**Functional analysis of leukemia-associated PTPN11 mutations in primary hematopoietic cells**

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*PTPN11* encodes the protein tyrosine phosphatase SHP-2, which relays signals from growth factor receptors to Ras and other effectors. Germline *PTPN11* mutations underlie about 50% of Noonan syndrome (NS), a developmental disorder that is associated with an elevated risk of juvenile myelomonocytic leukemia (JMML). Somatic *PTPN11* mutations were recently identified in about 35% of patients with JMML; these mutations introduce amino acid substitutions that are largely distinct from those found in NS.

**Introduction**

The *PTPN11* gene encodes SHP-2, a nonreceptor tyrosine phosphatase (PTPase) that relays signals from activated growth factor receptors to Ras and other effectors. Germline *PTPN11* mutations contribute to leukemogenesis. Indeed, somatic *PTPN11* mutations occur in about 35% of JMML samples from children without NS and have been detected at a lower frequency in other lymphoid and myeloid malignancies.22-25 Interestingly, almost all of these leukemia-associated mutations introduce amino acid substitutions within the N-SH2 and PTPase domains.4,5 Molecular modeling and biochemical data infer that exon 3 mutations dominantly activate SHP-2 phosphatase activity by altering critical N-SH2 amino acids that lie on the interface with the PTPase domain.4,5

Infants with NS show a spectrum of hematologic abnormalities that includes isolated monocytosis as well as myeloid disorders with features of chronic myelomonocytic leukemia that may remit spontaneously.5,6 Patients with NS are also predisposed to juvenile myelomonocytic leukemia (JMML), an aggressive myeloproliferative disorder (MPD) characterized by leukocytosis, tissue infiltration, and hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF).5,9,10 Studies of JMML specimens and experiments in mutant strains of mice strongly implicate aberrant Ras signaling in response to GM-CSF and other hematopoietic growth factors in the pathogenesis of the MPD.15-16 Approximately 50% of JMML bone marrows demonstrate a Ras gene mutation or inactivation of the *NF1* tumor suppressor, which encodes a guanosine triphosphatase (GTPase)-activating protein that negatively regulates Ras output.17-20 The association of NS with JMML and the known role of SHP-2 as a positive effector of Ras signaling in many systems2,21 suggested that *PTPN11* mutations might contribute to leukemogenesis. Indeed, somatic *PTPN11* mutations occur in about 35% of JMML samples from children without NS and have been detected at a lower frequency in other lymphoid and myeloid malignancies.22-25

The E76K substitution is the most common somatic *PTPN11* mutation identified in JMML.22-25 E76 lies within the N-SH2 domain and forms key contacts with the PTPase domain that are thought to inhibit its catalytic activity.5 An E76A mutant SHP-2 protein displays elevated phosphatase activity and exhibits gain-of-function activity in a Xenopus animal cap assay.21 The leukemia-associated E76K SHP-2 mutation conferred a stronger phenotype than a germline mutation found in patients with NS. Mutant SHP-2 proteins induce aberrant growth in multiple hematopoietic compartments, which supports a primary role of hyperactive Ras in the pathogenesis of JMML. (Blood. 2005;106: 311-317)

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protein shows elevated phosphatase activity when expressed in COS-7 cells and enhances interleukin 3 (IL-3)-independent survival of transduced Ba/F3 cells. We have investigated the effects of E76K SHP-2 expression in primary fetal liver and bone marrow cells. We found that this mutant SHP-2 protein confers hypersensitivity to GM-CSF and IL-3 in immature and committed progenitors that is dependent on SHP-2 PTPase activity. Fetal liver cells that were transduced with E76K SHP-2 also showed increased proliferation in liquid cultures. Together, these studies and 2 recent articles provide extensive data regarding the in vitro and in vivo effects of leukemia-associated PTPN11 mutations in primary hematopoietic cells.

Materials and methods

SHP-2 expression constructs

Wild-type (WT) and mutant Ptpn11 cDNAs were cloned into a vector derived from the murine stem cell virus (MSCV) backbone as described elsewhere. These plasmids also contain a green fluorescent protein (GFP) cassette driven by an internal ribosomal entry site (IRES) downstream of the Ptpn11 sequence. Each construct was verified by sequencing.

