Functional Analysis of Leukemia-Associated PTPN11 Mutations in Primary Hematopoietic Cells

Running Title: PTPN11 Mutations and Hematopoietic Growth

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ABSTRACT

PTPN11 encodes the protein tyrosine phosphatase SHP-2, which relays signals from growth factor receptors to Ras and other effectors. Germline PTPN11 mutations underlie ~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 ~35% of JMML patients; these mutations introduce amino acid substitutions that are largely distinct from those found in NS. We assessed the functional consequences of leukemia-associated PTPN11 mutations in murine hematopoietic cells. Expressing an E76K SHP-2 protein induced a hypersensitive pattern of colony-forming unit granulocyte-macrophage (CFU-GM) colony growth in response to granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 3 (IL-3) that was dependent on SHP-2 catalytic activity. E76K SHP-2 expression also enhanced the growth of immature progenitor cells with high replating potential, perturbed erythroid growth, and impaired normal differentiation in liquid cultures. In addition, leukemia-associated SHP-2 mutations 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.
INTRODUCTION

The *PTPN11* gene encodes SHP-2, a non-receptor tyrosine phosphatase (PTPase) that relays signals from activated growth factor receptors to p21ras (Ras), Src family kinases, and other signaling molecules (reviewed in\(^1,2\)). SHP-2 contains two src homology 2 (SH2) domains and a catalytic PTPase domain. The SHP-2 crystal structure predicts that binding of the N-SH2 domain to phosphotyrosyl peptides results in a conformational shift that relieves inhibition of the PTPase and activates SHP-2 function\(^3\). Missense mutations in *PTPN11* underlie \(~50\%\) of cases of Noonan syndrome (NS), a developmental disorder characterized by cardiac defects, facial dysmorphism, and skeletal malformations\(^4\). Most of the *PTPN11* mutations found in NS 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 (CMML) that may remit spontaneously\(^6-8\). NS patients 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)\(^9,10\). 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\(^11-16\). Approximately 50\% of JMML bone marrows demonstrate a *RAS* gene mutation or inactivation of the NF1 tumor suppressor, which encodes a 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 systems\textsuperscript{2,21} suggested that \textit{PTPN11} mutations might contribute to leukemogenesis. Indeed, somatic \textit{PTPN11} mutations occur in \textasciitilde35\% of JMML samples from children without NS and have been detected at a lower frequency in other lymphoid and myeloid malignancies\textsuperscript{22-25}. Interestingly, almost all of these leukemia-associated mutations introduce amino acid substitutions within the N-SH2 domain that are largely distinct from those found in NS\textsuperscript{22-25}. Araki and colleagues\textsuperscript{26} recently generated a mouse model of NS by constitutively expressing an amino acid substitution (D61G) identified in human patients from the endogenous murine \textit{Ptpn11} locus. Heterozygous \textit{Ptpn11\textsuperscript{D61G}} mutant mice show cardiac and skeletal defects and develop a subacute MPD that models some aspects of JMML\textsuperscript{26}.

The E76K substitution is the most common somatic \textit{PTPN11} mutation identified in JMML\textsuperscript{22-25}. Glutamate 76 lies within the N-SH2 domain, and forms key contacts with the PTPase domain that are thought to inhibit its catalytic activity\textsuperscript{3}. An E76A mutant SHP-2 protein displays elevated phosphatase activity and exhibits gain of function activity in a \textit{Xenopus} animal cap assay\textsuperscript{21}. The leukemia-associated E76K SHP-2 protein shows elevated phosphatase activity when expressed in COS-7 cells\textsuperscript{22} and enhances interleukin 3 (IL-3)-independent survival of transduced Ba/F3 cells\textsuperscript{23}. We have investigated the effects of E76K SHP-2 expression in primary fetal liver and bone marrow cells. We find 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 formed many more burst forming unit-erythroid (BFU-E) colonies. We also find that expressing E76K SHP-2 results in more pronounced growth factor hypersensitivity than another leukemia-associated SHP-2 mutation (D61Y), and that both of these mutations confer stronger hematopoietic phenotypes
than a N308S substitution that occurs in patients with NS. Cells that were transduced with E76K
SHP-2 also showed persistent progenitor activity, aberrant differentiation, and increased
proliferation in liquid cultures. Together, these studies and two recent articles\textsuperscript{27,28} provide
extensive data regarding the \textit{in vitro} and \textit{in vivo} effects of leukemia-associated \textit{PTPN11}
mutations in primary hematopoietic cells.

