Shp2\textsuperscript{E76K} Mutant Confers Cytokine-independent Survival of TF-1 Myeloid Cells by Up-regulating Bcl-X\textsubscript{L}*

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Shp2 has been known to mediate growth factor-stimulated cell proliferation, but its role in cell survival is less clear. Gain-of-function Shp2 mutants such as Shp2\textsuperscript{E76K} are associated with myeloid leukemias. We found that Shp2\textsuperscript{E76K} could transform cytokine-dependent human TF-1 myeloid cells into cytokine-independent survival. Roscovitine, which down-regulated mitotic/apoptosis pathway, which is controlled by the Bcl-2 family proteins. Analysis of Bcl-2 family proteins showed that Bcl-X\textsubscript{L} and Mcl-1 were up-regulated in Shp2\textsuperscript{E76K}-transformed TF-1 cells. Knockdown of Bcl-X\textsubscript{L} but not Mcl-1 with short hairpin RNAs prevented Shp2\textsuperscript{E76K}-induced cytokine-independent survival. Roscovitine, which down-regulated Mcl-1, also did not prevent cytokine-independent survival of TF-1/Shp2\textsuperscript{E76K} cells, whereas the Bcl-X\textsubscript{L} inhibitor HA14-1 did. Ras and mitogen-activated protein kinases Erk1 and Erk2 (Erk1/2) were constitutively activated in TF-1/Shp2\textsuperscript{E76K} cells, whereas little active Akt was detected under cytokine-free conditions. Shp2\textsuperscript{E76K}-induced Bcl-X\textsubscript{L} expression was suppressed by Mek inhibitors and by a dominant-negative Mek1 mutant but not by the phosphoinositide 3-phosphate inhibitor LY294002 and the Akt inhibitor API-2. Inhibition of Erk1/2 blocked cytokine-independent survival of TF-1/Shp2\textsuperscript{E76K} cells, whereas inhibition of Akt had a minimal effect on cytokine-independent survival of TF-1/Shp2\textsuperscript{E76K} cells, whereas the Bcl-X\textsubscript{L} inhibitor HA14-1 did.

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Shp2 is a non-receptor protein-tyrosine phosphatase (PTP)\textsuperscript{2} encoded by the PTPN11 gene (1). It contains two Src homologies-2 (SH2) domains (N-SH2, C-SH2), a PTP domain, and a carboxyl-terminal region. In resting cells, Shp2 PTP has a low basal PTP activity due to autoinhibition by its N-SH2 domain (2). In growth factor-stimulated cells, Shp2 binds to tyrosine-phosphorylated docking proteins such as Gab1 and Gab2 through its SH2 domains (3). Binding of Shp2 SH2 domains to these docking proteins relieves the autoinhibition, resulting in activation of Shp2 PTP activity (1, 4). Growth factor-activated Shp2 is known to play a positive role in activation of the Erk1 and Erk2 (Erk1/2) mitogen-activated protein kinases (1, 5, 6) and to mediate growth factor-stimulated cell proliferation (7–10). Although few studies have addressed the role of Shp2 in cell survival, a recent study (11) provided evidence that Shp2 is involved in fibroblast growth factor-4 (FGF4)-regulated survival of murine trophoblast stem cells.

In addition to being activated transiently by growth factors, Shp2 can be activated constitutively through point mutations (12–14). These gain-of-function Shp2 mutants have been found in Noonan syndrome, juvenile myelomonocytic leukemia (JMML), childhood myelodysplastic syndrome and myelo proliferative disorder, B-cell acute lymphoblastic leukemia, acute myelogenous leukemia, and in some cases of solid tumors (12, 13, 15–18). In particular, PTPN11 is frequently mutated in JMML patients, associating with ~35% of JMML cases (19). JMML is an aggressive disease characterized by overproduction of tissue-infiltrating myeloid cells. A hallmark of bone marrow and peripheral blood mononuclear cells from JMML patients is their ability to form granulocyte-macrophage colony-forming units in the absence of exogenous cytokines or at very low concentrations of granulocyte-macrophage colony-stimulating factor (GM-CSF) (20, 21). Autocrine and paracrine were ruled out in cytokine-independent formation of myeloid colonies (20).

Somatic PTPN11 mutations in hematologic malignancies occur most frequently in exon 3 that encodes amino acid residues of the N-SH2 domain (12, 13). It was reported that murine bone marrow or fetal liver cells transduced with retroviruses encoding the leukemia-associated Shp2\textsuperscript{E76K}, Shp2\textsuperscript{D61Y}, or Shp2\textsuperscript{E76K} mutant could evoke cytokine-independent myeloid colonies and display hypersensitivity to GM-CSF in methylcellulose cultures (22–24), suggesting that these Shp2 mutants

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2 The abbreviations used are: PTP, protein-tyrosine phosphatase; GM-CSF, granulocyte-macrophage colony-stimulating factor; Erk, extracellular signal-regulated kinase; SH2, Src homology-2; JMML, juvenile myelomonocytic leukemia; GFP, green fluorescence protein; 7-AAD, 7-aminoactinomycin D; PARP, poly(ADP-ribose) polymerase; FBS, fetal bovine serum; shRNA, short hairpin RNA; Mek, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; RNAi, RNA-mediated interference; HA, hemagglutinin; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
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have oncogenic potential. However, attempts to transform murine cytokine-dependent cell lines such as Ba/F3 cells with Shp2E76K and other Shp2 mutants have been unsuccessful (22, 25, 26).

