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Activating *PTPN11* Mutants Promote Hematopoietic Progenitor Cell Cycle Progression and Survival

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Abstract

Objective—Mutations in *PTPN11*, which encodes the protein tyrosine phosphatase Shp2, are commonly found in juvenile myelomonocytic leukemia (JMML). We hypothesized that *PTPN11* mutations promote cell cycle progression and confer enhanced survival to hematopoietic progenitors.

Methods—Murine bone marrow low density mononuclear cells were transduced with pMIEG3, pMIEG3-WT Shp2, pMIEG3-Shp2D61Y, or pMIEG3-Shp2E76K followed by cell cycle and survival functional analysis as well as biochemical analysis for key cell cycle and programmed cell death regulatory proteins.

Results—A higher proportion of hematopoietic progenitors bearing the gain-of-function Shp2 mutants were residing in the S or G2 phase of the cell cycle in response to low doses of GM-CSF compared to cells transduced with empty vector (MIEG3) or with WT Shp2. Likewise, Shp2D61Y- or Shp2E76K-expressing hematopoietic cells demonstrated reduced apoptosis based on annexin V staining and produced increased progenitor colonies after 48 hours in minimal media compared to cells transduced with empty vector or WT Shp2. To differentiate enhanced survival v. hyperproliferation, cells were stained with PKH26 to distinguish undivided cells from divided progeny. Shp2D61Y- or Shp2E76K-expressing PKH26+ cells similarly demonstrated reduced apoptosis. Upon biochemical analysis, expression of Akt- and Erk-responsive cell cycle and programmed cell death regulatory proteins were altered, including increased levels of cyclin D1, Bcl2, and BclXL and reduced levels of p27, p21, and Bim.

Conclusion—Collectively, these data demonstrate that gain-of-function Shp2 mutants promote hematopoietic progenitor cell cycle progression and survival and imply that agents targeting the cell cycle or promoting apoptosis may have therapeutic potential in JMML.

Juvenile myelomonocytic leukemia (JMML) is a lethal childhood disease characterized by spontaneous growth of peripheral blood hematopoietic progenitors consisting predominantly of macrophages and monocytes[1,2] and is particularly insensitive to chemotherapy[1]. Approximately 35% of children with JMML bear mutations in *PTPN11*, which encodes the

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protein tyrosine phosphatase, Shp2[3–5]. The observed mutations disrupt non-covalent interactions between the N-SH2 and phosphatase domains maintaining Shp2 in an inactive conformation and, thus, signify gain-of-function mutations[6,7]. We and others demonstrated that Shp2 mutations E76K, D61 Y, and D61 V induce hematopoietic progenitor hypersensitivity to GM-CSF in terms of hematopoietic progenitor colony formation[8–10]. Unfortunately, however, the pathophysiology underlying the development, progression, and chemoresistance of JMML remains elusive.

Previous studies from our lab revealed that mutant Shp2-bearing hematopoietic cells demonstrate constitutively elevated and sustained activation of the mitogen protein activation kinase (MAPK), Erk[8] and that these cells hyperproliferate in response to GM-CSF, implicating hyperactivation of the Ras-MAPK cascade in driving the hyperproliferative phenotype. Multiple previous studies have demonstrated the central role of Ras activation in cell cycle progression[11–14], suggesting that the gain-of-function Shp2 mutations likely promote the hyperproliferative phenotype via aberrant regulation of cell cycle checkpoints. Furthermore, multiple lines of evidence implicate Shp2 in promoting Ras-mediated activation of phospho-inositol-3-kinase (PI3K), including that Shp2 co-immunoprecipitates with p85 α , the regulatory subunit of PI3K, in response to GM-CSF stimulation[15] and that Ras activation leads to PI3K activation via binding to p110, the catalytic subunit of PI3K[16,17]. Thus, these findings suggest that GM-CSF-stimulated MAPK and PI3K pathways in mutant Shp2-bearing cells may work in concert to mediate dysregulated expression of cell cycle and apoptogenic regulatory proteins, leading to subversion of cell cycle regulation and programmed cell death, respectively, and contributing to the observed chemoresistance of JMML.

