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PAK5 is a potential target in myelodysplastic syndrome through interacting with LMO2 and GATA1

Xiaoyan Pan[1], Dawei Liu[2], Minchao Ying[3], Gaoming Zheng[3], Chaoming Fan3, Feng Pan[3], Qiang Ke[3,4*]

[1] Department of Laboratory Medicine, The Second Affiliated Hospital of Zhejiang University School of Medicine, 310009 Hangzhou, China
[2] Department of Cell Biology, China Medical University, 110122 Shenyang, China
[3] Department of Laboratory Medicine, Hangzhou Normal University Affiliated Hospital, 310015 Hangzhou, China
[4] Department of Diagnostics, Hangzhou Normal University Medical School, 311121 Hangzhou, China

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ABSTRACT

Myelodysplastic syndrome (MDS) is a neoplastic heterogeneous and pre-leukemic disease with the poor clinical outcome due to the failure of current chemotherapeutic strategies to target leukemic stem cells. Recently, we find that p21-activated kinase 5 (PAK5) overexpressed in MDS patients and leukemia cell lines. The clinical and prognostic value of PAK5 in MDS is unclear although it has an anti-apoptosis ability and can promote cell survival and mobility in solid tumors. In this study, we find that LMO2 is co-expressed with PAK5 in the aberrant cells from MDS, and mitochondria-localized PAK5 can translocate into cell nucleus upon fetal bovine serum stimulation to interact with LMO2 and GATA1, which are important transcription regulators in hematological malignancies. Interestingly, without LMO2, PAK5 fails to bind GATA1 and facilitate GATA1 Ser161 site phosphorylation, indicating that PAK5 may be a key kinase in LMO2-associated hematopoietic diseases. Moreover, we find that the PAK5 protein level in MDS is significantly higher than in leukemia, and the data of 2095 leukemia samples from the 'BloodSpot' database show that the PAK5 mRNA level in MDS is also increased obviously. Taken together, our findings suggest that PAK5-targeted strategies in clinical therapy have a potential value in MDS intervention.

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Introduction

The myelodysplastic syndromes (MDS) represent a clonal hematopoietic stem cell disorder characterized by ineffective hematopoiesis, manifested by morphologic dysplasia in hematopoietic cells and by peripheral blood-cytopenia, it is considered as preleukemic condition. Although disease course and prognosis are highly variable in MDS, approximately 30% of high-risk patients eventually progress to acute myeloid leukemia (AML), and the other low-risk patients frequently develop red blood cell transfusion dependence (1). Current chemotherapeutic approaches target rapidly dividing cells, have limited effects in maintaining remission and improving survival in MDS. Due to the complexity of pathogenesis and clinical manifestations of MDS, more comprehensive studies are needed to stratify risks and determine treatment directions.

We recently found that p21-activated kinase 5 (PAK5) are co-expressed with LMO2 in clinical MDS patient samples and leukemic cell lines at a high level. And we identified LMO2 and GATA1 as downstream effectors of PAK5, the last uncovered member of the PAK family of serine/threonine kinases that act downstream of the Rho family small GTPases Cdc42 and Rac1 in a variety of signaling pathways. There are six members in the PAK family including group I (PAK1-3) and group II (PAK4-6) based on their structural similarities, they are known to be important signaling effectors which interact with a wide array of intracellular proteins to mediate cell proliferation and motility, as well as cell transformation and tumor progression (2). Overexpressed PAK5 has been implicated in cancer phenotype in several malignancies including breast, lung, kidney, liver, colon, and ovarian cancers by interacting with AIF (3), SOX2 (4), SMUC (5), β-catenin (6), Integrin β1 (7), and PI3K/AKT (8). However, the underlying role of PAK5 and its substrate in leukemic cells still waits to be fully elucidated.

In this study, we overviewed the expression of PAK5 and LMO2 in peripheral blood cells from MDS, AML, acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML), chronic lymphocytic leukemia (CLL) patients, PAK5 protein increased significantly in MDS only, and the PAK5 mRNA level in 2095 leukemia samples also elevated obviously. Then we explored the relationship of PAK5 with LMO2 and its transcriptional cofactors, such as GATA1 and E47, which were important for hematopoiesis. Here, we identified that LMO2 was a novel direct-binding protein, and transcription factor GATA1 was an indirect kinase substrate of PAK5. Interestingly, without LMO2, PAK5 fails to interact with GATA1 and facilitate GATA1 phosphorylation on the Ser161 site, which will abolish its transcriptional regulated function in hematopoietic cells, indicating that PAK5 may be a key kinase in LMO2-associated hematopoietic diseases. Furthermore, we demonstrated the interaction capacity among PAK5, LMO2, and GATA1. Our findings suggest that PAK5-targeted strategies in clinical therapy have a potential value in hematopoietic disease intervention.

* Corresponding author. Email: keq@hznu.edu.cn

Materials and Methods

Cells and clinical samples

HEK-293, K562, THP-1, MV4-11, and MOLM-13 cell lines were purchased from American Type Culture Collection (ATCC) and cultured according to ATCC recommendations. All cell lines were maintained in an incubator at 37°C and 5%CO2. In this study, fresh EDTA-anti-coagulated blood samples were drawn from the median cubital vein of 20 patients with blood disease and 5 healthy volunteers from The Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou, China. After clinical routine tests, the residual blood samples were used for flow cytometry. There were 7 samples from MDS patients, 4 samples from AML patients, 3 samples from ALL patients, 3 samples from CML patients, 3 samples from CLL patients, and 5 samples from healthy volunteers. both patient and volunteer were informed of the experimental purposes and methods, they all understood the clinical research tests and signed the consents, and this study was conducted from March to July of 2022 and approved by the Institutional Review Board (2022-0194) of The Second Affiliated Hospital of Zhejiang University School of Medicine.

