Increased c-Jun Expression and Reduced GATA2 Expression Promote Aberrant Monocytic Differentiation Induced by Activating *PTPN11* Mutants^{∇§}

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Juvenile myelomonocytic leukemia (JMML) is characterized by myelomonocytic cell overproduction and commonly bears activating mutations in *PTPN11*. Murine hematopoietic progenitors expressing activating Shp2 undergo myelomonocytic differentiation, despite being subjected to conditions that normally support only mast cells. Evaluation of hematopoietic-specific transcription factor expression indicates reduced GATA2 and elevated c-Jun in mutant Shp2-expressing progenitors. We hypothesized that mutant Shp2-induced Ras hyperactivation promotes c-Jun phosphorylation and constitutive c-Jun expression, permitting, as a coactivator of PU.1, excessive monocytic differentiation and reduced GATA2. Hematopoietic progenitors expressing activating Shp2 demonstrate enhanced macrophage CFU (CFU-M) compared to that of wild-type Shp2-expressing cells. Treatment with the JNK inhibitor SP600125 or cotransduction with GATA2 normalizes activating Shp2-generated CFU-M. However, cotransduction of Δ GATA2 (lacking the C-terminal zinc finger, needed to bind PU.1) fails to normalize CFU-M. NIH 3T3 cells expressing Shp2E76K produce higher levels of luciferase expression directed by the macrophage colony-stimulating factor receptor (*MCSFR*) promoter, which utilizes c-Jun as a coactivator of PU.1. Coimmunoprecipitation demonstrates increased c-Jun-PU.1 complexes in mutant Shp2-expressing hematopoietic progenitors, while chromatin immunoprecipitation demonstrates increased c-Jun binding to the *c-Jun* promoter and an increased c-Jun-PU.1 complex at the *Mcsfr* promoter. Furthermore, JMML progenitors express higher levels of c-JUN than healthy controls, substantiating the disease relevance of these mechanistic findings.

Juvenile myelomonocytic leukemia (JMML) is a lethal myeloproliferative disorder characterized by pathological overproduction of myelomonocytic cells. Despite the use of multiple chemotherapeutic agents, the only curative therapy available is allogeneic stem cell transplantation (6, 35, 41, 49, 73). Approximately 70% of JMML patients bear loss-of-function mutations in *NF1* (~15 to 20%) (9, 57, 60), gain-of-function mutations in *KRAS* or *NRAS* (~15 to 20%) (58), or gain-of-function mutations in *PTPN11* (~35%) (36, 42, 66). Typically, these mutations are nonoverlapping, with the net effect being hyperactivation of Ras-mediated signaling. Gain-of-function Shp2 mutants clearly promote hyperproliferation of myeloid progenitors (14, 45, 55); however, given the profound chemoresistance of JMML, it is evident that merely targeting rapidly dividing cells is insufficient to effectively combat this disease. As an alternative, we have pursued studies to

examine the contribution of enhanced survival (71) as well as aberrant differentiation, the focus of this report, to the pathogenicity of JMML.

JMML is distinguished clinically by increased numbers of circulating and tissue immature myeloid cells and characterized in vitro by profound cytokine-independent progenitor colony growth (24, 27, 32). In contrast to normal subjects, the composition of progenitor colonies from JMML patients is almost exclusively macrophages and monocytes (26). Furthermore, the ratio of pure monocyte/macrophage colonies to granulocyte-containing colonies is >2/1 for JMML patients, compared to a ratio of pure monocyte/macrophage colonies to granulocyte-containing colonies of 1/3.5 for normal controls (2). Given that progenitor colonies from JMML patients contain monocytic cells along the full spectrum of differentiation, JMML is a disease distinguished phenotypically by the shunting of hematopoietic differentiation toward the monocytic pathway, in contrast to a complete block in hematopoietic differentiation, as found in acute myeloid leukemia. Considering this unique phenotypic characteristic of JMML, the central role of Ras deregulation in JMML, and previous studies demonstrating mutant Ras-induced skewed myelomonocytic differentiation (22, 31, 33, 44, 61), we hypothesized that activating *PTPN11* mutations promote commitment to the myelomonocytic lineage at the expense of other myeloid lineages, and as a

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corollary, we predicted that expression of hematopoietic-specific transcription factors is altered accordingly.

Transcription factors work either cooperatively or antagonistically (53, 68, 75, 76), suggesting that absolute levels (17, 54) as well as specific combinations of transcription factors (16) are needed for normal differentiation. The proto-oncogene *c-Jun* has been shown to function as a coactivator of PU.1 in promoting monocyte-specific gene expression (5, 29). Structure-function studies indicate that amino acids 265 to 283 within the basic domain of murine *c-Jun* interact physically with the Ets domain of PU.1 (4, 40, 75). Additionally, the C-terminal zinc finger (CZnF) of both GATA1 and GATA2 also interacts with the Ets domain of PU.1 (40, 75). Functional studies demonstrate that GATA1 and GATA2 compete with *c-Jun* for interaction with PU.1 and transactivation of PU.1-responsive promoters (75), clearly yielding a model whereby competition between GATA1 or GATA2 and *c-Jun* for binding to PU.1 underlies hematopoietic cell fate decisions.

Along with excessive monocytic differentiation, Ras hyperactivation is a cornerstone aberrancy leading to the pathogenesis of JMML. Oncogenic Ras leads to *c-Jun* activation by promoting hyperphosphorylation of activating sites (S63 and S73), leading to enhanced *c-Jun* transactivation capacity and enhanced *c-Jun* expression (3, 7, 62). The key kinase that phosphorylates *c-Jun* S63 and S73 (activating sites) is Jun N-terminal protein kinase (JNK) (19), a mitogen-activated protein kinase (MAPK) downstream of Ras (20, 37). In chronic myelogenous leukemia, a disease with pathological similarities to JMML, JNK hyperactivation and increased *c-Jun* expression promote BCR-ABL-induced cellular transformation (10, 52). We have demonstrated hyperphosphorylation of JNK1 and JNK2 in serum- and growth factor-deprived macrophage progenitors expressing Shp2 gain-of-function mutations (71), suggesting that increased *c-Jun* phosphorylation may promote constitutive *c-Jun* expression, favoring the *c-Jun*-PU.1 interaction and promoting monocytic differentiation.

We present data demonstrating that activating *PTPN11* mutants augment monocytic differentiation in response to growth factor signals that normally support only mast cell development. Consistently, we find that mutant Shp2-expressing cells express very low levels of *GATA2* and significantly increased levels of *c-Jun* compared to those of wild-type (WT) Shp2-expressing cells. Furthermore, we demonstrate increased *c-Jun* expression and increased *c-Jun* phosphorylation following short-term culture, implying that enhanced *c-Jun* function is a primary aberrancy contributing to the pathogenesis of JMML. Upon treatment with the JNK inhibitor SP600126, we find normalization of the mutant Shp2-induced monocytic differentiation to levels near that of progenitors transduced with empty vector or WT Shp2. Upon coexpression of GATA2 with mutant Shp2, the increased monocytic differentiation also is normalized; however, coexpression of GATA2 lacking the CZnF, the domain needed for interaction with PU.1, is unable to normalize the mutant Shp2-induced increased monocytic differentiation. Mechanistically, we find that the macrophage colony-stimulating factor receptor (*MCSFR*) promoter, characterized to be activated by the *c-Jun*-PU.1 complex (5), is expressed at higher levels in NIH 3T3 cells expressing Shp2 E76K than in those expressing WT Shp2. Consistently, increased *c-Jun*-PU.1 complexes are detected in mutant Shp2-

expressing hematopoietic progenitors. Furthermore, using chromatin immunoprecipitation (ChIP) assays, increased *c-Jun* is detected at the *c-Jun* promoter in mutant Shp2-expressing progenitors, implying enhanced *c-Jun* autoregulation, and an increased association of the *c-Jun*-PU.1 complex is detected at the murine *Mcsfr* promoter. These findings suggest that one fundamental sequela of Ras hyperactivation in mutant Shp2-expressing cells is elevated *c-Jun* expression and an increased *c-Jun*-PU.1 interaction, promoting monocytic differentiation and, thus, overproduction of myelomonocytic cells. Consistent with these observations, CD34⁺ cells from JMML patients express significantly increased levels of *c-JUN* compared to those of healthy controls. These findings imply that pharmaceutical interventions that inhibit *c-Jun* phosphorylation or expression could inhibit mutant Shp2-induced monocytic differentiation and, thus, serve as a novel approach for treatment of JMML.