Hematopoietic cell isolation and retroviral transduction

All experimental procedures involving mice were reviewed and approved by the UCSF Committee on Animal Research. Pregnant WT C57Bl/6 females were humanely killed by CO2 inhalation at E14.5 and fetal liver cells were isolated and prepared as described. Bone marrow cells were collected by humanely killing C57Bl/6 mice by CO2 inhalation that had previously been injected with a single dose of 5-fluorouracil (150 mg/kg), and flushing out marrow from the tibia with Iscove modified Dulbecco medium (IMDM; Gibco-BRL, Gaithersburg, MD) supplemented with 20% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT). Fetal liver cells were cultured in a stimulation medium containing StemSpan SFEM (StemCell Technologies, Vancouver, BC, Canada), 15% FBS, 100 ng/mL stem cell factor (SCF; Peprotech, Rocky Hill, NJ), 50 ng/mL FLT-3 ligand (Peprotech), and 100 ng/mL IL-11 (R&D Systems, Minneapolis, MN). Bone marrow cells were cultured in a stimulation medium containing StemSpan SFEM, 15% FBS, 100 ng/mL SCF, 50 ng/mL IL-6, and 10 ng/mL IL-3 (both from Peprotech). MSCV-Ptpn11-IRES-GFP plasmids engineered to express WT or mutant SHP-2 proteins were cotransfected with plasmids encoding retroviral gag-pol and env proteins into Phoenix cells using Lipofectamine2000 (Invitrogen, Carlsbad, CA). Supernatants from transfected cells were used to transduce fetal liver or bone marrow cells 24 to 72 hours after harvest. Expression levels of WT and mutant SHP-2 proteins were evaluated by Western blot as described previously.

Colony assays

After transduction, GFP+ fetal liver and bone marrow cells were sorted using a FACSVantage SE flow cytometer (BD Biosciences, San Jose, CA). GFP+ fetal liver and bone marrow mononuclear cells were seeded in methylcellulose medium (M3231; StemCell Technologies) containing recombinant murine GM-CSF or IL-3 (Peprotech). Granulocyte-macrophage colony-forming unit (CFU-GM) colonies were counted on day 8 by indirect microscopy. For erythroid progenitor assays, cells were seeded in methylcellulose medium containing a saturating concentration of human erythropoietin (EPO; M3334, StemCell Technologies) or in medium that contained varying amounts of recombinant murine EPO (R&D Technolo-

gies). BFU-E colonies were enumerated on day 7. Cellular content was evaluated by harvesting colonies into phosphate-buffered saline (PBS) followed by counting, cytospin preparations, and Wright-Giemsa staining. The growth of low-potential colony-forming cells (LPP-CFCs) and high-potential colony-forming cells (HP-CFCs) from c-kit+ bone marrow cells was assessed as described elsewhere. Briefly, growth factors used for culture of HPP-CFCs and LPP-CFCs included SCF, IL-1, CSF-1, and GM-CSF or IL-3. Cultures for growth of LPP-CFCs were cultured in 8% CO2 and 5% O2 and were scored on day 7 of culture, whereas those for HPP-CFCs were scored on day 14 of culture. Colonies were scored by indirect microscopy. Images were acquired using a Nikon Coolpix. 5000 camera (Torrance, CA) and imported into Adobe Photoshop (Adobe Systems, San Jose, CA).

