High expression of PIM2 induces HSC proliferation in myelodysplastic syndromes via the IDH1/HIF1-α signaling pathway

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Abstract. PIM2 proto-oncogene, serine/threonine kinase (PIM2) is a serine/threonine protein kinase that is upregulated in different types of cancer and serves essential roles in the regulation of signal transduction cascades, which promote cell survival and cell proliferation. The present study demonstrated that PIM2 was highly expressed in $CD34^+$ cells derived from the bone marrow of patients with myelodysplastic syndromes (MDS)/acute myeloid leukemia. The mRNA expression level of PIM2 was quantified in MDS cell lines and mRNA expression was significantly decreased compared with that in KG-1 cells. *In vitro*, downregulation of PIM2 by short interfering RNA (siRNA) inhibited cell proliferation and delayed G_0/G_1 cell cycle progression in the MDS cell line SKM-1. Western blotting revealed that cyclin dependent kinase 2 was markedly downregulated and cyclin dependent kinase inhibitor 1A was markedly upregulated following transfection with PIM2 siRNA. Cell Counting Kit-8 analysis demonstrated that cell proliferation of si-PIM2-transfected cells was significantly decreased compared with control cells. Reverse-transcription quantitative polymerase chain reaction and western blotting revealed that PIM2 expression was negatively correlated with isocitrate dehydrogenase [NADP(+)]1 cytosolic (IDH1) and positively correlated with hypoxia inducible factor 1 subunit α (HIF1A) in $CD34^+$ MDS cells. Collectively, these results suggested that the expression of PIM2 induced increased expression of HIF1A by decreasing the expression of IDH1, resulting in increased $CD34^+$ cell proliferation. Therefore, PIM2 may be a potential biomarker for the diagnosis of MDS and AML or a target for novel therapeutic agents.

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Introduction

PIM2 proto-oncogene, serine/threonine kinase (PIM2) was first identified in mice with lymphoma. PIM2 belongs to the proviral integration of Moloney virus (Pim) family of serine/threonine kinases, which also includes PIM1 and PIM3 (1). PIM2 serves an essential role in cell cycle regulation and cell proliferation and is involved in the malignant phenotypes of different types of cancer cells; in particular, PIM2 phosphorylates a wide range of cellular proteins and it is highly expressed in different types of tumors, including solid tumors (tumors of the prostate (2) and digestive system) (3) and hematological diseases including leukemia, lymphoma (4) and multiple myeloma (5). A previous study demonstrated that the PIM2 expression level was increased in cell lines derived from solid tumors (the A549 and H1299 lung cancer cell lines) and hematopoietic malignancies (the K562 leukemia cell line and RPMI-8226 multiple myeloma cell line) (6). The aforementioned study revealed that the downregulation of PIM2 resulted in cell cycle arrest in the G_0/G_1 phase. PIM2 regulates the cell cycle by inhibiting cyclin-dependent kinase 2 (CDK2) and phosphorylated retinoblastoma protein (pRb) expression levels via upregulation of cyclin dependent kinase inhibitor 1A (CDKN1A), which is a negative modulator of cell cycle progression. In addition, downregulation of PIM2 led to the downregulation of nuclear factor κβ (NF-κβ) which serves several roles in inflammatory responses, cell proliferation and tumorigenesis (6). PIM2 activates apoptosis inhibitor 5 (API-5) to inhibit apoptosis in hepatocellular carcinoma cells via the NF-κβ signaling pathway (7). Thus, NF-κβ may be a key downstream target of PIM2, which is consistent with the results of other studies (4). PIM2 may serve a role in the pathogenesis of myelodysplastic syndromes (MDS), a group of clonal malignant hematopoietic disorders characterized by ineffective hematopoiesis and an increased risk of malignant transformation. MDS are stem-cell disorders in which blockade of hematopoietic stem cell (HSC) maturation may give rise to different diseases, including acute myeloid leukemia (AML) (8,9).

Isocitrate dehydrogenase (IDH) mutations occur in a small proportion of patients with acute AML (10) and MDS (11). A previous study revealed an adverse prognostic effect for IDH1 mutants in MDS (12). The IDH family consists of three catalytic isozymes: IDH1, IDH2 and IDH3 (13). Overexpression of IDH1 mutants in cultured U-87MG cells

suppressed the activity of wild-type IDH1 via the formation of heterodimers, resulting in decreased levels of the enzyme product, α-ketoglutarate (α-KG), which is essential for prolyl hydroxylase (PHD) activity, which promotes hypoxia inducible factor 1 subunit α (HIF1A) degradation (14). The aforementioned results were consistent with those of a previous study, which revealed that the expression of the IDH1 R132H mutant increased HIF1A levels in cells and increased cell proliferation (14). Furthermore, it was demonstrated that the IDH1 R132H mutant activated the NF-κβ signaling pathway, which is frequently dysregulated in cancer (15). Wang *et al* (16) reported that IDH1 mutants promoted the proliferation of glioma cells via activation of NF-κβ in a HIF1A-dependent manner. HIF1A expression was correlated with poor overall survival and worse disease progression in MDS (17). In addition, the correlation analysis revealed that the expression of HIF1A was associated with the percentage of bone marrow blasts; i.e., the group exhibiting HIF1A expression had a greater percentage of bone marrow blasts (17). The results obtained in these studies suggest that there may be a correlation between HIFIA and PIM2 in the pathogenesis of MDS. The aim of the current study was to investigate the association between PIM2 and the IDH1/HIF1A signaling pathway in regulating the proliferation of HSCs in MDS.