MATERIALS AND METHODS

Retroviral vectors and retroviral transduction. The WT *Ptpn11* cDNA was mutated at nucleotide 226 (G→A) or 181 (G→T) using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) to yield amino acid change E76K or D61Y, respectively, as previously described (14). The WT *Gata2* cDNA was subjected to site-directed mutagenesis using primers 5'-GAT GAA TGG ACA GAA CAA GAA AGG GGC TGA ATG-3' (upper) and 5'-CAT TCA GCC CCT TTC TTG TTC TGT CCA TTC ATC-3' (lower) to remove nucleotides coding for amino acids 330 to 407 to generate Δ *Gata2*. The *Ptpn11* cDNAs were subcloned into the murine bicistronic retroviral vector pMIEG3 in tandem with the enhanced green fluorescent protein (EGFP) to generate pMIEG3-WT Shp2, pMIEG3-Shp2E76K, and pMIEG3-Shp2D61Y. The *Gata2* cDNAs were subcloned into pMIEG3 in tandem with human CD4 (hCD4) (pMIEG3-hCD4) to generate pMIEG3-GATA2 and pMIEG3- Δ GATA2. All ecotropic retroviral supernatants were prepared using Eco-Phoenix packaging cells. Bone marrow low-density mononuclear cells (LDMNCs) from C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were purified using a Ficoll gradient. Enrichment of lineage-negative cells was performed using SpinSep (StemCell Technologies, Vancouver, British Columbia). Cells were transduced, as previously described (14). Following transduction, cells were sorted for EGFP-positive (EGFP⁺) cells, or for cotransductions, cells were stained with anti-hCD4 conjugated to allophycocyanin (APC; BD Biosciences, San Jose, CA) and sorted for the EGFP⁺/APC-positive (APC⁺) populations using fluorescence-activated cell sorting (FACS).

Hematopoietic progenitor analysis. Transduced, EGFP⁺, or EGFP/APC⁺ cells were plated at a concentration of 8,000 cells/ml in 0.9% methylcellulose-based media containing Iscove's modified Dulbecco's medium, 2 mM glutamine, 1% penicillin-streptomycin, 80 μ M β -mercaptoethanol, 30% fetal bovine serum, 200 U/ml interleukin-3 (IL-3), 4 U/ml erythropoietin, and 100 ng/ml stem cell factor. Cultures were incubated in a humidified incubator at 37°C in 5% CO₂, and colonies were scored based on morphology after 7 days for granulocyte-macrophage CFU (CFU-GM), macrophage CFU (CFU-M), granulocyte CFU (CFU-G), or granulocyte-erythroid-megakaryocyte-monocyte CFU (CFU-GEMM or CFU-Mix). Assays were conducted on at least three independent occasions and plated in triplicate for each experiment. The data are represented as percent total colonies, which is calculated by dividing the number of each progenitor type by the number of total colonies in each plate.

Mast cell differentiation and analysis. Sorted, EGFP⁺, or EGFP/APC⁺ cells were plated into RPMI 1640, 10% fetal calf serum, 14 mM HEPES, 1% penicillin-streptomycin, and 10 U/ml IL-3 and cultured at 37°C in 5% CO₂ for selection of mast cell development. Nonadherent cells were retained and passaged into new media approximately two times each week. In some experiments, rat anti-mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) (anti-GM-CSF, ab7368; Abcam, Cambridge, MA) was included at a concentration of 4 μ g/ml. To examine mast cell versus monocytic differentiation, cells were collected using cell dissociation buffer (Gibco, Invitrogen Corporation), stained with anti-Mac-1 conjugated to APC (BD Pharmingen, San Jose, CA), anti-F4/80 conjugated to phycoerythrin (PE; Serotec, Oxford, United Kingdom), or anti-kit conjugated to APC (BD Pharmingen), and evaluated using flow cytometry.

Quantitative reverse transcription-PCR (RT-PCR). For the real-time PCR, each cDNA was mixed with SYBR green master mix; forward and reverse primers for murine *PU.1*, *GATA2*, Δ *GATA2*, *c-Jun*, *GM-CSF*, the gene for the α subunit GM-CSF receptor (*GM-CSFR α*), or human *c-JUN*; uracil *N*-glycosylase; and water and placed in an ABI Prism 96-well plate. Reactions were analyzed in an ABI Prism 7900HT sequence detection system (Applied Biosystems) which collects the data at the end of each cycle and exports cycle threshold (C_T) values onto Excel spreadsheets for analysis. Amplification of the *PU.1*, Δ *GATA2*, *GATA2*, and *c-Jun* cDNA was normalized to amplification of the internal control L27 cDNA (reactions run on the same plate) to calculate relative expression using the $2^{-\Delta\Delta C_T}$ method. Each reaction was run in triplicate to quadruplicate within each independent experiment. The following primer sets were used: WT *GATA2*, 5'-AGA CGA CAA CCA CCT TA-3' and 5'-TCC TTC TTC ATG GTC AGT GG-3'; Δ *GATA2*, 5'-CAG CAG TCT CTT CCA TCC AG-3' and 5'-GCA CCA CAG TTG ACA CAC TC-3'; *PU.1*, 5'-GCT ATA CCA ACG TCC AAT GC-3' and 5'-GTT GTT GTG GAC ATG GTG TG-3'; *c-Jun*, 5'-GTT GCG GCC GCG AAA CTT-3' and 5'-CAT TGC CCT CGA GCC CTG-3'; *GM-CSFR α* , 5'-CAG ACG GAC GGA CAC AGA C-3' and 5'-GGT GAT GTT CAT GGC ATG TG-3'; *GM-CSF*, 5'-GCA TGT AGA GGC CAT CAA AGA-3' and 5'-CGG GTC TGC ACA CAT GTT A-3'; human *c-JUN*, 5'-CCA AAG GAT AGT GCG ATG TTT-3' and 5'-CTG TCC CTC TCC ACT GCA AC-3'.

Generation and transfection of NIH 3T3-WT Shp2 and NIH 3T3-Shp2E76K cells. NIH 3T3 cells were transduced with ecotropic retroviral supernatants (pMIEG3, pMIEG3-WT Shp2, pMIEG3-Shp2E76K) in the presence of Polybrene at 8 μ g/ml. Following transduction, cells were sorted for EGFP using FACS to enrich for transduced cells. NIH 3T3-WT Shp2 and NIH 3T3-Shp2 E76K cells were transfected with pMCSFR-L (the M-CSF receptor promoter driving luciferase expression, as previously described) (5, 11, 74) with and without pMIEG3-PU.1 (generously provided by Mark Kaplan), pMIEG3-GATA2, or pMIEG3- Δ GATA2. As a negative control, cells were transfected with equal molar amounts of pMIEG3. Differences in total transfected micrograms of plasmid DNA were equalized by pUC19. As an internal control, cells were transfected with pTK-RL (*Renilla* luciferase driven by the thymidine kinase promoter; Promega). Forty-eight hours following transfection, protein lysates were prepared, and assays for luciferase and *Renilla* luciferase activity were conducted (dual-luciferase reporter assay system; Promega).

Immunoblot and coimmunoprecipitation analysis. Sorted, EGFP⁺ cells were cultured for 2 days (to examine phospho-c-Jun levels) or for 7 to 10 days (for all other analyses), followed by total cellular protein extract preparation, as previously described (59), or followed by nuclear protein extract preparation (nuclear complex co-IP kit; Active Motif, Carlsbad, CA). For coimmunoprecipitation assays, 1 mg of nuclear protein extract was incubated with anti-c-Jun antibody (H-79; Santa Cruz Biotechnology) and protein A Sepharose beads (CL-4B; GE Healthcare) for 2 h at 4°C. Blots were probed with anti-PU.1 antibody (D-19; Santa Cruz Biotechnology) and developed by enhanced chemiluminescence. Blots were reprobed with anti-c-Jun antibody (H-79; Santa Cruz Biotechnology). To measure total GATA2, PU.1, and c-Jun expression levels, total cellular protein or nuclear extracts were immunoprecipitated with anti-GATA2 (CG2-96), anti-PU.1 (T-21), or anti-c-Jun (H-79), all from Santa Cruz Biotechnology, and blots were probed with the same antibodies. To examine phospho-c-Jun levels, total cellular extracts were probed with anti-phospho-c-Jun (no. 420114; EMD Biosciences), and total c-Jun levels were determined by blotting with anti-c-Jun (ab31419; Abcam). Total Shp2 levels were examined, as previously described (13, 14). For all immunoblot analyses, quantitation of cellular and nuclear protein extracts was verified by probing with anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) (Bioscience International, Saco, ME) or with anti-TBP (ITBP18; Abcam, Cambridge, MA), respectively.