Hyperactivation of the MAPK, Erk, in response to multiple cytokines in cells bearing gain-of-function Shp2 mutants is well established[3,8,9,18]. However, the potential role of the other MAPKs, c-Jun NH2-terminal kinase (JNK) and p38, has not been examined. While hyperactivation of the Erk pathway is firmly established in both acute and chronic leukemias [19], the roles of JNK and p38 activation in leukemias, in general, are less clear and apparently depend on cellular context and circumstances[20]. However, in chronic myelogenous leukemia, a disease with relevant pathological similarities to JMML, JNK hyperactivation appears to promote BCR-ABL-induced cellular transformation[21,22] while p38 activation is required for the inhibitory effects of interferon- α and for differentiation of BCR-ABL-expressing cell lines[23,24]. Furthermore, the role of GM-CSF-stimulated Akt activation in the pathophysiology of JMML is not well documented. While IL-3-stimulated hyperactivation of Akt in primary mast cells or in Baf3 cells expressing gain-of-function Shp2 mutants is well established[9,18], recent studies utilizing the myeloid cell line, TF-1, expressing mutant Shp2E76K, failed to demonstrate GM-CSF-stimulated hyperactivation of Akt[25].

We hypothesized that Shp2 gain-of-function mutations permit evasion of cell cycle control checkpoints and of programmed cell death, similar to that observed in other myeloid leukemias [26–32]. Furthermore, we reasoned that mutant Shp2-induced dysregulation of the Ras-MAPK and Ras-PI3K signaling cascades cause inappropriate expression of cell cycle and programmed cell death regulatory proteins, leading to aberrant cell cycle progression and enhanced survival, respectively. To address these hypotheses, we have utilized pathophysiologically relevant primary progenitor cultures to examine cell cycle status and survival capacity of mutant Shp2-bearing cells in response to GM-CSF and to examine expression of key cell cycle and programmed cell death regulatory proteins. Clarification of aberrantly activated or expressed key molecules in these pathways is crucial for the definition of novel therapeutic targets for improved treatment of JMML.

MATERIALS and METHODS

Retroviral Transduction

Ecotropic retroviral supernatants (pMIEG3, pMIEG3-WT, pMIEG3-E76K, pMIEG3-D61V, and pMIEG3-D61Y) were prepared using Eco-Phoenix packaging cells. Bone marrow low density mononuclear cells from C57/Bl6 mice (Jackson Laboratories, Bar Harbor, ME) were purified using a Ficoll gradient. Cells were pre-stimulated at a concentration of 2×10^6 cells/mL in IMDM, 20% fetal bovine serum (FBS, Hyclone, Logan, UT), 2 mM glutamine, 1% penicillin/streptomycin, stem cell factor (SCF), granulocyte-colony stimulating factor (G-CSF), and thrombopoietin (Tpo) all at 100 ng/mL (growth factors from Peprotech, Rocky Hill, NJ) for 24 hours. Cells were then transferred to retroviral supernatant on plates coated with fibronectin fragments (Retronectin, Takara, Madison, WI) with SCF, G-CSF, and Tpo 100 ng/mL for an additional 48 hours. Cells were placed back in the pre-stimulation media for 24 hours prior to sorting for EGFP positive cells using fluorescence activated cell sorting (FACS). For biochemical assays, cells were cultured in IMDM, 10% FBS, and M-CSF 50 ng/mL for approximately one week, as described[8,33].

Cell Cycle Analysis

Transduced cells were subjected to growth factor- and serum-deprivation for 6 hours followed by culture in α -MEM, 10% serum, and GM-CSF 0.1, 1, or 10 ng/mL for 16 hours. Cells were collected and stained in 0.05 mg/mL propidium iodide (PI), 0.3% NP-40, and 1 mg/mL RNase A in phosphate buffered saline (PBS) at 4° C for 30 minutes followed by flow cytometry and analysis using ModFit LT (Verity Software House).

Survival Analysis

Transduced cells stained were with annexin V-allophycocyanin (APC, BD Pharmingen, San Diego, CA) followed by flow cytometry, as described[34]. To distinguish proliferation from survival, EGFP+ cells were stained with PKH26 (Sigma, St. Louis, MO) according to manufacturer's protocol, followed by culture in GM-CSF and evaluation by annexin V-APC staining.