Liquid cultures

After transduction with WT or E76K SHP-2 vectors, sorted GFP+ fetal liver cells were seeded into 12-well dishes in IMDM with 15% FBS and 2 ng/mL GM-CSF (Peprotech) at 1.2 × 106 cells/well. After 48 hours, cells were washed in IMDM with 15% FBS and subsequently cultured in medium without GM-CSF. Viable cells were counted by trypsin blue exclusion. Cytospins were stained with Wright-Giemsa (Sigma, St Louis, MO). To assess progenitor activity in liquid cultures, fetal liver cells were removed at various time points and plated in methylcellulose medium supplemented with saturating doses of IL-3, IL-6, SCF, and EPO (M3434, StemCell Technologies). Progenitor colonies were counted on days 7 and 8. The incorporation of 5-bromo-2-deoxyuridine (BrdU) was measured by incubating cells with 50 μM BrdU (Sigma) for 2 to 4 hours. The cells were then fixed in 70% ethanol, treated with 2N HCl/Triton X-100, and stained with fluorescein isothiocyanate (FITC)-conjugated mouse anti-BrdU antibody and 7-amino-actinomycin D (7-AAD; both from BD Pharmingen, Mountain View, CA).

Adoptive transfer

WT C57Bl/6 recipient mice that received a single dose of 900 cGy were injected with transduced bone marrow or fetal liver cells immediately after radiation via the dorsal tail vein. Recipients received prophylactic antibiotics consisting of polymyxin sulfate and neomycin sulfate for 2 weeks after radiation.

Results

GFP+ fetal liver cells that were infected with WT or E76K virus expressed similar amounts of SHP-2 protein, which were approximately 2- to 3-fold above endogenous levels (Figure 1A). E76K SHP-2 induced a hypersensitive pattern of CFU-GM growth in fetal liver cells, which was manifest by the appearance of colonies in the absence of GM-CSF and enhanced myeloid colony formation at low concentrations of growth factor (Figure 1B). Furthermore, E76K SHP-2–expressing cells formed significantly larger CFU-GM colonies than cells transduced with the WT virus, which showed the spreading morphology of large macrophage colonies (Figure 1C). Consistent with this, Wright-Giemsa staining revealed a high percentage of monocytes and macrophages and relatively few neutrophils in E76K SHP-2 cultures relative to WT colonies (data not shown). E76K SHP-2–expressing fetal liver cells are also hypersensitive to IL-3 and show aberrant morphology (Figure 1D-E). Transduced adult murine bone marrow demonstrated a similar pattern of progenitor colony formation as fetal liver cells (data not shown). The addition of a nonsaturating dose of SCF (10 ng/mL) to methylcellulose medium with GM-CSF or IL-3 did not enhance the hypersensitive pattern of CFU-GM formation of E76K-expressing cells, but induced a modest increase in the
require concentrations. These studies showed that E76K SHP-2 does not support PTPase-independent roles of SHP-2.32-35 In human SHP-2, C459 is required for catalytic activity. Because C459 corresponds to C463 in murine SHP-2, we expressed E76K SHP-2 with a saturating concentration of GM-CSF (B) or IL-3 (D) concentrations. The number of colonies formed at low and intermediate growth factor concentrations in WT SHP-2–expressing cells (data not shown).

The SHP-2 PTPase is activated by binding to phosphoryl residues on target proteins, where it may also serve as an adapter to recruit other signaling molecules.3 Whereas catalytic activity is essential for relaying signals to downstream effectors and for most of the known biologic effects of SHP-2, some evidence also supports PTPase-independent roles of SHP-2.32-35 In human SHP-2, C459 is required for catalytic activity. Because C459 corresponds to C463 in murine SHP-2, we expressed a doubly mutant SHP-2 protein with E76K and C463S substitutions in fetal liver cells and assessed myeloid progenitor colony formation in response to GM-CSF. Ablating PTPase activity abolished hypersensitive CFU-GM growth (Figure 2A). Importantly, cells expressing WT SHP-2, the PTPase C463S mutant alone, or the E76K-C463S double mutation all demonstrated a normal pattern of myeloid progenitor colony growth (Figure 2A). Thus, C463S and E76K-C463S SHP-2 proteins do not exhibit dominant-negative activity in this system.

The GM-CSF, IL-3, and IL-5 receptors share a common β subunit (βc) that associates with unique α chains to mediate biologic responses to these cytokines.36 In mice, a second gene called βIL-3 encodes a β subunit that can only associate with the IL-3α chain. To determine if hypersensitivity to IL-3 requires βc, we expressed E76K SHP-2 in WT and βc-deficient bone marrow cells and compared CFU-GM colony growth over a range of IL-3 concentrations. These studies showed that E76K SHP-2 does not require βc to induce hypersensitivity to IL-3 (Figure 2B).