Materials and methods

Subjects. The study included 77 participants (35 males and 42 females). A total of 23 healthy donors, 36 patients with MDS and 18 patients with AML were enrolled at the Tianjin Medical University General Hospital (Tianjin, China) between July 2017 and April 2018. According to the World Health Organization (WHO) (18), the MDS cases included 1 case of single lineage dysplasia (SLD), 1 case of 5q-syndrome, 5 cases of ring sideroblasts (RS), 5 cases of multilineage dysplasia (MLD), 7 cases of excess blasts-1 (EB-1) and 17 cases of excess blasts-2 (EB-2). The patients with MDS were divided into two groups according to the blast count: Group A, which included patients with <5% blasts (SLD, MLD, RS and 5q-syndrome) and group B, which included patients with 5-19% blasts (EB-1 and EB-2) (Table I). The present study was approved by the Ethics Committee of Tianjin Medical University General Hospital. Written informed consent was obtained from each patient.

Isolation of $CD34^+$ cells by magnetic absorption cell sorting. Bone marrow samples (10 ml) were collected from the patients and healthy donors. Mononuclear cells were isolated using lymphocyte separation medium (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and washed twice. A total of $1x10^8$ cells were resuspended in 300 μl autoMACS Running Buffer (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Hemopoietic stem and progenitor cells were purified from mononuclear cells with the CD34 MicroBeads kit (Miltenyi Biotec GmbH) according to the manufacturer's protocol. Bone marrow mononuclear cells were cultured with the CD34 MicroBeads (monoclonal mouse anti-human CD34 antibodies; isotype, mouse IgG1) and Fc receptor blocking reagent (human IgG) from the kit, for 30 min at 4˚C in the dark. $CD34^+$ cells were obtained using a magnetic absorption cell sorter, and the purity of the $CD34^+$ cells was analyzed using the CytExpert Pro analysis software (version 2.0; Beckman Coulter, Inc., Brea, CA, USA).

Cell culture and transfection. The MDS cell line SKM-1 was obtained from the National Institute of Biomedical Innovation, Osaka, Japan (19). The AML cell line KG-1 was obtained from the American Type Culture Collection (Manassas, VA, USA). These two cell lines were cultured in RPMI 1640 medium (Boehringer Ingelheim, Ingelheim am Rhein, Germany) containing 10% heat-inactivated fetal calf serum (Boehringer Ingelheim), 100 μg/ml penicillin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 100 U/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) in a humidified atmosphere at 37.5˚C and 5% CO_2. PIM2 si-RNA was transfected into the MDS cell line SKM-1 to silence PIM2 expression. The transfections were performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol with silencer-validated PIM2 siRNA (Silencer™ Select validated siRNA; Thermo Fisher Scientific, Inc.). A total of three PIM2-specific siRNAs were tested in the current study. The sequences were the following: siRNA-1 sense, 5'-CCAGCTCCACCTTCGACACC-3' and antisense, 5'-CCGGTAGTGGTCCTCATCAG-3'; siRNA-2 sense, 5'-ACCGTCACTATGGACCAGC-3' and antisense, 5'-TTC AGAGCTGGACTACATCC-3' and siRNA-3 sense, 5'-GUG CCAAACUCAUUGAUUUTT-3' and antisense, 5'-AAAUCA AUGAGUUUGGCACTT-3'. siRNA-3 resulted in the greatest knock-down efficiency and was selected for subsequent experimentation. A scrambled siRNA sequence was used as a control and had the following sequence: Sense, 5-AUCCGC GCGAUAGUACGUATT-3' and antisense, 5'-UACGUACUA UCGCGCGGAUTT-3'. The siRNAs were diluted to 20 μM with diethyl pyrocarbonate-treated water and aliquoted into a six-well plate. The siRNA master mix, which consisted of 5 μl siRNA (20 μM), 5 μl Lipofectamine 2000 and 100 μl Opti-MEM™ I Reduced Serum Medium (Gibco; Thermo Fisher Scientific, Inc.), was gently agitated and incubated for 15 min at room temperature to allow complex formation between the siRNA and lipids. The medium was removed from the wells and 1,900 μl of fresh RPMI 1640 culture medium was added to each well. The siRNA mixture (110 μl per well) was added drop by drop while gently swirling the plate. Cells were cultured for 48 h at 37.5˚C and harvested for analysis. The interference efficiency was assessed by western blotting.

Proliferation assay. Cells were seeded into 96-well plates at a density of $1.5x10^5$ cells/ml in 200 μl complete medium and incubated at 37˚C in 5% CO_2 for 1.5 h. A total of 20 μl Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) reagent was added to the wells and incubated for 1.5 h at 37˚C, and the optical density (OD) was read at a wavelength of 450 nm. This test was repeated three times with three replicates per sample. Cell proliferation was calculated using the following equation: Proliferation (%)=(OD_{450} of the experimental group/OD_{450} of the control group) x100%.