ChIP assay. ChIP assays were performed using a ChIP assay kit (Upstate Biotechnology, Lake Placid, NY). Briefly, following culture for 1 week, transduced cells were treated with 1% formaldehyde for cross-linking, followed by preparation of cellular extracts using lysis buffer (1% sodium dodecyl sulfate, 10 mM EDTA, and 50 mM Tris-HCl [pH 8.1]). Extracts were sonicated to shear chromatin DNA to produce 500- to 1,000-bp DNA fragments, followed by incubation with anti-c-Jun (H79, sc-1694X; Santa Cruz Biotechnology) or anti-PU.1 (T21, sc-352X) and salmon sperm DNA/protein A agarose in ChIP dilution buffer (0.01% sodium dodecyl sulfate, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl [pH 8.1], and 167 mM NaCl) overnight at 4°C. Immunoprecipitates were collected by gentle centrifugation, washed, and eluted with low- and high-stringency buffers. Histone-DNA cross-links were reversed by heating for 4 h at 65°C, proteins were digested by treatment with proteinase K, and DNA was recovered by phenol-chloroform extraction. PCRs were conducted using forward primer 5'-GCT TTG TTT TCT TCT AGA GAC-3' and reverse primer 5'-AGC

CTC CAG GGG ATA GCG-3', which generates a 214-bp band and spans the murine M-CSR receptor (*Mcsfr*) promoter PU.1 binding site homologous to the PU.1 binding site within the human M-CSR receptor (*MCSFR*) promoter found to be coactivated by c-Jun (5, 74). To look at c-Jun binding to the murine *c-Jun* promoter, PCRs were conducted using forward primer 5'-GCC TCC GTC ACT AGA CAG-3' and reverse primer 5'-CTC CTG CCA GGC TGT CTC-3', which generates a 398-bp band spanning from -350 to +40 relative to the murine *c-Jun* transcription start site (3, 30, 69). For internal control, total input DNA was amplified using primers 5'-CTG CCT CTG CCT CCT AAA TG-3' and 5'-CTC CCA GAG GAT TCC CAG AT-3' to amplify a region of the *HPRT* promoter, as previously described (72).

c-JUN, GATA2, PU.1, and C/EBP α mRNA expression in hematopoietic progenitor cells of patients with JMML. After receiving informed guardian consent, spleen cells were obtained from seven children with JMML who underwent splenectomy because of clinical necessity. Mononuclear cells were enriched by Ficoll density centrifugation and cryopreserved in liquid nitrogen. After being thawed, CD34-positive progenitor cells were stained with PE-conjugated antibodies (BD Biosciences) and sorted on a MoFlo high-speed cell sorter (Dako, Carpinteria, CA). CD34-positive hematopoietic cells from the cord blood of three healthy newborns were purchased from Lonza (Basel, Switzerland). RNA was isolated using the Trizol reagent (Invitrogen). Hybridization solutions for U133A expression microarrays (Affymetrix, Santa Clara, CA) were prepared according to the manufacturer's protocols using Affymetrix reagents. The hybridized arrays were stained with PE-conjugated streptavidin and read using a laser confocal scanner (Affymetrix). Fluorescence intensities and detection calls were determined from the scanner image data using Affymetrix GeneChip operating software. Expression values were normalized between arrays to a standard target value of 500. For validation studies, total cellular RNA was isolated from peripheral blood mononuclear cells from four JMML patients and four healthy controls, followed by quantitative RT-PCR for human *c-JUN*, as described above.

Statistical analysis. For the progenitor assays, quantitative RT-PCR assays, and reporter gene transfection assays, comparisons were conducted using random effects analysis of variance to deal with repeated measurements within each independent assay. The *P* value for pairwise comparisons was adjusted using Tukey's method (47). For all other comparisons (Mac-1, c-kit, F4/80 expression, and patient sample microarray *c-JUN*, *GATA2*, *PU.1*, and *C/EBP α* expression), unpaired Student's *t* test was used.

RESULTS

Activating Shp2 mutants promote monocytic differentiation in a nonautocrine manner. Based on the observation that CFU-M are disproportionately overrepresented in JMML peripheral blood (2), we examined the capacity of activating *PTPN11* mutants to promote monocytic differentiation. Using saturating concentrations of growth factors, (IL-3 at 200 U/ml, stem cell factor at 100 ng/ml, and erythropoietin at 4 U/ml), we observed that progenitor colonies derived from mutant Shp2-expressing cells were much larger than cells transduced with vector (MIEG3) or WT Shp2 (Fig. 1A), similar to the observations of others (45, 55) and consistent with the increased proliferative phenotype previously defined for mutant Shp2-expressing hematopoietic progenitors (14, 55, 71). Indicative of differentiation potential, several very large pure monocyte-macrophage colonies derived from Shp2D61Y- and Shp2E76K-transduced cells were observed (Fig. 1B). Like the human JMML data, a significantly greater number of CFU-M were generated from progenitors expressing Shp2D61Y or Shp2E76K, with a concomitant reduction in CFU-GM (Fig. 1C).

Normally, media containing low concentrations of IL-3 (10 U/ml) support only the development and expansion of mast cells, not of monocytes. However, when cultured in media containing IL-3 at 10 U/ml, cells transduced with the activating *PTPN11* mutants morphologically demonstrated increased adherence to the tissue culture plate, suggestive of monocytic

FIG. 1. (A) Representative CFU-GM from MIEG3- and Shp2E76-transduced hematopoietic progenitors. (B) Representative CFU-M from mutant Shp2-expressing hematopoietic progenitor. (C) Transduced and sorted bone marrow LDMNCs were plated into colony assays; colony morphology was assessed by light microscopy. Data were compiled from three independent experiments, plated in triplicate. *, $P \leq 0.0001$ for Shp2D61Y CFU-M versus MIEG3 or WT Shp2 CFU-M; **, $P \leq 0.0001$ for Shp2E76K CFU-M versus MIEG3 or WT Shp2 CFU-M. (D) Cellular morphology following culture of transduced and sorted LDMNCs in mast cell media for 1 to 2 weeks. (E) Flow cytometry analysis for c-kit (*, n of 4 and $P \leq 0.04$ for Shp2E76K or Shp2D61Y versus MIEG3 or WT Shp2), Mac-1 (*, n of 4 and $P \leq 0.001$ for Shp2E76K or Shp2D61Y versus MIEG3 or WT Shp2), and F4/80 (*, $P \leq 0.05$ [$n = 2$] for Shp2E76K or Shp2D61Y versus MIEG3 or WT Shp2).

differentiation, compared to cells transduced with empty vector or WT Shp2 (Fig. 1D). We next examined cells for myelomonocytic and mast cell phenotypic markers by staining with anti-Mac-1-APC, anti-F4/80-PE, or anti-c-kit-APC, followed by flow cytometry. A significantly higher level of Mac-1 and F4/80 was observed in cells expressing the activating *PTPN11* mutations (Fig. 1E). In contrast, expression of c-kit, which is highly expressed in mast cells, was significantly lower in cells expressing activating *PTPN11* mutations (Fig. 1E).