CFU-GMs comprise a relatively differentiated, lineage-restricted myeloid progenitor population. To investigate the effects of E76K SHP-2 expression in other compartments, we assayed the growth of HPP-CFCs, primitive myeloid progenitors with extensive replating potential, and LPP-CFCs from c-kit+ bone marrow cells that were infected with E76K SHP-2 or WT SHP-2 virus. Transduced cells were isolated by sorting and cultured in methylcellulose medium supplemented with saturating concentrations of GM-CSF or IL-3. Under these conditions, E76K SHP-2–expressing cells formed significantly more HPP-CFC and LPP-CFC colonies than cells transduced with WT SHP-2 (Figure 3). In addition, the individual HPP-CFC and LPP-CFC colonies formed from E76K SHP-2 cells were abnormally large. These data demonstrate that E76K SHP-2 perturbs the growth of both lineage-restricted and multilineage hematopoietic progenitors.

To compare the effects of expressing E76K or WT SHP-2 in bulk populations of hematopoietic cells, freshly transduced GFP+ fetal liver cells were placed in liquid cultures that were supplemented with a saturating concentration of GM-CSF to promote the survival of myelomonocytic cells. These cells were washed after 48 hours, transferred to medium without added cytokines, and counted every 2 days. E76K SHP-2–expressing cultures demonstrated significantly higher cell counts (Figure 4A) and a distinctive pattern of differentiation characterized by an abundance of monocytes and macrophages with a subpopulation of cells with blastlike morphology (Figure 4B). By contrast, most of the cells that were transduced with WT SHP-2 differentiated into mature neutrophils (Figure 4B). E76K SHP-2 cultures also retained significant numbers of BFU-E and CFU-GM colonies even after 1 week (Figure 4C). To assess the proliferative potential of myeloid progenitors in E76K SHP-2 and WT cultures, we isolated GFP+ fetal liver cells as described, maintained them in GM-CSF, and measured BrdU incorporation.
higher concentrations of EPO (Figure 5B). By contrast, cells expressing WT SHP-2 formed rare BFU-E colonies at saturating doses of EPO that were much smaller than the corresponding E76K SHP-2 erythroid colonies (Figure 5A).

A G>T transversion at nucleotide position 181, which results in a D61Y amino acid substitution in the N-SH2 domain, is another common somatic PTPN11 mutation found in JMML specimens.22,23 Like E76, D61 mediates contacts between the N-SH2 and PTP domains.3 Interestingly, whereas the D61Y mutation has elevated phosphatase activity in COS-7 cells, it is less active than E76K,22 and this mutant has less potent prosurvival effects in Ba/F3 cells.23 We therefore compared the effects of the D61Y SHP-2 and E76K SHP-2 proteins on CFU-GM growth. Like E76K SHP-2, fetal liver cells transduced in parallel with D61Y SHP-2 exhibited a hypersensitive pattern of myeloid progenitor growth in response to GM-CSF and IL-3 and formed colonies in the absence of exogenous cytokine (Figure 6A). However, D61Y-expressing cells consistently were less hypersensitive than the fetal liver cells that were transduced in parallel with the E76K virus. N308 is the most commonly mutated amino acid in individuals with NS.4,5 This amino acid resides in the PTPase domain of SHP-2 and mutations at this codon have not been identified in JMML specimens. We expressed a N308S mutation found in NS individuals in fetal liver cells and investigated the ability of these cells to form CFU-GM colonies. Whereas N308S SHP-2–expressing fetal liver cells also displayed a hypersensitive pattern of CFU-GM colony growth in response to GM-CSF, this mutant protein is markedly less potent in vitro than E76K (Figure 6B). Moreover, cells engineered to express N308S SHP-2 never formed colonies in the absence of added cytokine.

