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PRL2/PTP4A2 phosphatase is important for hematopoietic stem cell self-renewal

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Abstract

Hematopoietic stem cell (HSC) self-renewal is tightly controlled by cytokines and other signals in the microenvironment. While stem cell factor (SCF) is an early acting cytokine that activates the receptor tyrosine kinase KIT and promotes HSC maintenance, how SCF/KIT signaling is regulated in hematopoietic stem cells is poorly understood. The protein tyrosine phosphatase 4A (PTP4A) family [*aka* PRL (phosphatase of regenerating liver) phosphatases], consisting of PTP4A1/PRL1, PTP4A2/PRL2 and PTP4A3/PRL3, represents an intriguing group of phosphatases implicated in cell proliferation and tumorigenesis. However, the role of PTP4A in hematopoiesis remains elusive. To define the role of PTP4A in hematopoiesis, we analyzed HSC behavior in *Ptp4a2 (Prl2)* deficient mice. We found that *Ptp4a2* deficiency impairs HSC self-renewal as revealed by serial bone marrow transplantation assays. Moreover, we observed that *Ptp4a2* null hematopoietic stem and progenitor cells (HSPCs) are more quiescent and show reduced activation of the AKT and ERK signaling. Importantly, we discovered that the ability of PTP4A2 to enhance HSPC proliferation and activation of AKT and ERK signaling depends on its phosphatase activity. Furthermore, we found that PTP4A2 is important for SCF-mediated HSPC proliferation and loss of *Ptp4a2* decreased the ability of oncogenic KIT/D814V mutant in promoting hematopoietic progenitor cell proliferation. Thus, PTP4A2 plays critical roles in regulating HSC self-renewal and mediating SCF/KIT signaling.

Keywords

PRL2; PTP4A2; hematopoietic stem cell; self-renewal; cytokine signaling; SCF; KIT; KIT/D814V

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INTRODUCTION

In order to maintain hematopoietic homeostasis throughout the life of an animal, the hematopoietic stem cell (HSC) pool must be maintained through the process of self-renewal [1]. HSC self-renewal requires the integration of survival and proliferation signals to maintain an undifferentiated state. This demands a complex crosstalk between extrinsic signals from the microenvironment and the cell-intrinsic regulators of HSCs [2–3]. Stem cell factor (SCF) is a dimeric molecule that exerts its biological functions by binding to and activating the receptor tyrosine kinase KIT [4]. KIT is highly expressed in HSCs and SCF/KIT signaling plays a critical role in HSC maintenance [5–14]. SCF is an early acting cytokine that promotes HSC proliferation and survival [5–8]. Studies in mice with either partial or complete loss-of-function KIT mutations, have revealed severe hematopoietic deficiencies of multiple lineages, as well as an important role of KIT in sustaining rapidly cycling HSCs post transplantation when competing with wild-type HSCs [9–14]. Despite the wealth of knowledge on SCF/KIT signaling, it is poorly understood how KIT signaling is regulated in HSCs [4].

Gain-of-function mutations in KIT receptor in humans are associated with gastrointestinal stromal tumors (GIST), systemic mastocytosis (SM), and acute myelogenous leukemia (AML) [15–17]. These mutations results in altered substrate recognition and constitutive tyrosine autophosphorylation leading to promiscuous and constitutive signaling [18–20]. Consequently, cell lines and primary bone marrow (BM) cells that express the oncogenic KIT mutant demonstrate ligand-independent proliferation *in vitro* and myeloproliferative disease (MPD) *in vivo* [18–22]. However, the intracellular signals that contribute to mutant KIT-induced MPD are not known.

The PRL (Phosphatase of Regenerating Liver) phosphatases constitute a novel class of small, prenylated phosphatases (PRL1, 2, and 3) that share a high degree (>76%) of sequence identity [23–25]. The PRLs are relatively small proteins of about 20 kDa. In addition to the phosphatase domain, there are no regulatory domains except that all PRLs contain a consensus C-terminal prenylation motif CaaX, which is important for their localization to the plasma membrane and early endosomal compartments [23–25]. This family of phosphatases is also known as protein tyrosine phosphatase 4As (PTP4As). *PTP4A1/PRL1* was originally identified as an immediate early gene induced during liver regeneration after partial hepatectomy [23]. Subsequently, *PTP4A1/PRL1* as well as the closely related *PTP4A2/PRL2* and *PTP4A3/PRL3* were found to be elevated in numerous tumor cell lines, and cells expressing high levels of PTP4As exhibit enhanced proliferation and anchorage-independent growth [24–29]. Unlike most protein phosphatases that counteract the activity of protein kinases, the PTP4As play a positive role in signaling and possess oncogenic properties [30–31]. Consistent with their oncogenic potential, we recently revealed that PTP4A2/PRL2 promotes placenta development by downregulating PTEN, leading to AKT activation [32].

Ptp4a2/Prl2 is located on human chromosome 1p35 [30], a region often rearranged or amplified in malignant lymphoma and B-cell chronic lymphocytic leukemia (B-CLL) [33–34]. While *Ptp4a2/Prl2* mRNA is highly expressed in pediatric acute myeloid leukemia cells

[35–36], its role in normal and malignant hematopoiesis is largely unknown. Here we report a functional requirement of PTP4A2/PRL2 in hematopoietic stem cell self-renewal. We further demonstrate that PTP4A2/PRL2 is an important mediator of SCF/KIT signaling in HSCs.

MATERIALS AND METHODS

Mice

The generation of *Ptp4a2/Prl2* knockout mice (*Ptp4a2*$^{-/-}$, C57BL6/129P2 mixed background) has been described previously [32]. *Ptp4a2/Prl2* knockout mice were backcrossed with C57BL6 mice for at least 8 generations. Wild type C57BL/6 (CD45.2^{+}), B6.SJL (CD45.1^{+}) and F1 mice (CD45.2^{+} CD45.1^{+}) mice were purchased from the Jackson Laboratories. All mice were maintained in the Indiana University Animal Facility according to IACUC-approved protocols, and kept in Thorensten units with filtered germ-free air.

