

PIM3 Promotes the Proliferation and Migration of Acute Myeloid Leukemia Cells

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Purpose: Acute myeloid leukemia (AML) is associated with a poor overall prognosis. PIM family genes, including *PIM1*, *PIM2*, and *PIM3*, are proto-oncogenes that are aberrantly overexpressed in different types of human cancers. In this study, we aimed to explore and clarify the function of *PIM3* in AML.

Patients and Methods: The expression of the three *PIM* genes in AML was detected using the Gene Expression Omnibus. The expression of *PIM3* and PIM3 in patient samples and AML cell lines was measured using quantitative real-time polymerase chain reaction or Western blot analyses. The cellular behaviors of *PIM3*-overexpressing AML cell lines were detected using a CCK-8 assay, flow cytometry, Western blotting, immunofluorescence staining, and a cell migration assay. The interactions between PIM3 and phosphorylated CXCR4 (pCXCR4) were explored via immunoprecipitation.

Results: Higher *PIM3* expression was detected in primary AML cells than in healthy donor cells. Second, *PIM3* overexpression promoted AML cell proliferation and protected against spontaneous apoptosis by phosphorylating BAD (pBAD) at Ser112. Furthermore, *PIM3* overexpression might promote the migration of AML cells via CXCR4. *PIM3*-overexpressing AML cell lines exhibited increased CXCR4 phosphorylation at Ser339, and pCXCR4 interacted with PIM3.

Conclusion: Our findings suggest that PIM3 regulates the proliferation, survival, and chemotaxis of AML cell lines. Moreover, pCXCR4 might mediate the regulation of PIM3-induced chemotaxis. Therefore, the inhibition of *PIM3* expression may be a promising therapeutic target in AML.

Keywords: pBAD, pCXCR4, *PIM1*, *PIM2*, *PIM3*

Introduction

Acute myeloid leukemia (AML), a malignancy with a poor overall prognosis, is characterized by the expansion of malignant myeloid precursor cells in the peripheral blood and bone marrow.¹ Provirus integrating site moloney murine leukemia virus (PIM) family genes, including *PIM1*, *PIM2*, and *PIM3*, encode serine/threonine kinases that integrate into cell signaling networks and regulate various cell behaviors, such as proliferation, survival, and migration.²⁻⁵ Previous research findings suggest that PIM family genes are proto-oncogenes and are expressed aberrantly in different human cancers.

Of the three known *PIM* genes, Pim1 has been intensively investigated. Overexpression of this gene has been reported in prostate cancer,⁶ pancreatic cancer,⁷ squamous cell carcinoma,⁸ gastric cancer,⁹ diffuse large B-cell lymphoma,¹⁰ acute myeloid leukemia (AML),¹¹ multiple myeloma, and other malignancies. High *PIM1*

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expression has been linked to cancer progression and poor treatment outcomes.¹² *PIM2* expression is strongly elevated in AML cells and multiple B-cell tumors, including chronic lymphocytic leukemia and nested cell lymphoma.^{13–15} In multiple myeloma, *PIM2* is highly expressed and regulates mTOR-C1 by phosphorylating TSC2 to promote the proliferation of MM cells.^{16,17} Therefore, PIM kinases, particularly PIM1, are considered promising anti-cancer therapeutic targets.

PIM3 shares a high level of amino acid sequence similarity with PIM1 and PIM2.^{3,18,19} Forced PIM3 overexpression also promotes cell growth, and aberrant expression of this kinase has been observed in many human cancers. However, PIM3 expression appears to exhibit a different pattern of tissue specificity; for example, PIM1, but not PIM3, is expressed in human colon, pancreas, liver, and small intestine.^{19,20} This expression pattern suggests that PIM3 has unique functions under physiological conditions. However, the expression and function of PIM3 in leukemia are relatively less understood. Previously, Ishikawa et al observed that PIM3 knockdown suppressed cell growth in T cell leukemia cell lines,²¹ whereas Zhou et al compared PIM3 expression in AML patients before and after chemotherapy and observed that changes in this parameter during the treatment correlated with the patient's remission status.²² In this study, we performed both clinical data analyses and cell line studies to further elucidate the function of PIM3 in AML, and observed regulatory effects on proliferation, survival and chemotaxis.

Patients and Methods

Patient Samples

Bone marrow samples were collected from 40 patients with AML who were hospitalized at West China Hospital, Sichuan University, China, as well as from 26 healthy volunteers. All the patients with AML had bone marrow blast frequencies >50%. Mononuclear cells were isolated from the samples by Ficoll density gradient centrifugation. These specimens were collected in the early time. Informed consent was obtained and the study protocol was approved by the Ethical Committee of West China Hospital of Sichuan University and conformed to the Declaration of Helsinki.