To investigate the consequences of mutant SHP-2 expression in vivo, primary fetal liver and bone marrow cells from C57Bl/6 donors were transduced with retroviruses encoding E76K, D61Y, or WT SHP-2 proteins were transplanted into lethally irradiated congenic recipients. These mice were monitored for signs of MPD, which included obtaining monthly complete blood counts. The percentage of transduced GFP+ donor-derived cells was assessed by flow cytometry. Unfortunately, many mice died between 2 and 5 months after transplantation for unknown reasons. Excess mortality was not due to overt MPD and mice that received cells transduced with empty vector, WT SHP-2, or mutant SHP-2 displayed similar survivals. Moreover, the blood leukocyte counts of mice that received E76K- or D61Y-transduced bone marrow or fetal liver cells were not consistently higher 1 to 5 months after adoptive transfer than the counts of mice given transplants with cells infected with either WT SHP-2 or empty vector constructs. Although the transduced cells that we injected were consistently

![Image](318x115 to 547x215)

**Figure 4.** E76K SHP-2 expression enhances proliferation and perturbs differentiation of fetal liver cell growth in liquid cultures. (A) GFP+ cells that had been transduced with WT or E76K SHP-2 were isolated by sorting and plated in quadruplicate at 1.2 × 10^6 cells/well in medium containing 15% FBS and 2 ng/mL GM-CSF. GM-CSF was removed from the culture medium after 48 hours (on day 3), and live cells were counted every other day. Error bars reflect standard error of the mean. (B) Cytospin preparations of cells removed after 5 days in culture (original magnification, ×200). Whereas most of the cells in WT cultures are mature neutrophils, E76K SHP-2 cultures show a predominance of monocyte-macrophage cells with some blastlike elements. (C) Progenitor colony growth of cells isolated from WT and E76K SHP-2–transduced fetal liver cells and cultured with IL-3, IL-6, SCF, and EPO. (D) BrdU incorporation by liquid culture cells maintained in GM-CSF over time. Data shown are for 2 hours of labeling. Similar differences were observed in WT and E76K SHP-2 cultures that were labeled for 4 hours. (E) Flow cytometry analysis of BrdU incorporation by WT and E76K SHP-2–transduced fetal liver cells on day 6. All of the data shown in panels A-E are representative of 3 independent experiments. The numbers on the plots indicate the percentage of BrdU-positive cells.

![Image](67x357 to 295x728)

**Figure 5.** Effects of E76K SHP-2 expression on erythroid progenitor growth. (A) Fetal liver (FL) and bone marrow (BM) cells transduced with E76K SHP-2 form large numbers of BFU-E colonies in methylcellulose cultures supplemented with a saturating concentration of recombinant EPO alone (Figure 5A). E76K SHP-2–expressing cells also generated EPO-independent BFU-E colonies. We observed increases in both the number of colonies and the size of the individual BFU-E colonies at over time. The number of proliferating cells was increased in E76K SHP-2 cultures when assayed after 4 to 7 days (Figure 4D-E). Interestingly, despite the persistence of greater numbers of live cells that included viable progenitors, E76K expression did not immortalize primary fetal liver cells.