Flow cytometry

Flow cytometry analysis of hematopoietic stem and progenitor cells was performed as described previously [37–38]. Murine hematopoietic stem and progenitor cells were identified and evaluated by flow cytometry using a single cell suspension of bone marrow mononuclear cells (BMMCs). Hematopoietic stem and progenitors are purified based upon the expression of surface markers: LT-HSC (Lin^{-}Sca1^{+}Kit^{+}CD34^{-}CD48^{-}CD150^{+}), ST-HSC (Lin^{-}Sca1^{+}Kit^{+}CD34^{+}CD48^{+}CD150^{+}), MPP (Lin^{-}Sca1^{+}Kit^{+}CD34^{+}CD48^{+}CD150^{-}), CMP (Lin^{-}Sca1^{-}IL7R^{-}Kit^{+}FcγRII/III^{low}CD34^{high}), GMP (Lin^{-}Sca1^{-}IL7R^{-}Kit^{+}FcγRII/III^{high}CD34^{high}), MEP (Lin^{-}Sca1^{-}IL7RKit^{+}FcγRII/III^{low}CD34^{low}) and CLP (Lin^{-}IL7R^{+}Sca1^{low}Kit^{low}). BMMCs were obtained from both tibias and femurs by flushing cells out of the bone using a syringe and DMEM plus 10% FBS. Cells were first stained with a lineage (Lin) cocktail of antibodies from BD Biosciences (biotinylated anti-mouse antibodies directed against CD3e, CD11b, CD45R/B220, Gr-1, Ter119) as well as Sca-1 PE and c-KIT APC (Pharmingen), and a streptavidin Cychrome conjugate (Pharmingen). c-KIT-APC, Sca-1-PE-Cy7, Flt3-PE, CD34-FITC and streptavidin APC-Cy7 were used for analysis using a FACSLSR II cytometer (BD Biosciences). FITC-CD41, FITC-CD48 and FITC-CD34 were purchased from eBioscience and PE-CD150 purchased from Biolegend for SLAM marker analysis. All other antibodies were from BD Biosciences. For immunophenotypic analysis, approximately 3×10^6 BMMCs were stained with antibodies for 30 minutes on ice in dark. Nuclear staining of Ki67 was assessed by using an FITC-anti-human Ki67 antibody (BD Pharmingen) and fixation and permeabilization solutions from BD Biosciences. Experiments were performed on FACSAria and FACSLSR II cytometers (BD Biosciences) and analyzed by using the FlowJo Version 9.3.3 software (TreeStar).

Bone marrow transplantation

For the competitive repopulation assays, we injected 5×10^5 BMMCs from wild type and *Ptp4a2* null mice (CD45.2^{+}) plus 5×10^5 competitor bone marrow cells (CD45.1^{+}) into lethally irradiated F1 mice (CD45.1^{+}CD45.2^{+}). Peripheral blood was obtained by tail vein bleeding every month, the RBC lysed, and the PBMCs stained with anti-CD45.2 FITC and anti-CD45.1 PE, and analyzed by flow cytometry. 16 weeks following transplantation, we

harvested bone marrow cells from mice reconstituted with wild type or *Ptp4a2* null bone marrow cells and transplanted 3×10^6 bone marrow cells into lethally irradiated F1 mice ($CD45.1^+CD45.2^+$).

For HSC transplantation, we injected 200 $CD48^-CD150^+$KSL cells from wild type or *Ptp4a2* null mice ($CD45.2^+$) plus 3×10^5 competitor bone marrow cells ($CD45.1^+$) into lethally irradiated F1 mice ($CD45.1^+CD45.2^+$). Peripheral blood was obtained by retro-orbital eye bleeding every month, the RBC lysed, and the PBMCs stained with anti-CD45.2 FITC and anti-CD45.1 PE, and analyzed by flow cytometry. 16 weeks following transplantation, we harvested bone marrow cells from recipient mice and performed flow cytometry analysis to evaluate HSC repopulating capability.

Homing assays

The homing ability of wild type and *Ptp4a2* null HSPCs were analyzed in irradiated recipient mice. 1×10^7 wild type or *Ptp4a2* null bone marrow cells ($CD45.2^+$) were injected into lethally irradiated recipient ($CD45.1^+$) mice. Bone marrow cells were harvested 18 hours following injection and donor-derived cells were evaluated by flow cytometry as $CD45.2^+$ cells, simultaneously identifying $Lin^-Sca1^+CD150^+$ cells.

Stem and progenitor cell assays

Clonogenic progenitors were determined in methylcellulose medium (MethoCult GF M3234, StemCell Technologies) with cytokines (SCF, TPO, EPO, IL-3 and GM-CSF) using 2×10^4 BMMCs per well (6-well plate). Colonies were scored after 7 days of the initial culture, and all cells were collected and washed twice in phosphate-buffered saline. Subsequently cells were cultured at 2×10^4 per well in the same medium. Colony scoring and replating were repeated every 7 days for at least two times, or until no colonies were observed in the cultures.

Immunoblotting

Cells were washed with ice-cold phosphate-buffered saline, and lysed on ice for 30 min in lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 10% glycerol) supplemented with protease inhibitor (Roche) and phosphatase inhibitor (Roche). Cell lysates were cleared by centrifugation at 13,200 rpm for 10 min, and boiled with Laemmli (SDS)-sample buffer, separated by SDS-PAGE, transferred electrophoretically to a nitrocellulose membrane, and immunoblotted with pAKT (Cell Signaling), AKT (Cell Signaling), pERK1/2 (Cell Signaling), ERK1/2 (Cell Signaling), PTEN (Cell Signaling), PTP4A2/PRL2 (a generous gift from Dr. Qi Zeng) and β-Actin (Santa Cruz) followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The blots were developed by the enhanced chemiluminescence technique using the SuperSignal West Pico Chemiluminescent substrate (Pierce) or SuperSignal West Femto Maximum Sensitivity Chemiluminescent Substrate (Pierce). Data shown are representation of multiple repeat experiments.

Statistical analysis

We used either student t test or Two-way ANOVA to determine statistical significance. *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant.

RESULTS

Ptp4a2 deficiency results in inefficient hematopoiesis

To investigate the role of PTP4A2 in hematopoiesis, we analyzed the peripheral blood and bone marrow of 8 to 12 week-old wild type and *Ptp4a2*$^{-/-}$ mice. *Ptp4a2*$^{-/-}$ mice show decreased white blood cell, neutrophil and lymphocyte counts, whereas hemoglobin levels are normal in the peripheral blood (Supplementary Fig. 1A). While *Ptp4a2* null mice show decreased body size as we previously reported (Supplementary Fig. 1B) [32], the bone marrow cellularity is normal when normalized to total body weight (supplementary Fig. 1C). We also found that *Ptp4a2*$^{-/-}$ mice have smaller spleen and thymus compared with wild type mice (Supplementary Fig. 2). These data indicate that PTP4A2 plays an important role in hematopoiesis.

Ptp4a2 null mice have normal number of immunophenotypic hematopoietic stem cells

While *Ptp4a1* and *Ptp4a2* are broadly expressed in adult tissues including liver [25, 39–40], the expression pattern of these *Ptp4as* in the hematopoietic system is unknown. We examined the expression of *Ptp4a1, Ptp4a2* and *Ptp4a3* during different stages of hematopoietic stem cell differentiation by using real-time RT-PCR assays. *Ptp4a1* and *Ptp4a2* are ubiquitously expressed in the hematopoietic compartment, whereas *Ptp4a3* expression is most pronounced in megakaryocyte-erythroid progenitor cells (MEPs). *Ptp4a2* is highly expressed in hematopoietic cells and its expression is enriched in differentiated cells compared to long-term HSCs and committed progenitor cells, suggesting that it could play an important role in lineage commitment (Fig. 1A). We then examined the number of primitive hematopoietic stem/progenitor cells in the bone marrow of *Ptp4a2*$^{-/-}$ mice. Loss of *Ptp4a2* slightly increased the frequency (*p<0.05, n=9; Fig. 1B), but not the absolute number of $Kit^{+}Sca1^{+}Lin^{-}$ cells (KSLs) compared to wild-type mice (Fig. 1C); however, *Ptp4a2* deficiency neither affects the frequency nor the absolute number of long-term HSCs (LT-HSCs) based on SLAM and CD34 expression (Fig. 1D, 1E). Thus, loss of *Ptp4a2* does not affect immunophenotypic HSCs.