Given the low concentration of exogenous cytokines in mast cell media (IL-3 at 10 U/ml), we asked if the mutant Shp2-expressing progenitors express increased levels of endogenous GM-CSF, thus yielding an autocrine mechanism producing excessive myelomonocytic differentiation. Transduced and sorted cells were cultured for either 2 days or 7 days in mast cell media, followed by preparation of total cellular RNA and examination of *GM-CSF* using quantitative RT-PCR. At day 2 of culture, *GM-CSF* expression was relatively similar for all cell types (Fig. 2A); however, by day 7 of culture, *GM-CSF* expression was reduced in mutant Shp2-expressing cells. In a similar fashion, expression of *GM-CSFR α* was also reduced, at both day 2 and day 7 of culture, in the mutant Shp2-expressing cells (Fig. 2A). To examine this question functionally, we cultured all cell types in mast cell media in the presence of blocking antibody to GM-CSF (anti-GM-CSF, 4 μ g/ml, changed every other day for 1 week) and examined differentiation phenotypically. While the presence of anti-GM-CSF antibody modestly reduced total cell numbers of mutant Shp2-expressing cells (Fig. 2B, compare i and ii), the cellular morphology demonstrated persistent adherence to the plate (Fig. 2B, compare iii and iv). Upon phenotypic analysis, culture in the presence of anti-GM-CSF antibody did not result in reduced myelomonocytic differentiation of the mutant Shp2-expressing cells, as shown by flow cytometry using anti-Mac1 and anti-F4/80 antibodies (Fig. 2C). Thus, while blocking GM-CSF function modestly reduced total cell numbers of mutant Shp2-expressing cells, consistent with these cells being hypersensitive to even very low concentrations of GM-CSF (14), the differentiation status remained unchanged. Collectively, these findings suggest that the skewed myelomonocytic differentiation does not result from an autocrine mechanism of enhanced *GM-CSF* or *GM-CSFR α* expression or function.

Activating Shp2 mutants induce c-Jun expression and promote c-Jun phosphorylation. We next focused on potential alterations in transcription factor expression and function that may lead to enhanced myelomonocytic differentiation of mutant Shp2-expressing cells. Using the defined mechanistic roles of PU.1 and GATA2 in macrophage and mast cell differentiation as a premise (68), we anticipated elevated *PU.1* and reduced *GATA2* levels in cells bearing the activating Shp2 mutants. Total cellular RNA was prepared from cells cultured

in mast cell media for 1 to 2 weeks, and quantitative RT-PCR was conducted. *GATA2* expression in cells bearing the activating Shp2 mutants was only 5 to 10% of that in cells transduced with MIEG3 or WT Shp2 (Fig. 3A); however, no significant elevation of *PU.1* was observed in mutant-bearing cells (Fig. 3B), suggesting that interactions with cooperating transcription factors are critical for promoting monocytic differentiation in mutant Shp2-expressing cells. Given the role of c-Jun in promoting monocytic differentiation (28, 39, 43, 46, 65) and the capacity of oncogenic Ras to increase c-Jun activation and expression (3, 7, 62), we predicted that *c-Jun* expression would be elevated in mutant Shp2-expressing cells. Consistently, a fivefold-higher level of *c-Jun* expression was observed in activating Shp2 mutant-bearing cells (Fig. 3C). Evaluation of GATA2, PU.1, and c-Jun protein levels very similarly matched those of the RT-PCR findings (Fig. 3A to C).

The substantial difference observed in *c-Jun* and *GATA2* expression in the mutant Shp2-expressing cells may be the result of the skewed monocytic differentiation observed after a prolonged time in culture. Therefore, we examined *c-Jun* and *GATA2* expression following culture for only 1 day postsorting, when cells appear morphologically similar. Nevertheless, at this early time point, the *c-Jun* expression level was significantly elevated in the mutant Shp2-expressing cells (Fig. 3E), while *GATA2* expression levels were similar in all the cells (Fig. 3D). These findings indicate that increased *c-Jun* expression is a primary molecular aberrancy that contributes to mutant Shp2-induced monocytic differentiation and that the reduced *GATA2* expression is a secondary effect of the skewed monocytic differentiation.

Based on our previous finding of JNK hyperactivation in mutant Shp2-expressing cells (71), we predicted increased c-Jun phosphorylation in mutant Shp2-expressing cells, leading to increased *c-Jun* gene expression. To address this hypothesis, we prepared protein lysates from transduced and sorted LDMNCs that were cultured in IL-3 at 10 U/ml for 2 days. As anticipated, c-Jun phosphorylation was significantly elevated in mutant Shp2-expressing progenitors (Fig. 3F). The finding of increased levels of phosphorylated c-Jun as well as c-Jun transcripts suggests that increased c-Jun function in mutant Shp2-expressing progenitors provides positive feedback, leading to the constitutive c-Jun expression.

JNK inhibition reduces the enhanced monocytic differentiation induced by activating Shp2 mutants. We next hypothesized that inhibition of JNK, the key protein kinase that phosphorylates c-Jun S63 and S73 (19), would lead to reduced monocytic differentiation. Transduced and sorted murine bone marrow LDMNCs were plated into progenitor assays, as described in Fig. 1, in the presence or absence of 15 μ M SP600125, an ATP-competitive anthrapyrazolone inhibitor of JNK. While this concentration of SP600125 did not signifi-

FIG. 2. (A) Quantitative RT-PCR for *GM-CSF* and *GM-CSFR α* following 2 and 7 days in mast cell media. Bar graph for *GM-CSF*, day 2, shows two independent experiments in triplicate, while the graph for *GM-CSF*, day 7, shows three independent experiments in triplicate. #, P is <0.003 for Shp2D61Y or Shp2E76K versus WT Shp2. Bar graph for *GM-CSFR α* , day 2, shows three independent experiments in triplicate. *, P is ≤ 0.002 for Shp2D61Y or Shp2E76K versus MIEG3 or WT Shp2. Bar graph for *GM-CSFR α* , day 7, shows three independent experiments in triplicate. **, P is ≤ 0.004 for WT Shp2, Shp2D61Y, or Shp2E76K versus MIEG3. (B) Morphology of cells transduced with Shp2E76K in the absence and presence of anti-GM-CSF antibody at 4 μ g/ml. α , anti. (C) Flow cytometry using anti-Mac-1 and anti-F4/80 antibodies, comparing myelomonocytic differentiation in the absence and presence of anti-GM-CSF antibody.

FIG. 3. Quantitative RT-PCR and corresponding immunoblot analysis for *GATA2* (A), showing three independent experiments in duplicate to quadruplicate (*, P is 0.01 for WT Shp2 versus MIEG3; **, P is <0.005 for Shp2E76K or Shp2D61Y versus MIEG3 or WT Shp2), *PU.1* (B), showing three independent experiments in duplicate to quadruplicate, and *c-Jun* (C), showing two independent experiments in triplicate (*, P is ≤ 0.0001 for Shp2E76K or Shp2D61Y versus MIEG3 or WT Shp2). (D) Quantitative RT-PCR analysis for *GATA2* following 1 day of culture after sorting. Results are from three independent experiments in triplicate. (E) Quantitative RT-PCR analysis for *c-Jun* following 1 day of culture after sorting. Results are from three independent experiments in triplicate. (*, P is <0.0001 for Shp2E76K or Shp2D61Y versus MIEG3 or WT Shp2). (F) Immunoblot analysis examining phospho-c-Jun and total c-Jun following culture of sorted hematopoietic progenitors in mast cell media for 2 days.

FIG. 4. (A) Transduced and sorted murine bone marrow LDMNCs were plated into colony assays in the presence and absence of 15 μ M SP600125; colony morphology was assessed by light microscopy. Data were compiled from three independent experiments, plated in triplicate. *, P is <0.0001 for Shp2D61Y CFU-GM plus SP600125 versus Shp2D61Y CFU-GM without (-) SP600125; **, P is <0.0001 for Shp2D61Y CFU-M plus SP600125 versus Shp2D61Y CFU-M without SP600125; #, P is <0.0001 for Shp2E76K CFU-GM plus SP600125 versus Shp2E76K CFU-GM without SP600125; ##, P is <0.0001 for Shp2E76K CFU-M plus SP600125 versus Shp2E76K CFU-M without SP600125. (B) Representative CFU-GM from Shp2E76K-transduced hematopoietic progenitors in the absence and presence of 15 μ M SP600125.

cantly reduce total colony numbers of the MIEG3-, WT Shp2-, Shp2D61Y-, or Shp2E76K-expressing progenitors (suggesting the absence of nonspecific toxicity), CFU-M derived from the Shp2D61Y- or Shp2E76K-expressing progenitors was significantly reduced to levels similar to that derived from MIEG3- or WT Shp2-expressing progenitors (Fig. 4A). In addition to reduced absolute numbers of CFU-M, CFU-GM colonies derived from Shp2D61Y- and Shp2E76K-transduced progenitors in the presence of SP600125 demonstrated a reduced monocytic and increased granulocytic component, indicated morphologically by increased nonadherent and smaller cells (Fig. 4B, compare i and ii, iii and iv, and v and vi and vii and viii, respectively). These findings support the notion that JNK hyperactivation, elevated c-Jun phosphorylation, and constitutive c-Jun expression promote enhanced monocytic differentiation induced by activating Shp2 mutants. Based on the model of GATA2 and c-Jun competition for binding to PU.1 (75), we next examined if the introduction of GATA2 would normalize the promonocytic phenotype induced by the activating Shp2 mutants.