BFU-E colony formation normally requires both EPO and a source of burst-promoting activity such as GM-CSF or IL-3. Interestingly, GFP+ fetal liver cells that were infected with the E76K SHP-2 virus formed large numbers of BFU-E colonies in methylcellulose cultures supplemented with a saturating concentration of recombinant EPO alone (Figure 5A). E76K SHP-2–expressing cells also generated EPO-independent BFU-E colonies. We observed increases in both the number of colonies and the size of the individual BFU-E colonies at
Hypersensitivity to GM-CSF is an in vitro hallmark of JMML. The observation that hematopoietic cells from Nf1 and Kras mutant mice also demonstrate a hypersensitive pattern of CFU-GM colony growth in methylcellulose links aberrant Ras activation to this cellular phenotype. In work performed to date, Kras2, Nras, Nf1, and PTPN11 mutations have largely been identified in mutually exclusive subsets of patients with JMML, which infers that the encoded proteins are components of the same growth control network. We find that expressing leukemia-associated SHP-2 proteins in primary hematopoietic cells induces hypersensitivity of myeloid progenitors to GM-CSF and IL-3. Like CFU-GM colonies grown from Nf1 and Kras mutant mice, colonies generated from E76K SHP-2–expressing fetal liver cells contain a high percentage of monocytes and macrophages. These data, which are in agreement with 2 recent reports, infer that hyperactive Ras perturbs myeloid differentiation by actively driving cells toward a macrocyte-macrophage fate or by interfering with granulocytic maturation. Similarly, regulated expression of mutant H-Ras in human myeloid progenitors promotes monocytic differentiation, and monocytes is a major diagnostic criterion for JMML. The overall pattern of CFU-GM growth induced by expressing leukemia-associated SHP-2 proteins is similar to that of Kras mutant bone marrow cells, which form numerous CFU-GM colonies in the absence of any exogenous cytokine and are hypersensitive to IL-3. By contrast, Nf1-deficient cells, which do not form abnormal numbers of CFU-GM colonies in the presence of low doses of IL-3 and are less hypersensitive to GM-CSF, most closely reproduce the in vitro growth of human JMML cells. The differential effects of expressing mutant SHP-2 versus loss of Nf1 on CFU-GM growth are unlikely to be explained by the use of retroviral-mediated gene transfer because Araki and colleagues also found that myeloid progenitors from Ptpn11 D61G mice are hypersensitive to both GM-CSF and IL-3. Our studies further show that the effects of E76K SHP-2 are not restricted to CFU-GMs, but that this mutation also perturbs the growth of immature myeloid progenitors (HPP-CFCs and LPP-CFCs). Similarly, the bone marrows of mice that had been reconstituted with Nf1−/− fetal liver cells also contain increased numbers of HPP-CFCs and LPP-CFCs.

Adoptive transfer of bone marrow cells transduced with E76K SHP2 or D61Y SHP2 did not consistently induce MPD, although some recipient mice developed anemia in association with splenic infiltration by myeloid and erythroid cells. By contrast, Mohi et al observed MPD and T-cell leukemias in mice that were given transplants with bone marrow cells engineered to express E76K or D61Y SHP-2. Importantly, we used the same MSCV retroviral backbone as these investigators, transduced a similar percentage of bone marrow cells and a substantially higher proportion of fetal liver cells, and found identical effects on CFU-GM progenitor colony growth in methylcellulose. However, we expressed SHP-2 proteins in C57Bl/6 hematopoietic cells, whereas Mohi and colleagues used Balb/c mice. Interestingly, the ability of BCR-ABL to induce MPD is highly dependent on the background strain, with Balb/c bone marrow sensitive to transformation and C57Bl/6 mice resistant. Our data therefore suggest that expressing leukemia-associated SHP-2 proteins from retroviral vectors requires one or more modifying genes that are present in the Balb/c strain, but are absent in C57Bl/6 mice, to efficiently induce MPD or T-cell leukemia in vivo.

In addition to myelomonocytic proliferation, JMML is characterized by anemia, ineffective erythropoiesis, splenic infiltration by erythroid cells, and elevated fetal hemoglobin levels. Increased numbers of

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**Figure 6. Comparative effects of E76K, D61Y, and N308S SHP-2 expression on CFU-GM colony growth.** (A) Fetal liver cells engineered to express D61Y SHP-2 show cytokine-independent CFU-GM colony formation but are less hypersensitive to GM-CSF than cells expressing E76K SHP-2. Error bars indicate standard deviation of duplicate plates. (B) Fetal liver cells expressing N308S SHP-2 do not demonstrate GM-CSF–independent colony formation and display modest hypersensitivity. Error bars indicate standard error of the mean of 3 independent experiments.