Ptp4a2 deficiency impairs the long-term repopulating ability of HSCs

To examine whether *Ptp4a2* deficiency affects HSC function, we performed serial competitive bone marrow transplantation assays. Equal numbers of donor and competitor bone marrow mononuclear cells (BMMCs) were transplanted into lethally irradiated recipient mice. Sixteen weeks after primary transplantation, the repopulating ability of *Ptp4a2* null cells was significantly lower than wild-type cells (**p<0.01, n=7; Fig. 2A). Moreover, we found that the percentage of donor-derived T cells was significantly decreased in mice transplanted with *Ptp4a2* null cells than that with wild-type cells (11.0 ± 1.9% vs. 4.1 ± 1.7% at 16 weeks, Fig. 2B). Analysis of the bone marrow revealed a striking reduction in the number of phenotypically defined HSCs in the recipients repopulated with *Ptp4a2*

null bone marrow cells (**p<0.01, n =5; Fig. 2C). We then transplanted 3×10^6 BMMCs isolated from the primary recipient mice repopulated with wild-type or *Ptp4a2* null cells into lethally irradiated secondary recipients. Sixteen weeks after transplantation, *Ptp4a2* null cells continued to show decreased repopulating ability (***p<0.001, n=7; Fig. 2D).

To further establish that the self-renewal defect is HSC intrinsic, we purified wild-type and *Ptp4a2* null LT-HSCs ($CD48^-CD150^+$KSLs) and transplanted 200 of each into lethally irradiated recipient mice along with 300,000 wild-type competitor BMMCs. In this context, *Ptp4a2* null HSCs exhibited a substantially lower contribution to peripheral blood production compared to control HSCs (***p<0.001, n=7; Fig. 2E), demonstrating that Ptp4a2 plays an important role in HSC maintenance and loss of *Ptp4a2* impairs HSC self-renewal.

The decreased self-renewal of *Ptp4a2* null HSCs in transplantation assays could be due to abnormalities in homing following transplantation. To determine whether *Ptp4a2* null bone marrow cells are defective in homing, we performed homing assays and observed similar numbers of donor-derived $Lin^-Sca\text{-}1^+Slam^+$ cells from the bone marrow of recipient mice repopulated with wild-type or *Ptp4a2* null bone marrow cells (Supplementary Fig. 3A), suggesting the absence of homing defects. The impaired HSC self-renewal in *Ptp4a2* null mice could also be due to increased apoptosis. Therefore, we evaluated HSC survival by Annexin-V staining and observed no significant difference in the number of apoptotic HSCs (Annexin-V^+/PI^-CD48^-KSLs) in the absence of cytokines (Supplementary Fig. 3B). Thus, the impaired self-renewal of *Ptp4a2* null HSC is likely due to an inability to properly self-renew, rather than homing defects or an increase in apoptosis.

PTP4A2 promotes hematopoietic stem and progenitor cell proliferation

To assess the effect of *Ptp4a2* deficiency on proliferation, we stained *Ptp4a2* null hematopoietic stem and progenitor cells with proliferation marker Ki67. While we detected normal number of Ki67 negative LT-HSCs, we found more Ki67 negative multipotent progenitor cells (MPPs) than normal (**p<0.01, n=6; Fig. 3A), indicating *Ptp4a2* null MPPs are more quiescent and less proliferative. We then performed methylcellulose colony-forming unit (CFU) assays to quantify myeloid progenitor cells. While *Ptp4a2* deficiency does not affect the total number of colonies formed, *Ptp4a2* null bone marrow cells formed significantly fewer immature GEMM colonies compared with wild type cells (*p<0.05, n=3; Fig. 3B). Consistent with the cell cycle data, *Ptp4a2* null BMMCs show decreased replating potential compared to wild-type cells (**p<0.01, n=3; Fig. 3C), further demonstrating that hematopoietic progenitor cells show decreased proliferation in the absence of *Ptp4a2*. To decipher the molecular mechanisms underlying the HSPC proliferation defects seen in the *Ptp4a2* null mice, we examined the expression of several known HSC regulators by using real time RT-PCR assays. Several negative regulators of cell cycle, including *p21* and *p57*, are upregulated in *Ptp4a2* null HSCs and MPPs respectively (**p<0.01, n=3; Fig. 3D, 3E). In addition, the expression of C*yclin D1* (*CCND1*) and C*yclin E1* (*CCNE1*) are downregulated in MPPs (**p<0.01, n=3; Fig. 3E). We further confirmed the changes in p57 and CCND1 expression in MPPs by flow cytometry (Supplementary Fig. 4).

The ability of PTP4A2 to enhance hematopoietic progenitor cell proliferation depends on its phosphatase activity

While we showed that PTP4A2 is important for hematopoietic progenitor cell proliferation, whether this effect depends on its phosphatase activity is not clear. To this end, we overexpressed wild-type or the catalytic inactive mutant form of *Ptp4a2* (*Ptp4a2/C101S*) in Lin^- cells isolated from wild-type and *Ptp4a2* null mice and performed colony forming unit (CFU) assay [41]. While both wild-type and *Ptp4a2* null mononuclear cells form similar number of colonies in CFU assays (Fig. 3B), *Ptp4a2* null Lin-Sca1+ cells formed significantly fewer colonies compared with wild-type cells following retroviral transduction (*$p<0.05$, n=3; Fig. 4A). Lin-Sca1+ cells are enriched for hematopoietic stem and progenitor cells and we showed that *Ptp4a2* null progenitor cells are less proliferative (Fig. 3A). Therefore, the difference between the two CFU assays we observed is likely due to distinct composition of cells used in the experiments. Ectopic expression of wild-type or mutant *Ptp4a2* did not affect the colony forming potential of wild-type cells. However, we found that overexpressing wild-type *Ptp4a2*, but not the mutant form, significantly increase the colony formation of *Ptp4a2* null cells (**$p<0.01$, n=3; Fig. 4A). We also overexpressed wild-type or the mutant form of *Ptp4a2* in Lin^- cells isolated from wild-type and *Ptp4a2* null mice and monitored their proliferation. Consistent with data in the CFU assays (Fig. 4A), mock-transduced *Ptp4a2* null Lin^- cells show decreased proliferation compared with that of mock-transduced wild type cells (**$p<0.01$, n=3; Fig. 4B). Wild-type *Ptp4a2* transduced *Ptp4a2* null cells showed enhanced proliferation compared with mock and mutant *Ptp4a2* transduced cells (**$p<0.01$, n=3; Fig. 4B).