Cell Lines

The K562, U937, and THP-1 human leukemia cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). All cells were maintained in RPMI-

1640 medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) at 37°C and 5% CO₂.

Cell Treatments

The pCDNA4 plasmid backbone ligated with *PIM3* cDNA was kindly provided by Kanazawa University, Japan. *PIM3* cDNA, including the open reading frame, was subcloned into the pIRES2-EGFP vector. The resulting construct was transfected into K562 cells to induce PIM3 overexpression (Pim3-OE) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. K562 cells were also transfected with a vector overexpressing GFP only as a control (CTR-OE). The cells were incubated with normal growth medium for another 24 hours prior to Western blotting, immunoprecipitation, immunofluorescence staining, cell proliferation, and apoptosis assays. The lentiviral vector LV-Pim3 (engineered to overexpress *PIM3*) and a negative control vector (LV-NC) were purchased from Western (Chongqing, China). U937 cells were infected with LV-Pim3 or LV-NC using polybrene (Sigma, St. Louis, MO, USA) to establish U937-CTR and U937-Pim3 cell lines, as previously described.²³

Real-Time Quantitative PCR

Total RNA was isolated from the cells using TRIzol agent (Life Technologies, Carlsbad, CA, USA) According to the manufacturer's protocol. Next, cDNA was synthesized using the PrimerScript RT Kit (Takara, Shiga, Japan). For each real-time quantitative PCR (RT-PCR), 50 ng cDNA were mixed with SYBR Green PCR MasterMix (Takara). The reactions were run in a CFX96 real-time PCR thermocycler (Bio-Rad, US). The following oligonucleotides were used as RT-PCR primers:

Pim3 forward, 5'-GCACACACAATGCAAGTCCT-3'

Pim3 reverse, 5'-AGAGGCAGACTGCTCAGAGG-3'

GAPDH forward, 5'-ACCACAGTCCATGCCATCAC-3'

GAPDH reverse, 5'-TCCACCACCCTGTTGCTGTA-3'

Western Blotting and Immunoprecipitation

The cells were harvested, washed with ice-cold phosphate-buffered saline (PBS) and lysed in lysis buffer (Beyotime, Shanghai, China). Target proteins were analyzed by Western blotting as previously described.¹⁰ Antibodies specific for phosphorylated BAD (pBAD)(Ser112) and total BAD were purchased from Cell Signaling, Inc. (Danvers, MA, USA).

Antibodies specific for pCXCR4 (Ser339) and CXCR4 were purchased from Abcam, Inc. (Cambridge, UK). A Pim3-specific antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

For immunoprecipitation, the cells were lysed as described above.²⁰ The lysates were pre-cleaned by incubation with protein A/G agarose beads for 3 hours at 4°C and then incubated with a pCXCR4 (Ser339) antibody overnight at 4°C. The protein A/G beads were added to the mixture, which was incubated at 4°C for 3 hours. The beads were washed 5 times with lysis buffer, resuspended in 2×loading buffer, and boiled for 10 min before Western blotting analyses.

Immunofluorescence Staining and Confocal Microscopy

The cells were adhered to a glass slide, fixed with 4% formaldehyde for 5 min on ice, and washed 3 times with ice-cold PBS. Next, the cells were permeabilized with 0.1% Triton X-100 for 5 min and blocked with 5% bovine serum albumin in PBS at room temperature. After a washing step, the cells were incubated with anti-pBAD (Ser112), and anti-PIM3 antibodies overnight at 4°C. After washing, the cells were incubated with fluorescently labeled secondary antibodies (Invitrogen). The cell nuclei were stained with DAPI. Fluorescence staining was visualized using a confocal microscope (Carl Zeiss, Oberkochen, Germany).

Flow Cytometry Analysis

Flow cytometry was used to examine the expression of cellular proteins, as previously described.²⁴ Antibody specific for pCXCR4 (Ser339) was purchased from Abcam, Inc. (Cambridge, UK). The labeled cells were analyzed using a Navios flow cytometer (Beckman Coulter, Brea, CA, USA).

Cell Proliferation and Apoptosis Assays

The cells were seeded in 96-well plates at a density of 4000 cells in 100 μ L medium/well, and were cultured for 24 hours, 48 hours, 72 hours, 96 hours and 120 hours. To examine cell proliferation, 10 μ L of CCK-8 solution were added to each well, and incubated for 2 hours. The absorbance in each well at 450 nm was examined using a microplate reader. To evaluate cell apoptosis, the cells were harvested and stained using an Annexin-V Alexa Fluor 647/PI kit according to the manufacturer's instructions (4A Biotech, Beijing, China). The cells were analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA).