Hematopoietic Progenitor Assays

Transduced cells were plated into IMDM with 2% fetal bovine serum (FBS, Hyclone, Logan, UT) in the absence or presence of GM-CSF (0.1 ng/mL or 1 ng/mL) for 48 hours. Cells were then plated into progenitor assays containing 1% methylcellulose, 30% fetal bovine serum, 2% penicillin/streptomycin, 1% glutamine, 80 μ M β -mercaptoethanol, IL-3 200 U/mL, erythropoietin 4 U/mL, and SCF 100 ng/mL. Progenitors were scored after culture in a humidified incubator for 7 days at 37° C, 5% CO₂.

Immunoblot Analysis

Sorted cells were differentiated into macrophage progenitors and protein extracts were prepared, as described[8,35]. Blots were probed with anti-phospho-Erk, anti-phospho-Akt, anti-phospho-JNK, anti-phospho-p38 (Cell Signaling Technology, Beverly, MA), anti-Bim (H-191), anti-Bcl2 (C-2), anti-cyclin D1(A-12) (Santa Cruz Biotechnology), anti-BclX_L, anti-p21, anti-p27 (BD Transduction Laboratories), and anti-GAPDH (Biodesign International, Saco, ME). Signals were detected by enhanced chemiluminescence.

RESULTS

Gain-of-Function Shp2 Mutants Induce Increased Activation of JNK and Akt and Reduced Activation of p38

Commonly, aberrant regulation of cell cycle control checkpoints or subversion of stress-induced apoptosis render leukemias and cancers non-responsive to chemotherapeutic agents. JMML is a particularly chemoresistant disease with few patients achieving a meaningful clinical remission. We have conducted experiments to define the aberrant cellular and molecular mechanisms that potentially induce the observed chemoresistance in JMML in an effort to define novel rational therapeutic targets in this disease. Although hyperactivation of the Ras effector, Erk, in mutant Shp2-bearing cells at baseline or in response to multiple cytokines is well-established[3,8,9,18], the activation state of the other MAPKs, JNK and p38, has not previously been examined. Furthermore, examination of GM-CSF-stimulated Akt has not been examined in physiologically relevant cells. To examine the effect of Shp2 gain-of-function mutations on activation of JNK, p38, and Akt, bone marrow LDMNCs were subjected to retronectin-assisted retroviral transduction with pMIEG3, pMIEG3-WT Shp2, pMIEG3-Shp2D61Y, or pMIEG3-Shp2E76K. Following transduction, cells were cultured for differentiation to macrophage progenitors, as previously described. Previous studies from our lab demonstrated that each of these cultures express similar levels of the GM-CSF receptor [8]. Cells were subjected to serum- and growth factor-deprivation followed by stimulation with GM-CSF for various times. At baseline, mutant Shp2-expressing macrophage progenitors demonstrated constitutively active Erk, as previously described[8](Figure 1A). Notably, we similarly observed hyperactivation of JNK at baseline, but reduced activation of p38 in the mutant-bearing cells (Figure 1A). Upon stimulation with GM-CSF, we observed minimal additional activation of either JNK or p38 above that observed following serum deprivation (baseline), consistent with the activation of these MAPKs being more commonly stress- or inflammatory cytokine-induced rather than growth factor-induced[36] (data not shown).

To examine the potential contribution of GM-CSF-stimulated phospho-Akt to aberrant cellular function of mutant Shp2-bearing cells, macrophage progenitors were serum-deprived for 24 hours followed by stimulation with GM-CSF (10 ng/mL). Modestly elevated levels of phospho-Akt at baseline in the mutant Shp2-bearing cells were observed compared to cells transduced with MIEG3 or WT Shp2) and approximately a 2-fold higher level of phospho-Akt in the mutant Shp2-expressing cells was observed following GM-CSF stimulation for 60 minutes (Figure 1B). These findings define GM-CSF-stimulated hyperactivation of Akt, in addition to hyperactivation of Erk[8], as relevant in mediating GM-CSF hypersensitivity observed in JMML progenitors.