\textbf{METHODS}

\textit{SHP-2 Expression Constructs}. Wild-type and mutant \textit{Ptpn11} cDNAs were cloned into a vector
derived from the murine stem cell virus (MSCV) backbone\textsuperscript{29} as described elsewhere\textsuperscript{23}. These
plasmids also contain a green fluorescent protein (GFP) cassette driven by an internal ribosomal
entry site (IRES) downstream of the \textit{Ptpn11} sequence. Each construct was verified by
sequencing.

\textit{Hematopoietic Cell Isolation and Retroviral Transduction}. All experimental procedures
involving mice were reviewed and approved by the UCSF Committee on Animal Research.
Pregnant wild-type C57Bl/6 females were killed by CO\textsubscript{2} inhalation at E14.5 and fetal liver cells
were isolated and prepared as described\textsuperscript{30}. Bone marrow cells were collected by killing C57Bl/6
mice by CO\textsubscript{2} inhalation that had previously been injected with a single dose of 5-fluorouracil
(150 mg/kg), and flushing out marrow from the tibias with Iscove’s Modified Dulbecco’s
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), 15\% FBS, 100
ng/mL stem cell factor (SCF) (Peprotech, Rocky Hill, NJ), 50 ng/mL FLT-3 ligand (Peprotech),
and 100 ng/mL interleukin 11 (R&D Technologies, Minneapolis, MN). Bone marrow cells were
cultured in a stimulation medium containing StemSpan SFEM, 15% FBS, 100 ng/mL SCF, 50 ng/mL interleukin 6, and 10 ng/mL IL-3 (both from Peprotech). MSCV-Ptpn11-IRES-GFP plasmids engineered to express wild-type or mutant SHP-2 proteins were co-transfected 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 – 72 hours after harvest. Expression levels of wild-type and mutant SHP-2 proteins were evaluated by Western blot as described previously23.

**Colony Assays.** After transduction, GFP-positive fetal liver and bone marrow cells were sorted using a FACSVantage SE flow cytometer. GFP-positive fetal liver and bone marrow mononuclear cells were seeded in methylcellulose medium (M3231, Stem Cell Technologies) containing recombinant murine GM-CSF or IL-3 (Peprotech). 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, Stem Cell Technologies) or in medium that contained varying amounts of recombinant murine EPO (R&D Technologies). BFU-E colonies were enumerated on day 7. Cellular content was evaluated by harvesting colonies into PBS followed by counting, cytospin preparations, and Wright-Giemsa staining. The growth of low proliferating potential colony forming cells (LPP-CFC) and high proliferating potential colony forming cells (HPP-CFC) from c-kit positive bone marrow cells was assessed as described elsewhere31. Briefly, growth factors used for culture of HPP-CFC and LPP-CFC included SCF, IL-1, CSF-1, and GM-CSF or IL-3. Cultures for growth of LPP-CFC were cultured in 8% CO₂, 5% O₂, and were scored on day 7 of culture, whereas those for HPP-CFC were scored on day 14 of culture. Colonies were scored by indirect microscopy.
**Liquid Cultures.** After transduction with WT or E76K SHP-2 vectors, sorted GFP-positive fetal liver cells were seeded into 12 well dishes in IMDM with 15% FBS and 2 ng/ml GM-CSF (Peprotech) at 1.2 × 10^6 cells per 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 trypan 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 methycellulose medium supplemented with saturating doses of IL-3, IL-6, SCF, and EPO (M3434, Stem Cell 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 2-4 hours. The cells were then fixed in 70% ethanol, treated with 2N HCl/Triton X-100, and stained with FITC conjugated mouse anti-BrdU antibody and 7-AAD (both from BD Pharmingen, Mountain View, CA).