Cell Migration Assay

To examine cell migration, 2×10^4 cells were loaded into the upper chamber of a two-chamber Transwell with a pore size of 8.0 μ m (Costar, Corning, NY, USA) and allowed to migrate toward a SDF1a (10 nM) gradient for 4 hours as previously described.²⁵ The cells that migrated to the lower layer were then subjected to a CCK-8 viability analysis.

Bioinformatics and Statistics

The AML GEP dataset GSE13159 was downloaded from the NCBI Gene Expression Omnibus database.²⁶ Clinical information about the patients in the dataset was downloaded from Oncomine (www.oncomine.org). All expression values were reported as means \pm standard errors of the means of multiple experiments or as means \pm standard deviations from representative experiments. Student's *t* test was used to compare two experimental groups, and a *p* value of <0.05 was considered statistically significant.

Results

PIM3 Expression in Adult AML

We initially performed a microarray-based analysis using the GSE13159 dataset to examine the expression of *PIM3* in adult AML cells. Notably, stronger *PIM3* expression was observed in these cells than in peripheral blood mononuclear cells (PBMCs) collected from healthy donors (Figure 1A, $p < 0.001$). Next, we examined the expression of *PIM1* and *PIM2* using the same dataset and sorted the results by *PIM3* expression. As shown in Figure 1B, we observed no correlation between the expression of *PIM3* and that of *PIM1* or *PIM2*, suggesting that the gene expression each PIM family member might be regulated differently. Finally, we validated our microarray-based analysis findings using AML patient samples from West China Hospital. As shown in Figure 1C, stronger *PIM3* expression was observed in patient samples than in bone marrow mononuclear cells from healthy volunteers (The fold-changes: 2.227 ± 0.4998 versus 0.8667 ± 0.09480 ; $p < 0.05$). Overall, our results demonstrate increased *PIM3* expression in AML.

Forced PIM3 Overexpression in AML Cell Lines

Next, we examined PIM3 expression in human leukemia cell lines to investigate the potential role of this protein in AML. Quantitative real-time PCR (qRT-PCR) and Western blotting analyses revealed that the K562, U937 and THP-1

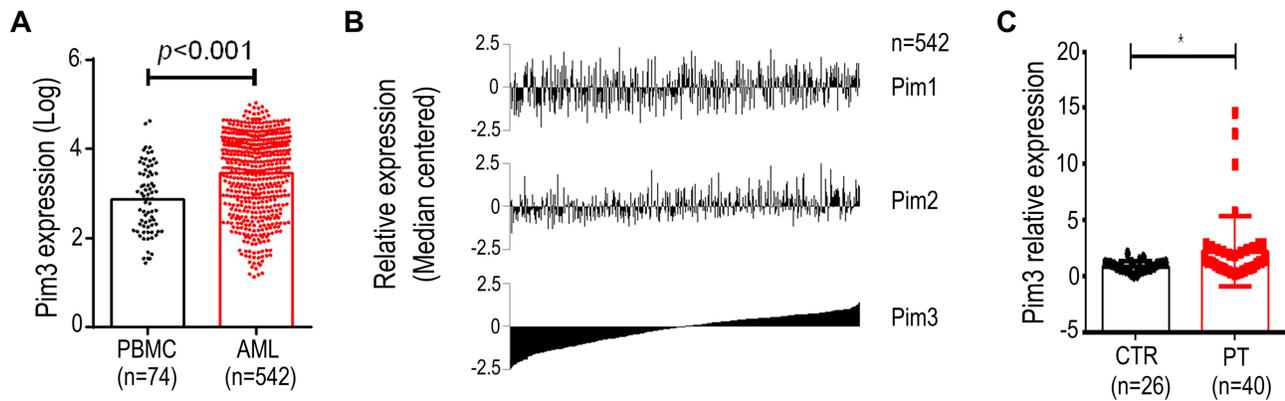


Figure 1 PIM3 expression in adult acute myeloid leukemia (AML). Microarray-based analysis of PIM family gene expression in AML patient samples. **(A)** PIM3 expression in peripheral blood mononuclear cells from healthy donors versus AML cells. **(B)** Expression of *PIM1*, *PIM2*, and *PIM3* in AML patient samples. **(C)** Expression of *PIM3* mRNA in AML cells from patients (PT) and normal bone marrow mononuclear cells (CTR) from healthy donors via qRT-PCR analysis. * $p < 0.05$.

