressed in normal bone marrow (Fig. 6B; see also Fig. S1 in the supplemental material). Importantly, eight of these also expressed elevated levels of *MYC* (two- to sixfold higher than normal bone marrow), and two others expressed elevated levels of *MYCN* (Fig. 6B). Only 2 of the 13 patients with elevated TEL2 expressed neither elevated *MYC* nor *MYCN*. Therefore, increased coexpression of *MYC*/*MYCN* and *TEL2* occurs in pediatric B-cell malignancies.

DISCUSSION

TEL2 is an oncogene that cooperates with c-Myc in lymphomagenesis. TEL1 is currently the only member of this subfamily of ETS transcriptional repressors associated with human malignancy (23). Here we report that TEL2 behaves as an oncogene that promotes the growth, survival, and immortalization of B cells and that cooperates with Myc in lymphoma development. Further, the coincident, high levels of *TEL2* and *MYC*/*MYCN* in a cadre of B-ALL suggest that TEL2 also cooperates with MYC in the development of human B-cell malignancies.

TEL2 is located on chromosome 6p21, a region implicated in a number of cancers, including chondroid hamartomas, thyroid adenomas, ductal carcinoma in situ of the breast, B-cell non-Hodgkin's lymphoma, cervical cancer, astrocytoma, non-small cell lung carcinomas, and ovarian carcinomas (20, 24, 26). Elevated levels of TEL2 have been reported in some cancer cell lines (15) and in various leukemia samples, including B-ALL (19, 29). However, despite these correlative data, there was heretofore no causal link between TEL2 expression and tumor development in humans.

TEL2 is expressed at very low levels in most tissues (29) but is expressed at higher levels in hematopoietic tissues (30). TEL2 promotes Ras-induced transformation and inhibits vitamin D_3-induced differentiation of HL60 and U937 myeloid cells (19). In addition, enforced TEL2 expression in mouse bone marrow results in a myeloproliferative disease with a long latency period and its expression in *Arf*-null bone marrow promotes B-cell lymphomagenesis (C. Carella and G. Grosveld, unpublished results), further supporting the notion that TEL2 functions as an oncogene. Indeed, our results establish that the antiapoptotic and growth-promoting activities of TEL2 cooperate with c-Myc in B lymphomagenesis and that TEL2 impairs Myc-induced apoptosis. One expectation from our TEL2/Eμ-*Myc* studies was that *TEL2* would also show involvement in BL and, indeed, many BL carry missense point mutations in *P53* or other defects in the $p14^{ARF}$-HDM2-p53 pathway (36). However, connections between *TEL2* and *MYC* in BL were rare. Nonetheless, there is simultaneous up-regulation of *TEL2* and *MYC*/*MYCN* in pediatric patients with B-lymphoid malignancies, and we postulate this combination contributes to lymphomagenesis in these cases. There was no correlation of elevated TEL2 expression with disease prognosis, survival rate, FAB subtype, or white cell counts.

TEL2 promotes B-cell survival. The data presented herein suggest that TEL2 transcription functions cooperate with Myc, at least in part, through its ability to inhibit Myc-induced apoptosis. However, the emergence of tumorigenic clones in Eμ-*Myc*/TEL2 mice only occurred when Myc's apoptotic pathways were disabled through p53 mutations. We therefore propose that TEL2's effects on enhancing B-cell proliferation contribute to accelerated disease by enlarging the cycling B-cell compartment in Eμ-*Myc* mice, thereby increasing chances for p53 mutations. Similar effects can also be accomplished by overexpressing antiapoptotic proteins, such as Bcl2 or Bcl-X_L (6, 43), and it is thus also notable that a hallmark of Eμ-*Myc*/TEL2 lymphomas was a marked up-regulation in Bcl-2, which is suppressed in precancerous B cells of Eμ-*Myc* transgenic mice (12). The *Bcl-2* promoter harbors several elements that respond to Ets transcription factors (8), but at present it is unclear whether TEL2 directly regulates *Bcl-2*. However, a widespread role for Ets transcription factors regulating apoptosis has been proposed. For example, Ets2 inhibits apoptosis following growth factor deprivation by inducing *Bcl-X* (41), whereas TEL1 promotes apoptosis by down-regulating *Bcl-X* (17). Up-regulation of Bcl-2 by TEL2 is particularly relevant in pre-B cells, where Bcl-2 expression is normally low and Bcl-X_L is high (14). Thus, TEL2's ability to augment Bcl-2 expression in this compartment would be predicted to spare pre-B cells at risk of suicide, particularly those that overexpress Myc, and elevated levels of Bcl-2 are also likely responsible for the delayed programmed cell deaths of TEL2-expressing pre-B cells following the withdrawal of IL-7 and stromal contacts (Fig. 5C).

TEL2 accelerates B-cell proliferation. Enforced expression of TEL2 in cultured pre-B cells increases the expression of both c-Myc and E2f1 (Fig. 5D). Since the growth-promoting effects of Myc are dependent upon E2f1 in pre-B cells (5), increases in the thresholds of these two transcription factors likely mediate TEL2's ability to augment pre-B-cell proliferation. This fits well with the simultaneous up-regulation of Arf in TEL2-expressing cells, which responds to proliferative signals coming from Myc (11, 48) or E2f1 (4, 9). We favor this over a direct induction of *Arf* by TEL2, as TEL2 has only been shown to repress transcription. Finally, the combined effects of c-Myc and E2f1 put TEL2-expressing B cells under proliferative stress, a condition under which cells would be predisposed to losing and/or silencing *Arf*, a scenario operational during TEL2-directed B-cell immortalization.

MYC and TEL2 in human B-cell lymphomas. Although cooperation between MYC and TEL2 appears infrequent in sporadic human BL, approximately one-third of B-ALL samples displayed coordinated elevated expression of *MYC*/*MYCN* and *TEL2*. The frequency of elevated *TEL2* expression in this cohort of pediatric patient samples (34.8%) is considerably higher than in a previous cohort of pediatric ALL (8.7%) which was, however, biased towards t(12;21) pre-B-ALL patients (19). Given our experience with the mouse model, the *MYC*/*TEL2* combination is likely important in human lympholeukemia, and perhaps TEL2 should be considered as a diagnostic and/or therapeutic target in these malignancies.

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