Plasmids construction and Yeast two-hybrid screen

The Human PAK5 cDNA-containing vector was a kind gift from Dr. Jonathan Chernoff (Fox Chase Cancer Center, Philadelphia, USA). Full-length and nuclear exclusion sequence (NES, aa400-411) deletion of PAK5 (UniProt: Q9P286) were obtained by PCR and subcloned into pcDNA3.1/Myc-His (Invitrogen), pGEX-5X-1 (GE Healthcare) and pEGFP-C1 (ClonTech) vectors to get Myc/GST/GFP-tagged proteins respectively. The cDNAs of LMO2 (UniProt: P25791), E47 (UniProt: P15923), and GATA1 (UniProt: P15976) were obtained by PCR from human fetal brain cDNA library (ClonTech) and cloned into corresponding vectors, GST-tagged LMO2 and GATA1 deletions were subcloned into pGEX-5X-1 vector. As described previously (9), the human brain cDNA library was screened by yeast two-hybrid and yeast mating assay using the Matchmaker system III (Clontech) following the manufacturer's instructions.

GST pull-down assay

In vitro transcription and translation of goal, proteins were performed by using the TNT-coupled transcription and translation system (Promega Biotech Co., Ltd). Using a T7-TNT kit, we translated 1μg of pcDNA3.1 vector in the presents of methionine or ^{35}S-methionine in a reaction volume of 50μl. An aliquot of 10~20μl was used for each GST pull-down assay. Translation protein size was verified by subjecting 1~5μl reaction mixture to SDS-PAGE and western blot or autoradiography. The GST-pull down assay was performed by incubating equal amounts of GST, GST-fusion proteins immobilized by GST sepharose beads (Amersham Biosciences) with in vitro translated protein. Bound proteins were isolated by incubating the mixture for 2 h at 4°C, washing 3 times with binding buffer (20mM Tris, pH7.5, 50mM NaCl, 10% Glycerol, 1% NP-40). Proteins were eluted by 2×SDS loading buffer and separated by SDS-PAGE. Ponceau or Coomassie brilliant blue stain indicated the loading amounts of the GST-fusion proteins. The bound proteins were then visualized by western blot or autoradiography.

PAK5 kinase assay

The GST-fused proteins were expressed in BL-21 bacteria and purified with GST Sepharose beads (Amersham) and used for PAK5 kinase assay as described previously (10). GST-fusion proteins were purified in vitro and washed three times with kinase buffer (50mM HEPES, pH 7.5, 10mM MgCl2, 2mM MnCl2 and 0.2mM DTT). And afterward for 60min at 30°C with commercialized PAK5 Kinase (Cell Signaling Technology) or immunoprecipitated cell synthesized PAK5 kinase domain for kinase assay in 50μl of kinase buffer added with 10μCi of [γ-^{32}P] ATP (5,000 Ci/mM) and 2.5μM cold ATP. Reactions were stopped by the addition of a 6×SDS loading buffer. After 10% SDS-PAGE and transferred onto PVDF membranes ^{32}P-labeled proteins were visualized by radioautography with Molecular Imager RX (BIO-RAD). Histone H3 (Invitrogen) was used as a positive control. Ponceau stain indicated the loading amounts of GST-fusion proteins.

Immunofluorescence

The attached human embryonic kidney (HEK) 293 cells that were transfected with GFP- or Flag-tagged protein-expressing plasmids, grown on glass coverslips for 24 h and then were starved for 36 h and incubated in fetal bovine serum (FBS) if necessary. The GFP-PAK5 was observed directly under a fluorescence microscope or immunostained with anti-GFP primary antibody following fixation with methanol at room temperature for 20 min and then blocked with normal goat serum for 30 min, these cells were incubated with anti-GFP and anti-Flag primary antibody overnight at 4°C and subsequent secondary antibody conjugated with Alexa Fluor 488 (green) and Alexa Fluor 546 (red) dye from Molecular Probes after washed three times in PBST (PBS with 1‰ triton x-100). The DNA dye TO-PRO-3 (Molecular Probes) was used to stain the DNA (blue). The suspension of leukemic cell lines K562 and THP-1 cells were cultured in RPMI-1640 medium supplemented with 10% FBS. Following starvation for 24 hours, the cells were stimulated with or without FBS for 60 min. Then the cells were fixed with 4% of paraformaldehyde for 20 min, spread onto the anti-slip glass evenly, and dried naturally. After permeabilization and blocking, the cells were incubated with an anti-PAK5 antibody (1:50, PAB2302, Abnova) overnight at 4°C. After washing, the cells were incubated with goat anti-rabbit IgG the second antibody conjugated with Cy3 (1:100, BA1032, Boster) at room temperature for one hour. Then the cells were blocked with goat serum for 30 min at room temperature again and incubated with anti-LMO2 antibody (1:50, MAB2336, Abnova) overnight at 4°C. After washing, the cells were incubated with goat anti-mouse IgG second antibody conjugated with FITC (1:100, BA1101, Boster) at room temperature for one hour. Then the nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI) (Molecular Probes) for 5 min. A LEIKA laser confocal scanning microscope was used for the analysis of the cellular localization of proteins.