TF-1 is a CD34+ human myeloid precursor cell line that requires GM-CSF or interleukin-3 for cell survival and proliferation. We report here that the leukemia-associated Shp2E76K mutant can transform TF-1 cells into cytokine-independence. We further analyzed Shp2E76K-induced cytokine-independent cell survival mechanism and found that up-regulation of Bcl-XL via the Erk1/2 pathway plays a critical role in the Shp2 mutant-induced cytokine-independent survival.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Monoclonal (M2) and polyclonal anti-FLAG antibodies, antibody to active Bax (6A7), and α-tubulin were from Sigma. Antibodies to the following proteins were from Santa Cruz Biotechnology: β-actin, Shp2, phospho-Erk1/2, Erk1/2, Akt, Ras, Stat5, Mcl-1, and Bax. Antibodies to poly(ADP-ribose) polymerase (PARP), cytochrome c, and Hsp60 were from BD Pharmingen. Other antibodies were from Cell Signaling Technology. GM-CSF was from Immunix. Roscovitine was from Calbiochem. HA14-1 was from Tocris Bioscience. U0126 and PD98059 were from Biomol. Doxorubicin and etoposide were from Sigma. API-2 (27) was obtained from the National Cancer Institute, National Institutes of Health.

Shp2 Retroviruses and Generation of Stable TF-1 Cell Lines—MSCV-P is a bicistronic retroviral vector derived from MigR1 (28) in which the green fluorescence protein (GFP) coding region has been replaced with a puromycin-resistance gene. MSCV-Shp2 and MSCV-Shp2E76K retroviral vectors were made by subcloning FLAG-tagged human wild type Shp2 and Shp2E76K-coding sequences into MSCV-P. MSCV, MSCV-Shp2, and MSCV-Shp2E76K retroviruses were prepared with Phoenix AmphiPack293 cells by transient transfection. Viruses containing supernatants were collected and filtered through a 0.45-μm filter.

TF-1 cells were cultured in RPMI 1640, 10% fetal bovine serum (FBS), 2–5 ng/ml human GM-CSF. For viral infection, TF-1 cells (3 × 10⁶) were incubated with retrovirus (8 ml) in the presence of Polybrene (5 μg/ml) and GM-CSF (5 ng/ml) for 24 h. After infection, cells were cultured in RPMI 1640, 10% FBS, 5 ng/ml GM-CSF for another 24 h before puromycin (0.5 μg/ml) was added to the medium. Puromycin selection for retrovirus-transduced cells (TF-1/V, TF-1/Shp2, and TF-1/ Shp2E76K) cells were performed in the presence of GM-CSF.

Knockdown of Bcl-XL and Mcl-1—The retroviral vector for human Bcl-XL shRNA (SINeG-XRi) and the control vector SINeG-Ri were kindly provided by Dr. Jirong Bai (29). VSV-G pseudotyped retroviral particles were prepared by co-transfection of Phoenix293 packaging cells with pVPack-GP, pVPack-VSV-G, and SINeG-XRi or SINeG-Ri. Supernatants (10 ml/each) were collected, filtered, and centrifuged at 25,000 rpm for 2 h with a Beckman SW41 rotor to concentrate the viral particles. Each pellet was resuspended in 0.5 ml of RPMI 1640, 10% FBS.

Two retroviral Mcl-1 shRNAs, Mcl1-RNAi692 and Mcl1-RNAi908, were prepared using the MSCV-LMP shRNA vector (Open Biosystems) that carries a GFP gene. The targeting sequences were GCTTCGAAAACGTGACATCAA (for Mcl1-RNAi692) and GGGACTGGCTAGTTAAAACAAAG (for Mcl1-RNAi908). The Mcl1-RNAi908 targeting sequence was the same as that used by others (30). VSV-G pseudotyped viruses were prepared as above.

For retrovirus infection, 0.5 ml TF-1/Shp2E76K cells (1 × 10⁶ cells/ml) in RPMI 1640, 10% FBS, 10 ng/ml GM-CSF, 6 μg/ml Polybrene were incubated with 0.5 ml of retrovirus in a 12-well plate and centrifuged at 2500 rpm at 30 °C for 1.5 h. After centrifugation, cells were transferred to 6-well plates containing 1 ml of RPMI 1640, 10% FBS, 5 ng/ml GM-CSF medium in each well and incubated for 24 h. For selection of GFP+ cells, cells were cultured in 10 ml of RPMI 1640, 10% FBS, 5 ng/ml GM-CSF for 48 h and then processed for cell sorting with a flow cytometer. For analysis of apoptosis, cells were washed twice with GM-CSF-free medium and incubated in 10 ml of RPMI 1640, 10% FBS for 4 days.

pLKO.1-based lentiviral Mcl-1 shRNA vectors, TRCN 0000055515 (designated here Mcl1-RNAi55515, targeting sequence GCAGAAAGTATCACAGACGTT) and TRCN 0000055517 (designated Mcl1-RNAi5517, targeting sequence GCTAAACACTTGAAGACCAT), were obtained from Open Biosystems. The non-target pLKO.1-scrambled shRNA (designated non-target-RNAi) was from Sigma. To prepare viral particles, 293FT cells were co-transfected with 10 μg of ViraPower™ Packaging Mix (Invitrogen) and 5 μg of vector using the calcium phosphate precipitation method. Supernatants containing lentivirus particles were collected 48–72 h post-transfection and filtered.

For lentivirus infection, TF-1/Shp2E76K cells (3 × 10⁶ cells/ml) in RPMI 1640, 10% FBS, 10 ng/ml GM-CSF, 3 μg/ml Polybrene were incubated with an equal volume of lentivirus for 24 h. Cells were then washed and diluted with a 1× volume of RPMI 1640, 10% FBS without GM-CSF and incubated for 4 days and then analyzed for Mcl-1 knockdown and apoptosis. A parallel experiment using a GFP-encoding lentivirus indicated that >80% of TF-1/Shp2E76K cells were transduced by the virus in our experiments.