To decipher the molecular mechanisms underlying the HSPC proliferation defects seen in the *Ptp4a2* null mice, we examined the activation of AKT and ERK signaling in freshly purified Lin^- cells. We observed that the levels of pAKT and pERK1/2 are significantly lower in *Ptp4a2* null Lin- cells compared with those in wild-type cells (Fig. 4C). To investigate the mechanism by which *Ptp4a2* deficiency attenuates AKT and ERK activation, we analyzed PTEN (phosphatase and tensin homologue deleted on chromosome ten) expression in both wild-type and *Ptp4a2* null cells. PTEN is an antagonist of PI3K signaling pathway [42]. In addition, PTEN has also been shown to negatively regulate the activation of the ERK pathway [42]. We previously reported that in placenta, *Ptp4a2* deficiency led to elevated PTEN expression [32]. To examine whether this is also the case in HSPCs, we measured PTEN protein level in wild-type and *Ptp4a2* null Lin- cells. As shown in Figure 4C, PTEN expression was 1.4 ± 0.15 fold higher in *Ptp4a2* null samples, consistent with our previous observation in placenta [32]. Therefore, impaired AKT and ERK activation in *Ptp4a2* null Lin- cells is likely due to increased PTEN level. Furthermore, we found that expression of wild-type *Ptp4a2*, but not the catalytically inactive mutant *Ptp4a2*, significantly augments pAKT and pERK1/2 levels in *Ptp4a2* null Lin^- cells (Fig. 4D and Supplementary Fig. 5), demonstrating that the ability of PTP4A2 to promote hematopoietic progenitor cell proliferation and activation of AKT and ERK signaling depends on its phosphatase activity.

PTP4A2 is important for SCF/KIT signaling in hematopoietic stem and progenitor cells

Given that *Ptp4a2* null bone marrow mononuclear cells show decreased colony formation in serial replating assays, PTP4A2 may play an important role in cytokine signaling. To test this hypothesis, we cultured both wild-type and *Ptp4a2* null Kit^+ cells in the presence or absence of cytokines, including SCF, G-CSF and IL-3, and monitored their proliferation. While wild-type and *Ptp4a2* null Kit^+ cells exhibited similar proliferative behaviors in response to G-CSF or IL-3 stimulation, *Ptp4a2* null hematopoietic progenitor cells showed decreased proliferation in response to SCF (*$p<0.05$, n=3; Fig. 5A). In CFC assays, *Ptp4a2* null KSL cells formed significantly fewer colonies compared with wild-type KSL cells in response to SCF stimulation (**$p<0.01$, n=3; Fig.5B). Importantly, *Ptp4a2* null KSL cells showed marked reduction of GEMM colony formation in response to decreased concentration of SCF compared with wild-type KSL cells, demonstrating that PTP4A2 is essential for the proliferation of hematopoietic progenitor cells following SCF stimulation. We then examined whether *Ptp4a2* deficiency affects HSPC survival in the absence of cytokine. As shown in Figure 5C, *Ptp4a2* null Kit^+ cells showed decreased cell counts compared with wild-type cells (*$p<0.05$, $n = 3$), indicating that PTP4A2 is essential for HSPC survival in the absence of cytokine. Furthermore, we observed that the levels of pAKT and pERK1/2 are also significantly lower in *Ptp4a2* null Kit^+ cells compared with those in wild-type cells in the basal state (Figure 5D). Stimulation of HSPCs with SCF can activate several signaling pathways through KIT, including PI3K/AKT and ERK1/2 pathways [4–6]; therefore, it is possible that *Ptp4a2* deficiency impairs the activation of these signaling pathways in response to SCF stimulation. This is indeed the case, as *Ptp4a2* null cells displayed decreased activation of both pAKT and pERK1/2 at 30 minutes following SCF stimulation (Figure 5D), demonstrating that PTP4A2 is important for sustaining SCF signaling in hematopoietic stem and progenitor cells.

PTP4A2 is important for oncogenic KIT signaling in hematopoietic progenitor cells

Hematopoietic cell lines and primary BM cells expressing the oncogenic KIT/D814V mutant display ligand-independent proliferation *in vitro* and myeloproliferative disease (MPD) *in vivo* [18–22]. Based on our finding that PTP4A2 is important for SCF/KIT signaling in HSPCs, we speculated that PTP4A2 may also enhance KIT/D814V-mediated ligand-independent hyperproliferation and activation of signaling pathways. To test this, we introduced wild-type KIT and KIT/D814V mutant into Lin^- cells purified from wild-type and *Ptp4a2* null mice and performed serial replating assays. In primary (*$p<0.05$, n=3), secondary (**$p<0.01$, n=3) and tertiary replating experiments (***$p<0.001$, n=3), *Ptp4a2* null Lin^- cells expressing KIT/D814V formed significantly fewer colonies compared with wild-type Lin^- cells expressing the KIT mutant (Fig. 6A). Both wild-type and *Ptp4a2* null cells expressing wild-type KIT did not form any colonies in the tertiary replating experiments (Fig. 6A). To determine whether PTP4A2 is essential for KIT/D814V-mediated ligand-independent growth, we cultured *Ptp4a2* wild-type and null cells expressing wild-type or KIT/D814V in the absence of cytokine or the presence of SCF. As shown in Figure 6B (**$p<0.01$, n=3), *Ptp4a2* deficiency significantly decreased the ability of KIT/D814V to promote cell proliferation both in the absence and presence of SCF. Furthermore, we observed decreased phosphorylation of AKT and ERK1/2 in *Ptp4a2* null cells expressing

KIT/D814V mutant compared with wild-type cells (Figure 6C). Thus, PTP4A2 is essential for KIT/D814V-mediated hematopoietic progenitor cell hyperproliferation and activation of signaling pathways.

DISCUSSION

The PTP4A family of phosphatases, consisting of PTP4A1, PTP4A2, and PTP4A3, represents an intriguing group of proteins implicated as biomarkers and therapeutic targets in cancer [23–25]. Individual PTP4As are overexpressed in a variety of cancer cell lines and tissues when compared with their normal counterparts [26–29]. However, the exact biological functions of PTP4As are largely unknown [23–25]. Herein we report that PTP4A2/PRL2 plays a critical role in hematopoietic stem cell self-renewal and SCF/KIT signaling.