**Adoptive Transfer.** Wild-type 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 polymixin sulfate and neomycin sulfate for two weeks after radiation.

**RESULTS**

GFP-positive fetal liver cells that were infected with wild-type (WT) or E76K virus expressed similar amounts of SHP-2 protein, which were approximately 2-3 fold above endogenous levels (Fig. 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 (Fig. 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 (Fig. 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 (Figs. 1D and 1E). Transduced adult murine bone marrow demonstrated a similar pattern of progenitor colony growth as fetal liver cells (data not shown). The addition of a non-saturating dose of stem cell factor (SCF; 10 ng per 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 number of colonies formed at low and intermediate growth factor concentrations in WT SHP-2-expressing cells (data not shown).
Figure 1. E76K SHP-2 expression results in a hypersensitive pattern of CFU-GM colony growth. (A) SHP-2 expression levels in fetal liver cells transduced with retroviruses encoding wild-type (WT), D61Y, or E76K SHP-2 proteins. The blot was also probed with an anti-ERK2 antibody to confirm equal protein loading. (B, D) CFU-GM colony growth of cells expressing either WT or E76K SHP-2 over a range of GM-CSF (B) or IL-3 (D) concentrations. Graphs display the average growth of three independent experiments of cells plated in duplicate. (C, E) Morphology of representative CFU-GM colonies grown in saturating concentrations of GM-CSF (C) or IL-3 (E) from fetal liver cells expressing WT or E76K SHP-2 proteins (40x magnification).

The SHP-2 PTPase is activated by binding to phosphotyrosyl residues on target proteins, where it may also serve as an adapter to recruit other signaling molecules. 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\textsuperscript{32-35}. In human SHP-2, cysteine 459 is required for catalytic activity. As 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 (Fig. 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 (Fig. 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 \(\beta\) subunit (\(\beta_c\)) that associates with unique \(\alpha\) chains to mediate biological responses to these cytokines\textsuperscript{36}. In mice, a second gene called \(\beta_{IL-3}\) encodes a \(\beta\) subunit that can only associate with the IL-3\(\alpha\) chain. To determine if hypersensitivity to IL-3 requires \(\beta_c\), we expressed E76K SHP-2 in WT and \(\beta_c\)-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 \(\beta_c\) to induce hypersensitivity to IL-3 (Fig. 2B).
CFU-GM 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-CFC, a primitive myeloid progenitor with extensive replating potential, and LPP-CFC from c-kit-positive 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 (Fig. 3). In addition, the individual HPP-CFC and LPP-CFC colonies formed from E76K SHP-2 cells were abnormally large. These

Figure 2. Hypersensitive CFU-GM colony growth induced by E76K SHP-2 is dependent on PTPase activity, but does not require the \( \beta_c \) subunit. (A) CFU-GM colony growth of fetal liver cells expressing WT SHP-2, WT-C463S (catalytically inactive WT SHP-2), E76K SHP-2, or E76K-C463S (a catalytically inactive E76K mutant SHP-2) over a range of GM-CSF concentrations. Cells expressing WT, WT-C463S, and E76K-C463S SHP-2 proteins all demonstrate a normal pattern of CFU-GM colony growth. (B) CFU-GM colony growth of WT and \( \beta_c \)-deficient (\( \beta_c^{-/-} \)) bone marrow cells transduced with WT or E76K SHP-2 vectors over a range of IL-3 concentrations. \( \beta_c^{-/-} \) bone marrow cells expressing E76K SHP-2 demonstrate a hypersensitive response that is similar to WT bone marrow cells.
data demonstrate that E76K SHP-2 perturbs the growth of both lineage restricted and multi-lineage hematopoietic progenitors.