Ectopic GATA2 expression normalizes the enhanced monocytic differentiation induced by activating Shp2 mutants. Previous studies demonstrated that ectopic expression of GATA2 reduces hematopoietic progenitor-derived colonies (51) and has the capacity to redirect embryonic stem cell-derived mono-

cytes (34). To perform cotransduction experiments, we employed a second retroviral vector, expressing GATA2 in tandem with hCD4 (pMIEG3-hCD4) (15). Using the empty pMIEG3-hCD4 vector in pilot studies, we did not observe any differences functionally comparing the singly and doubly transduced cells in terms of total colony formation (see Fig. S1A in the supplemental material) or in terms of the skewed monocytic differentiation induced by Shp2E76K (see Fig. S1B in the supplemental material). Murine LDMNCs were cotransduced, stained with anti-hCD4 conjugated to APC, sorted for the EGFP⁺ APC⁺ populations (Fig. 5A, top right quadrant), and plated into progenitor assays. Cells cotransduced with Shp2D61Y plus pMIEG3-hCD4 generated significantly more CFU-M than cells transduced with WT Shp2 plus pMIEG3-hCD4 (Fig. 5B, compare lanes 12 and 2), as observed previously. In a compensatory fashion, fewer CFU-GM were obtained from cells transduced with Shp2D61Y plus pMIEG3-hCD4 (Fig. 5B, compare lanes 11 and 1). Upon introduction of WT GATA2, cells cotransduced with either WT Shp2 or Shp2D61Y produced significantly fewer CFU-M colonies (Fig. 5B, compare lanes 7 and 2 and lanes 17 and 12). Consistent with previous findings that GATA2 is expressed in immature hematopoietic progenitors and is able to redirect monocytic differentiation toward erythroid and megakaryocytic differentiation, enforced GATA2 expression promoted the develop-

ment of CFU-GEMM (or CFU-Mix) and megakaryocyte CFU (CFU-Meg) from cells transduced with either WT Shp2 or Shp2D61Y (Fig. 5B, compare lanes 9 and 10 to 4 and 5 and lanes 19 and 20 to 14 and 15). These findings demonstrate that GATA2 not only is able to redirect physiologic monocytic differentiation (WT Shp2-expressing progenitors) but also retains the capacity to redirect enhanced, oncogene-induced monocytic differentiation (Shp2D61Y-expressing progenitors).

When examining total colony numbers, ectopic expression of GATA2 reduced the total colony number and absolute number of CFU-M derived from Shp2D61Y-expressing progenitors to levels similar to those observed with WT Shp2-expressing progenitors (Fig. 5C and D). Additionally, sorted cells were cultured in mast cell media (IL-3, 10 U/ml) and assessed for differentiation. Like previous findings, expression of Shp2D61Y induced increased adherence to the tissue culture plate, suggestive of macrophage differentiation (Fig. 5E, compare i and iii). However, upon introduction of GATA2, the adherent phenotype was normalized to a nonadherent phenotype, similar to that observed with WT Shp2 (Fig. 5E, compare iv and i). Consistently, quantitative RT-PCR studies demonstrated restored *GATA2* expression in cells transduced with Shp2D61Y (Fig. 5F).

Introduction of GATA2 lacking the CZnF fails to normalize the enhanced monocytic differentiation induced by activating Shp2 mutants. Two lines of evidence led us to hypothesize that the GATA2 CZnF domain is critical for directing cells away from monocytic differentiation and toward multilineage hematopoiesis. First, structure-function studies demonstrated that the CZnF domain alone, although not as efficiently as the full-length GATA2, is able to direct the myeloid cell line 416B toward megakaryocytic differentiation (67). Corroborating these findings, biochemical studies demonstrate that the CZnF of GATA2 is needed for the physical interaction with PU.1, supporting a model that competition between GATA2 and c-Jun for binding to PU.1 contributes to hematopoietic lineage-specific cell fate determination (75). Thus, we hypothesized that GATA2 lacking the CZnF (Δ GATA2, lacking amino acids 330 to 407) would fail to redirect mutant Shp2-enhanced monocytic differentiation. The GATA2 cDNA lacking nucleotides encoding amino acids 330 to 407 was subcloned into the retroviral vector pMIEG3-hCD4 (Fig. 6A). Murine lineage-negative enriched bone marrow cells were cotransduced, sorted (Fig. 6B), and plated into progenitor assays or mast cell

media (IL-3, 10 U/ml). Expression of activating Shp2 mutant Shp2E76K similarly promoted monocytic differentiation, as demonstrated by increased CFU-M and ectopic GATA2-reduced CFU-M (Fig. 6C). However, introduction of GATA2 Δ 330-407 (pMIEG3- Δ GATA2) failed to reduce the number of CFU-M (Fig. 6C). Importantly, despite expression of Shp2E76K, ectopic expression of GATA2 normalized the progenitor colony-type distribution to a profile very similar to that of cells coexpressing WT Shp2 and GATA2 (Fig. 6D, compare lanes 5 and 2). In contrast, introduction of Δ GATA2 failed to reduce Shp2 E76K-induced CFU-M development (Fig. 6C) or to alter appreciably the skewed progenitor colony-type distribution (Fig. 6D, compare lanes 6 and 4). Consistently, when plated into mast cell media, cells cotransduced with Shp2E76K plus Δ GATA2 persisted in demonstrating increased adherence, similar to that of cells cotransduced with Shp2E76K plus empty vector (Fig. 6E, compare iv and vi), while introduction of the full-length GATA2 restored the nonadherent phenotype and permitted megakaryocytic differentiation (Fig. 6E, compare v to iv and vi).

Based on the elevated levels of *c-Jun* observed in mutant Shp2-expressing progenitors (Fig. 3), we predicted that the introduction of GATA2 would result in reduced *c-Jun* expression, despite the presence of Shp2E76K, to levels observed in WT Shp2-expressing cells and that the introduction of Δ GATA2 would fail to result in reduced *c-Jun* expression. As anticipated, ectopic expression of GATA2 in Shp2E76K-expressing cells resulted in significantly reduced *c-Jun* expression compared to that of Shp2E76K-expressing cells cotransduced with empty vector or with Δ GATA2 (Fig. 6F). Primers designed to amplify only WT *GATA2* (primer set 1) or both WT *GATA2* and Δ GATA2 (primer set 2) were used to examine WT *GATA2* and Δ GATA2 expression following transduction. Upon cotransduction of WT Shp2 and Shp2E76K with empty vector, significantly reduced WT *GATA2* expression was detected in the Shp2E76K-expressing cells using both primer sets, similar to that observed in Fig. 3A and 5F (Fig. 6G). Introduction of WT *GATA2* resulted in a great increase of *GATA2* expression in the WT Shp2-expressing cells and a modest increase in the Shp2E76K-expressing cells to levels similar to that of WT Shp2-expressing cells cotransduced with pMIEG3-hCD4, again detected by both primer sets and similar to that demonstrated in Fig. 5F. Upon introduction of Δ GATA2 into either WT Shp2- or Shp2E76K-expressing cells,