While loss of *Ptp4a2/Prl2* modestly increased the frequency of multipotent progenitor cells, *Ptp4a2* deficiency does not affect immunophenotypic hematopoietic stem cell numbers. To further define the role of PTP4A2 in regulating hematopoietic stem cell self-renewal, we performed serial bone marrow transplantation assays and found that loss of *Ptp4a2* impairs the ability of hematopoietic stem cells to repopulate the lethally irradiated recipient mice. These observations were confirmed by HSC transplantation. Furthermore, we observed no homing defects following transplantation and no increased HSC apoptosis, demonstrating that PTP4A2 functions as a positive regulator of hematopoietic stem cell self-renewal. We also found that the percentage of donor-derived T cells was significantly lower in mice transplanted with *Ptp4a2* null cells than that with wild-type cells, indicating that PTP4A2 may also regulate T cell differentiation under stress conditions.

Among the signal transduction pathways that have attracted considerable attention as possibly being involved in HSC maintenance is the phosphoinositide 3-kinase (PI3K)-AKT pathway [43]. As an antagonist of the PI3K pathway, PTEN has been implicated as a regulator of HSC self-renewal and loss of PTEN in the HSC compartment results in hyperproliferation of hematopoietic stem and progenitor cells [44]. Recently, we showed that in mouse placenta PTP4A2/PRL2 promotes cell proliferation by activating the AKT kinase through downregulation of the tumor suppressor PTEN [32]. It appears that *Ptp4a2* overexpression enhances the degradation of PTEN protein in these cells, indicating that PTP4A2/PRL2 regulates the stability of PTEN in placenta [32]. In addition, we observed that loss of *Ptp4a2* decreases hematopoietic stem and progenitor cell proliferation and *Ptp4a2* null hematopoietic progenitor cells show decreased phosphorylation of AKT and ERK1/2 in steady state. To investigate how PTP4A2 regulates PI3K/AKT signaling in hematopoietic progenitor cells, we examined the level of PTEN in *Ptp4a2* null HSPCs. As seen in placenta, we observed moderate but statistically significant elevation of PTEN protein in *Ptp4a2* null HSPCs compared to that in wild-type HSPCs, whereas PTEN RNA level is identical between *Ptp4a2* wild-type and null cells. Therefore, it is possible that PTP4A2 regulates the stability and turnover of PTEN protein in hematopoietic stem and progenitor cells as well. PTEN has been shown to regulate cell proliferation and cancer progression in a dosage-dependent manner and a slight reduction in PTEN levels dictates cancer susceptibility [45–46]. Thus, it is likely that increased level of PTEN we observed

contributes to the decreased activation of AKT and ERK1/2 signaling pathways in *Ptp4a2* null HSPCs. Moreover, consistent with PTEN's established role in sustaining HSPC proliferation, our data suggest that increased PTEN expression in *Ptp4a2* deficient mice leads to reduced proliferation of HSPCs.

Ectopic expression of *Ptp4a2* in nontumorigenic cells has been shown to enhance cell cycle progression and promotes proliferation [28, 36]. This process may be dependent on the down-regulation of cyclin dependent kinase inhibitor p21 [28]. The cell cycle defects seen in *Ptp4a2* null mice may also be mediated by cell cycle regulators as we observed decreased expression of p21 and p57 in hematopoietic stem and progenitor cells. Both p21 [47] and p57 [48] have been shown to regulate HSPC proliferation; therefore, PTP4A2 plays an important role in promoting HSPC proliferation, at least in part by controlling the level of cell cycle regulators. How PTP4A2 regulates the expression of these cell cycle regulators is not clear. However, we note that PTEN controls cell cycle entry and progression through inhibiting PI3K-AKT activity [49–50] and PTEN loss leads to enhanced G0–G1 cell cycle transition, which yields a short-term expansion of HSCs [43].

Given that *Ptp4a2* null hematopoietic progenitor cells show decreased colony formation in serial replating assays, it is possible that PTP4A2 regulates cytokine signaling in hematopoietic progenitor cells. While *Ptp4a2* null hematopoietic progenitor cells proliferate normally in response to IL-3 or G-CSF stimulation, these cells are less proliferative in response to SCF stimulation. Furthermore, *Ptp4a2* null hematopoietic progenitor cells show decreased colony formation in response to increased concentrations of SCF compared with wild-type cells. The role of PTP4A2 in mediating SCF/KIT signaling appears modest, as SCF still activates a remarkable amount of AKT and ERK in *Ptp4a2* null cells (Fig. 5D). Therefore, PTP4A2 may be one of many factors that contribute to KIT function in hematopoietic stem and progenitor cells.

As the receptor for SCF, the biologic significance of KIT in hematopoiesis was first revealed in the white spotting (*W*) mutant mice [51]. Several *W* mutations with different levels of KIT kinase deficiency were found to correlate with the phenotype severity [9–14]. Studies in the viable primary *W* mutant mice or in mice after transplantation indicate the importance of KIT signaling in maintaining HSC quiescence and survival [9–14]. The hematopoietic defects seen in the *Ptp4a2* null mice recapitulate some hematopoietic phenotype of KIT mutant mice, providing additional evidence that PTP4A2 plays an important role in mediating SCF/KIT signaling in hematopoietic progenitor cells.

While *Ptp4a2* is highly expressed in acute myeloid leukemia cells [35–36], its role in leukemogenesis is largely unknown. We discovered that loss of *Ptp4a2* decreased the ability of KIT/D814V mutant in promoting hematopoietic progenitor cell proliferation, demonstrating that PTP4A2 is an important effector molecule of oncogenic KIT signaling in hematopoietic stem and progenitor cells and positively contributes to KIT/D814V-induced hematopoietic progenitor hyperproliferation. Therefore, pharmacological inhibition of PTP4A2 in KIT/D814V-expressing cells *in vivo* could delay leukemia progression and decrease the severity of the disease.