To compare the effects of expressing E76K or WT SHP-2 in bulk populations of hematopoietic cells, freshly transduced GFP-positive 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 (Fig. 4A), and a distinctive pattern of differentiation characterized by an abundance of monocytes and macrophages with a subpopulation of cells with blast-like morphology (Fig 4B). By contrast, most of the cells that were transduced with WT SHP-2 differentiated into mature neutrophils (Fig. 4B). E76K SHP-2 cultures also retained significant numbers of BFU-E and CFU-GM even after 1 week (Fig. 4C).

To assess the proliferative potential of myeloid progenitors in E76K SHP-2 and WT cultures, we isolated GFP-positive fetal liver cells as above, maintained them in GM-CSF, and measured BrdU incorporation over time. The number of proliferating cells was increased in E76K SHP-2 cultures when assayed after 4-7 days (Figs. 4D, 4E). Interestingly, despite the persistence of
greater numbers of live cells that included viable progenitors, E76K expression did not immortalize primary fetal liver cells.

Figure 4. E76K SHP-2 expression enhances proliferation and perturbs differentiation of fetal liver cell growth in liquid cultures. (A) GFP-positive cells that had been transduced with WT or E76K SHP-2 were isolated by sorting, and plated in quadruplicate at $1.2 \times 10^6$ cells per well in medium containing 15% FBS and 2 ng/ml of GM-CSF. GM-CSF was removed from the culture medium after 48 hours (on day 3), and live cells were counted every other day. (B) Cytospin preparations of cells removed after 5 days in culture (200x original magnification). Whereas most of the cells in WT cultures are mature neutrophils, E76K SHP-2 cultures show a predominance of monocyte-macrophage cells with some blast like elements. (C) Progenitor colony growth of cells isolated from WT and E76K SHP-2 liquid cultures in
methylcellulose medium supplemented 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 liquid cultures on day 6. All of the data shown in panels A-E are representative of 3 independent experiments.

BFU-E colony formation normally requires both EPO and a source of burst promoting activity such as GM-CSF or IL-3. Interestingly, GFP-positive 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 (Fig 5A). E76K SHP-2-expressing cells also generated EPO-independent BFU-E. We observed increases in both the number of colonies and the size of the individual BFU-E colonies at higher concentrations of EPO (Fig. 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 (Fig. 5A).

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 burst-forming unit erythroid (BFU-E) colonies in methylcellulose medium supplemented with a saturating dose of erythropoietin (EPO). In contrast, cells expressing WT SHP-2 form few colonies. (B) E76K SHP-2-expressing fetal liver cells show EPO-independent BFU-E colony growth. The addition of EPO increases both the number and the size of the BFU-E colonies.

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 \textit{PTPN11} mutation found in
JMMML specimens\textsuperscript{22,23}. Like glutamic acid 76, aspartic acid 61 mediates contacts between the N-SH2 and PTP domains\textsuperscript{3}. Interestingly, while the D61Y mutation has elevated phosphatase activity in COS-7 cells, it is less active than E76K\textsuperscript{22}, and this mutant has less potent pro-survival effects in Ba/F3 cells\textsuperscript{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 (Fig. 6A). However, D61Y-expressing cells consistently were less hypersensitive than the fetal liver cells that were transduced in parallel with the E76K virus. Asparagine 308 is the most commonly mutated amino acid in individuals with NS\textsuperscript{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 \textit{in vitro} than E76K (Fig 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 and 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-positive donor-derived cells was assessed by flow cytometry. Unfortunately, many mice died between 2 and 5 months after transplant 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 1-5 months after adoptive transfer than
the counts of mice transplanted with cells infected with either WT SHP-2 or empty vector constructs. Although the transduced cells that we injected were consistently 20-40% GFP-positive, these levels varied widely in recipient animals and were frequently <10% (data not shown). Surviving recipients were sacrificed for analysis ~5 months post transplant. Interestingly, whereas splenocytes harvested from several mice transplanted 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 transplanted 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 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-positive cells in blood, marrow, and spleen (30 - 60%).