Dominant-negative Mek1K97A Retroviruses—cDNA encoding a HA-tagged rat Mek1K97A mutant was subcloned from pCMV-HA vector (31) into MigR1 to make MigR1-Mek1K97A retroviral vector. MigR1 and MigR1-Mek1K97A retroviruses were generated with Phoenix AmphiPack293 cells. TF-1/ Shp2E76K cells (2 × 10⁶ cells) were infected with MigR1 or MigR1-Mek1K97A viral supernatants. Forty-eight hours after viral infection, GFP+ cells were selected by cell sorting with a flow cytometer. GFP+ cells were used immediately for methylcellulose colony formation assay in the absence of GM-CSF or used for immunoblotting analyses of cell lysates after starvation of GM-CSF for 24 h.

Immunoblotting and Immunoprecipitation—Cells were lysed in lysis buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25 mM NaF, 5 mM Na₂P₂O₇, 1% Triton X-100, 1 mM Na₃VO₄, 20 mM p-nitrophenyl phosphate, 2 mg/ml leupeptin, 2 mg/ml aprotinin, and 1 mM phenylmethyl-
obtained in 0.5% Nonidet P-40 lysis buffer. Protein concentration to obtain the cytosolic fraction. Whole cell lysate was centrifuged further at 100,000 g to pellet the mitochondria-enriched heavy membrane fraction.

GM-CSF were washed twice with RPMI 1640, 10% FBS and then diluted with 400 µl of 1 × binding buffer. Flow cytometric data were acquired from 1 × 10^6 cells with a FACSCalibur cytometer (BD Pharmingen), and data were analyzed by the Flowjo software. For analysis of apoptosis of retroviral-based shRNA knockdown cells, GFP+ cells (1 × 10^4) were gated and analyzed for alloglycocyanin-conjugated annexin-V and 7-AAD staining.

**Bcl-X₁-promoter-luciferase Reporter Assay**—A Bcl-X₁-promoter-luciferase reporter plasmid (Bcl-X₁-Luc) was provided by Dr. Zuoming Sun (34). TF-1/V, TF-1/Shp2, and TF-1/Shp2E76K cells were co-transfected with 1.8 µg of Bcl-X₁-Luc and 0.2 µg of pCMV-βgal using FuGENE 6 transfection reagent (Roche Applied Science). Twenty-four hours after transfection, cells were washed and incubated in RPMI 1640, 10% FBS without or with 10 µM U0126, 50 µM PD98059, 50 µM LY294002, or 10 µM API-2 for 24 h. Cell lysates were prepared for determination of luciferase and β-galactosidase activities as described (35).

**Cell Growth Assay**—TF-1/V, TF-1/Shp2, and TF-1/Shp2E76K cells were maintained in RPMI 1640, 10% FBS, 5 ng/ml GM-CSF, 0.5 µg/ml puromycin. To determine cytokine-independent and cytokine-dependent cell growth, cells were washed with RPMI 1640 and plated at 2 × 10^5 cell/ml in RPMI 1640, 10% FBS without or with indicated amounts of GM-CSF in 6-well plates. Viable cell number was determined by trypan blue exclusion assay every 24 h.

**Methylcellulose Colony Formation Assay**—Methylcellulose medium was prepared using MethoCult H4100 (StemCell Technologies) and RPMI 1640, 10% FBS, 0.5 µg/ml puromycin without or with the indicated amounts of GM-CSF according to the supplier’s instruction. Cells (500 or 1000 cells in 0.1 ml) were mixed with 1 ml of methylcellulose medium and plated in 6-well plates. On day 7 cell colonies were stained with thiazolyl blue tetrazolium bromide and enumerated.

**RESULTS**

**Shp2E76K Mutant Transforms Human Cytokine-dependent TF-1 Cells**—TF-1 is a human myeloid cell line that requires GM-CSF or interleukin-3 for survival and proliferation. To determine whether the leukemia-associated Shp2E76K mutant (Fig. 1A) could confer cytokine independence transformation of TF-1 cells, we expressed the wild type Shp2 (as a control) and Shp2E76K in TF-1 cells through retroviral transduction. TF-1 cells were infected with bicistronic retrovirus MSCV-P or the virus encoding FLAG-tagged wild type Shp2 or Shp2E76K. Puromycin-resistant cells (TF-1/V, TF-1/Shp2, TF-1/Shp2E76K) were selected in cell culture medium containing puromycin and GM-CSF. Immunoblot analysis of these retrovirus-transduced cells showed that a similar amount of FLAG-tagged Shp2 and Shp2E76K were expressed in TF-1/Shp2 and TF-1/Shp2E76K cells, respectively (Fig. 1B). Immunoprecipitation of FLAG-tagged Shp2 and Shp2E76K from GM-CSF-starved cells followed by PTP assay indicated that Shp2E76K had constitutively elevated PTP activity, which was ~25 times that detected in the wild type Shp2 (Fig. 1C).

We next incubated these cells in the absence or presence of various concentrations of GM-CSF and measured viable cell number by the trypan blue exclusion assay. Viable TF-1/V and
TF-1/Shp2 cell numbers started to decrease after 2 days in GM-CSF-free medium that contained 10% FBS (Fig. 2A). This property was the same as that of the parental cells (not shown). In contrast, the viable TF-1/Shp2E76K cell number continued to increase in the absence of GM-CSF (Fig. 2A). GM-CSF caused concentration-dependent stimulation of proliferation of these cell lines. At low concentrations of GM-CSF (≤0.01 ng/ml), TF-1/Shp2E76K was more sensitive to GM-CSF than that of TF-1/V and TF-1/Shp2 cells (Fig. 2).