CONCLUSION

In summary, this study reveals a critical role of PTP4A2/PRL2 in hematopoietic stem cell self-renewal and SCF/KIT signaling. Our results suggest that the PTP4A2 phosphatase may be a druggable target in myeloproliferative disease (MPD) and acute myeloid leukemia (AML) with oncogenic KIT mutations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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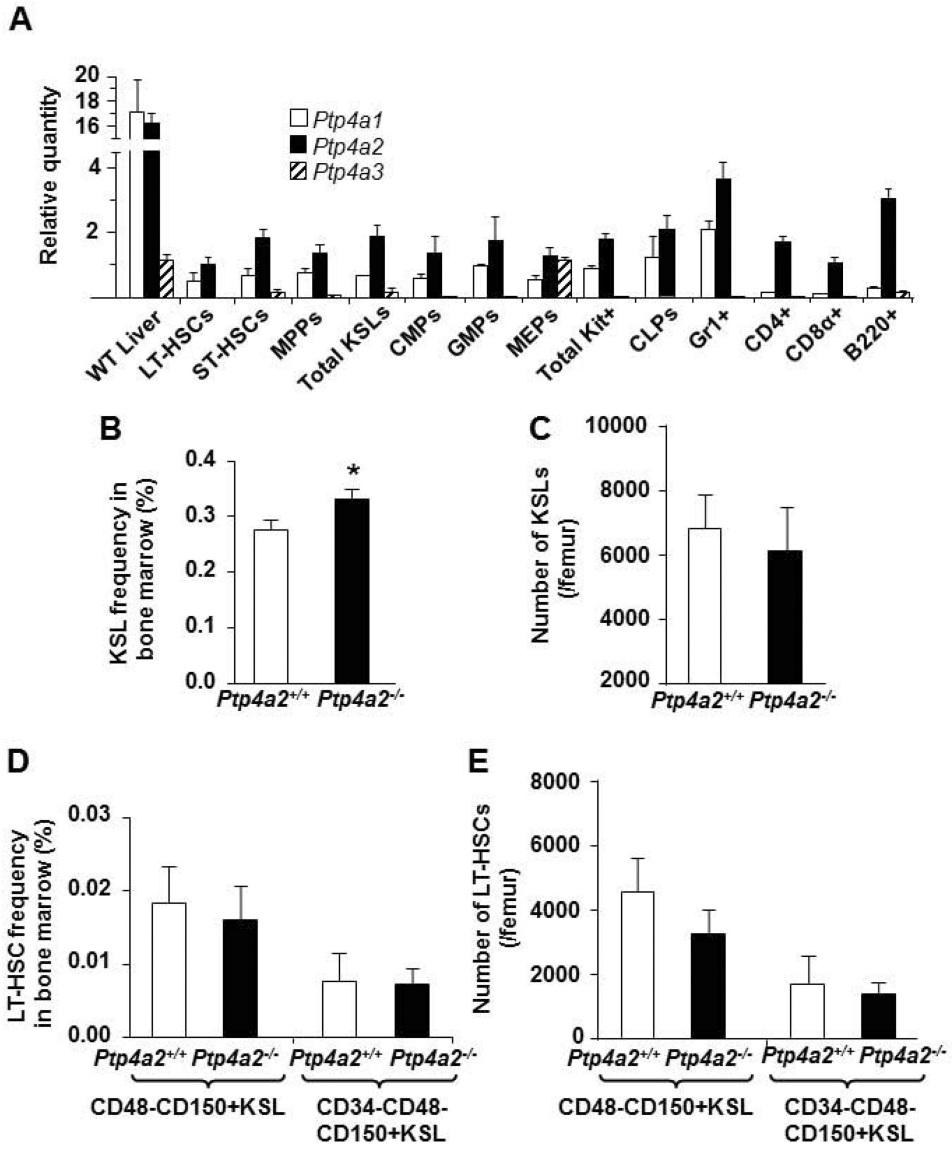

Figure 1.

Ptp4a2 null mice have normal number of immunophenotypic hematopoietic stem cells. (A): Real-time RT-PCR analysis of *Ptp4a* mRNAs in long-tern HSCs (LTHSCs), short-term HSCs (ST-HSCs) and representative committed progenitors and differentiated cells. MPPs, multi-potential progenitors; CLPs, common lymphoid progenitors; CMPs, common myeloid progenitors; MEPs, megakaryocyte-erythroid progenitors; GMPs, granulocyte-macrophage progenitors. Data shown are the mean values ± SD (n = three biological replicates). (B): The frequency of $Kit^{+}Sca1^{+}Lin^{-}$ cells (KSLs) were quantified by flow cytometry. Data shown are the mean percentage (± SD) of KSL cells in the bone marrow (*$p<0.05$, n=9). (C): The absolute number of KSLs per femur is shown. Graphs represent the mean ± SD (n=9). (D): The frequency of LT-HSCs ($CD34^{-}CD48^{-}CD150^{+}$KSLs) was defined by flow cytometry analysis of SLAM cell surface markers. Data shown are the mean percentage (± SD) of LT-

HSCs in the bone marrow (n=9). (E): The absolute number of LT-HSCs per femur is shown. Graphs represent the mean ± SD (n=9).

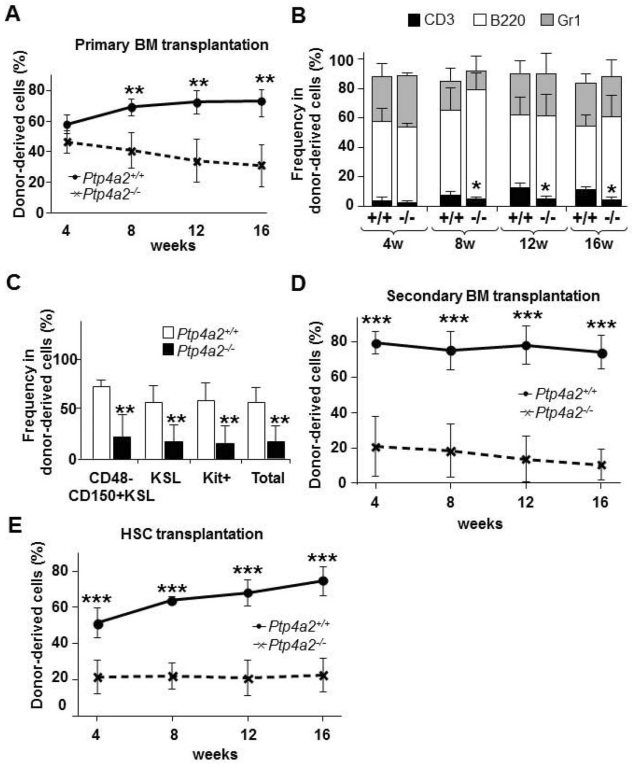

Figure 2.

Ptp4a2 deficiency impairs the long-term repopulating ability of HSCs. (A): Lethally irradiated recipient mice ($CD45.1^+$ and $CD45.2^+$) were transplanted with 5×10^5 bone marrow mononuclear cells (BMMCs) from wild-type and *Ptp4a2* null mice ($CD45.2^+$) plus 5×10^5 competitor cells ($CD45.1^+$) in competitive repopulation assays. Graph shows the mean percentage (±SD) of donor-derived ($CD45.2^+$) cells in the peripheral blood posttransplantation, measured at monthly intervals (**$p<0.01$, n = 7). (B): Contribution of donor-derived cells ($CD45.2^+$) from A, for myeloid cells ($Gr1^+$), B cells ($B220^+$) and T cells ($CD3^+$) in peripheral blood from recipient mice at different time points after transplantation (*$p<0.05$, n = 7). (C): Quantification of donor-derived ($CD45.2^+$) HSC frequency in the bone marrow of primary recipient mice 18 weeks following transplantation by three phenotypic definitions. Mean ± SD values are shown (**$p<0.01$, n = 5). (D): Contribution of wild type and *Ptp4a2* null bone marrow mononuclear cells (BMMCs) to recipient mouse peripheral blood in secondary competitive transplants, measured at monthly intervals (***$p<0.001$, n = 7). (E): Lethally irradiated recipient mice ($CD45.1^+$ and $CD45.2^+$) were transplanted with 200 LT-HSCs ($CD48^-CD150^+$KSLs) from wild-type and *Ptp4a2* null mice ($CD45.2^+$) plus 3×10^5 competitor cells ($CD45.1^+$) in competitive repopulation assays. Graph shows the mean percentage (±SD) of donor-derived ($CD45.2^+$) cells in the peripheral blood posttransplantation, measured at monthly intervals (***$p<0.001$, n = 7).