Co-immunoprecipitation and western blot analysis

For co-immunoprecipitation, cultured HEK-293, K562 and THP-1 cells were washed by cold PBS twice before being lysed in IP lysis buffer [25mM Tris/HCl (pH 7.4),

150mM NaCl, 1%Nonidet P-40, 1mM EDTA], supplemented with proteinase and phosphatase inhibitors (2mM dithiothreitol, 1mM phenylmethylsulfonyl fluoride, 10μg/ml leupeptin, 10μg/ml aprotinin, 20mM glycerophosphate, 1mM Na3VO4). The cell lysates were collected, rotated at 4°C for 1 h, and cleaned by centrifugation to obtain whole cell extracts. Then 50μl of protein A/G-agarose slurry (GE Healthcare Bio Sciences) preloaded with antibodies or normal IgG was added to equal amounts of cell extracts and rotated overnight at 4°C. The immune complexes were precipitated by centrifugation and washed twice with lysis buffer. The precipitated proteins were denatured in 2×SDS loading buffer, separated by SDS-PAGE, transferred to PVDF membrane, and analyzed by western blot. The membrane was blocked with 5% nonfat dry milk in TBST [20mM Tris/HCl (pH 7.4), 137mM NaCl, 0.05% Tween-20] for 3h at room temperature, and the proteins were probed with specific antibodies. All PVDF membranes were detected by chemiluminescence (ECL, Pierce Technology) after 2nd antibody was incubated for 2h at room temperature. The samples were incubated with anti-PAK5 (PAB2302, Abnova), anti-LMO2 (MAB2336, Abnova), and anti-GATA1/E47/c-Myc (Santa Cruz), anti-GFP (Genescript), anti-Flag (Ruixing) antibodies.

Flow cytometry

A complete blood count was obtained with the XN-B4 hematology analyzer (Sysmex). One million white cells containing whole blood were mixed and incubated at room temperature in dark 10 minutes in 2mL of BD Pharm Lyse™ Lysing Buffer for red blood cell lysis. After centrifugation and washing one time with PBS, the residual white cells were fixed, permeabilized, and washed with BD Fixation/Permeabilization Solution Kit according to the manufacturer protocol, 100μL pre-titrated antibodies PAK5 (1:100, PA5-82725, Invitrogen), LMO2 (1:200, MA5-17110, Invitrogen), CD45-Pacific Blue (1:200, 304022, BioLegend), goat-anti-rabbit Alexa Fluor® 488 (1:1000, A11008, Invitrogen), goat-anti-mouse-Alexa Fluor® 633 (1:1000, A21052, Invitrogen) were added to the cells for targets staining. Each tube was then washed in sheath fluid twice and finally, 500μL of sheath fluid was used to suspend the cells for acquisition in a three-laser Navois flow cytometer (Beckman Coulter) and Kaluza analysis software (Beckman Coulter). After gating singlets, living cells and ruling out the autofluorescence, CD45-positive white blood cells were analyzed, and fluorescence-minus-one (FMO) control was set to discriminate the PAK5 or LMO2 negative gating.

Statistical Analysis

All data were presented as mean ± standard deviation (SD). Student's t-test was used to compare the difference between the control and the experimental groups. Any value of P < 0 05 was considered a significant difference. Each experiment was repeated in triplicate.

Results

The interaction between PAKs and LMOs

To find novel PAKs-interacting proteins, yeast two-hybrid was performed as previously described (9). LMO2, a critical transcription cofactor in hematopoietic development (11), was identified as one of the PAK4C-terminal-interacting proteins after sequencing positive clones. To confirm the interaction, full-length PAK4 was cloned into the pGBKT7 vector and LMO2 cDNA into the pGADT7 vector to do a yeast mating assay. The results showed that PAK4 and LMO2 yeast colonies could grow on His/Leu/Trp/Ade defect SD (+X-α-gal) plate, indicating that PAK4 could interact with LMO2 in eukaryotes (Figure 1a). To further confirm the binding of PAK4 with LMO2, in vitro translated ^{35}S-labeled PAK4 was incubated with GST-LMO2 fusion protein for GST pull-down assay. The data indicated that PAK4 could bind LMO2 directly (Figure 1b). To explore the binding specificity of LMO2 with the PAK family, ^{35}S-labeled LMO2 protein was incubated with GST-PAKs, except for GST-PAK3, due to the failure of protein purification. The data revealed that LMO2 not only bound to PAK1 and PAK4, the two best-characterized PAK members, but also bound to PAK5, the least studied PAK member, and the binding of PAK5 with LMO2 was very strong (Figure 1c). Since LMO2 is a well-known transcription cofactor in hematopoiesis, and the research about the nuclear function of PAK5 is limited, our target was shifted from PAK4 to PAK5. Because there are 4 members in the LMO family, a GST pull-down assay was performed to check the specificity of PAK5 binding to LMO2. The data showed that in vitro translated PAK5 could bind to LMO1/2/4 but not LMO3 (Figure 1d). Taken together, these results indicate that LMO2 is a newly identified protein that interacts with PAK5 directly in vitro.

PAK5 interacts with LMO2 in the cell nucleus

As reported, PAK5 constitutively localizes to mitochondria as it contains a mitochondrial targeting sequence, and it plays an important role in cell survival (12-14). But

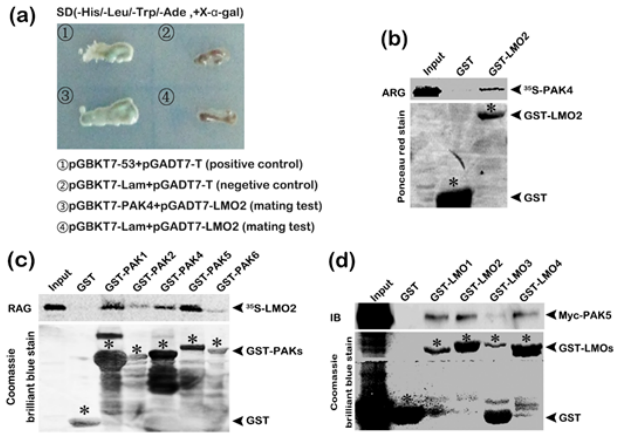

Figure 1. Screening of new PAK-interacting proteins. (a) Y187 yeast cells were transfected with pGBKT7-53/Lam control and pGBKT7-PAK4 vector, AH109 yeast cells were transfected with pGADT7-T control and pGADT7-LMO2 vector respectively, and the yeast mating colonies were grown on His/Leu/Trp/Ade defect SD (+X-α-gal) plate. pGBKT7-53 and pGADT7-T containing cells were positive controls, and pGBKT7-Lam and pGADT7-T containing cells were negative controls. (b) In vitro-translated, ^{35}S-labelled PAK4 was incubated with GST-LMO2, then analyzed by SDS-PAGE and radioautography. The ponceau red stain shows the loading amounts of GST-fused proteins. (c, d) ^{35}S-LMO2 or Myc-PAK5 was incubated with GST-PAKs or GST-LMOs, then analyzed by SDS-PAGE and autoradiography. The Coomassie brilliant blue stain and stars show the loading amounts of GST-fused proteins. RAG: radioautography. IB: immunoblot.