Cell cycle analysis by flow cytometry. Cell cycle analysis was performed using a FACSCalibur C6 flow cytometer (BD Biosciences, San Jose, CA, USA). Cells were fixed in 70%

Table I. Characteristics of the patients and healthy donors in the present study.

Category	n	Mean age (range), years	Sex	
			Male	Female
Patients with MDS	36	59.4 (30-84)	19	17
World Health Organisation Classification (2016)				
SLD	1	49	1	0
MLD	5	63.6 (59-67)	4	1
RS	5	67.4 (56-84)	4	1
5q-syndrome	1	63	0	1
EB-1	7	51.9 (30-66)	2	5
EB-2	17	59.1 (44-75)	8	9
Patients with AML	18	51.9 (20-78)	10	8
Healthy donors	23	47.7 (23-79)	6	17

MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; SLD, single lineage dysplasia; RS, ring sideroblasts; MLD, multilineage dysplasia; EB, excess blasts.

ethanol at 4˚C for at least 4 h and then stained with PI/RNase staining buffer (20 μg/ml propidium iodide (PI) containing 10 μg/ml RNase; BD Biosciences, San Jose, CA, USA) for 30 min at room temperature. The DNA distributions in the cells were analyzed by Modifit (v.4.0; Verity Software House, Inc., Topsham, ME, USA) to determine the proportions of cells in each phase of the cell cycle.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the $CD34^+$ cells, and the SKM-1 and KG-1 cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA (1 μg) was reverse transcribed using the SuperScript First-Strand Synthesis system (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. qPCR was subsequently performed using SYBR® Premix Ex Taq™ II (Tiangen Biotech, Co., Ltd., Beijing, China) and the Thermal Cycler Dice Real Time system (Tiangen Biotech Co., Ltd.) in a 96-well plate according to the manufacturer's protocol. The primers used were synthesized by Sangon Biotech, Co., Ltd., (Shanghai, China) and are presented in Table II. The optimized parameters for PCR were: 95˚C for 2 min, 94˚C for 10 sec, 61.5˚C for 30 sec and 72˚C for 40 sec (40 cycles). The expression level of each gene was calculated by the $2^{-\Delta\Delta Cq}$ method (20) using GAPDH as an internal control.

Western blotting. Western blot analysis was used to evaluate the PIM2, CDKN1A, CDK2, IDH1 and HIF1A protein concentrations in the SKM-1 cell extracts 48 h after siRNA transfection. Cells were lysed with a lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 100 mM NaF, 1% NP40, 1 μg/ml leupeptin, 1 μg/ml anti-pain and 1 mM phenyl methyl sulfonyl fluoride), and the protein concentration was determined using a bicinchoninic acid assay (Pierce; Thermo Fisher Scientific, Inc.). The extracted proteins (30 μg/lane) were separated via SDS-PAGE on an 8% gel. The separated proteins were subsequently transferred to polyvinylidene difluoride membranes (BioRad Laboratories, Inc., Hercules, CA, USA). PVDF membranes were blocked using a solution containing 5% skimmed milk in double distilled water (95%) and incubated for 1 h at room temperature. The membranes were incubated with primary antibodies against CDKN1A (1:1,000; cat. no. ab2947), CDK2 (1:1,000; cat. no. ab2546), IDH1 (1:1,000; cat. no. ab172964), HIF1-α (1:1,000; cat. no. ab179483), PIM2 (1:1,000; cat. no. ab97475) (all Abcam, Cambridge, UK) and GAPDH (1:1,000; cat. no. 5174; Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4˚C. The membranes were washed with Tris-buffered saline containing Tween-20 and incubated with horseradish peroxidase-conjugated anti-rabbit IgG sheep antibody (1:2,000; cat. no. ab6721; Abcam) or horseradish peroxidase-conjugated anti-mouse IgG sheep antibody (1:2,000; cat. no. ab6785; Abcam) diluted in PBS for 1 h at room temperature. Protein bands were visualized with the Immobilon Western Horseradish Peroxidase Chemiluminescence kit (EMD Millipore, Billerica, MA, USA).

Statistical analysis. All experiments were performed at least three times in triplicate for each group. SPSS software (v.19; IBM Corp., Armonk, NY, USA) was used for statistical analysis. Multiple groups were analyzed using a one-way analysis of variance (ANOVA) followed by the Tukey's test. The Student's t-test was used to compare two groups. The Spearman's correlation test was used for correlation analysis between PIM2 and IDH-1 or HIF1A. The data are presented as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

PIM2 expression is reduced in the $CD34^+$ cells of patients with MDS and the MDS cell line, compared with patients with AML and the AML cell line. Patients with MDS were stratified into two groups according to the WHO 2016 classification: Group A (SLD, MLD, RS, and 5q-syndrome, n=12) and group B (EB-1 and EB-2, n=24). The ratio of the $2^{-\Delta\Delta Cq}$

Table II. Primers used for reverse transcription-quantitative polymerase chain reaction.