To determine whether cytokine independence of TF-1/Shp2E76K cells was mediated by autocrine secretion of cytokine(s), we tested if conditional medium from TF-1/Shp2E76K cells could support survival and growth of TF-1 cells. The result was negative (not shown). Thus, similar to that reported in human JMML cells (20), transformation of TF-1 cells by Shp2E76K is not mediated by an autocrine mechanism.

In methylcellulose culture, TF-1/Shp2E76K cells formed GM-CSF-independent colonies and displayed hypersensitivity to low concentrations of GM-CSF (Fig. 2B, left panel). Cytokine-independent methylcellulose colony was not observed with TF-1/V or TF-1/Shp2 cells. To determine whether Shp2E76K was expressed in GM-CSF-independent TF-1/Shp2E76K cell colonies, we randomly isolated GM-CSF-independent TF-1/Shp2E76K colonies from methylcellulose culture and analyzed the expression of Shp2E76K in these cells by immunoblotting. As shown in Fig. 2B (right panel), all GM-CSF-independent colonies were Shp2E76K-positive. This result and the observation that cytokine-independent colonies were never observed with TF-1/V and TF-1/Shp2 cells indicate that cytokine independence transformation of TF-1/Shp2E76K cells is due to Shp2E76K expression.

**Shp2E76K Induces Cytokine-independent Survival of TF-1 Cells**—To assess if Shp2E76K supports cytokine-independent survival of TF-1 cells, TF-1/V, TF-1/Shp2, and TF-1/Shp2E76K cells were cultured in the presence or absence of GM-CSF for 3 days. Cell death was then analyzed by annexin-V/7-AAD binding assay. Approximately 10–15% cells were stained annexin-V-positive (annexin-V−/7AAD− plus annexin-V+/7AAD+ cells) in the presence of GM-CSF (Fig. 3A). Three days after GM-CSF withdrawal, ~70% of TF-1/V and TF-1/Shp2 cells became annexin-V positive, whereas no increase in annexin-V binding was observed in TF-1/Shp2E76K cells (Fig. 3A).

Bax activation mediates the intrinsic/mitochondrial apoptosis pathway (36). The monoclonal antibody 6A7 specifically recognizes the active form of Bax (37, 38). Active Bax was not detected in TF-1/V, TF-1/Shp2, or TF-1/Shp2E76K cells when they were cultured in the presence of GM-CSF (Fig. 3B). After deprivation of GM-CSF for 1–2 days, active Bax was readily detectable in TF-1/V and TF-1/Shp2 cells, indicating that cyto-

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**FIGURE 1. Expression of Shp2 and Shp2**

**FIGURE 2.** Shp2E76K confers cytokine independence transformation of TF-1 cells. A, TF-1/V (V), TF-1/Shp2 (WT), and TF-1/Shp2E76K (E76K) cells (2 × 10^4 cells/ml) were incubated in RPMI 1640, 10% FBS, 0.5 μg/ml puromycin without or with indicated concentrations of GM-CSF. Viable cell numbers were determined every day (left panel) or on day 4 (right panel) by the trypan blue exclusion assay. B, TF-1/V, TF-1/Shp2, and TF-1/Shp2E76K cells (500 cells/well) were incubated in methylcellulose medium in the absence or the presence of GM-CSF for 7 days, and cell colonies were enumerated (left panel). The data were from two separate experiments performed in duplicate. GM-CSF-independent colonies of TF-1/Shp2E76K cells were randomly isolated from the methylcellulose culture. After expansion of cell number in RPMI 1640, 10% FBS, 0.5 μg/ml puromycin, cell lysates were prepared and analyzed by immunoblotting (IB) with antibodies to FLAG-tag and β-actin (right panel).
Kine withdrawal triggered the intrinsic apoptosis pathway in these cells. In contrast, no active Bax was observed in TF-1/Shp2E76K cells after GM-CSF withdrawal even though there was no decrease in the amount of total Bax protein in the cell lysate (Fig. 3B). Consistent with the Bax activation data, cytosolic cytochrome c was detected in TF-1/V and TF-1/Shp2 cells, but not in TF-1/Shp2E76K cells, after deprivation of GM-CSF for 2 days (Fig. 3C). These results show that TF-1/Shp2E76K cells have acquired the cytokine-independent survival activity by preventing cytokine withdrawal-induced intrinsic apoptosis pathway.

Up-regulation of Bcl-X 

**Is Essential for Cytokine-independent Survival of TF-1/Shp2E76K Cells**—The intrinsic/mitochondrial apoptosis pathway is controlled by the interplay between pro-survival and pro-apoptotic Bcl-2 family proteins (36). To understand how Shp2E76K induces cytokine-independent survival of TF-1 cells, we examined expression of pro-survival Bcl-2 family proteins (Bcl-2, Bcl-X 

L, Bcl-w, Mcl-1, and A1) and a number of BH3-only pro-apoptotic Bcl-2 family proteins observed in TF-1/Shp2E76K cells (Fig. 4). Because Bad is a pro-apoptotic Bcl-2 family protein, the lower amount of Bad in TF-1/V and TF-1/Shp2 cells under GM-CSF-free conditions could not be a contributing mechanism for TF-1/V and TF-1/Shp2 cell death while TF-1/Shp2E76K cells survived in GM-CSF-free medium.