upon growth factor signal transduction, it can shuttle into the nucleus to execute transcription regulative function (10). So, normally, mitochondrial localized PAK5 can shuttle between mitochondria and the nucleus to perform different biological functions. Here, GFP-PAK5 was generated to monitor the process of PAK5 translocation upon FBS stimulation. As immunofluorescence data showed, GFP-PAK5 was distributed as dots in the cytoplasm of starved HEK-293 cells. After adding FBS to the medium and culturing for 30 min, the dot-distributed GFP-PAK5 was partially translocated into the nucleus and mostly shuttled into the nucleus in 60 min (Figure 2a left). Subsequently, GFP-PAK5 and Flag-LMO2 were co-expressed in HEK-293 cells. After starvation, PAK5 was found to be colocalized with LMO2 when the cells were stimulated with FBS for 60 min, indicating that PAK5 may interact with LMO2 in the nucleus (Figure 2a right upper). To confirm this interaction, co-immunoprecipitation was performed with HEK-293 cells expressing Myc-PAK5 and Flag-LMO2, and LMO2 was precipitated with PAK5 using anti-Myc antibody (Figure 2a right lower). Meanwhile, the nuclear exclusion sequence (NES) domain of PAK5 was deleted to make GFP-PAK5ΔNES, which should be localized in the nucleus only. As expected, PAK5ΔNES was observed to colocalize with LMO2 in the nucleus and LMO2 was precipitated with PAK5 using an anti-Myc antibody (Figure2b). LMO2 is a well-known transcription cofactor and very important for blood cell development, but PAK5 has not been reported to be expressed in hematopoietic cells. So, the expression and colocalization of endogenous PAK5 and LMO2 were examined by immunoblotting and immunocytochemistry in leukemia cell lines K562 and THP-1. As the data shows, not only LMO2 but also PAK5 was expressed in K562 and THP-1 cells. Upon FBS stimulation, colocalization of endogenous PAK5 and LMO2 was observed (Figure 2c, d left). The endogenous LMO2 was immunoprecipitated by anti-PAK5 antibody in K562 and THP-1 cells, and more precipitated LMO2 protein was observed for the starved cells stimulated with FBS for 60 min (Figure2c, d right). These observations indicated that endogenous PAK5 and LMO2 could interact with each other under normal physiological conditions, and PAK5 may play a critical role in the regulation of gene expression in hematopoietic cells. Altogether, these results indicated that PAK5 could translocate to the nucleus from mitochondria and interact with transcription cofactor LMO2 upon FBS stimulation, and PAK5 may participate in the regulation of gene expression.

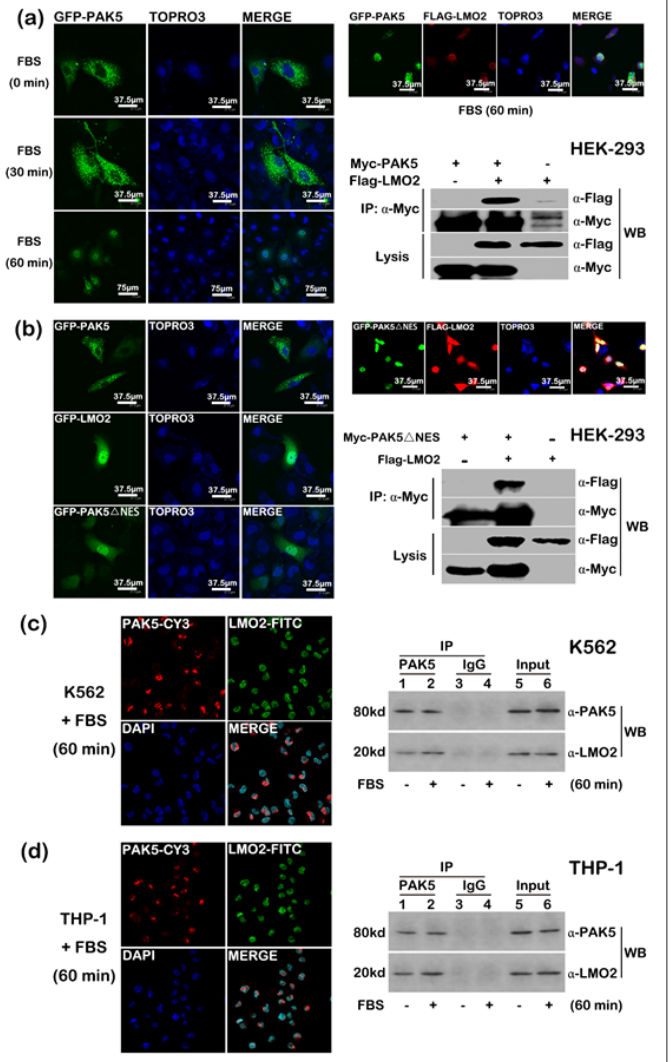

Figure 2. Interaction between PAK5 and LMO2 in the nucleus. (a, b) HEK-293 cells transfected with GFP-PAK5/PAK5ΔNES and Flag-LMO2 vectors were stimulated with FBS for a different time after 36h of no serum starvation, followed by immunofluorescence assay and detected by confocal scanning. Yellow indicates co-localization. And HEK-293 cells transfected with or W/O Myc-PAK5/PAK5ΔNES and Flag-LMO2 vectors were harvested to perform co-IP with anti-Myc antibody. The co-immunoprecipitated proteins were immuno-blotted with anti-Myc/Flag antibody. (c, d) K562 and THP-1 cells were immuno-stained with anti-PAK5/LMO2 primary antibody and CY3 (Red) or FITC (Green) conjugated goat-anti-rabbit/mouse second antibody to detect endogenous PAK5 and LMO2, the nucleus was stained with DAPI. And K562 and THP-1 cell lysates were immunoprecipitated with PAK5 antibody or IgG. Precipitates were analyzed by western blot using the indicated antibodies.