leukemia lines all expressed PIM3, although K562 cells expressed a relatively lower level (Figure 2A and B). In addition, the patterns of *PIM1* and *PIM3* expression differed among the tested cell lines (Figure 2B). Next, we established transient control-overexpressing (CTR-OE, overexpressing GFP only) and PIM3-overexpressing (Pim3-OE) K562 cells (Figure 2C, left panel) and compared these to normal K562 cells (blank CTR). The construction and transfection of vectors are described in the Material and Methods section. Meanwhile, U937 cells were infected with lentiviral to generate U937-Pim3 cells with consistent overexpressing PIM3 (Figure 2C, right panel).

PIM3 Overexpression Promotes AML Cell Proliferation

Next, we examined cell proliferation in PIM3-overexpressing cells using the CCK8 method. Notably, PIM3 overexpression promoted the proliferation of K562 cells in a time-dependent manner, as demonstrated by a higher proliferation rate relative

to the control group after 72 hours of culture (Figure 3A). Similar results were observed in U937 cell line (Figure 3B). Next, we examined the effect of PIM3 on the spontaneous apoptosis of K562 cells. As shown in Figure 3C, leukemia cells exhibited increased apoptosis during culture. However, Pim3-OE cells exhibited a significant lower rate of cell apoptosis than CTR-OE cells, as presented quantitatively in Figure 3D ($p < 0.05$).

Data from previous studies of other cells suggest that PIM kinases promote cell survival by regulating the activity of BAD,^{27–29} a known pro-apoptotic factor that interacts with and attenuates the anti-apoptotic activities of BCL-2 family members. BAD can be phosphorylated at different residues, which blocks BCL-2 binding activity and thus promotes cell survival.^{4,29} However, it remained unclear whether PIM3 promoted BAD phosphorylation in AML. We observed that in AML cells, PIM3-overexpression was associated with an increased level of pBAD (Ser112) (Figure 4A), but did not affect the levels of total Bad and cMyc. Interestingly, PIM3

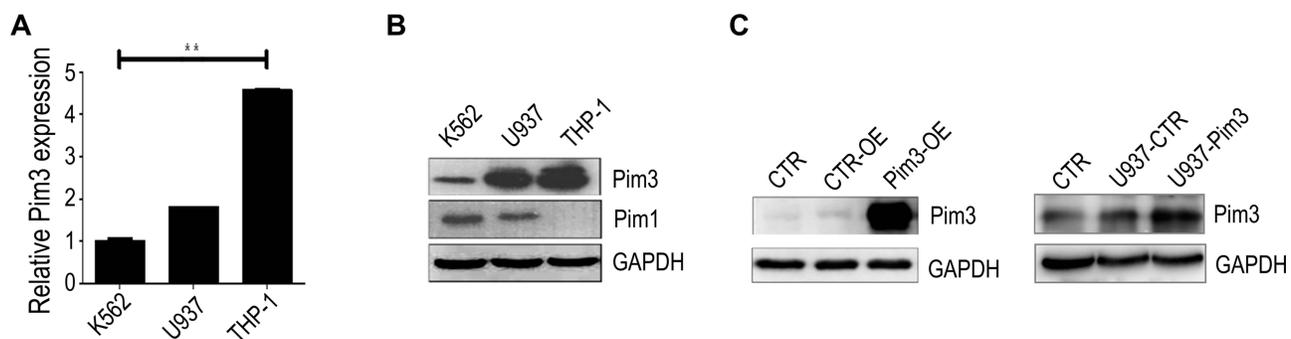


Figure 2 PIM3 expression in human acute myeloid leukemia (AML) cell lines. **(A)** Expression of *PIM3* mRNA in K562, U937, and THP-1 cells analyzed by qRT-PCR. **(B)** Western blotting of PIM3 and PIM1 expression in different human AML cell lines. **(C)** Left panel: Western blotting of PIM3 in control (CTR-OE) versus PIM3-overexpressing (Pim3-OE) K562 AML cells. Blank CTR: normal K562 cells. Right panel: Western blotting of PIM3 in control (U937-CTR) versus PIM3-overexpressing (U937-Pim3) U937 AML cells. Blank CTR: normal U937 cells. ** $p < 0.01$.