Gain-of-Function Shp2 Mutants Promote Hematopoietic Progenitor Cell Cycle Progression

Previous studies have clearly demonstrated increased myeloid colony development from mutant Shp2-bearing hematopoietic progenitors as well as increased proliferation in response to GM-CSF[8–10]; however, the contribution of cell cycle dysregulation to the hyperproliferative phenotype and of hematopoietic progenitor survival to the increased myeloid colony number is unknown. To examine the effect of Shp2 gain-of-function mutations on hematopoietic progenitor cell cycle, bone marrow LDMNCs were subjected to retronectin-assisted retroviral transduction with pMIEG3, pMIEG3-WT Shp2, pMIEG3-Shp2D61Y, or pMIEG3-Shp2E76K and sorted for enrichment of transduced cells. Following transduction and sorting, cells were serum- and growth factor-deprived for 6 hours followed by stimulation in α -MEM with 10% FBS and GM-CSF 0.1, 1, or 10 ng/mL for 16 hours. Cells were collected and stained with PI followed by flow cytometry for cell cycle analysis. Consistent with the observed hyperproliferative phenotype, a higher proportion of cells bearing the gain-of-function Shp2 mutants were consistently residing in the S or G2 phase of the cell cycle

compared to cells transduced with empty vector or with WT Shp2 (Figure 2A and B). The difference progressively diminished with increasing doses of GM-CSF, consistent with the characteristic JMML progenitor phenotype of hypersensitivity to low concentrations of GM-CSF (Figure 2A and B).

Gain-of-Function Shp2 Mutants Induce Aberrant Expression of Cell Cycle Regulatory Proteins

Given the hyperproliferative phenotype of mutant Shp2-bearing hematopoietic progenitors and the observed increased proportion of mutant Shp2-bearing hematopoietic progenitors in the mitotically active S and G2 phases of the cell cycle, we next examined the molecular mechanism(s) underlying this aberrant cell cycle phenotype. Progression from G1 to the S phase of the cell cycle is regulated in a collaborative fashion by cyclin dependent kinases, regulatory cyclin subunits, and cyclin dependent kinase inhibitors[37]. Multiple studies have demonstrated that Erk activation induces increased expression of the positive cell cycle regulator, cyclin D1[38,39], while activation of p38 has been shown to reduce cyclin D1 expression[39]. Consistent with constitutive increased phospho-Erk and reduced phospho-p38 levels, cyclin D1 is elevated at baseline in mutant Shp2-bearing cells compared to cells transduced with MIEG3 or WT Shp2 (Figure 3). Expression of cyclin D1 was modestly increased in the MIEG3- and WT Shp2-bearing cells following treatment with GM-CSF for 5 hours, but the level did not increase further in the mutant-bearing cells.

In addition to being controlled by positive regulators, cell cycle progression is also controlled by the negative regulatory cyclin dependent kinase inhibitors, p27 and p21. Multiple studies have demonstrated that Erk activation leads to reduced expression of p27, thereby promoting cell cycle progression[40–43]. Consistent with the observed increased portion of Shp2 mutant-bearing cells in the S + G2 phase of the cell cycle, expression of p27 was significantly reduced in the mutant Shp2-bearing cells both at baseline and following treatment with GM-CSF (Figure 3). Although high expression of p21 was originally described to inhibit cyclin dependent kinase activities and induce cell cycle arrest[44–48], further studies demonstrated that at appropriate concentrations, p21 promotes assembly of cyclin dependent kinases and regulatory cyclins and activation of cyclin dependent kinases, thus promoting cell cycle progression rather than arrest[49–51]. However, most consistently, we observed reduced levels of p21 in mutant Shp2-expressing cells, both at baseline and in response to GM-CSF stimulation (Figure 3). Collectively, these studies demonstrate that the gain-of-function Shp2 mutants promote cell cycle progression in part by upregulating cyclin D1 and by down-regulating p27 and p21.

Gain-of-Function Shp2 Mutants Promote Hematopoietic Progenitor Survival

The previous data demonstrate that dysregulation of cell cycle progression likely contributes to the increased number and larger size of myeloid colonies derived from hematopoietic progenitors expressing gain-of-function Shp2 mutants[8–10]; however, the contribution of hematopoietic progenitor survival has not been examined. To address this question, transduced cells were cultured in minimal media (IMDM with 2% FBS in the absence or presence of GM-CSF, 0.1 ng/mL or 1 ng/mL) for 48 hours followed by analysis with annexin V staining. Following culture for 48 hours, microscopic morphological analysis revealed increased numbers of viable cells expressing the Shp2 mutants D61Y or E76K compared to cells transduced with MIEG3 or WT Shp2 at various doses of GM-CSF (Figure 4A). Consistently, flow cytometry analysis demonstrated increased viable cells expressing the Shp2 mutants D61Y or E76K (Figure 4B, gated by R1 on the light scatter plots) and fewer non-viable cells (Figure 4B, gated by R2, likely a combination of late-apoptotic and necrotic cells), compared to cells transduced with MIEG3 or WT Shp2. The ratio of viable to apoptotic/necrotic cells is shown quantitatively in Figure 4C and demonstrates that Shp2D61Y- and Shp2E76K-