PAK5 significant high-expressed in MDS

To further assess the relevance of PAK5 and LMO2 in hematological system diseases, we evaluated PAK5 expression in hematopoietic cells. Firstly, we examined the percentage of PAK5 and LMO2 double-positive cells in human leukemic cell lines (K562, THP-1, MV4-11, and MOLM-13) by flow cytometry (FCM). According to the guideline for the use of FCM, negative staining with the isotype control does not infer that the staining one observes with the experimental antibody is specific (15). To determine the optimal gate position, we set a fluorescence-minus-one (FMO) control for PAK5 positive gating (16), and the expression of PAK5 in these four cell lines were considerably high. The percentages of PAK5+LMO2+ cells were 94.44%, 97.16%, 97.68%, and 98.77% in K562, THP-1, MV4-11, and MOLM-13 cell lines respectively (Figure3a). To further investigate whether PAK5 is overexpressed in primary human mononuclear cells of healthy controls or clinical leukemia patients, we checked its expression in peripheral blood mononuclear cells (PBMC) from MDS, AML, ALL, CML, and CLL patients. Flow cytometry analysis revealed that PAK5 protein was significantly high-expressed in PBMC of MDS compared with healthy controls and other leukemia patients, and LMO2 was co-expressed with PAK5 in these aberrant cells. In contrast, no significant difference in AML, ALL, CML, and CLL patient samples compared with healthy donor samples (Figure3b). Meanwhile, we overviewed PAK5

mRNA log2 expression of 2095 leukemia samples from the 'Leukemia MILE study' database of 'BloodSpot', in which all datasets were generated using oligonucleotide microarray chips, and the data showed that PAK5 significant high-expressed in MDS compared with AML, ALL, CLL, and healthy controls, except for CML (Figure3c), this might be due to the fact that protein and RNA expression are not completely consistent.

PAK5 interacts with LMO2-involved transcription cofactors

LMO2 is a well-known trans-cofactor that participates in the transcription process with its partners GATA1/E47 by forming a transcriptional complex. To investigate the role of PAK5 in this complex, in vitro transcribed and translated Myc-PAK5 was incubated with purified GST-tagged LMO2/GATA1/E47. The data indicated that PAK5 could bind to LMO2/E47 directly, but not GATA1 (Figure 4a). To further illustrate the participation of PAK5 in the LMO2-involved transcription complex, Myc-LMO2/GATA1/E47 were co-immunoprecipitated with GFP-PAK5 using the anti-GFP antibody in HEK-293 cells. The results showed that PAK5 could interact with these partners accordantly (Figure4b). Considering that the relationship of PAK5 with LMO2 involves transcription complex, Myc-PAK5 was incubated with GST-LMO2 or -GATA1 in the presence or absence of Myc-LMO2 for GST-pull down assay. Interestingly, PAK5 bound GATA1 stronger once LMO2 was added to the reaction solution, it indicated that LMO2 may be a linker molecule between PAK5 and GATA1 (Figure 4c). To confirm this interesting phenomenon, PAK5 and GATA1 were co-expressed with or without LMO2 in HEK-293 cells, and co-IP was performed with anti-GATA1 or anti-PAK5 antibodies. It recurred that the binding of PAK5 and GATA1 was stronger in the presence of LMO2 (Figure 4d). These data partially support that LMO2 interlinks PAK5 and GATA1 to execute the gene-express regulation function.

The interactional manner among PAK5, LMO2 and GATA1

Since the indirect binding of PAK5 and GATA1 depends on the LMO2 presence, the interacting regions among PAK5, LMO2 and GATA1 were examined. A series of GST-LMO2 truncated fusion proteins were incubated with ^{35}S-PAK5 or Myc-GATA1, which were transcribed and translated in vitro for GST-pull-down assay. The data showed that PAK5 and GATA1 not only bound the LIM1 domain but also the LIM2 domain of LMO2 because the binding bands of GST-LMO2ΔD/E were positive (Figure 5a, 5b). However, the N-terminal region of LMO2 (1-