Gene	Primer sequence (5'→3')	
	Forward	Reverse
Hypoxia inducible factor 1 subunit α	ACGTTCCTTCGATCAGTTGTCACC	GGCAGTGGTAGTGGTGGCATTAG
Isocitrate dehydrogenase [NADP(+)] 1, cytosolic	TCAGTGGCGGTTCTGTGGTAGAG	CATCCTTGGTGACTTGGTCGTTGG
Pim-2 proto-oncogene, serine/threonine kinase	TTGGGAAGGAATGGTAGATG	CAGGAGAACAAACAG CAAGC
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

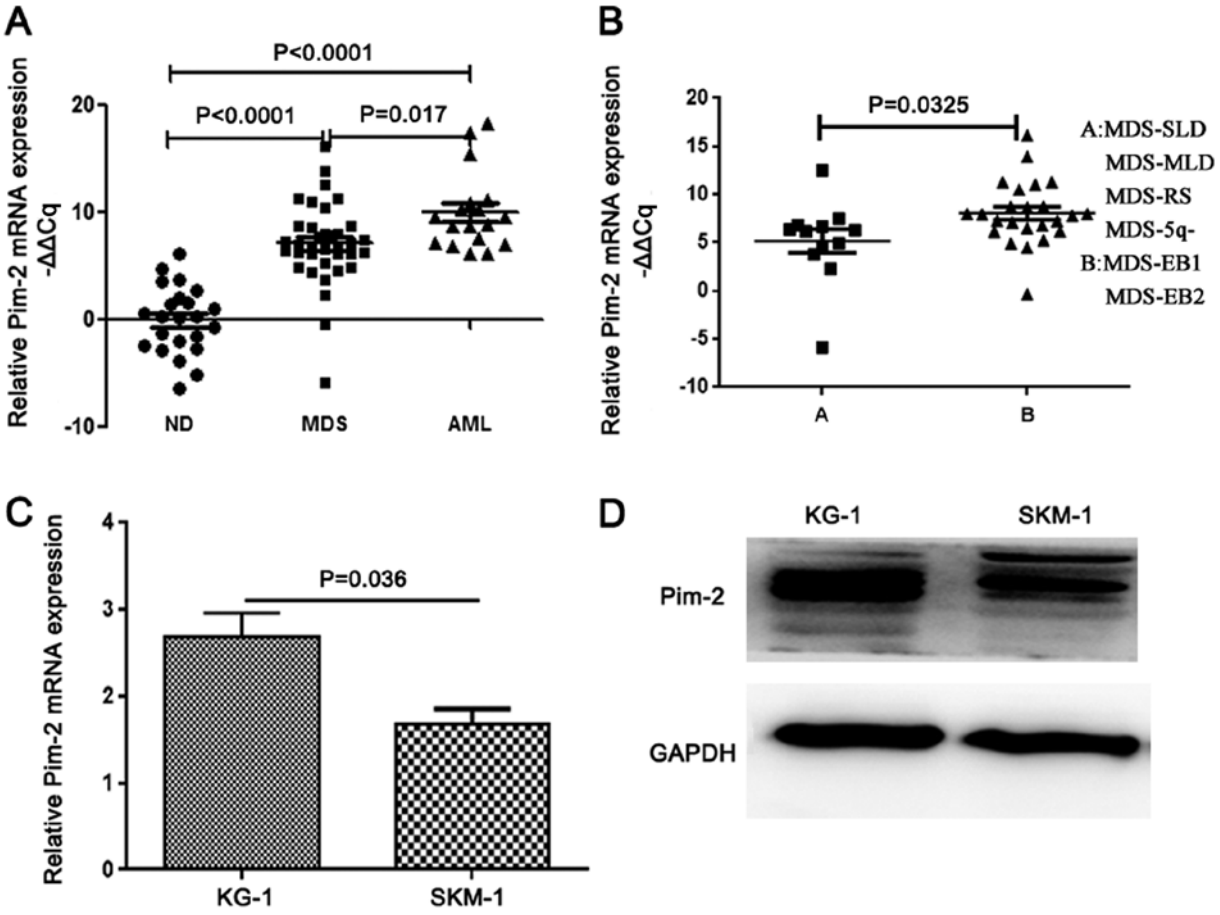

Figure 1. PIM2 expression is decreased in the CD34+ cells of patients with MDS and an MDS cell line, compared with patients with AML and an AML cell line, respectively. (A) RT-qPCR analysis of the expression level of PIM2 in CD34+ cells obtained from the bone marrow of healthy donors and newly diagnosed patients with MDS/AML. (B) RT-qPCR analysis of the expression level of PIM2 in CD34+ cells obtained from the bone marrow of newly diagnosed patients with MDS (A and B groups). (C) RT-qPCR analysis of the expression level of PIM2 in an MDS cell line (SKM-1) and an AML cell line (KG-1). (D) Western blot analysis of the PIM2 expression levels in the KG-1 and SKM-1 cell lines. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; PIM2, Pim-2 proto-oncogene, serine/threonine kinase; SLD, single lineage dysplasia; RS, ring sideroblasts; MLD, multilineage dysplasia; EB, excess blasts; ND, healthy donors.