To determine whether up-regulation of Bcl-X 

L and Mcl-1 plays a critical role in cytokine-independent survival of TF-1/Shp2E76K cells, we analyzed the effects of Bcl-X 

L and Mcl-1 knockdown on cytokine-independent survival of TF-1/Shp2E76K cells. TF-1/Shp2E76K cells were infected with a retrovirus (SINeG-XRi) that encodes Bcl-X 

L shRNA and GFP or with the control virus (SINeG-Ri) (29). Immunoblot analysis of GFP 

/H11001 cells indicated Bcl-X 

L was knocked down by the shRNA in infected cells (Fig. 5A). The effect of Bcl-X 

L knockdown on cytokine-independent survival of TF-1/Shp2E76K cells was then determined by flow cytometric analysis of annexin-V/7-AAD staining in GFP-gated cell population after these cells were incubated in GM-CSF-free medium. As shown in Fig. 5B, TF-1/V, TF-1/Shp2, and TF-1/Shp2E76K cells cultured in the presence or the absence of GM-CSF. TF-1/V, TF-1/Shp2, and TF-1/Shp2E76K cells had a similar amount of Bcl-X 

L in the presence of a high concentration of GM-CSF (Fig. 4). After GM-CSF withdrawal, the Bcl-X 

L level in TF-1/V and TF-1/Shp2 cells was decreased by 5-fold. However, TF-1/Shp2E76K cells continued to maintain a high level of Bcl-X 

L. TF-1/V, TF-1/Shp2, and TF-1/Shp2E76K cells contained a similar amount of Mcl-1 in the presence of GM-CSF (Fig. 4). A smaller, 2-fold decrease in Mcl-1 protein level was detected after GM-CSF deprivation in TF-1/V and TF-1/Shp2 cells but not in TF-1/Shp2E76K cells.

No difference in pro-apoptotic Bcl-2 family proteins BimEL, Noxa, and Puma was observed among the three cell lines in the presence or absence of GM-CSF (Fig. 4). The amount of pro-apoptotic Bad protein was markedly decreased in TF-1/V and TF-1/Shp2 cells after GM-CSF withdrawal, whereas a lesser reduction in Bad protein was observed in TF-1/Shp2E76K cells (Fig. 4). Because Bad is a pro-apoptotic Bcl-2 family protein, the lower amount of Bad in TF-1/V and TF-1/Shp2 cells under GM-CSF-free conditions could not be a contributing mechanism for TF-1/V and TF-1/Shp2 cell death while TF-1/Shp2E76K cells survived in GM-CSF-free medium.

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L. TF-1/V, TF-1/Shp2, and TF-1/Shp2E76K cells contained a similar amount of Mcl-1 in the presence of GM-CSF (Fig. 4). A smaller, 2-fold decrease in Mcl-1 protein level was detected after GM-CSF deprivation in TF-1/V and TF-1/Shp2 cells but not in TF-1/Shp2E76K cells.
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| GM-CSF | + | - |
|--------|---|---|
| V      | WT | EK |
| Bcl-XL |   |   |
| Bcl-XL level: | 1 | 0.8 | 1 | 0.2 | 0.2 | 1.3 |
| Actin  |   |   |
| Mcl-1  |   |   |
| Mcl-1 level: | 1 | 1 | 1 | 0.5 | 0.6 | 1.2 |
| Actin  |   |   |
| A1     |   |   |
| Bad    |   |   |
| BimEL  |   |   |
| Noxa   |   |   |
| Puma   |   |   |
| Actin  |   |   |

**FIGURE 4. Expression of Bcl-2 family proteins in TF-1/V (V), TF-1/Shp2E76K (WT), and TF-1/Shp2E76K (E76K) cells.** Cells were cultured in RPMI 1640, 10% FBS, 0.5 μg/ml puromycin with 5 ng/ml GM-CSF or without GM-CSF for 24 h. Cell lysate supernatants were analyzed by immunoblotting with antibodies to pro-survival or pro-apoptotic Bcl-2 family proteins or to β-actin (loading control) as indicated.

Shp2E76K cells infected with the control SINeG-Ri virus were stained 11.2% positive for annexin-V, whereas TF-1/Shp2E76K cells infected with the SINeG-Rxi Bcl-XL shRNA virus were stained 39.1% positive for annexin-V. Thus, knockdown of Bcl-XL prevented cytokine-independent survival of TF-1/Shp2E76K cells.

To knock down Mcl-1 in TF-1/Shp2E76K cells, we employed two lentiviral shRNAs (Mcl1-RNAi5515 and Mcl1-RNAi5517) obtained from a commercial source. As shown in Fig. 5C, knockdown of Mcl-1 was apparent in non-selected cell populations after lentivirus infection, indicating that most of cells were infected by these shRNA-encoding lentiviruses. However, flow cytometric analysis of apoptosis showed that knockdown of Mcl-1 by these two lentiviral shRNAs did not cause apoptosis of TF-1/Shp2E76K cells cultured in GM-CSF-free medium (Fig. 5D).

To rule out the possibility that a small fraction of cells not infected by lentiviruses might obscure the result of our apoptosis analysis, we prepared two retroviral Mcl-1 shRNAs (Mcl1-RNAi692 and Mcl1-RNAi908) using the MSCV-LMP vector. MSCV-LMP is a shRNA vector that carries a GFP gene, so that infected cells can be selected for flow cytometric analysis. Fig. 5E shows that both Mcl1-RNAi692 and Mcl1-RNAi908 were able to knock down Mcl-1 in GFP+ TF-1/Shp2E76K cells. Apoptotic analysis of GFP-gated TF-1/Shp2E76K cells showed that knockdown of Mcl-1 with Mcl1-RNAi692 or Mcl1-RNAi908 did not result in apoptosis of TF-1/Shp2E76K cells cultured in GM-CSF-free medium (Fig. 5F).