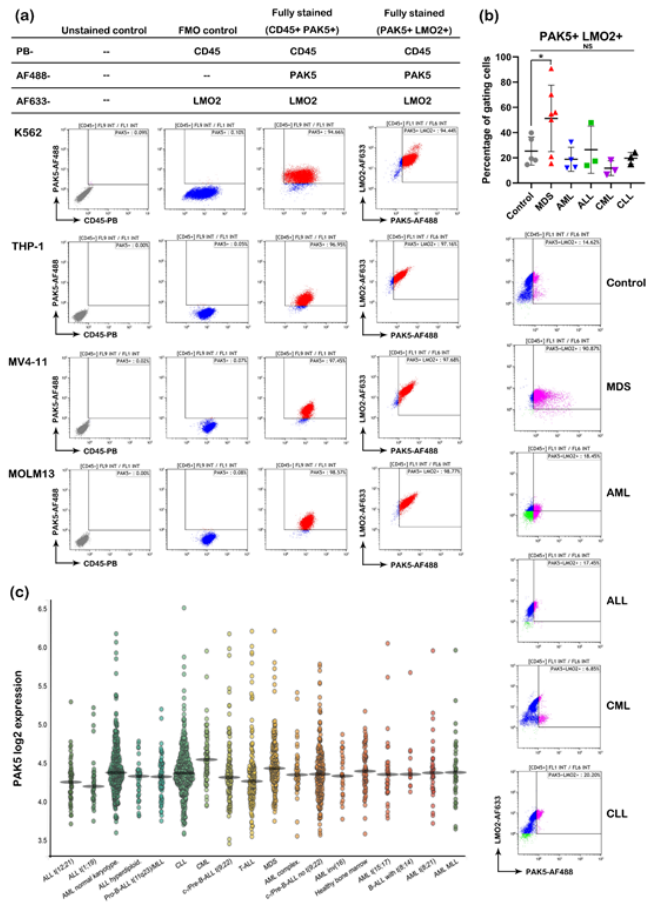

Figure 3. PAK5 overexpressed in MDS. (a) Human leukemic cell lines K562, THP-1, MV4-11, and MOLM-13 were stained with or W/O CD45-PB, PAK5-AF488, LMO2-633 antibodies and analyzed by flow cytometry. Fluorescence Minus One (FMO) control was set as the PAK5 positive gating, and the far right fully stained group showed PAK5 and LMO2 double positive population. (b) Peripheral blood cells from different hematological disease patients were stained with or W/O CD45-PB, PAK5-AF488, LMO2-633 antibodies and analyzed by flow cytometry, the gating strategy was the same as before. (c) Autogenerate graph from 'Leukemia MILE study' database of 'BloodSpot' website by input PAK7 (also named PAK5).

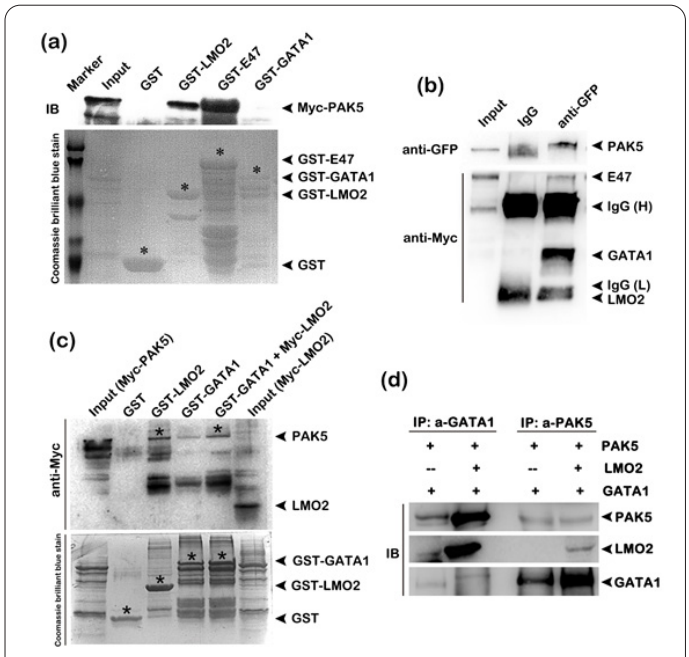

Figure 4. PAK5 binds GATA1 with LMO2 ligation. (a) Myc-PAK5 was incubated with GST-fused proteins and separated by SDS-PAGE, then immunoblotted with anti-Myc antibody. The Coomassie brilliant blue stain and stars show the loading amounts of GST-fused proteins. (b) HEK-293 cells transfected with GFP-PAK5, Myc-LMO2/E47/GATA1 vectors were harvested to perform co-IP with anti-GFP antibody. The co-immunoprecipitated proteins were immuno-blotted with anti-GFP and Myc antibodies. (c) Myc-PAK5 with or W/O Myc-LMO2 protein was incubated with GST-LMO2/GATA1 for GST-pull down assay. (d) HEK-293 cells transfected with Myc-PAK5/LMO2/GATA1 vectors were harvested to perform co-IP with anti-PAK5 or anti-GATA1 antibodies.

30aa) abolished the binding activity without the LIM2 domain. These results indicated that PAK5 preferred to binding the LIM2 domain of full-length LMO2. Oppositely, LMO2 (1-30aa) region does not affect GATA1 binding to the LIM1 domain of LMO2. Considering that PAK5 has occupied the LIM2 domain, GATA1 very likely binds the LIM1 domain of LMO2 in the PAK5/LMO2/GATA1 complex. To check which one was the PAK5 kinase substrate, a kinase assay was performed. The results indicate that GATA1 and E47 were positive, except LMO2 (Figure 5c). Due to LMO2, PAK5 could phosphorylate GATA1 indirectly. To find out the phosphorylate sites of GATA1 by PAK5, a series of GATA1 truncated fusion proteins were generated for PAK5 kinase assay, and the radioautography photo indicated the phosphorylate sites should be in 161-240aa region as 1-240aa was positive but 1-160aa was negative (Figure5d upper). Then several serine/threonine sites of GATA1 were mutated to alanine for PAK5 kinase assay, and the Ser161Ala mutation interfered distinctly with PAK5 phosphorylation of GATA1 (Figure 5d lower). It indicated that PAK5 phosphorylates GATA1 on the Ser161 site with LMO2 ligation.