20% to 40% GFP+, these levels varied widely in recipient animals and were frequently less than 10% (data not shown). Surviving recipients were humanely killed for analysis about 5 months after transplantation. Interestingly, whereas splenocytes harvested from several mice given transplants with cells engineered to express E76K or D61Y SHP-2 formed CFU-GM colonies in response to GM-CSF, myeloid colonies were never detected in the spleens of mice given transplants with cells transduced with WT SHP-2 or the empty vector. Some of the mice with abnormal splenic CFU-GM colony growth were anemic (hemoglobin level, 3.3-6.1 g/dL) and also showed disrupted splenic architecture with effacement of germinal centers and myeloid infiltration (data not shown). These hematopoietic abnormalities correlated with relatively high levels of GFP+ cells in blood, marrow, and spleen (30%-60%).
BFU-Es are present in the blood and bone marrows of patients with JMML, some of which form EPO-independent colonies in methylcellulose.39 Similarly, we found that expressing E76K SHP-2 in bone marrow and fetal liver cells generated BFU-E colonies in the absence of EPO or a source of burst-promoting activity beyond the amount available in normal serum. Adding exogenous EPO further augmented BFU-E colony formation. We also observed anemia and splenic erythroid infiltration in some of the irradiated mice that were given transplants with these cells. Interestingly, somatic activation of oncogenic Kras results in anemia and splenomegaly with erythroid infiltration.15,16 Furthermore, fetal liver cells transduced with oncogenic Hras display a block in terminal erythroid differentiation that can be reversed by expressing a dominant-negative allele of the Ras effector Raf-1.40,41 Our data provide further evidence that hyperactive Ras contributes to aberrant erythropoiesis in JMML.

The germline PTPN11 mutations found in patients with NS are largely distinct from the somatic mutations identified in JMML specimens, which suggests that leukemia-associated mutations encode more severe gain-offunction alleles that might not be compatible with normal development. The observation that E76K SHP-2 confers profound in vitro CFU-GM hypersensitivity extends previous studies showing that this mutation has relatively high phosphatase activity and promotes Ba/F3 cell survival.22,23 Our data showing that the leukemia-associated D61Y mutation has less potent effects on progenitor colony growth, and that the NS-associated N308S mutation had markedly reduced activity, are also consistent with biochemical analysis of these mutant proteins in cell lines.22 Two recent papers also compared the effects of expressing E76K and D61Y SHP-2 on myeloid progenitor colony growth.27,28 One group described no differences between these mutant proteins; however, their data showed that D61Y SHP-2 induced a lower percentage of maximal CFU-GM colony formation than E76K SHP-2 at GM-CSF concentrations between 0 and 0.1 ng/mL.27 Moreover, Mohi and coworkers28 found that bone marrow cells transduced with the E76K SHP-2 formed more cytokine-independent CFU-GM colonies than cells expressing the D61Y mutant protein. Our observation that the N308S mutation is remarkably less potent in inducing hypersensitive CFU-GM colony growth over a range of GM-CSF concentrations is also in agreement with their finding that other NS mutations that are not associated with JMML caused little or no cytokine-independent colony formation.28 At the time Araki et al26 reported D61G SHP-2 mutant mice, this mutation had not been associated with JMML. Interestingly, recent data indicate that D61G is the only PTPN11 mutation that is detected in NS patients with and without leukemia as well as in sporadic JMML. (C. Kratz, C. Niemeyer, M. Tartaglia, and M.L.L., unpublished data, April 2005). D61G SHP-2 has lower phosphatase activity than the E76K SHP-2 and it is of interest that CFU-GMs from these animals are less hypersensitive to GM-CSF and IL-3 than cells transduced with E76K SHP-2.2,26 Taken together, the existing data argue in favor of biologic differences between leukemia-associated mutant proteins that correlate with levels of phosphatase activity and, more importantly, demonstrate that these SHP-2 proteins are reminiscent of the phenotypes seen in murine Nf1 and Kras mutant hematopoietic cells and in JMML patient samples and suggest that hyperactive Ras contributes to leukemogenesis. We were therefore surprised to discover that expressing D61Y or E76K SHP-2 did not deregulate signaling through Ras effectors in Ba/F3 cells.27 Mohi et al28 confirmed these observations and went on to investigate bone marrow cells from mice given transplants that had been cultured with IL-3 to generate bone marrow mast cells. Interestingly, cells expressing E76K or D61Y SHP-2 proliferated extensively under these conditions, which is also characteristic of Kras mutant hematopoietic cells and in JMML patient samples and suggest that hyperactive Ras contributes to leukemogenesis.