**HA14-1 but Not Roscovitine Induces Apoptosis of TF-1/Shp2E76K Cells Cultured in GM-CSF-Free Medium**—Roscovitine is a cyclin-dependent kinase inhibitor known to downregulate Mcl-1 expression (30, 39). HA14-1 was discovered originally as a Bcl-2 inhibitor (40–43). Recent data indicated that it cross-inhibited Bcl-XL and Bcl-w (44). Consistent with previous reports (30, 39), treatment of TF-1/Shp2E76K cells with roscovitine resulted in down-regulation of Mcl-1, whereas it had no effect on Bcl-XL (Fig. 6A). To further analyze the requirement of Bcl-XL and Mcl-1 in Shp2E76K-induced cytokine-independent survival of TF-1 myeloid cells, we examined the effects of roscovitine and HA14-1 on apoptosis of TF-1/Shp2E76K cells cultured in GM-CSF free medium. As shown in Fig. 6B, incubation of TF-1/Shp2E76K cells with roscovitine at concentrations sufficient to down-regulate Mcl-1 did not lead to cleavage of PARP. This result indicates that down-regulation of Mcl-1 does not prevent Shp2E76K-induced cytokine-independent survival of TF-1 cells. In the parallel experiment, treatment of TF-1/Shp2E76K cells with HA14-1 resulted in PARP cleavage. Because Bcl-2 and Bcl-w are not expressed in TF-1 cells, data from the HA14-1 experiment suggest that inhibition of Bcl-XL is sufficient to block cytokine-independent survival of TF-1/Shp2E76K cells.

**The Ras-Erk1/2 Pathway Is Constitutively Activated in Shp2E76K-transformed TF-1 Myeloid Cells**—To determine which signaling pathway(s) is activated by Shp2E76K in GM-CSF-free medium, TF-1/V, TF-1/Shp2, and TF-1/Shp2E76K cells were starved of GM-CSF for 18 h and then left untreated or stimulated with GM-CSF (1 ng/ml) for 10–60 min. Active Erk1/2, Akt, and Stat5 in cell lysates were analyzed by immunoblotting with phospho-specific antibodies. Active Erk1/2 was readily detected in TF-1/Shp2E76K cells in the absence of GM-CSF, whereas active Akt and Stat5 were not (Fig. 7A). The amount of constitutively active Erk1/2 in TF-1/Shp2E76K cells under GM-CSF-free conditions was similar to that detected in GM-CSF-stimulated TF-1/V and TF-1/Shp2 cells. Consistent with Erk1/2 activation in TF-1/Shp2E76K cells, Ras was constitutively activated in TF-1/Shp2E76K cells (Fig. 7B). To increase the sensitivity of detecting active Akt and Stat5 under GM-CSF-free conditions, we immunoprecipitated Akt and Stat5 and analyzed the presence of phosphorylated Akt and Stat5 in these immunoprecipitates. A small amount of active Akt was detectable in Akt immunoprecipitates from TF-1/V and TF-1/Shp2 cells (Fig. 7C). The amount of active Akt was increased marginally in Akt immunoprecipitates from TF-1/Shp2E76K cells. Similarly, a small amount of active Stat5 was detected after immunoprecipitation of Stat5 from TF-1/Shp2E76K cells (Fig. 7D). However, this did not result in an increase in Stat5 luciferase reporter activity (data not shown).

Stimulation of cells with GM-CSF markedly activated Erk1/2, Akt, and Stat5 in TF-1/V and TF-1/Shp2 cells (Fig. 7A). A higher level of active Erk1/2 was observed in GM-CSF-stimulated TF-1/Shp2E76K cells than that in TF-1/V and TF-1/Shp2 cells at a late time point (30 min). In contrast, GM-CSF-stimulated Akt activation was suppressed by Shp2E76K. These results
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Shp2<sup>E76K</sup> Up-regulates Bcl-X<sub>L</sub> via the Erk1/2 Pathway—To evaluate the contribution of Erk1/2 and Akt pathways in Shp2<sup>E76K</sup>-induced Bcl-X<sub>L</sub> expression in TF-1 cells, we determined the effects of Mek1/2 inhibitors U0126 and PD98059, the phosphoinositide 3-kinase inhibitor LY294002, and the Akt inhibitor API-2 on Bcl-X<sub>L</sub> expression in TF-1/Shp2<sup>E76K</sup> cells. TF-1/Shp2<sup>E76K</sup> cells were incubated in GM-CSF-free medium containing U0126 (10 μM), PD98059 (50 μM), LY294002 (50 μM), API-2 (10 μM), or the solvent for 24 h. Cell lysates were analyzed by immunoblotting for Bcl-X<sub>L</sub>, U0126 and PD98059 effectively reduced the Bcl-X<sub>L</sub> protein level in TF-1/Shp2<sup>E76K</sup> cells, whereas LY294002 and API-2 did not (Fig. 8A). The Shp2<sup>E76K</sup>-regulated Mcl-1 expression was also reduced by U0126, PD98059, and LY294002 but not by API-2 treatment (Fig. 8A).

We compared Bcl-X<sub>L</sub>-promoter activity in TF-1/V, TF-1/Shp2, and TF-1/Shp2<sup>E76K</sup> cells using a Bcl-X<sub>L</sub>-promoter-Luc reporter. Under GM-CSF-free conditions, Bcl-X<sub>L</sub>-promoter transcription activity was similar in TF-1/V and TF-1/Shp2 cells, whereas the Bcl-X<sub>L</sub>-promoter activity was elevated ~3-fold in TF-1/Shp2<sup>E76K</sup> cells (Fig. 8B). U0126 and PD98059 treatments reduced Bcl-X<sub>L</sub>-promoter activity in TF-1/Shp2<sup>E76K</sup> cells to a level similar to that detected in TF-1/V and TF-1/Shp2 cells. In contrast, LY294002 and API-2 treatments did not reduce the Bcl-X<sub>L</sub>-promoter activity in TF-1/Shp2<sup>E76K</sup> cells (Fig. 8B). These results indicate that up-regulation of Bcl-X<sub>L</sub> by Shp2<sup>E76K</sup> in TF-1 cells is mediated primarily by the Erk1/2 pathway.