FIG. 5. (A) Representative FACS of cotransduced cells. (B) Progenitors were plated into colony assays, and colony morphology was assessed by light microscopy. Data were compiled from two independent experiments, plated in triplicate. *, P is 0.07 for Shp2D61Y plus pMIEG3-hCD4 CFU-GM versus WT Shp2 plus pMIEG3-hCD4 CFU-GM (lane 11 versus 1); **, P is <0.0001 for Shp2D61Y plus pMIEG3-hCD4 CFU-M versus WT Shp2 plus pMIEG3-hCD4 CFU-M (lane 12 versus 2); ***, P is <0.0001 for Shp2D61Y plus GATA2 CFU-M versus Shp2D61Y plus pMIEG3-hCD4 CFU-M (lane 17 versus 12); $\wedge$, P is 0.02 for Shp2D61Y plus GATA2 CFU-Mix versus Shp2D61Y plus pMIEG3-hCD4 CFU-Mix (lane 19 versus 14); $\wedge$, P is 0.04 for Shp2D61Y plus GATA2 CFU-Meg versus Shp2D61Y plus pMIEG3-hCD4 CFU-Meg (lane 20 versus 15); #, P is 0.03 for WT Shp2 plus GATA2 CFU-M versus WT Shp2 plus pMIEG3-hCD4 CFU-M (lane 7 versus 2); ##, P is 0.06 for WT Shp2 plus GATA2 CFU-Mix versus WT Shp2 plus pMIEG3-hCD4 CFU-Mix (lane 9 versus 4); ###, P is 0.04 for WT Shp2 plus GATA2 CFU-Meg versus WT Shp2 plus pMIEG3-hCD4 CFU-Meg (lane 10 versus 5). Total colonies (C) and total CFU-M (D) derived from cotransduced cells; data were compiled from two independent experiments, plated in triplicate for both datasets. (C) *, P is 0.001 for total colonies from WT Shp2 plus pMIEG3-GATA2 versus WT Shp2 plus pMIEG3-phCD4; **, P is 0.001 for total colonies from Shp2D61Y plus pMIEG3-GATA2 versus Shp2D61Y plus pMIEG3-hCD4. (D) *, P is 0.006 for total CFU-M from Shp2D61Y plus pMIEG3-hCD4 versus WT Shp2 plus pMIEG3-hCD4. (E) Morphology of cells cotransduced with WT Shp2 or Shp2D61Y with or without GATA2 and cultured in mast cell media. (F) Quantitative RT-PCR for GATA2 from cotransduced cells cultured in mast cell media for 1 week. Data were compiled from two independent experiments, with reactions performed in quadruplicate.

no additional increase in WT *GATA* expression was detected by primer set 1; however, increased total *GATA2* expression was detected by primer set 2 compared to that of cells cotransduced with the empty vector pMIEG3-hCD4. These data demonstrate proficient WT *GATA2* and Δ *GATA2* expression following cotransduction. The inert functional capacity of Δ *GATA2* to normalize the skewed monocytic differentiation supports the idea that Ras hyperactivation in mutant Shp2-expressing cells produces constitutive c-Jun expression, permitting in collaboration with PU.1 excessive monocytic differentiation.

Expression directed by the *MCSFR* promoter is higher in cells expressing Shp2E76K than in cells expressing WT Shp2. We have previously conducted studies using the human *MCSFR* promoter driving luciferase expression (pMCSFR-L) (11), the promoter originally used to define c-Jun as a coactivator of PU.1 (5, 74). We reasoned that if the c-Jun-PU.1 interaction contributes to mutant Shp2-induced aberrant monocytic differentiation, we would observe increased c-Jun expression and increased *MCSFR* promoter-directed luciferase expression in cells transduced with Shp2E76K compared to those of cells transduced with WT Shp2, independent of hematopoietic cell differentiation status. To conduct these studies, we utilized the nonhematopoietic cell line NIH 3T3 to remove the variable of hematopoietic differentiation. NIH 3T3 cells were transduced with WT Shp2 or with Shp2E76K and sorted for EGFP⁺ cells (Fig. 7A). Total Shp2 expression was approximately threefold greater in the WT Shp2- and Shp2E76K-transduced cells compared to that of vector-transduced or parental NIH 3T3 cells (Fig. 7B). As predicted, exponentially growing Shp2E76K-transduced NIH 3T3 cells expressed higher levels of c-Jun than WT Shp2-transduced cells (Fig. 7C). NIH 3T3-WT Shp2 and NIH 3T3-Shp2E76K cells were transfected with pMCSFR-L (5, 11, 74) with and without pMIEG3-PU.1. As a negative control, cells were transfected with equal molar amounts of pMIEG3, and as an internal control, cells were transfected with pTK-RL (*Renilla* luciferase driven by the thymidine kinase promoter). As expected, transfection of PU.1 increased expression directed by the *MCSFR* promoter in both WT Shp2- and Shp2E76K-expressing cells (Fig. 7D, compare lanes 2 and 1 and lanes 7 and 6). Additionally, consistent with our hypothesis that increased c-Jun expression augments monocyte-specific gene expression in mutant Shp2-expressing cells, expression directed by the *MCSFR* promoter induced by PU.1 was significantly higher in the NIH 3T3-Shp2E76K cells than in NIH 3T3-WT Shp2 cells (Fig. 7D, compare lanes 7 and 2).

Previous studies demonstrated that transfection of *GATA1* or *GATA2* is able to inhibit c-Jun-PU.1-induced *MCSFR* promoter-directed expression (75). Consistently, introduction of *GATA2* significantly reduced *MCSFR* promoter-driven expression in Shp2E76K-expressing cells to levels similar to that observed in the WT Shp2-expressing cells (Fig. 7D, compare lanes 9 and 7 and lanes 9 and 4). Given that the *GATA2* CZNf is needed for interaction with PU.1, we predicted that, in contrast to WT *GATA2*, Δ *GATA2* would fail to effectively reduce *MCSFR* promoter-directed expression in the Shp2E76K-expressing cells. As anticipated, introduction of Δ *GATA2* only modestly inhibited PU.1-induced *MCSFR* promoter-directed expression (Fig. 7D, compare lanes 10 and 7), and expression remained significantly higher than that observed in Shp2E76K-expressing cells transfected with *GATA2* (compare lanes 10 and 9) or in WT Shp2-expressing cells transfected with Δ *GATA2* (compare lanes 10 and 5). These findings, using a well-characterized promoter previously defined to be activated by the c-Jun-PU.1 complex, support a molecular mechanism whereby gain-of-function Shp2 mutants lead to constitutive c-Jun expression and yield an augmented level of the c-Jun-PU.1 complexes, thus supporting enhanced monocytic differentiation.

Hematopoietic progenitors expressing activating Shp2 mutants demonstrate increased c-Jun-PU.1 protein complexes and increased association of the c-Jun-PU.1 complex at the *Mcsfr* promoter. We next examined the hypothesis that mutant Shp2-expressing hematopoietic progenitors would have an increased interaction between PU.1 and c-Jun. Nuclear protein extracts were prepared from transduced, sorted hematopoietic progenitors cultured in mast cell media for 1 week, and coimmunoprecipitation studies were performed. Upon immunoprecipitation with anti-c-Jun and immunoblotting with anti-PU.1, we observed a strong interaction between c-Jun and PU.1 in mutant Shp2-expressing hematopoietic progenitors but no detectable interaction in vector or WT Shp2-expressing cells (Fig. 8A). Consistent with the RT-PCR findings and immunoblot data (Fig. 3C), the c-Jun protein was expressed at significantly higher levels in the mutant Shp2-expressing cells, as determined by stripping the blot and reprobing with anti-c-Jun (Fig. 8A).

To evaluate the hypothesis that c-Jun induces increased transactivation of its own promoter in mutant Shp2-expressing cells, which would be predicted by the higher level of phosphorylated c-Jun (Fig. 3F), we conducted ChIP assays examining the murine *c-Jun* promoter immediately proximal to the transcription start site (30) and found increased amplification of this region

FIG. 6. (A) Schematic diagram of *GATA2* and *GATA2* Δ 330–407. (B) Representative FACS of cotransduced cells. (C) Cotransduced cells, as indicated, were plated into colony assays and scored for CFU-M. The experiment was conducted three times and plated in triplicate. *, P is <0.0001 for Shp2E76K plus pMIEG3-hCD4 versus WT Shp2 plus pMIEG3-hCD4; **, P is <0.0001 for Shp2E76K plus *GATA2* versus Shp2E76K plus pMIEG3-hCD4. (D) Progenitor colony-type distribution comparison between cells cotransduced with WT Shp2 or Shp2E76K with or without empty vector, *GATA2*, or Δ *GATA2*. (E) Morphology of cells cotransduced with WT Shp2 or Shp2E76K with or without empty vector, *GATA2*, or Δ *GATA2* and cultured in mast cell media. Black arrows indicate megakaryocytes. (F) Quantitative RT-PCR for *c-Jun* expression from cotransduced cells cultured in mast cell media for 1 week. The experiment was conducted on three independent occasions in triplicate. *, P is <0.0001 for Shp2E76K plus pMIEG3-hCD4 versus WT Shp2 plus pMIEG3-hCD4; **, P is <0.0001 for Shp2E76K plus *GATA2* versus Shp2E76K plus pMIEG3-hCD4. (G) Quantitative RT-PCR for WT *GATA2* (primer set 1) and for both WT *GATA2* and Δ *GATA2* (primer set 2) from cotransduced cells cultured in mast cell media for 1 week. Data were compiled from three independent experiments, with reactions performed in triplicate.