DISCUSSION

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, NFI, and PTPN11 mutations have largely been identified in mutually exclusive subsets of JMML patients, 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 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 two recent reports, infer that hyperactive Ras perturbs myeloid differentiation by actively driving cells toward a monocyte-macrophage fate or by interfering with granulocytic maturation. Similarly, regulated expression of mutant H-Ras in human myeloid progenitors promotes monocytic differentiation, and monocytosis 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 as 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-GM, but that this mutation also perturbs the growth of immature myeloid progenitors (HPP-CFC and LPP-CFC). Similarly, the bone marrows of mice that had been reconstituted with Nf1-/- fetal liver cells also contain increased numbers of HPP-CFC and LPP-CFC.

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 transplanted 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, while 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 marrow resistant\(^3\\)\(^8\). 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 \textit{in vivo}.

In addition to myelomonocytic proliferation, JMML is characterized by anemia, ineffective erythropoiesis, splenic infiltration by erythroid cells, and elevated fetal hemoglobin levels\(^9\)\(^,\)\(^10\). Increased numbers of BFU-E are present in the blood and bone marrows of JMML patients, some of which form EPO-independent colonies in methylcellulose\(^3\\)\(^9\). 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 transplanted with these cells. Interestingly, somatic activation of oncogenic \textit{Kras} results in anemia and splenomegaly with erythroid infiltration\(^15\)\(^,\)\(^16\). Furthermore, fetal liver cells transduced with oncogenic \textit{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-of-function alleles that might not be compatible with normal development. The observation that E76K SHP-2 confers profound *in vitro* CFU-GM hypersensitivity extend 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 - 0.1 ng/mL\(^{27}\). Moreover, Mohi and coworkers\(^{28}\) 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 al.*\(^{26}\) 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). D61G SHP-2 has lower phosphatase activity than the E76K SHP-2 and it is of interest that CFU-GM from these animals
are less hypersensitive to GM-CSF and IL-3 than cells transduced with E76K SHP-2\textsuperscript{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 profoundly alter the growth of primary myeloid cells whereas common germline \textit{PTPN11} mutations found in NS patients encode SHP-2 proteins that have relatively subtle effects.

\textit{In vitro} data infer a catalytic-independent role of SHP-2 in IL-3 and platelet derived growth factor signaling\textsuperscript{33-35}. By contrast, we found that expressing WT SHP-2 did not alter CFU-GM colony growth and that ablating SHP-2 phosphatase activity in the context of the E76K mutation abolished GM-CSF hypersensitivity. These data directly linking a JMML-associated cellular phenotype to SHP-2 catalytic activity are consistent with extensive structure-function studies performed by Mohi \textit{et al.}\textsuperscript{28}, which also defined essential roles of phosphotyrosyl binding by the SH2 domain and of tyrosine residues that link SHP-2 to Grb2 in cytokine hypersensitivity.

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 \textit{et al.}\textsuperscript{27} reported lower levels of F4/80 expression in cultured macrophage progenitors that were induced to differentiate \textit{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 clinico-pathologic 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 replating 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-positive fetal liver cells that were cultured without growth factors for 2-24 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\(^23\). Mohi *et al.*\(^28\) confirmed these observations, and went on to investigate bone marrow cells from transplanted mice that had been cultured with IL-3 to generate bone marrow mast cells (BMMCs). Interestingly, cells expressing E76K or D61Y SHP-2 proliferated extensively under
these conditions, which is also characteristic of Kras mutant cells\textsuperscript{15}. Biochemical analyses revealed elevated levels of phosphorylated ERK, Akt, and STAT5 in cells from mice that had been transplanted with cells expressing E76K or D61Y SHP-2\textsuperscript{28}. Chan and coworkers\textsuperscript{27} 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\textsuperscript{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\textsuperscript{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.

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

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