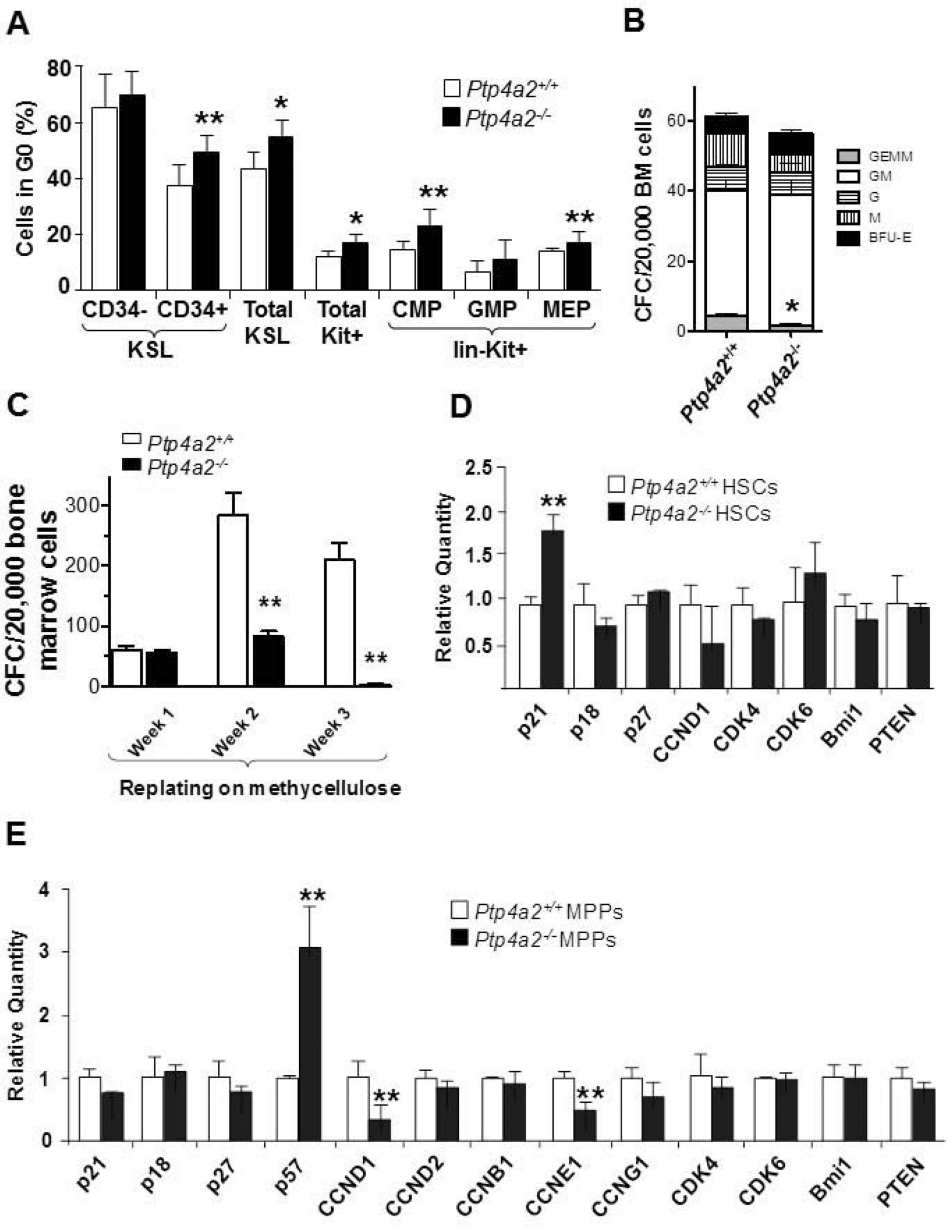

Figure 3.

PTP4A2 regulates hematopoietic stem and progenitor cell proliferation. (A): Cell-cycle analysis of hematopoietic stem and progenitor cells was performed by staining with DAPI and Ki67 and analyzed by FACS. Data shown are the mean values ± SD (**p<0.01, n = 6). (B): Myeloid progenitors were quantified by methylcellulose culture using bone marrow mononuclear cells from wild-type and *Ptp4a2* null mice (*p<0.05, n = 3). (C): Serial replating studies. Myeloid progenitors were quantified by methylcellulose culture using BMMCs from wild-type and *Ptp4a2* null mice. The methylcellulose cultures were serially replated, weekly, for 3 weeks. Mean values (±SD) are shown (**p<0.01, n = 3). (D and E): *Ptp4a2* deficiency results in upregulation of cell cycle regulators in HSCs (D) and MPPs

(E). Real-time RT-PCR analysis of some known HSC regulators in LT-HSCs and MPPs. Data shown are the mean values ± SD (**p<0.01, n = three biological replicates).

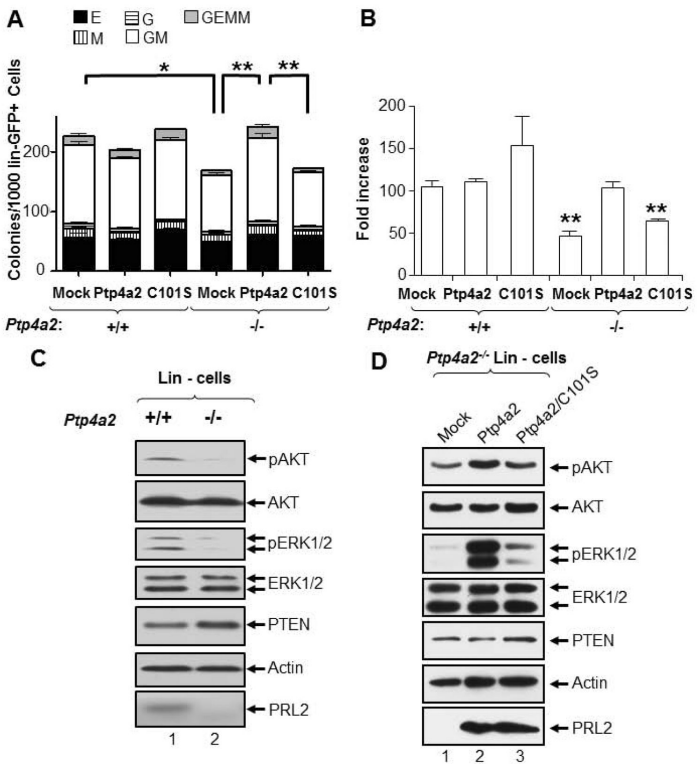

Figure 4.