values of PIM2 to GAPDH was reported as the relative PIM2 mRNA level. The comparisons of the PIM2 mRNA level in the different groups were performed by a one-way ANOVA followed by the Tukey's test. The expression levels of PIM2 transcripts were significantly increased in patients with MDS and *de novo* AML compared with healthy donors (P<0.0001; Fig. 1A). The PIM2 expression level was reduced in patients with MDS compared with patients with AML. Furthermore, there was a significant difference in the relative PIM2 mRNA levels between groups A and B among patients with MDS (P=0.0325; Fig. 1B). The expression level of PIM2 was also measured in the MDS cell line SKM-1 and the AML cell line

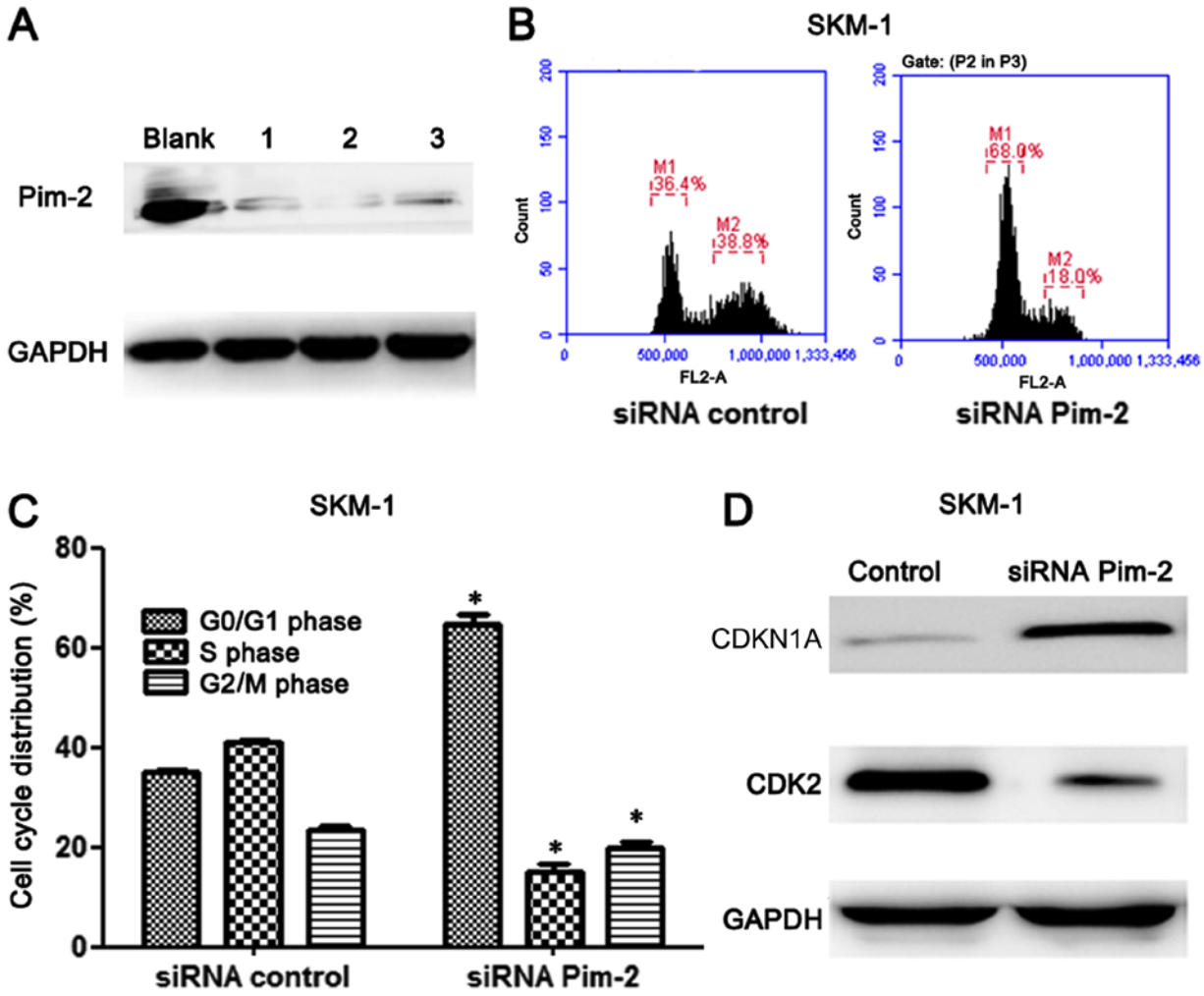

Figure 2. Silencing of PIM2 arrests MDS cells at the G_0/G_1 phase. (A) Inhibition of PIM2 expression by specific siRNAs. Western blot analysis revealed that all PIM2 siRNAs exhibited efficient inhibition of PIM2 expression in SKM-1 cells. (B) The results of cell cycle analysis by flow cytometry in SKM-1. M1 presents the cells in G_0/G_1 phase and M2 presents the cells in G2/S phase. (C) PIM2 inhibition arrested the cell cycle in G_0/G_1 phase in SKM-1 cells. The P=0.0002, P=0.00007 and P=0.049 for the G_0/G_1, S and G2/M phases, respectively). (D) The effect of PIM2 inhibition in SKM-1 cells on cell cycle regulatory protein expression levels. Western blot analysis of the cell cycle regulatory proteins CDKN1A and CDK2 in siRNA control and PIM2-silenced cells. CDKN1A was highly expressed in SKM-1 cells. GAPDH was used as an endogenous control. MDS, myelodysplastic syndrome; PIM2, Pim-2 proto-oncogene, serine/threonine kinase; siRNA, small interfering RNA; CDKN1A, cyclin dependent kinase inhibitor 1A; CDK2, cyclin dependent kinase 2 (*P<0.05 vs. siRNA control at the respective phase of the cell cycle).