Clonogenic assays measure the growth of rare progenitors in methylcellulose medium supplemented with serum and growth factors. We therefore established liquid cultures to assess the effects of expressing E76K SHP-2 in a mixed population of primary hematopoietic cells. These studies uncovered increased numbers of myeloid and erythroid progenitors at early time points, higher cell numbers at later time points, and aberrant differentiation. Cultured E76K SHP-2–expressing cells that were maintained in GM-CSF also demonstrated an elevated rate of proliferation. These data provide direct evidence that leukemia-associated SHP-2 proteins perturb terminal differentiation programs while also driving the proliferation of myelomonocytic cells. Similarly, Chan et al27 reported lower levels of F4/80 expression in cultured macrophage progenitors that were induced to differentiate in vitro as well as hyperproliferation in response to GM-CSF. The in vitro phenotypes of primary cells engineered to express mutant SHP-2 proteins are consistent with clinicopathologic features of JMML, which is characterized by both prominent myeloproliferation and by trilineage dysplasia with anemia and thrombocytopenia.9,10 Importantly, E76K SHP-2–expressing fetal liver and bone marrow cells eventually die in liquid cultures, which argues that mutant SHP-2 proteins do not immortalize cells or enhance self-renewal capacity. This idea is consistent with data from Kras mutant bone marrow cells, which are profoundly hypersensitive to growth factors but show normal replate efficiency.15,16 We have reported that E76K SHP-2 enhances the IL-3–independent survival of Ba/F3 cells.23 We found no differences in the rate of annexin V staining (a measure of apoptosis) between WT and E76K SHP-2–expressing GFP+ fetal liver cells that were cultured without growth factors for 2 to 24 hours after undergoing transduction (data not shown). However, these data do not exclude the possibility that leukemia-associated SHP-2 proteins enhance the survival of specific subpopulations of hematopoietic cells, an idea that is consistent with the persistence of progenitor activity in E76K SHP-2 liquid cultures after growth factor withdrawal.

We have shown that expressing E76K SHP-2 in primary hematopoietic cells perturbs the growth of immature (LPP-CFC and HPP-CFC) and committed (CFU-GM and BFU-E) progenitors. These effects of mutant SHP-2 are reminiscent of the phenotypes seen in murine Nf1 and Kras mutant hematopoietic cells and in JMML patient samples and suggest that hyperactive Ras contributes to leukemogenesis. We were therefore surprised to discover that expressing D61Y or E76K SHP-2 did not deregulate signaling through Ras effectors in Ba/F3 cells.27 Mohi et al28 confirmed these observations and went on to investigate bone marrow cells from mice given transplants that had been cultured with IL-3 to generate bone marrow mast cells. Interestingly, cells expressing E76K or D61Y SHP-2 proliferated extensively under these conditions, which is also characteristic of Kras mutant hematopoietic cells.15 Biochemical analyses revealed elevated levels of phosphorylated extracellular-regulated kinase (ERK), Akt, and signal transducer and activator of transcription 5 (STAT5) in cells from mice that had been given transplants with cells expressing E76K or D61Y SHP-2.27 Chan and coworkers7 generated macrophage progenitors from retrovirally infected bone marrow and found that E76K, D61Y, or D61V SHP-2 induced elevated basal levels of phosphorylated ERK with prolonged activation in response to GM-CSF, but not M-CSF. This pattern of ERK activation in cells stimulated with GM-CSF is similar to our observations in Mac1+ bone marrow cells from Nf1 mutant mice.14 Together, the existing biochemical data infer that the effects of mutant SHP-2 proteins on Ras signaling are strongly modulated by both the responding cell type and the stimulus.23,26,28 Understanding the basis of this specificity and identifying critical effectors that are amenable to therapeutic intervention is a rational strategy for improving the outcome of patients with JMML.
Acknowledgments

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References

Functional analysis of leukemia-associated *PTPN11* mutations in primary hematopoietic cells

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