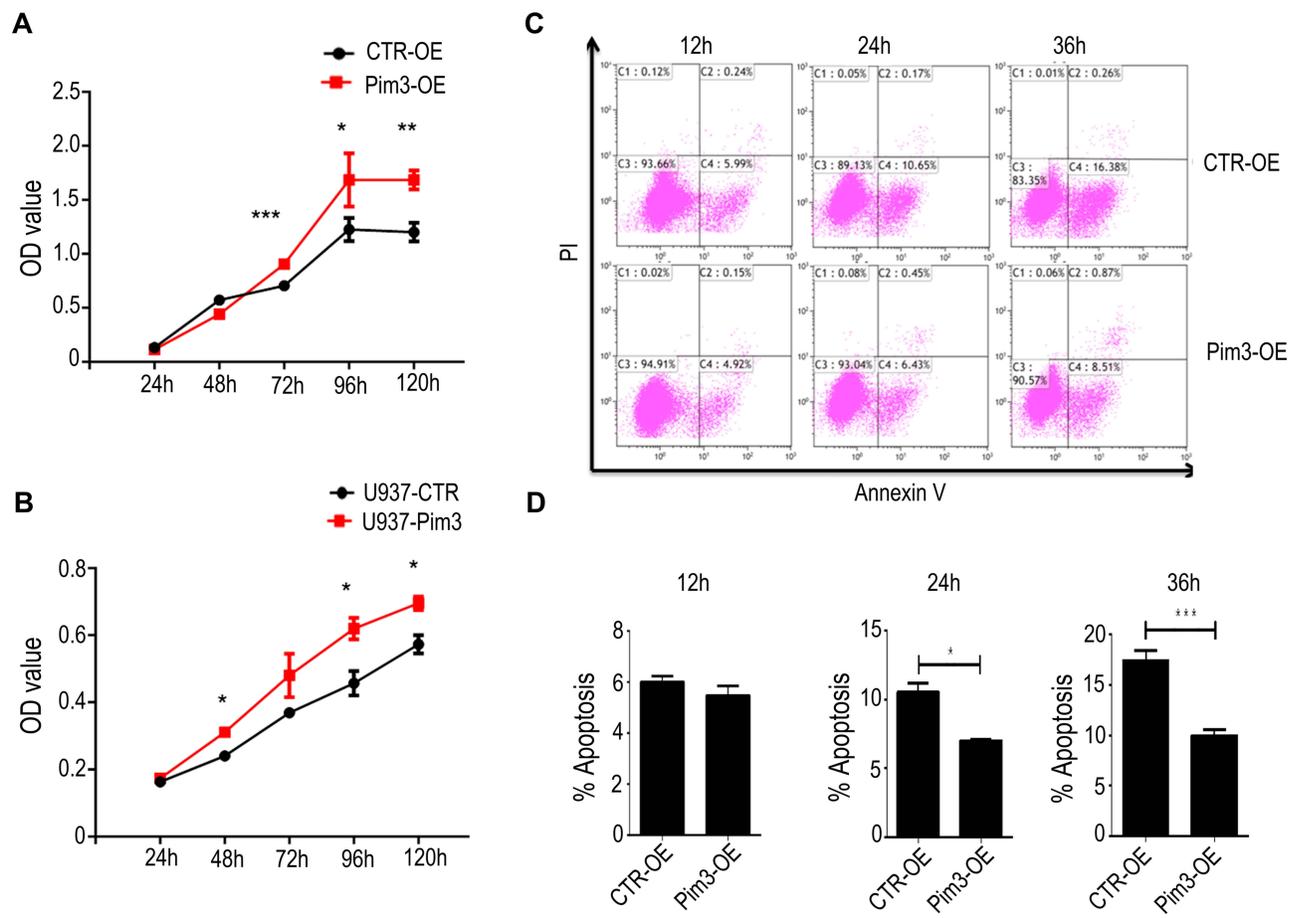


Figure 3 PIM3 overexpression promotes acute myeloid leukemia (AML) cell proliferation and survival. **(A)** Growth of control (CTR-OE) and PIM3-overexpressing (Pim3-OE) K562 AML cells. **(B)** Growth of control (U937-CTR) and PIM3-overexpressing (U937-Pim3) U937 AML cells. **(C)** The apoptotic cells were detected by staining with annexin V and PI and flow cytometry. The results are quantified in **(D)**. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

overexpression also increased the level of pCXCR4 (Ser339) but did not affect the level of total CXCR4. We further examined the level of pBAD using immunofluorescent staining. As shown in [Figure 4B](#), PIM3-overexpressing cells produced stronger PIM3 and pBAD (Ser112) signals, and these proteins were found to colocalize. In summary, we observed that the overexpression of PIM3 promoted the proliferation and survival of AML cells, possibly by inducing the phosphorylation of BAD.