KG-1 using RT-qPCR (Fig. 1C) and western blotting (Fig. 1D). Expression of PIM2 was detected in MDS cell lines; however, the levels of mRNA expression were significantly decreased compared with the AML cell line (P=0.036).

Downregulation of PIM2 kinase expression induces cell cycle arrest at the G_0/G_1 phase. To investigate the role of PIM2 in MDS, PIM2 was knocked down in SKM-1 cells. The degree of PIM2 expression knockdown by siRNA was assessed by western blotting. The PIM2-specific siRNAs markedly decreased the protein expression levels (Fig. 2A), and the third siRNA was chosen for further experiments, as it exhibited good efficiency in several cell lines (data not shown).

Cell cycle changes following inhibition of PIM2 were analyzed by flow cytometry. Cells in the G_0/G_1, S and G2/M phases were separated based on linear fluorescence intensity following staining with PI. The cell cycle analysis demonstrated a significant increase in the percentage of cells in the G_0/G_1 cell cycle phase after transfection with PIM2 siRNA compared with the percentage Pleasin the control cells (P<0.05; Fig. 2B and C; Table III). There was a significant decrease in the percentage of cells in the S phase and G2/M phase in the PIM2-silenced cells compared with the control cells (P<0.05). Therefore, downregulation of PIM2 led to an increased number of cells in the G_0/G_1 phase. A previous study revealed that the inhibition of CDK2 and pRb expression via upregulation of CDKN1A resulted in PIM2 downregulation and G_0/G_1 arrest in lung cancer and hematopoietic malignancies (6). The western blot results obtained in SKM-1 cells in the current study are consistent with the findings of previous studies. CDK2 was markedly downregulated, whereas CDKN1A was markedly upregulated following treatment with PIM2 siRNA (Fig. 2D). The CDKN1A protein, as a member of the CDK interacting protein/kinase inhibitory protein family of CDK2 inhibitors, binds to and inhibits CDK2/cyclin complexes during the G1 phase (21,22). Therefore, PIM2 may serve an important role in the regulation of the cell cycle.

Table III. Percentage of SKM-1 cells in each cell cycle phase.

siRNA	G_0/G_1 phase, %	S phase, %	G2/M phase, %
Control	35.07±1.35	41.30±0.30	23.63±1.22
PIM2	64.70±3.90	15.10±2.72	20.20±1.90

Data are presented as mean ± standard deviation. siRNA, small interfering RNA; PIM2, Pim-2 proto-oncogene, serine/threonine kinase.

Table IV. Effect of PIM2 silencing on the proliferation of SKM-1 cells. Cell proliferation was determined by the Cell Counting Kit-8 assay.

Parameter	Control siRNA	PIM2 siRNA	P-value
Proliferation,%	100±17.17	39.23±7.84	<0.0001

Data are presented as mean ± standard deviation. siRNA, small interfering RNA; PIM2, Pim-2 proto-oncogene, serine/threonine kinase.

PIM2 promotes cell proliferation via the IDH1/HIF1A signaling pathway. To determine whether downregulation of PIM2 by siRNA had an inhibitory effect on MDS cell growth, cell proliferation was assessed by the CCK-8 assay. Cell proliferation was significantly reduced in PIM2 siRNA-transfected cells when compared with control siRNA-transfected cells ($P<0.05$; Fig. 3A; Table IV). These data suggested that PIM2 may serve a role in the proliferation of MDS HSCs. To explore the underlying molecular mechanisms, the expression levels of IDH1 and HIF1A in bone marrow $CD34^+$ cells obtained from patients with MDS were investigated. The Spearman's correlation test indicated that PIM2 expression is negatively correlated with IDH1 (Fig. 3B) and positively correlated with HIF1A in patients with MDS (Fig. 3C). To further explore the molecular mechanisms, the levels of IDH1 and HIF1A proteins in an MDS cell line following treatment with PIM2 siRNA were analyzed by western blotting. IDH1 was markedly downregulated, whereas HIF1A was markedly upregulated following transfection with PIM2 siRNA (Fig. 3D).