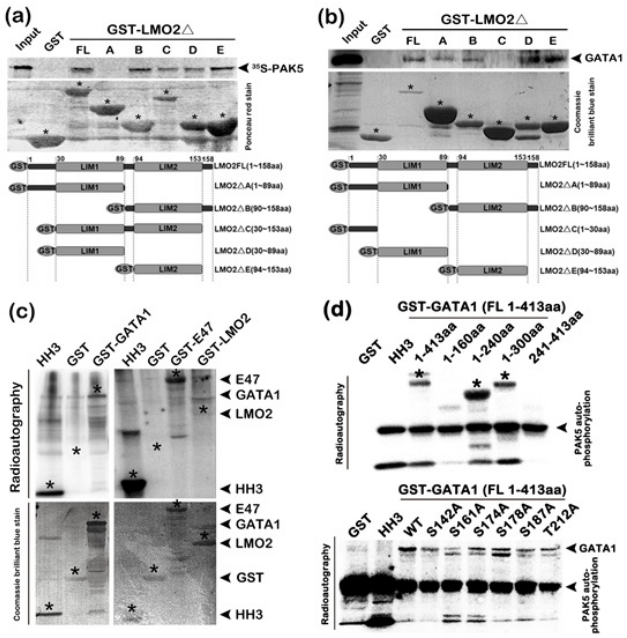

Figure 5. Interactional manner among PAK5, LMO2 and GATA1. (a) ^{35}S-PAK5 was incubated with GST-fused serial truncated LMO2 proteins for GST-pull down assay and analyzed by SDS-PAGE and radioautography. (b) Myc-GATA1 was incubated with GST-fused serial truncated LMO2 proteins for GST-pull down assay, separated by SDS-PAGE, and immunoblotted with anti-Myc antibody. (c) PAK5 kinase domain truncated protein (catalytic active PAK5) was mixed with GST-LMO2/GATA1/E47, histone 3 (HH3, positive control) and GST (negative control) for kinase assay. (d) GST fused GATA1 truncated or site mutations proteins were incubated with PAK5 for kinase assay.

Discussion

p21cdc42/rac1-activated kinases (PAKs), a binding protein of small GTPases, are known to be important signaling effectors which interact with a wide array of intracellular proteins to mediate various cellular processes. Upon binding of the upstream regulators, including the cell division control protein 42 (Cdc42) and Rho family GTPases RAC, PAKs activated persistently and participate in cell proliferation, cell motility, apoptosis evasion and gene transcription-dependent or independent of kinase activity (17). It's interesting that, as the last identified and the least understood PAK family member, PAK5 not only has the highly conserved kinase domain but also has a nuclear localization sequence (NLS), a nuclear exclusion sequence (NES) and a mitochondria localization sequence (MLS) (18), it could be activated as a downstream effector of growth-factor receptors (19). During cell cycle progression, the mitochondrial localization of PAK5 is important for cell apoptosis and survival (20). But what is the function of PAK5 in the nucleus? Is there any partner of PAK5 to interact with? Growing evidences indicate that PAK5 may play a critical role in regulating gene transcription since mitochondria-localized PAK5 can translocate into the cell nucleus upon growth factor treatment, and it no longer protects cells from apoptosis once it is retained in the nucleus (13). PAK5 is a brain mitochondrial kinase with declined expression in mature neurons, but in response to acute injury-ischemic stress in mature neurons and adult brains, PAK5 signaling remobilizes and replaces damaged mitochondria via the phosphorylation switch that turns off the axonal mitochondrial anchor syntaphilin, thereby maintaining local energy supply to support central nervous system (CNS) survival and regeneration (21). Although PAK5 plays a positive role in brain tissue, recent studies have shown that acceleration of cancer cell proliferation, migration, invasion and anti-apoptosis are correlated with PAK5 by interacting with downstream factors directly (18). In breast cancer, PAK5 phosphorylates apoptosis-inducing factor (AIF) at the Thr281 site to prevent the formation of AIF/importin α3 complex, leading to a decrease AIF nuclear translocation and inhibiting cancer cells' caspase-independent apoptosis (3). Previous studies have confirmed that activation of the PAK5 signaling pathway conduces activation or inhibition of several transcription factors, including EGR1 (22). RAF1 (23), etc., which subsequently enhance or suppress multiplex biological activities. However, PAK5 function has been poorly revealed so far, especially gene regulation in hematological system diseases.

To date, numerous interacting proteins of PAK5 have been identified, which act as downstream effectors in different cellular procedures, but it's limited, and the biological function of PAK5 has been poorly understood, especially its role in gene transcription, the underlying role of PAK5 and its substrate in leukemia cells still waits to be fully elucidated. Here, we primarily identified a novel PAK4-interacting protein LMO2 by yeast two-hybrid assay. Unexpectedly, we found that LMO2 not only binds PAK4, but also PAK5, and the binding capacity of PAK5 is stronger than PAK4 as shown in Figure 1c. Nevertheless, upon FBS stimulation, PAK5 could translocate to the nucleus and interact with nuclear transcription cofactor LMO2 directly. But PAK5 does not phosphorylate LMO2 although it is a kinase. Interestingly, with LMO2 ligation, PAK5 could phosphorylate GATA1, which is a GATA-frame binding transcription factor (24), on the Ser161 site. This implies that PAK5 interacts with LMO2 and GATA1, which may execute a more important physiological function in the regulation of gene expression, and the interaction is PAK5 kinase activity-dependent in the presence of LMO2. The LMO2 protein has a central and crucial role in

hematopoietic development and hematological diseases. LMO2 acts as a part of a bridge for DNA-binding proteins to create a bipartite multi-protein DNA-binding complex, although it does not bind DNA directly, to execute the transcription-regulated function. For example, the transcription complex that consists of LMO2, GATA1, LDB1, TAL1 and E47 recognizes and combines with E-box and GATA-frames of target genes to regulate erythropoiesis (25). According to our data, PAK5 not only binds LMO2, but also E47 directly in the nucleus, and PAK5 binds GATA1 indirectly in the LMO2-associated transcription complex. Meanwhile, our study discovered that LMO2 is a bridge molecule that links the interactions of PAK5 and GATA1, through the binding domains of PAK5/LMO2 and LMO2/GATA1. The relationship between PAK5 and LMO2-related transcription co-factors in hematological system diseases is partially uncovered.