If Bcl-X<sub>L</sub> is essential for Shp2<sup>E76K</sup>-induced cytokine-independent survival of TF-1 cells and if Erk1/2 mediates Shp2<sup>E76K</sup>-induced Bcl-X<sub>L</sub> expression, blocking Erk1/2 activation should prevent Shp2<sup>E76K</sup>-induced cytokine-independent survival of TF-1 cells. Consistent with this prediction, Fig. 8C shows that marked increases in apoptotic cells were detected after TF-1/Shp2<sup>E76K</sup> cells were treated with Mek inhibitors U0126 or PD98059 in GM-CSF-free medium. Inhibition of Akt, on the other hand, had a minimal effect on cytokine-independent survival of TF-1/Shp2<sup>E76K</sup> cells.

Shp2<sup>E76K</sup>-induced TF-1 Cell Transformation Is Blocked by a Dominant-negative Mek1 Mutant—To further evaluate the requirement of Erk1/2 in Shp2<sup>E76K</sup>-induced TF-1 cell transformation, we transiently expressed a kinase-dead Mek1 mutant (Mek1K97A) in TF-1/Shp2<sup>E76K</sup> cells using a bicistronic GFP reporter. TF-1/Shp2<sup>E76K</sup> cells were infected with MigR1 and Mig-R1-Mek1K97A retroviruses, respectively. GFP<sup>+</sup> cells were selected and analyzed. As shown in Fig. 9A, Mek1K97A effectively blocked Shp2<sup>E76K</sup>-induced Erk1/2 activation under GM-CSF-free conditions. Consistent with data obtained with U0126 and PD98059, Shp2<sup>E76K</sup>-regulated Bcl-X<sub>L</sub> and Mcl-1 expres-
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**FIGURE 6. Effects of HA14-1 and Roscovitine on cytokine-independent survival of TF-1/Shp2<sup>E76K</sup> cells.** TF-1/Shp2<sup>E76K</sup> cells were incubated in GM-CSF-free medium containing the indicated concentrations of roscovitine or HA14-1 for 24 h. Cell lysates were analyzed for down-regulation of Mcl-1 (A) or PARP cleavage (B) by immunoblotting.

**DISCUSSION**

Shp2<sup>E76K</sup> is the most frequently observed Shp2 mutant in human leukemias (16, 25, 45). We found here that Shp2<sup>E76K</sup> could transform cytokine-dependent human TF-1 myeloid cells into cytokine independence. This represents the first cytokine-independent cell line that can be transformed by a leukemia-associated Shp2 mutant and opens a new avenue for mechanistic studies of Shp2 mutant-associated leukemogenesis.

Cytokine independence transformation involves bypassing the requirement for cytokine-dependent cell survival and proliferation. In the present study, we addressed the mechanism by which Shp2 mutant induced cytokine-independent cell survival. Data from our Bax activation and cytochrome c release experiments indicate that Shp2<sup>E76K</sup> suppressed the intrinsic/mitochondrial apoptosis pathway caused by cytokine withdrawal in TF-1 cells. The intrinsic/mitochondrial apoptosis pathway is controlled by Bcl-2 family proteins. We found no decrease in pro-apoptotic Bcl-2 family proteins in TF-1/Shp2<sup>E76K</sup> cells among those that we have analyzed, suggesting that Shp2<sup>E76K</sup> is unlikely to cause cytokine-independent sur-
vival of TF-1 cells by suppressing expression of pro-apoptotic Bcl-2 family proteins.

A recent report found that Shp2 mediates FGF4-induced survival of murine trophoblast stem cells by degradation of the pro-apoptotic protein Bim in these cells (11). In murine trophoblast stem cells, deprivation of FGF4 results in Bim accumulation. Activation of Erk1/2 through Shp2 by FGF4 causes degradation of Bim and, thus, cell survival (11). Unlike these cells where Bim is minimal in the presence of FGF4 (11), TF-1 cells contain a high level of Bim even in the presence of GM-CSF (Fig. 4). We found no decrease in Bim protein levels in TF-1/Shp2E76K cells.

Apoptosis were determined by flow cytometric analysis of annexin-V/7-AAD binding (n ≥ 4). Annexin-V-positive cells include both 7-AAD-negative and 7-AAD-positive cell population. V, TF-1/V cells; WT, TF-1/Shp2 cells; EK, TF-1/Shp2E76K cells.
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Shp2E76K cells, indicating that the gain-of-function Shp2E76K mutant does not utilize the same mechanism as the wild type Shp2 in FGFR4-stimulated murine trophoblast stem cells to suppress apoptosis of TF-1 myeloid cells.

Among pro-survival Bcl-2 family proteins, Bcl-XL and Mcl-1 were up-regulated by Shp2E76K in TF-1 cells. Interestingly, knockdown of Bcl-XL by shRNAs was sufficient to prevent cytokine-independent survival of TF-1/Shp2E76K cells, whereas knockdown of Mcl-1 by shRNAs did not affect TF-1/Shp2E76K cell survival in cytokine-free medium. These data indicate that Bcl-XL and Mcl-1 are not functionally equivalent and that up-regulation of Bcl-XL is essential for cytokine-independent survival of TF-1/Shp2E76K cells.