Discussion

The current study revealed that the expression level of PIM2 was increased in cells from patients with MDS compared with the PIM2 levels in cells from healthy donors. Furthermore, the expression level of PIM2 was decreased in the $CD34^+$ cells of patients with MDS and in an MDS cell line, compared with patients with AML and the AML cell lines, respectively. PIM2 may serve a role in the pathogenesis of hematological malignancies (3). The PIM2 expression level was increased in patients with AML and acute lymphoblastic leukemia (ALL) (4). The results obtained in the present study suggest that PIM2 expression is increased in MDS. PIM2 expression was increased in patients with EB-1 and EB-2 compared with patients with other types of MDS. The downregulation of PIM2 in SKM-1 cells induced cell cycle arrest at the G_0/G_1 phase and was associated with changes in the expression of cell cycle-associated proteins, CDK2 and CDKN1A. This result is consistent with results obtained in a previously published study, which revealed that the inhibition of CDK2 and pRb expression via the upregulation of CDKN1A mediated the effects of PIM2 downregulation on G_0/G_1 arrest in lung cancer and hematopoietic malignancies. The current study demonstrated that PIM2 is required for the proliferation of MDS cells as the downregulation of PIM2 inhibited their proliferation. A previous study in AML cell lines suggested that NF-κβ may be a downstream factor in the PIM2 signaling pathway (6). Furthermore, PIM2 activated API-5 to inhibit apoptosis in hepatocellular carcinoma cells via the NF-κβ signaling pathway (7).

The present study aimed to investigate the action of PIM2 in the pathogenesis of MDS. MDS is a stem-cell disorder in which maturation blockade of HSCs results in the disease, similar to the pathology in AML (8,9). The present study analyzed the expression level of PIM2 in $CD34^+$ cells extracted from the bone marrow of healthy donors and patients with MDS and AML by RT-qPCR. These data suggested that PIM2 transcripts were significantly increased in patients with MDS and *de novo* AML compared with healthy donors, and the transcript levels were increased in patients with AML compared with patients with MDS. Furthermore, there was a significant difference between the two groups of patients with MDS, PIM2 levels were increased in patients with EB-1 and EB-2 compared with SLD, MLD, RS and 5q-. These data suggested that PIM2 serves an important role in the pathogenesis of MDS. Therefore, the present study tested this hypothesis.

Flow cytometry analysis revealed that a greater percentage of SKM-1 cells transfected with PIM2 siRNA were in the G_0/G_1 phase of the cell cycle compared with control siRNA-transfected SKM-1 cells. There was a significant decrease in the percentage of cells in S and G2/M phases in the PIM2 siRNA-transfected cells compared with control siRNA-transfected cells. This indicated that PIM2 may promote cell cycle arrest at the G_0/G_1 phase.

To explore the molecular mechanisms underlying the effects of PIM2 on the cell cycle, the current study investigated the protein expression levels of the cell cycle regulator CDK2 and its inhibitor CDKN1A (21,22) by western blotting. The results revealed that CDK2 expression was downregulated and CDKN1A was upregulated in the SKM-1 cells transfected with PIM2 siRNA. The decreased level of CDK2 and increased level of CDKN1A suggested a possible mechanism by which cell-cycle arrest is induced at the G_0/G_1 phase following deregulation of PIM2 expression. Downregulation of PIM2 may serve a key role in the G_0/G_1 arrest via upregulation of CDKN1A to inhibit the expression of CDK2.

The CCK-8 assay revealed a significant decrease in the proliferation rate of PIM2 siRNA-transfected SKM-1 cells compared with the proliferation of control siRNA-transfected SKM-1 cells. The effect of PIM2 on the cell cycle suggested increased cell proliferation; however, there may be another mechanism through which PIM2 influenced the proliferation of HSCs. Kapelko-Slowik *et al* (4) reported that the expression of PIM2 altered the NF-κβ pathway, resulting in the development of AML and ALL. A previous study on AML cell lines suggested