expressing cells have a significantly increased percentage of viable cells at 0.1 and 1 ng/mL GM-CSF. To examine apoptosis levels, cells were stained with annexin V-APC and, consistent with increased viability, cells expressing Shp2 mutant D61Y or E76K demonstrated significantly reduced apoptosis at 0.1 and 1 ng/mL GM-CSF (Figure 4D). These data support the hypothesis that the Shp2 gain-of-function mutants promote enhanced GM-CSF-stimulated hematopoietic progenitor survival.

To examine hematopoietic progenitor survival functionally, as well as to characterize the nature of the surviving progenitors, after 48 hours of culture, cells were plated in methylcellulose-based colony assays. Colonies were scored for colony forming unit (CFU)-granulocyte/macrophage (GM), -macrophage (M), or -granulocyte (G) based on morphologic appearance. Progenitors expressing Shp2 mutants D61Y or E76K demonstrated larger colonies as well as an increased total number of colonies compared to cells transduced with MIEG3 or WT Shp2 at all conditions tested, suggesting that Shp2 mutants do indeed confer a survival advantage to hematopoietic progenitors (Figure 5A). Based on progenitor morphology, CFU-M made up the majority of the total colonies from the D61Y and E76K plates (Figure 5A), consistent with the disease phenotype of JMML patients. Figures 5B and 5C demonstrate the standard colony morphology and cytospin analysis of CFU-GM and CFU-M, respectively.

Given the observation of mutant Shp2-induced hyperproliferation, even in the absence of or in very low concentrations of GM-CSF (above data and [8,10]), an additional conclusion potentially derived from the above data, however, is that enhanced GM-CSF-stimulated proliferation results in a lower portion of apoptotic cells at the time of annexin V staining, rather than actual enhanced GM-CSF-stimulated survival. This distinction is particularly important to clarify as some pharmacological agents specifically directed at the intrinsic apoptotic pathway are currently in clinical development. To address this question, we evaluated the level of apoptosis in an undivided or quiescent cell population to differentiate the contribution of increased proliferation v. survival to the increased number of viable cells observed by microscopy and by flow cytometry (Figure 4) and to the increased number of hematopoietic progenitor colonies (Figure 5). To perform this analysis, cells were stained with the fluorescent cell membrane dye PKH26 in order to distinguish divided from undivided cells. Immediately following PKH26 staining, an aliquot of cells was fixed with 1% formaldehyde and was utilized to set the gate defining undivided cells (Figure 6, top panel). The remainder of stained cells was cultured in various doses of GM-CSF for 16 hours, stained by annexin V-APC, and analyzed using flow cytometry. When gating EGFP positive, PKH26 positive cells (transduced, undivided cells), gain-of-function Shp2-expressing progenitors demonstrated less annexin V-APC staining when cultured in GM-CSF 0.1 and 1 ng/mL compared to cells transduced with pMIEG3 or with WT Shp2, with no difference observed when cultured in the absence of GM-CSF (Figure 6). These results are similar to the findings observed when examining the entire cell population (Figure 4) and support the hypothesis that mutant Shp2-bearing hematopoietic progenitors indeed possess enhanced GM-CSF-stimulated survival capacity.

Gain-of-Function Shp2 Mutants Induce Aberrant Expression of Pro-Survival and Pro-Apoptotic Proteins

Based on the increased hematopoietic progenitor survival induced by gain-of-function Shp2 mutations, we next examined expression levels of proteins within the intrinsic apoptosis pathway to define potential molecular mechanisms leading to this phenotype. Hyperactivation of phospho-Akt and phospho-Erk can both contribute to enhanced cellular survival by inducing expression of the pro-survival molecules, Bcl-2 and Bcl-X_L, and via suppression of the cell death activator, Bim[52]. Western blot analysis demonstrated that the mutant Shp2-bearing cells expressed higher Bcl2 and BclX_L levels and substantially reduced Bim levels compared

to cells transduced with empty vector or WT Shp2 (Figure 7). These data demonstrate that under minimal culture conditions, cells expressing the Shp2 gain-of-function mutants express persistently elevated levels of pro-survival molecules Bcl2 and BclX_L and reduced levels of the anti-apoptotic molecule, Bim. Collectively, these data demonstrate that gain-of-function Shp2 mutants induce dysregulated expression of proteins within the intrinsic apoptotic pathway and permit evasion of programmed cell death by alteration of the intrinsic cell death machinery.