This notion is supported by data from our experiments using pharmacological inhibitors. Consistent with previous observations in other cell lines (30, 39), roscovitine reduced Mcl-1 expression in TF-1/Shp2E76K cells. However, inhibition of Mcl-1 expression by roscovitine did not result in apoptosis of TF-1/Shp2E76K cells under GM-CSF-free conditions. In contrast, treatment of these cells with the Bcl-XL inhibitor HA14-1 led to apoptosis of TF-1/Shp2E76K cells. HA14-1 has been reported to inhibit Bcl-2, Bcl-XL, and Bcl-w (44), among which only Bcl-XL is expressed in TF-1 cells. Whether HA14-1 can cross-inhibit Mcl-1 is currently unknown. However, because down-regulation of Mcl-1 with shRNAs and roscovitine did not result in apoptosis of TF-1/Shp2E76K cells in cytokine-free medium, the apoptotic activity of HA14-1 is unlikely due to Mcl-1 inhibition even if HA14-1 could cross-inhibit Mcl-1. Moreover, we found that doxorubicin could deplete Mcl-1 in TF-1 cells. However, depletion of Mcl-1 by doxorubicin in TF-1/Shp2E76K cells did not result in apoptosis. Together, these molecular and pharmacological data indicate that Bcl-XL plays an essential role in the gain-of-function Shp2E76K mutant-induced cytokine-independent survival of TF-1 myeloid cells. Furthermore, these data suggest that Bcl-XL is a molecular target for therapeutic intervention of Shp2 mutant-associated leukemias.

Similar to that observed in Shp2E76K-transduced murine bone marrow cells (22, 24), Erk1/2 was constitutively activated in GM-CSF-starved TF-1/Shp2E76K cells to a level similar to that in GM-CSF-stimulated TF-1/V and TF-1/Shp2 cells. This property is consistent with the fact that Shp2 is a positive regulator of the Ras-Erk1/2 pathway. Importantly, it demonstrates that Shp2E76K is able to cause constitutive activation of the Ras-Erk1/2 pathway in TF-1 cells. Inhibition of Erk1/2 activation with Mek inhibitors or a dominant-negative Mek1 mutant blocked Shp2E76K-induced Bcl-XL expression and prevented cytokine-independent survival and transformation of TF-1/Shp2E76K cells. These data indicate the Erk1/2 activity is essential for Bcl-XL-dependent survival of Shp2E76K-transformed TF-1 cells in cytokine-free medium.

Akt was weakly activated in TF-1/Shp2E76K cells cultured in GM-CSF-free RPMI 1640, 10% FBS. Inhibition of Akt did not interfere with Bcl-XL expression in TF-1/Shp2E76K cells in GM-CSF-free medium and had a minimal effect on cytokine-independent survival of TF-1/Shp2E76K cells. These results suggest that Akt does not play a major role in Shp2E76K-induced cytokine-independent survival of TF-1 myeloid cells.

GM-CSF (1 ng/ml) markedly activated Akt in TF-1/V and TF-1/Shp2 cells but not in TF-1/Shp2E76K cells in RPMI 1640, 10% FBS medium. This result demonstrates that Shp2E76K could have dual effects on Akt activation. In the absence of GM-CSF, Shp2E76K could weakly activate Akt, but at higher concentrations of GM-CSF, Shp2E76K could negatively affect the GM-CSF-stimulated Akt activation. The suppressive effect of Shp2E76K on GM-CSF-stimulated Akt activity (Fig. 7A) may account for the slower growth rate of TF-1/Shp2E76K cells than that of TF-1/V and TF-1/Shp2 cells in the presence of higher concentrations of GM-CSF (≥1 ng/ml) (Fig. 2A, right panel).

Leukemia-associated Shp2 mutants such as Shp2E76K have been reported to induce cytokine-independent methylcellulose colonies in murine bone marrow and fetal liver cells (22–24). However, previous attempts (22, 25) have found that Shp2 mutants could not transform murine cytokine-dependent Ba/F3 and 32D cells into cytokine independence. Although leukemic oncogenes such as Bcr-Abl readily transforms cytokine-dependent Ba/F3 and 32D cells, the inability of a leukemic oncogene to transform Ba/F3 and 32D cells into cytokine independence is not unprecedented. Jak2V617F, which causes polycythemia vera and several other types of myeloid malignancies, also cannot transform Ba/F3 and 32D cells (46).

The reason that Shp2E76K cannot transform Ba/F3 or 32D cells is currently unknown. However, this may be due to (a) insufficiency of Shp2E76K to activate intracellular signaling pathways in these cells or (b) that the Shp2E76K-activated Ras-Erk1/2 signaling pathway is not sufficient to support cytokine-independent survival/proliferation of these cells. Interestingly, no increased level of active Erk1/2, Akt, or STAT5 was detected in GM-CSF-stimulated cell lines (30, 39), roscovitine reduced Mcl-1 expression in TF-1/Shp2E76K cells. However, inhibition of Mcl-1 by doxorubicin in TF-1/Shp2E76K cells did not result in apoptosis. Therefore, it appears that leukemia-associated Shp2 mutants require specific cellular context to activate intracellular signaling, which is present in TF-1 cells and in murine macrophage progenitor cells. Identification of the cellular basis required for Shp2E76K to activate intracellular signaling in future study should shed light on leukemogenic mechanism of Shp2 mutants.

In summary, we have found that the leukemia-associated Shp2E76K mutant can transform the cytokine-dependent TF-1 cells into cytokine independence, which is dependent upon the ability of Shp2E76K to cause constitutive Erk1/2 activation in TF-1 cells. Furthermore, our study has revealed that Shp2E76K confers cytokine-independent survival of TF-1 cells through a novel mechanism involving up-regulation of the anti-apoptotic Bcl-XL protein via the Erk1/2 signaling pathway.

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