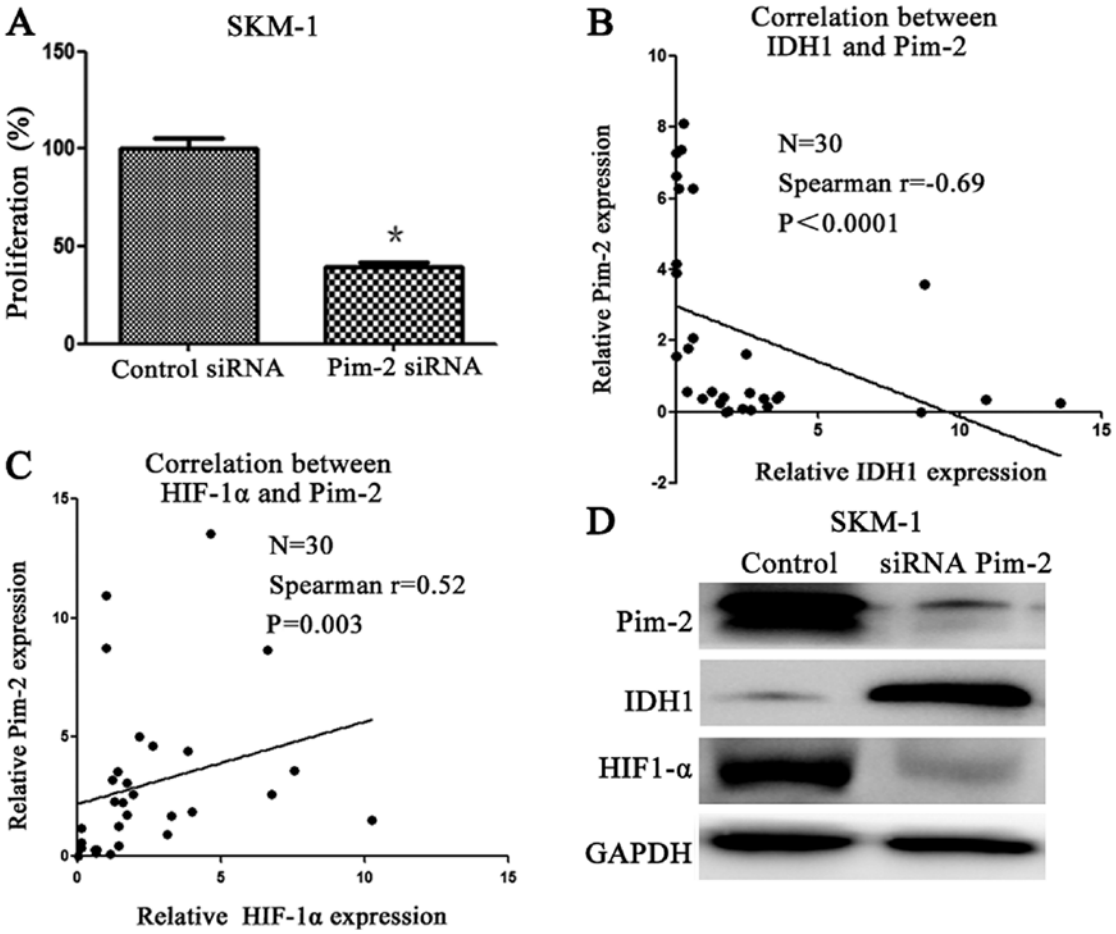

Figure 3. Downregulation of PIM2 inhibits the proliferation of MDS cell lines via the IDH1/HIF1A signaling pathway. (A) Cell proliferation was determined by the Cell Counting Kit-8 assay. Downregulation of PIM2 inhibited the proliferation of the SKM-1 cell line. The results are presented as the mean ± standard deviation of three replicates, and are representative of three independent experiments. *P<0.05 vs. control siRNA. Reverse transcription-quantitative polymerase chain reaction revealed that the expression of PIM2 is (B) negatively correlated with IDH1 and (C) positively correlated with HIF1A in patients with MDS. (D) Western blotting revealed that inhibition of PIM2 expression led to increased expression of IDH1 and decreased expression of HIF1A. MDS, myelodysplastic syndrome; PIM2, Pim-2 proto-oncogene, serine/threonine kinase; siRNA, small interfering RNA; IDH1, isocitrate dehydrogenase [NADP(+)]1, cytosolic; HIF1A, hypoxia inducible factor 1 subunit α.

that the NF-κβ signaling pathway may serve a role in the action of PIM2 on cell proliferation (6). The current study revealed that PIM2 expression is negatively correlated with IDH1 and positively correlated with HIF1A in patients with MDS. To further confirm the RT-qPCR results, protein expression in a PIM2 siRNA-transfected cell line was analyzed by western blotting. IDH1 was markedly downregulated and HIF1A was markedly upregulated in cells transfected with PIM2 siRNA. A previous study revealed that upregulated HIF1A was correlated with poor overall survival time and disease progression (17). In addition, correlation analysis revealed that the expression of HIF1A was positively correlated with the percentage of bone marrow blasts (17). This suggested that HIF1A may accelerate the proliferation of HSCs. The overexpression of IDH1 mutants in cultured U-87MG cells suppressed the activity of wild-type IDH1 via the formation of heterodimers, resulting in a decrease of the enzyme product, α-KG (14). α-KG is required for PHD activity, which promotes HIF1A degradation (14). The forced overexpression of the IDH1 mutant activated NF-κβ in a HIF1A-dependent manner and was involved in the regulation of cell proliferation (16). Previous studies have reported IDH1 mutants in patients with MDS and AML (12,23). However, the MDS patients in the aforementioned studies had a low frequency of IDH1 mutations, implying that different pathways may result in the reduction of IDH1 expression. The results obtained in the present study substantiated the initial hypothesis that PIM2 expression is negatively correlated with IDH1 and positively correlated with HIF1A in MDS HSCs. PIM2 upregulation may lead to increased expression of HIF1A via downregulation of IDH1. However, a limitation of the current study was that the direct role of PIM2 in IDH1 expression was not investigated. A future study is required to investigate the molecular mechanism, via chromatin immunoprecipitation or co-immunoprecipitation assays, for example.

Taken together, the present study demonstrated that the expression level of PIM2 was reduced in patients with MDS compared with patients with AML, but increased compared with the healthy donor group. These results suggested that PIM2 upregulated HIFA by downregulating IDH1, resulting in increased proliferation of HSCs. Novel therapeutic agents that suppress PIM2 may be effective for the treatment of MDS and hematopoietic malignancies, and therefore future studies are required to elucidate the underlying mechanisms behind PIM2 inhibitors and their effects on MDS cells.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

ZL and RF designed the study. ZL and MT performed the experiments, analyzed the data, generated the figures and drafted the manuscript. MT contributed to cell culture and western blotting. YW contributed to RT-qPCR and flow cytometry. KD and HL collected the data of patients and healthy donors. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Tianjin Medical University General Hospital (ethical no. IRB2017-YX-041). Written informed consent was obtained from each patient for any study-specific experiments being performed.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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