As mentioned before, PAK5 can also bind E47 directly and phosphorylate E47 in vitro. E47 is a member of the E protein family of helix-loop-helix transcription factors that activate transcription by binding to regulatory E-box sequences on target genes (26). Representatively, E47 plays a critical role in B and T lymphocyte development as well as in lymphoid malignancies (27). It implies that PAK5 may be involved in lymphoma pathogenesis as it is an oncogene in some neoplastic diseases. Moreover, PAK5 probably is an important messenger in blood cell development, and functions in gene expression based on its kinase activity. Up to now, the main function of PAK5 focuses on 1) cell motility via epithelial-to-mesenchymal transition on the cell surface, 2) cell survival via interaction with apoptosis-associated proteins in the cytoplasm, and 3) oncogene regulation via shuttling into the nucleus and interacting with transcription factors. With different subcellular locations, PAK5 has a different molecular function to promote tumor cell proliferation. Further research is required to elucidate the regulation of gene expression in which PAK5/LMO2/GATA1/E47 participates in hematological diseases.

PAK family is conserved in functional domains that regulate signaling pathways through their function as serine/threonine kinases. PAK5, the youngest member of PAKs, was first characterized for inducing microtubule stabilization by binding to actin and microtubule, and its subcellular localization was regulated during cell cycle progression (28). And it was shown that PAK5 was primarily localized in the mitochondria, and nucleus of brain tissue (29), it is a brain-specific kinase that is the downstream target of small GTPases Cdc42/Rac1, promoting neurite outgrowth and filopodia formation (30). RAS proteins are membrane-associated GTPases that regulate serine or threonine kinases of the mitogen-activated protein kinase (MAPK) cascade, they are encoded by three proto-oncogenes (H-RAS, K-RAS, and N-RAS) that regulate growth and differentiation of many cell types. PAK5 is an upstream regulator of MAPK signaling pathways during the re-arrangement of the cytoskeleton and apoptosis (31). Growing evidence shows that the RAS/Cdc42/PAK5/MAPK signaling axis plays a role in cell apoptosis and differentiation, including myeloid cells. Large-scale studies have consistently reported RAS signaling deregulation due to mutations of RAS genes in Myelodysplastic syndrome (MDS), and the patients with RAS mutations in particular N-RAS, have been found associated with shorter survival and higher risk of transformation into acute myeloid leukemia (AML) with higher white cell count and bone marrow blast percentage. It is shown that there is a connection between RAS and PAK5 in MDS or AML. The clinical heterogeneity of MDS is attributed to multiple genetic and epigenetic aberrations resulting in the dysplastic and proliferative features of MDS. In general, more than 80% of patients with MDS with distinct mutations resulting in hematopoietic stem cells and progenitor cells signal transduction, transcriptional regulation, genetic regulation and RNA splicing molecules disorder. In this study, we found that mitochondria-localized PAK5 translocated into the cell nucleus interact with and phosphorylate LMO2, GATA1, and E47, which are well-known transcriptional factors in hematopoietic development. And we found that PAK5 and LMO2 overexpressed in the peripheral blood of MDS patients, not in other leukemia patients. The expression pattern of PAK5 and LMO2 in MDS suggests that the interaction of PAK5 and LMO2 was consequential for the pathological process of MDS. And the next step, we will focus on this field.

MDS is a heterogeneous, clonal hematological disorder characterized by ineffective hematopoiesis, cytopenias, morphologic dysplasia, and predisposition to AML. To date, the main function of endogenous PAK5 remains largely unknown although it has an anti-apoptosis ability and can promote cell survival and mobility. PAKs are considered as promising therapeutic targets in hematological malignancies except for PAK5, because PAK5 is restrictedly expressed in brain tissue normally, and there are no evidences to prove PAK5 was expressed in clinical leukemic samples. But in our studies, we found that PAK5 not only is overexpressed in human AML cell lines (THP-1, MV4-11, and MOLM-13) and one CML cell line K562, but also in the peripheral blood of MDS patients. In these limited clinic samples, PAK5 expression was significantly increased only in MDS and not in AML, ALL, CML or CLL blood samples In a recent study, a total of 155 AML patients with relative expression of PAK5 from The Cancer Genome Atlas (TCGA) database were divided into $PAK5^{low}$ and $PAK5^{high}$ groups, found that $PAK5^{high}$ group was associated with poor event-free survival, overall survival, and poor prognosis, compared with $PAK5^{low}$ group (32). The reasons for this phenomenon may be as follows: 1) The cell lines were pure leukemic cells, and PAK5 protein was concentrated. 2) PAK5 is expressed in AML at a very low level actually, although it was divided into relatively low and high groups. 3) MDS is considered a preleukemic disease as MDS patients frequently progress to AML and PAK5 overexpression in MDS, not leukemia patients due to its particular functions in MDS progress. Then we overviewed PAK5 log2 expression of 2095 leukemic samples from the 'Leukemia MILE study' database of 'BloodSpot', and the data showed that PAK5 expression was higher in MDS compared with AML, ALL, CLL, and healthy controls, except for CML. These prompt that PAK5 is associated with MDS, and might be a potential precise target in MDS therapy. If that's true, how does PAK5 play a role in the course of MDS? And what are the functions of PAK5 in MDS? These are what we should uncover in future work.

Taken together, recent studies have significantly improved our understanding, and our scientific findings in this study provide insight that mitochondria-located PAK5

could shuttle into the nucleus, interact with transcriptional co-factors LMO2 and GATA1, forming a complex which may affect the transcription of genes associated with hematopoietic diseases It enriches the nuclear function of PAK5 and provides increased support for clinical strategies of targeting PAK5. Here, we highlight that PAK5 may be a potential target in myelodysplastic syndrome through interacting with LMO2 and GATA1.

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Interest conflict
The authors declare that they have no conflicts of interest.

Consent for publications
The author read and proved the final manuscript for publication.

Availability of data and material
All data generated during this study are included in this published article

Authors' Contribution
Xiaoyan Pan and Qiang Ke performed the cellular and clinical experiments and wrote the paper, Dawei Liu and Minchao Ying performed the molecular assay, Qiang Ke, Gaoming Zheng, Chaoming Fan, and Feng Pan designed, performed and supervised the research.

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This study was approved by the Institutional Review Board (2022-0194) of The Second Affiliated Hospital of Zhejiang University School of Medicine.

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