The ability of PTP4A2 to enhance hematopoietic progenitor cell proliferation depends on its phosphatase activity. (A): Myeloid progenitors were quantified by methylcellulose culture using wild type and *Ptp4a2* null Lin-Sca1+ cells transduced with retroviruses expressing the WT or the mutant form (Ptp4a2/C101S) of Ptp4a2. Data are means ± SD (**p<0.01, n = 3 independent experiments). (B): Proliferation of wild type and *Ptp4a2* null Lin$^-$ cells expressing the WT or the mutant form (Ptp4a2/C101S) of Ptp4a2 in response to SCF stimulation. Data are means ± SD (**p<0.01, n = 3 independent experiments). (C): Immunoblot analysis of AKT and ERK phosphorylation in freshly purified wild type and *Ptp4a2* null Lin$^-$ cells. *p < 0.05 compared with wild type cells by one-way analysis of variance (ANOVA) and Bonferroni post hoc test, n= three independent experiments. Representative Western blot analysis of indicated proteins is shown. (D): Immunoblot analysis of AKT and ERK phosphorylation in *Ptp4a2* null Lin$^-$ cells expressing the WT or the mutant form (Ptp4a2/C101S) of Ptp4a2. *p < 0.05 compared with mock transduced cells by one-way analysis of variance (ANOVA) and Bonferroni post hoc test, n= three independent experiments. Representative Western blot analysis of indicated proteins is shown. .

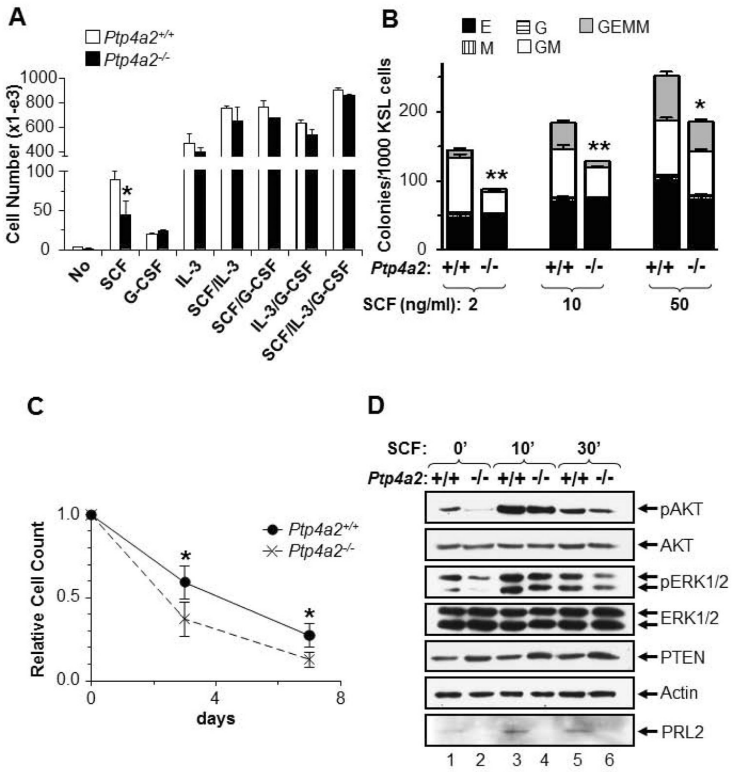

Figure 5.

PTP4A2 is important for SCF/KIT signaling in hematopoietic stem and progenitor cells. (A): Proliferation of wild type and *Ptp4a2* null Kit$^+$ cells in response to cytokine stimulation. Data shown are the mean values ± SD (*p<0.05, n = 3). (B): Colony Forming Unit (CFU) assays in the presence of increased concentration of SCF. Data shown are the mean values ± SD (**p<0.01, n = 3). (C): Proliferation of wild type and *Ptp4a2* null Kit$^+$ cells in the absence of cytokines. Data shown are the mean values ± SD (*p<0.05, n = 3). (D): Immunoblot analysis of AKT, and ERK phosphorylation in wild type and *Ptp4a2* null Kit$^+$ cells following SCF stimulation. Data represent three independent experiments.

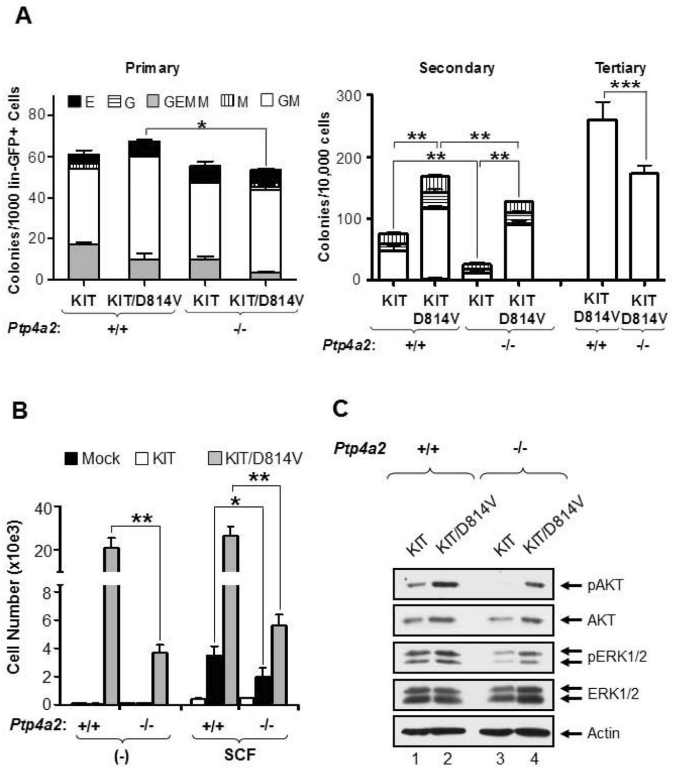

Figure 6.

PTP4A2 is important for oncogenic KIT signaling in hematopoietic progenitor cells. (A): Myeloid progenitors were quantified by methylcellulose culture in the presence of SCF using wild type and *Ptp4a2* null Lin$^-$ cells transduced with retroviruses expressing the WT or the mutant form (KIT/D814V) of KIT. The methylcellulose cultures were serially replated, weekly, for 3 weeks. Data are means ± SD (*p<0.05, **p<0.01, ***p<0.001, n = 3 independent experiments). (B): Proliferation of wild type and *Ptp4a2* null Lin$^-$ cells expressing the WT or the mutant form (KIT/D814V) of KIT in the presence or the absence of cytokine stimulation. Data are means ± SD (**p<0.01, n = 3 independent experiments). (C): Immunoblot analysis of AKT and ERK phosphorylation in wild type and *Ptp4a2* null Lin$^-$ cells expressing the WT or the mutant form (KIT/D814V) of KIT in response to SCF stimulation. Data represent three independent experiments.