PIM3 Overexpression Promotes AML Cell Migration

As shown in [Figure 4A](#), PIM3-overexpressing K562 cells contained an increased level of pCXCR4 (Ser339). Similar results were observed in U937 cells ([Supplementary Figure 1A](#)). We additionally performed flow cytometry to detect the expression of pCXCR4 (Ser339) on the cell surface. Our results demonstrated a significantly higher level of pCXCR4 (Ser339) in Pim3-OE cells ([Figure 5A](#);

quantitative results in [Figure 5B](#)). Next, we used Western blotting to detect the expression of PIM3 and pCXCR4 (Ser339) and further verify the relationship between these molecules in AML patients. We discovered significantly higher levels of pCXCR4 (Ser339) in Patient 5 and Patient 8 (PIM3-overexpressing) than in healthy volunteers (CTR) and other AML patients ([Figure 5C](#)), consistent with the results obtained in vitro. Furthermore, the results of an immunoprecipitation experiment suggested that Pim3 and pCXCR4 (Ser339) formed a complex in K562 cells and U937 cells ([Figure 5D, E](#) and [Supplementary Figure 1B, 1C](#)).

CXCR4 is the receptor that binds SDF-1 α . Based on the above results, we hypothesized that the regulatory effect of PIM3 on AML cell migration might be mediated by CXCR4. To investigate the exact biological role of PIM3 in AML, we also examined the migration of PIM3-overexpressing AML cells in response to a gradient of the chemotactic factor SDF-1 α , as described in the Material

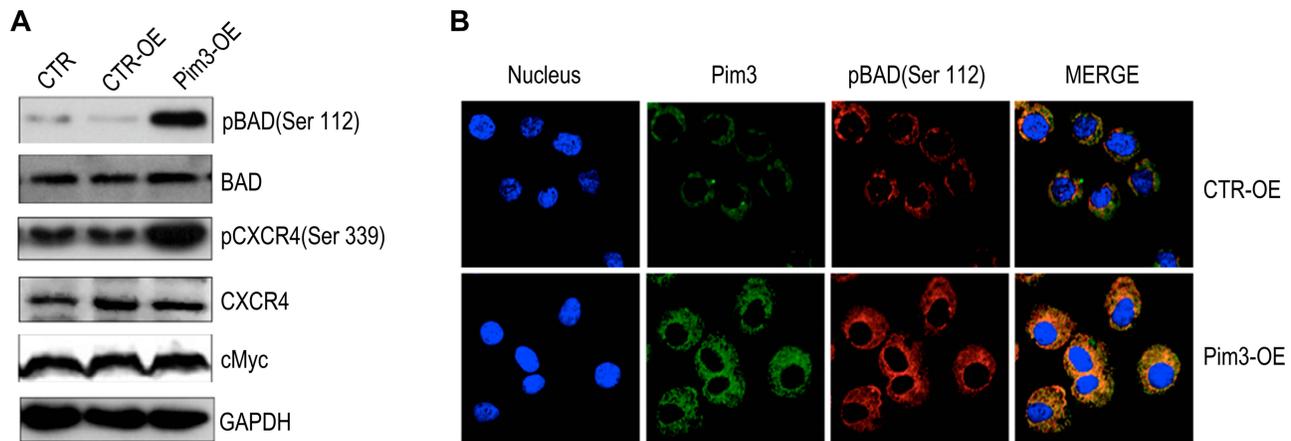


Figure 4 PIM3 promotes BAD phosphorylation in acute myeloid leukemia (AML) cells. **(A)** Phosphorylated BAD (pBAD) (Ser112), BAD, pCXCR4 (Ser339), CXCR4 and cMyc were detected in control (CTR-OE) versus PIM3-overexpressing (Pim3-OE) K562 AML cells by Western blotting. Blank CTR: normal K562 cells. **(B)** Confocal fluorescence microscopy analysis of immunofluorescently stained cells. Red, green, and blue correspond to pBAD (Ser112), PIM3 and DAPI (nuclear) staining.

and Methods section. However, U937 cells were used because K562 exhibited a poor chemotactic response. Representative photographs of the migrated cells are

shown in [Figure 5F](#). A CCK8 assay of cells in the lower chamber revealed that PIM3-overexpressing U937 cells exhibited increased migration ([Figure 5G](#)).