FIG. 7. (A) FACS of NIH 3T3 cells transduced with MIEG3-WT Shp2 or MIEG3-Shp2E76K. (B) Immunoblot analysis demonstrating Shp2 protein expression in parental NIH 3T3 cells and transduced cells. (C) Quantitative RT-PCR for c-Jun from NIH 3T3 cells transduced with WT Shp2 or with Shp2E76K. *, *n* is 3 and *P* is <0.05 for comparing WT Shp2 to Shp2E76K. (D) MCSFR promoter-directed expression of luciferase in the absence and presence of ectopic expression of PU.1 with or without GATA2 or ΔGATA2; data were compiled from three independent experiments, plated in triplicate. Δ, *P* is <0.0001 for NIH 3T3-WT Shp2 cells transfected with PU.1 versus vector (lane 2 versus 1); *, *P* is <0.0001 for NIH 3T3-Shp2E76K cells transfected with PU.1 versus vector (lane 7 versus 2); #, *P* is <0.0001 for NIH 3T3-Shp2E76K transfected with PU.1 versus NIH 3T3-WT Shp2 transfected with PU.1 (lane 7 versus 2); ##, *P* is <0.0001 for NIH 3T3-Shp2E76K transfected with PU.1 and GATA2 versus NIH 3T3-E76KShp2 transfected with PU.1 (lane 9 versus 7); **, *P* is 0.4 for NIH 3T3-Shp2E76K transfected with PU.1 and GATA2 versus NIH 3T3-WT Shp2 transfected with PU.1 and GATA2 (lane 9 versus 4); ###, *P* is 0.004 for NIH 3T3-Shp2E76K transfected with PU.1 and ΔGATA2 versus NIH 3T3-E76KShp2 transfected with PU.1 (lane 10 versus 7); ***, *P* is <0.0001 for NIH 3T3-Shp2E76K transfected with PU.1 and ΔGATA2 versus NIH 3T3-Shp2E76K transfected with PU.1 and GATA2 (lane 10 versus 9); ####, *P* is 0.002 for NIH 3T3-Shp2E76K transfected with PU.1 and ΔGATA2 versus NIH 3T3-WT Shp2 transfected with PU.1 and ΔGATA2 (lane 10 versus 5).

upon immunoprecipitation with anti-c-Jun (Fig. 8B). Furthermore, to test the hypothesis that the increased c-Jun-PU.1 interaction found in mutant Shp2-expressing cells functionally promotes monocyte-specific gene products, we conducted ChIP assays specifically examining the PU.1 binding site within the murine *Mcsfr* 5' untranslated region (within exon 2) homologous to the PU.1 binding site within the human *MCSFR* 5' untranslated region found to be coactivated by c-Jun (5, 74). Immunoprecipitation with PU.1 yielded similar levels of the amplified

Mcsfr promoter region in all cell types examined (Fig. 8B), consistent with similar levels of PU.1 expression among all cell types (Fig. 3B). However, immunoprecipitation with anti-c-Jun yielded increased levels of the amplified *Mcsfr* promoter region in cells expressing either Shp2D61Y or Shp2E76K (Fig. 8B). Together, findings from the coimmunoprecipitation and ChIP assays support the mechanism that increased c-Jun expression in mutant Shp2-expressing hematopoietic progenitors enhances its interaction with PU.1, promoting skewed monocytic differentiation.

FIG. 8. (A) Coimmunoprecipitation assays were performed by immunoprecipitation (IP) with anti-c-Jun and blotting with anti-PU.1 and anti-c-Jun. To control for nuclear protein extract concentrations, levels of the nuclear protein TATA binding protein (TBP) were examined. (B) For ChIP assays, isolated lysates were immunoprecipitated with either anti-PU.1 or anti-c-Jun, followed by amplification of purified DNA fragments using primers specific for the *c-Jun* promoter or *Mcsfr* promoter or by antiacetylated H4, followed by amplification of purified DNA fragments using primers specific for the *HPRT* promoter.

JMML patients express higher levels of *c-JUN* than healthy controls. We next examined if *c-JUN* expression, as well as other hematopoietic-specific transcription factors, is altered in hematopoietic progenitors obtained from JMML patients compared to healthy controls. CD34⁺ LDMNCs were isolated from splenic tissue of JMML patients following splenectomy, as indicated clinically, and *c-JUN*, *GATA2*, *PU.1*, and *C/EBPα* expression levels were compared to those in CD34⁺ cord blood cells from healthy infants. As seen in Fig. 9, the JMML samples expressed significantly higher levels of *c-JUN* than the healthy controls, consistent with findings in our murine in vitro model.

GATA2 expression was also significantly reduced, however, not to the extent observed in our in vitro system, further supporting our overall conclusion that dysregulated *c-JUN* expression is a primary aberrancy in JMML. *C/EBPα* expression was quite variable in the JMML samples examined, making it difficult to predict potential functional effects of *C/EBPα* in JMML. Surprisingly, in spite of the JMML phenotype of myelomonocytic cell overproduction, *PU.1* expression was reduced in JMML samples compared to that in healthy controls. To validate the upregulated *c-JUN* expression found in the microarray analysis, we used quantitative RT-PCR to examine *c-JUN* expression in peripheral blood mononuclear cells from four JMML patients and four healthy controls. Again, we observed significantly elevated *c-JUN* expression in the JMML samples. Collectively, these data support the notion that dysregulation of *c-JUN* expression is a primary molecular aberrancy that contributes to the etiology and pathogenesis of JMML.

DISCUSSION

JMML is a difficult-to-treat and too commonly lethal leukemia of young children. One notable and unique functional characteristic of JMML is the overproduction of monocytic cells along the full spectrum of differentiation, from immature progenitors to terminally differentiated tissue macrophages. At the molecular level, Ras hyperactivation is implicated in JMML based on patients bearing either loss-of-function *NFI* mutations or gain-of-function *RAS* or *PTPN11* mutations. Given these relevant cellular and molecular characteristics of JMML, we hypothesized that hyperactivation of the Ras-MAPK pathway within mutant Shp2-bearing hematopoietic progenitors leads to aberrant transcription factor expression and to skewed monocytic differentiation, thus contributing to the pathological overproduction of monocytic cells. As a corollary, we hypothesized that the reestablishment of normal transcription factor ratios would permit mutant Shp2-bearing cells to undergo nonbiased hematopoietic differentiation.

Consistent with the findings of other models of JMML (8, 12, 38, 45), we observed skewed monocytic differentiation of mutant Shp2-expressing hematopoietic progenitors in multiple assay systems. As some models of myeloproliferative disorder have demonstrated an autocrine component based on increased endogenous GM-CSF expression or increased expression of the GM-CSF receptor (21, 50), we investigated a potential autocrine mechanism in our in vitro system of mutant Shp2-expressing cells; however, GM-CSF and GM-CSFRα expression was not elevated, and was surprisingly reduced, in mutant Shp2-expressing cells. Furthermore, we observed no functional effect of blocking antibody to GM-CSF on mutant Shp2-skewed monocytic differentiation. These findings are consistent with previous studies demonstrating that while JMML samples appear to produce mediators promoting spontaneous progenitor growth that can be blocked with neutralizing antibody to GM-CSF, the absolute GM-CSF levels are not consistently elevated in JMML samples, implying the presence of JMML progenitor cell autonomous molecular aberrancies at the level of the GM-CSF receptor-stimulated signaling (25, 32). Additionally, our findings are consistent with the lack of an autocrine effect of a conditional D61Y knock-in allele (12).