DISCUSSION

Juvenile myelomonocytic leukemia is a lethal disease of young childhood. Multiple chemotherapeutic agents have been employed in this disease including intensive therapy utilized in acute myeloid leukemia, combinations of interferon- α , etoposide, cytarabine, and 6-mercaptopurine, and single agent use of interferon- α , etoposide, cytarabine, 6-mercaptopurine, and retinoids (see Bergstraesser *et al.* for review[53]). However, despite the utilization of multiple chemotherapeutic agents in various combinations and doses, few patients achieve a complete hematological remission. Currently, the only curative therapy is allogeneic hematopoietic stem cell transplantation; however, even following this rigorous intervention only approximately 50% of patients remain disease-free at 5 years following transplantation [54]. Second allogeneic hematopoietic stem cell transplantation in patients with relapsed JMML provides no improved therapeutic outcome[55]. In short, this disease has proven to be quite resistant to standard chemotherapeutic modalities.

A current tenet suggests that dysregulated cell cycle progression and/or abrogation of inherent apoptotic pathways in combination with enhanced oncogenic signaling pathways permits tumor chemoresistance[56,57]. Thus, exploitation of aberrancies in cell cycle control and survival capacity in transformed progenitors found in JMML patients may impart a novel therapeutic approach in this lethal disease. In the studies presented herein, we demonstrate that a significantly higher proportion of primary hematopoietic progenitors bearing gain-of-function Shp2 mutations reside in the mitotically active S and G2 phases of the cell cycle compared to cells transduced with empty vector or WT Shp2. This finding is expected based on the previously defined hyperproliferative phenotype of mutant Shp2-expressing cells[8, 10,18]; however, these findings for the first time clarify that activating Shp2 mutants promote dysregulated cell cycle progression utilizing primary cells and in response to low doses of GM-CSF. Importantly, we demonstrate that the MAPK, p38, is significantly less active in mutant Shp2-bearing progenitors. Based on the cell cycle phenotype, this biochemical finding is significant as p38 activation has been shown to inhibit cell cycle progression by reducing expression of cyclin D1[39]. Furthermore, some studies suggest that the cell cycle inhibitor, p27, may also be a target of activated p38 and, consistently, we observe reduced levels of p27 in mutant Shp2-bearing cells[58]. An additional role of activated p38 is to mediate oncogene-induced cellular senescence as well as in replicative senescence[59,60]; however, we do not observe cell cycle arrest or senescence in the mutant Shp2-expressing cells, consistent with low levels of activated p38. One explanation potentially accounting for the lack of oncogene-induced cell cycle arrest in the mutant Shp2-bearing cells is the concomitant hyperactivation of Akt, which has previously been demonstrated to effectively overcome hyperactivated Ras-induced cell cycle arrest[61]. Taken together, our biochemical and functional findings suggest that oncogenic Shp2 permits cellular avoidance of normal cell cycle control checkpoints and imply that pharmacologic modulators of cell cycle checkpoint machinery may provide a novel approach to improved treatment for JMML[62,63].

Additionally, we performed studies demonstrating that primary hematopoietic progenitors bearing activating Shp2 mutants display a GM-CSF-supported survival advantage. Colony assays demonstrate that monocyte/macrophage progenitors are the most significantly affected, consistent with the JMML phenotype. To rule out potentially confounding contribution of

hyperproliferation to the observed survival phenotype, we also evaluated the survival capacity of undivided hematopoietic progenitors using the cell membrane dye PKH26. Upon cellular division, this cell membrane dye is distributed between progeny, resulting in reduced PKH26 intensity with progressive cellular division. Although our experimental conditions were established to examine cellular survival (low serum and growth factor concentrations which do not promote cellular proliferation), to unambiguously evaluate the effect of Shp2 gain-of-function mutations on hematopoietic progenitor survival independent of proliferation, we evaluated the annexin V staining levels of PKH26 positive cells. Our experiments demonstrate that mutant Shp2-bearing cells bear increased survival capacity in response to low doses of GM-CSF and suggest that utilization of therapeutic agents targeting apoptotic signaling pathways may be a novel rational approach to improved therapy in JMML. These findings are relevant, as novel therapeutic agents are being developed which specifically target apoptotic signaling pathways in transformed cells.