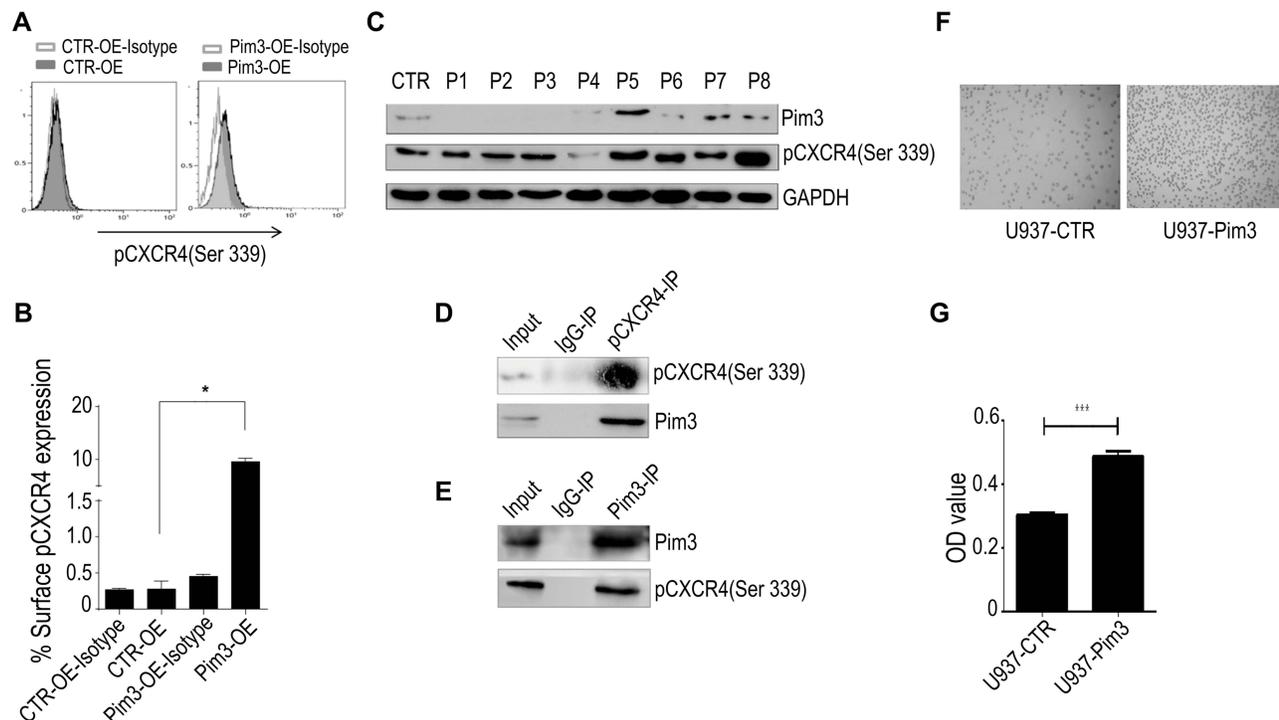


Figure 5 PIM3 promotes the chemotaxis of acute myeloid leukemia (AML) cells via CXCR4. **(A)** Flow cytometry analysis of surface pCXCR4 (Ser339) expression on CTR-OE and Pim3-OE K562 AML cells. **(B)** Quantitative analysis of results from **(A)** and **(C)** Western blotting of PIM3 and pCXCR4 (Ser339) in samples from AML patients and healthy volunteers. **(D)** Immunoprecipitation of PIM3 and pCXCR4 (Ser339) and Western blotting of pCXCR4 (Ser339) and PIM3 (upper and lower panels, respectively). **(E)** Immunoprecipitation of PIM3 and pCXCR4 (Ser339) and Western blotting of PIM3 and pCXCR4 (Ser339) (upper and lower panels, respectively). **(F)** Imaging of control (U937-CTR, left panel) and PIM3-overexpressing (U937-Pim3, right panel) U937 cells subjected to a migration assay. **(G)** The cells in the lower migration chambers were collected and subjected to a quantitative CCK8 assay. * $p < 0.05$, *** $p < 0.001$.