Upon examining potential endogenous molecular aberran-

FIG. 9. *c-JUN*, *GATA2*, *PU.1*, and *C/EBPα* expression levels from CD34⁺ spleen cells of JMML patients were compared to those of CD34⁺ cells from the cord blood (CB) of healthy newborns, using three cord blood samples and seven JMML samples. *, $P = 0.0001$; **, $P = 0.014$; ***, $P = 0.01$. *c-JUN* expression levels from peripheral blood mononuclear cells (PB MNC) of JMML patients were compared to those from peripheral blood mononuclear cells of healthy controls, using four healthy controls assayed in triplicate and four JMML samples assayed in triplicate. #, $P = 0.03$.

cies leading to skewed myelomonocytic differentiation, we found no difference in PU.1 expression at the mRNA or protein levels; however, we did observe a significant reduction in GATA2 and a significant increase in c-Jun at both the mRNA and protein levels in murine hematopoietic progenitors. Examination at earlier culture time points indicated increased *c-Jun* expression as well as c-Jun phosphorylation, implying that dysregulated c-Jun function is a primary molecular anomaly in JMML. Consistent with these findings as well as our previous report of JNK hyperactivation in mutant Shp2-bearing macrophage progenitors (71), we found that treatment with the JNK inhibitor SP600125 significantly reduced the increased mutant Shp2-induced CFU-M.

The finding of reduced GATA2 expression in mutant Shp2-expressing progenitors led us to hypothesize that reintroduction of GATA2 would redirect mutant Shp2-skewed monocytic differentiation. Indeed, GATA2 expression promoted the development of CFU-Mix and CFU-Meg in WT Shp2-expressing progenitors and, importantly, worked equally well in Shp2D61Y- and Shp2E76K-expressing progenitors (Fig. 5B and 6C). These findings indicate that GATA2 expression is capable of guiding differentiation away from the monocytic lineage as well as reducing total progenitor colony formation (Fig. 5C), even in the presence of activating mutations Shp2D61Y and Shp2E76K. However, given the similar effect of GATA2 on WT or mutant Shp2-expressing progenitors, it is apparent that GATA2 does not redirect monocytic differentiation in a mutant Shp2-specific manner. In complementary assays, introduction of GATA2 reduced *MCSFR* promoter-directed expression of luciferase (Fig. 7D), shown previously to be transactivated by the c-Jun–PU.1 complex. These findings, taken together with increased c-Jun expression found in mutant Shp2-expressing progenitors, led us to examine the hypothesis that GATA2 and c-Jun compete for binding to PU.1. Consistent with this hypothesis, the introduction of ΔGATA2, lacking the CZnF

which is needed for physical interaction with PU.1, failed to reduce the number of CFU-M (Fig. 6C) and only modestly reduced *MCSFR* promoter-directed luciferase expression (Fig. 7D).

Although c-Jun is a ubiquitously expressed transcription factor, it has been found to promote monocytic differentiation (28, 39, 43, 46, 65). One likely mechanism by which c-Jun exerts its monocyte tissue-specific effect is by functioning as a coactivator of PU.1 (5). Using coimmunoprecipitation assays, we determined that the interaction between PU.1 and c-Jun is significantly increased in the mutant Shp2-expressing cells. Furthermore, we demonstrated using ChIP assays that immunoprecipitation with anti-c-Jun yields increased amplification of the region of the murine *Mcsfr* promoter where the c-Jun–PU.1 complex has been defined to bind (5, 74). Importantly, gel shift assays demonstrated that c-Jun does not bind directly to this region of the *MCSFR* promoter (5), strongly suggesting that an increased c-Jun–PU.1 complex is functional at the *Mcsfr* promoter in the mutant Shp2-expressing hematopoietic progenitors.

As a pilot evaluation of key hematopoietic-specific transcription factor expression in primary JMML samples, we examined expression data comparing JMML samples to healthy controls obtained from U133A expression microarrays for *c-JUN*, *GATA2*, *PU.1*, and *C/EBPα*. Notably, CD34⁺ cells from JMML patients expressed significantly higher levels of *c-JUN* than CD34⁺ cord blood cells from healthy newborns, suggesting that our model of mutant Shp2-expressing murine progenitors correctly recapitulates the molecular aberrancies found in malignant JMML progenitors. The *c-JUN* expression findings were validated by quantitative RT-PCR, using total cellular RNA isolated from peripheral blood mononuclear cells from JMML patients and healthy controls. While the *c-JUN* expression was significantly elevated in the JMML peripheral blood mononuclear cells, the difference in those compared to healthy

FIG. 10. Proposed molecular mechanism accounting for mutant Shp2-induced aberrant monocytic differentiation as well as potential points of therapeutic targeting with JNK inhibitor SP600125 (inhibiting c-Jun phosphorylation) or with the epigenetic modifying agent trichostatin A (TSA; inhibiting c-Jun expression).

controls was not as great as that observed using the CD34⁺ cells. This smaller difference is likely accounted for by the heterogeneous cellular nature of peripheral blood mononuclear cells, in contrast to the relatively homogeneous CD34⁺ progenitor population. *GATA2* expression was also reduced in primary JMML samples; however, the reduction was modest compared to that of our findings following in vitro culture in mast cell media for 1 to 2 weeks (Fig. 3A). These data suggest that reduced *GATA2* expression may contribute to the pathogenesis of JMML, but at least from this initial evaluation, it appears unlikely that reduced *GATA2* expression is a central abnormality in JMML pathogenesis. The reduced *PU.1* expression was surprising as well as interesting. Several studies have indicated reduced *PU.1* expression and function in acute myeloid leukemia (48, 54, 64) as well as in chronic myelogenous leukemia (1). In the model of knockdown *PU.1*-induced acute myeloid leukemia, the introduction of c-Jun successfully normalized the myelomonocytic differentiation block (63). One possibility is that the elevated c-JUN expression in combination with reduced *PU.1* expression in the disease state of JMML yields the more chronic and indolent myeloproliferative disorder rather than full-blown acute leukemia. These findings clearly indicate that further examination of hematopoietic-specific transcription factor expression and function in primary JMML samples is in order.

Based on the findings presented herein, we propose a molecular model underlying mutant Shp2-enhanced monocytic differentiation and indicate potential pharmacologic interventions that may reduce c-Jun function or expression, thus providing a novel therapeutic approach for JMML (Fig. 10). We propose that Ras hyperactivation leads to increased JNK activation, increased c-Jun phosphorylation, and a positive feedback loop, leading to constitutive c-Jun expression. Due to constitutive c-Jun expression, we propose that the c-Jun-PU.1 interaction is favored over the interaction of PU.1 with potential alternative transcription factors such as *GATA2*, thus promoting excessive monocytic differentiation. As JNK inhibition as well as ectopic expression of *GATA2* normalizes mutant Shp2-induced aberrant monocytic differentiation, we propose that a pharmacologic agent with the capacity to inhibit JNK activity, reduce c-Jun expression, and maintain *GATA2* expression would be an ideal candidate. Thus, a JNK inhibitor may be of clinical utility in JMML patients. Notably, CC-401, an anthrapyrazolone JNK inhibitor based on the chemistry of SP600125, is currently in clinical development and has been administered to healthy volunteers and patients with acute myeloid leukemia in phase I clinical trials. Additionally, studies demonstrate that trichostatin A, a histone deacetylase (HDAC) inhibitor, is able to induce *GATA2* expression (18, 23), reduce c-Jun expression (70), and inhibit GM-CSF-stimulated macrophage proliferation (56), leading us to propose that an HDAC inhibitor may normalize hematopoietic lineage-specific transcription factor expression in activating Shp2 mutant-bearing cells. Importantly, trichostatin A is within the hydroxamate class of HDAC inhibitors, similar to suberoyl anilide hydroxamic acid (Vorinostat), which is currently approved by the U.S. Food and Drug Administration for treatment of cutaneous T-cell lymphoma.

Using functional, pharmacologic, genetic, and biochemical analyses, we have identified a novel molecular mechanism in gain-of-function *PTPN11* mutant-bearing progenitors, leading to enhanced monocytic differentiation and thus contributing to JMML disease progression. We believe these findings provide innovative insights into the pathogenesis of JMML and support an objective of targeting aberrant transcription factor expression and skewed differentiation in addition to targeting hyperactivated Ras-induced hyperproliferation.

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