The intrinsic apoptotic pathway is mediated by the regulatory interactions between three classes of Bcl2 family members including multi-domain anti-apoptotic proteins (Bcl2, BclXL, Mcl1), multi-domain pro-apoptotic proteins (Bax and Bak), and BH3-only pro-apoptotic proteins (Bim, Bid, Bad, Bik, Noxa, Puma, reviewed in Danial *et al.*[64]). Compounds currently in clinical development are designed to target the pro-survival or mimic the pro-apoptotic proteins in each of these classes[57,65]. To examine a potential molecular mechanism leading to the increased survival capacity of mutant Shp2-bearing cells, we examined expression of key Akt- and Erk-responsive molecules within various classes of the intrinsic programmed cell death pathway, including Bcl2, BclXL, and Bim. We observed significantly higher levels of the pro-survival molecule BclXL in the mutant Shp2-expressing cells. These results are consistent with the recent findings of Ren *et al.* who also observed increased BclXL, as well as increased Mcl1 levels, in Shp2E76K-expressing TF-1 cells[25]. In contrast to the findings of Ren *et al.*, however, we also observed significant differences in Bcl2 and Bim in mutant Shp2-expressing cells, suggesting that these Bcl2 family proteins may also contribute to the pro-survival phenotype of the mutant Shp2-expressing hematopoietic progenitors. In our experimental system, cells expressing WT Shp2 also demonstrated increased Bcl2 and BclXL, intermediate between that of MIEG3- and Shp2D61Y- or Shp2E76K-transduced cells; however, their survival was not significantly altered. Notably, expression of Bim was not reduced in the WT Shp2-expressing cells, suggesting that Bim is a crucial apoptogenic mediator needed for transformed hematopoietic progenitor chemosensitivity, consistent with that observed in chronic myeloid leukemia[27,29].

These biochemical findings provide the rationale to examine novel compounds currently in clinical development on the survival of mutant Shp2-expressing hematopoietic progenitors *in vitro* and *in vivo*. One promising compound currently in clinical development is ABT-737, which mimics the BH3-only containing pro-apoptotic molecules and specifically inhibits Bcl2, BclXL, and Bclw, but not Mcl1[66]. Normally, the BH3-only containing molecules interact with the pro-survival Bcl2 family of proteins, thus permitting interaction of the pro-apoptotic proteins Bax and Bak and promoting programmed cell death[67]. Therefore, in the absence of adequate amounts of the BH3-only pro-apoptotic proteins, cancerous and transformed cells remain potentially insensitive to apoptosis-promoting signals. Several studies have shown efficacy of ABT-737 against myeloid leukemia cell lines or primary myeloid leukemia samples *in vitro* and *in vivo*[68–70]. Notably, ABT-737 appears to be more effective in tumors in which Mcl1 is not overexpressed or is in some fashion down-regulated or neutralized[68,70,71]. Based on these considerations and the findings of increased Mcl1 expression in Shp2E76K-expressing TF-1 cells[25], it is possible that ABT-737 will need to be used in combination with agents that reduce Mcl1 expression, such as roscovitine[25,70], for efficient cytotoxicity to JMML cells. However, it is also noteworthy that although Ren *et al.* observed increased Mcl1 expression, they did not observe increased apoptosis of Shp2E76K-expressing TF-1 cells by

downregulating Mcl1 expression using short haripin RNA or by treatment with roscovitine [25]. These findings suggest that survival of JMML cells may be more dependent on increased BclX_L and Bcl2 or on reduced expression of Bim, rather than on increased expression of Mcl1. Future studies examining the treatment of primary JMML samples with the novel agents and/or combinations of agents that target the intrinsic apoptotic pathway are in order to address this question.