Discussion

Very few previous studies have evaluated the expression and function of PIM3 in AML.^{21,22} Here, we revealed a high level of PIM3 expression in AML cells relative to healthy donor PBMCs. Moreover, we observed distinctive expression patterns of PIM1, PIM2, and PIM3 in AML,^{10,11,13,22} and our results suggest that PIM3 expression is subject to regulation via a unique upstream process. A previous study of the PIM3 promoter revealed a canonical TATA box and binding sites for the transcription factors Stat3, Sp1, Ets1 and NF- κ B.³⁰ In contrast, PIM1 promoter activity is regulated by NF1,³¹ HOXA9,³² ERG,³³ and some other downstream factors in the Akt pathway. These differences in promoter regulatory elements might explain the differential regulation of these 2 PIM kinases in AML cells. Furthermore, the differential expression of PIM3 among different patients with AML indicated a transcription-level regulation of this gene. In summary, multiple transcription factors appear to determine the expression of PIM3. In this study, we focused mainly on the function of PIM3 in the regulation of AML cell proliferation and survival. Notably, forced overexpression of PIM3 promoted the proliferation and survival of PIM3^{L-ow} K562 AML cells. BAD is a member of the BCL-2 family of proteins, which play crucial roles in apoptosis regulation. Some proteins within this family have proapoptotic effects (eg, BIM, BAD, BAX, BAK), whereas others play antiapoptotic roles (eg, BCL-2, BCL-xL, MCL-1, A1, BAG-1).²⁸ Previous studies revealed that PIM kinases can inactivate BAD via phosphorylation, retain the expression of BCL-xL and ultimately inhibit the apoptosis of cancer cells. Furthermore, PIM kinases phosphorylate BAD at multiple sites, including the Ser112 gatekeeper site, Ser136, and Ser155.²⁷ Like other PIM kinases, PIM3^{high} promoted cell survival in a process mediated by BAD phosphorylation. In this study, we demonstrated for the first time that PIM3 overexpression stimulates BAD phosphorylation in AML, consistent with similar findings in many solid tumors, such as hepatocellular carcinoma,³⁴ pancreatic cancer,³⁵ colon cancer and gastric adenoma.²⁰ We further observed that PIM3 overexpression promoted AML cell migration. Previous studies demonstrated that PIM1 stimulated the surface expression of CXCR4 and promoted the migration and homing of AML cells to the bone marrow.^{36,37} The SDF1a-CXCR4 axis plays a critical role in AML cell migration,³⁸ and our findings indicate that PIM3 interacts

with pCXCR4 in AML cells. Notably, the overexpression of PIM3 upregulated the level of pCXCR4 (Ser339) without affecting the level of total CXCR4. CXCR4 contains many potential phosphorylation sites, each of which is responsible for different molecular functions. A previous study suggested that the phosphorylation of CXCR4 at Ser339 was catalyzed primarily by the kinase GRK6.⁵ Our observations that PIM3 overexpression increased pCXCR4 (Ser339) in AML cells and that these two proteins were co-immunoprecipitated strongly suggest a functional crosstalk between PIM3 and pCXCR4 (Ser339). We are conducting further investigations to demonstrate the mechanism by which PIM3 functionally interacts with and regulates CXCR4.

PIM family kinases are considered therapeutic targets in many human cancers.^{3,5,12,19} Currently, several PIM kinase inhibitors are under investigation in clinical or pre-clinical trials. In particular, increasing evidence suggests that PIM1 targeting may enhance the treatment of FLT3-ITD-positive AML.^{36,39} Other studies demonstrated that PIM1 plays a critical regulatory role in the homing and migration of hematopoietic stem cells via CXCR4,³⁶ which itself has been identified as a pivotal factor in the homing and maintenance of cancer stem cells. Therefore, PIM inhibitors may exert dual antitumor effects by targeting tumor cells and interfering with the microenvironment. Our findings suggest that PIM3 and other PIM kinases have partially overlapping and partially distinct patterns of expression and mechanisms of regulation and downstream function. Mikkers et al demonstrated the selective activation of PIM3 in Myc transgenic mice that lacked PIM1 and PIM2, and suggested that PIM3 may replace or compensate for the functions of the missing Pim kinases.⁴⁰ Other researchers believe that other PIM kinases or complementary mechanisms may remain to be discovered.² Collectively, these findings suggest that it may be more beneficial to target all the PIM genes and proteins, rather than selectively suppressing one or two family members. The development of an efficient PIM targeting strategy may rely on a better understanding of the function of each PIM kinase in AML.

Conclusion

In conclusion, our results demonstrate that PIM3 overexpression promoted AML cell proliferation by phosphorylating BAD. Moreover, we observed that the PIM3-induced regulation of chemotaxis might be mediated by the phosphorylation of CXCR4. Our findings suggest that PIM3 might represent

a potential therapeutic target in AML. Our future work aims to address the exact mechanism involved in this process.

Abbreviations

AML, acute myeloid leukemia; PBS, phosphate-buffered saline.

Ethics Approval and Informed Consent

The patient and healthy volunteer consents were written informed consent and the study protocol was approved by the Ethical Committee of West China Hospital of Sichuan University and conformed to the Declaration of Helsinki.

Acknowledgment

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Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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