Although not addressed by the experiments presented in this manuscript, the findings provided by these experiments may yield clues to the observed dysplastic nature and monocytic predominance found in JMML. For example, dysregulated cell cycle checkpoints may result in aberrant differentiation programs, producing dysplastic hematopoietic progeny. Additionally, JNK hyperactivation observed in the mutant Shp2-expressing cells may lead to increased levels of the AP-1 transcription factor, c-jun, which has been reported to collaborate with PU.1 to enhance monocytic differentiation[72,73]. However, collectively, the data presented in this manuscript imply that targeting cell cycle modulatory proteins or the intrinsic apoptotic pathway potentially may offer an innovative strategy for improved chemotherapeutic management of JMML.

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Figure 1.

A) Representative Western blot analysis demonstrating phospho-Erk, phospho-JNK, and phospho-p38 levels following serum and cytokine withdrawal for 24 hours; **B)** Representative Western blot showing 0, 60, and 120 minute time points of GM-CSF-stimulated phospho-Akt.

Figure 2.

A) Representative cell cycle flow cytometry analysis demonstrating cell cycle distribution of hematopoietic progenitors transduced with pMIEG3, WT Shp2, Shp2D61Y, or Shp2E76K in response to various doses of GM-CSF; **B)** Quantitation of cells within the S + G2 phases of the cell cycle. Representative of 3 independent experiments shown, * $p < 0.05$ for Shp2D61Y or Shp2E76K v. MIEG3 at 0.1, 1, or 10 ng/mL GM-CSF. Statistics performed using unpaired, two-tailed, Student's *t* test.

Figure 3. Representative Western blot analysis showing cyclin D1, p27, and p21 expression levels following serum and cytokine withdrawal for 24 hours and following stimulation with GM-CSF 50 ng/mL for 5 hours.

Figure 4.

A) Representative photomicrographs following culture of sorted and transduced bone marrow LDMNCs in minimal media for 48 hours; **B)** Representative forward/side scatter flow cytometry analysis following culture of sorted and transduced bone marrow LDMNCs in minimal media for 48 hours demonstrating increased Shp2D61Y or Shp2E76K-expressing viable cells (R1). **C)** Quantitation of viable cells to apoptotic/necrotic cells. Experiment conducted on 4 independent occasions, *p<0.01 for Shp2D61Y or Shp2E76K v. MIEG3 or WT Shp2 at 0.1 and 1 ng/mL GM-CSF. **D)** Analysis of apoptotic cells by annexin V staining. Viable cells (gated in R1) were analyzed for apoptosis by staining with annexin V-APC and normalized to MIEG3, no GM-CSF. Experiment conducted on 3 independent occasions, *p<0.01 for Shp2D61Y or Shp2E76K v. MIEG3 at 0.1 and 1 ng/mL GM-CSF. Statistics for all experiments performed using unpaired, two-tailed, Student's *t* test.

Figure 5.

A) Transduced and sorted bone marrow LDMNCs were placed in minimal media (IMDM and 2% FBS) in the absence or presence of GM-CSF, 0.1 or 1 ng/mL for 48 hours followed by plating into methylcellulose-based colony assays. Colonies were assessed based on morphology by light microscopy. Experiment conducted on 3 independent occasions and plated in triplicate; * $p < 0.05$ for Shp2D61Y or Shp2E76K v. MIEG3 or WT Shp2; ** $p < 0.001$ for Shp2D61Y or Shp2E76K v. MIEG3 or WT Shp2. **B)** Representative CFU-GM shown with cytopsin of plucked colony demonstrating both monocytes and granulocytes (indicated by arrows). **C)** Representative CFU-M shown with cytopsin of plucked colony demonstrating only monocytes.

Figure 6.

Transduced and sorted bone marrow LDMNCs were stained with the fluorescent cell membrane dye PKH26. An aliquot of cells fixed with 1% formaldehyde was utilized to set the gate defining undivided cells, which included approximately 68% of the most intensely stained cells for each sample (left-most panels). Cells bearing this PKH26 intensity following culture in GM-CSF were considered undivided and were evaluated for apoptosis using annexin V-APC staining. Numbers represent percent of PKH26-stained or annexin V-APC-stained cells within indicated gate. Representative of two independent experiments shown.

Figure 7. Representative Western blot analysis showing Bcl2, BclXL, and Bim expression levels following serum and cytokine withdrawal for 24 hours. All Western blots conducted on at least two independent occasions utilizing macrophage progenitors derived from independent transductions and sort collections.