FAM122A promotes acute myeloid leukemia cell growth through inhibiting PP2A activity and sustaining MYC expression

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Online Supplemental Materials and Figures for the role of FAM122A in AML

Material and methods

Public data collection and analysis

RNA expression profiling of *FAM122A* gene in AML cohorts were obtained from Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) and HemaExplorer (http://servers.binf.ku.dk/hemaexplorer/). GEO dataset (GSE48173) includs 17 $CD34^+$ human cord blood samples (n=5 of $CD34^+$; n=12 of $CD34^+$CD45RA), 43 AML, and 12 ALL patients. HemaExplorer dataset includes 8 normal hematopoietic stem cells from human bone marrow (HSC-BM) samples, 39 AML with t(8;21)/AML1-ETO, 37 AML with t(15;17)/PML-RARα, 28 AML with inv(16)/t(16;16) and 38 AML with t(11q23)/MLL patient samples. These two datasets were respectively used to compare the expression levels of *FAM122A* mRNA between AML with different subtypes and normal HSPC or HSCs. The correlation of *FAM122A* mRNA expression level and overall survival of AML patients (from TCGA database) were analyzed with PROGgeneV2 online software, in which a group of AML patients were divided into a high-expression group with 79 patients and a low-expression group with 78 patients according to the median expression level of *FAM122A*.

Plasmid construction and viral production

Human and mouse complementary oligonucleotides specifically against FAM122A were synthesized, annealed and ligated into pLKO.1 vector according to the

Addgene's pLKO.1 protocol. pLKO.1-GFP vector was constructed from pLKO.1-puro by replacing puro with GFP. The target sequences for FAM122A (FAM122A shRNAs) were shown respectively as following: 5′-TTGCACCAGATCAAACAAGAA-3′ for shFAM122A #1, 5′-GATTGCCTCCAAGCCCTATTC-3′ for shFAM122A#2, 5′-CAC CAGATCAAACAGGAGGAG-3′ for shFam122a#3, 5′-GAGGAAAGTTTCAGCCT GA-3′ for shFam122a#4, and 5′-CAACGAAGGAATTCTTCGGCC-3′ for shScramble or shControl. Human FAM122A cDNAs amplified from HEK293T cells by reverse transcription-PCR, were first cloned into pENTR1A donor vector (containing attL sites), followed by recombination with pINDUCER20 destination vector (containing attR sites) to generate pINDUCER20-FAM122A expression plasmid using Gateway LR Clonase II Enzyme mix kit (Invitrogen, USA). pINDUCER20-FAM122A-6m shRNA-resistant plasmid was generated using Quick change Lightning Site-Directed Mutagenesis Kit (Stratagen, USA) by introducing six mutations (-CATCAATTAAGCAGGAG-) into the pINDUCER20-FAM122A plasmid. For virus packaging, lentivirus constructs pLKO.1-GFP-shRNAs, pINDUCER20-FAM122A-6m, or control vectors, were respectively mixed with pSPAX2 and pMD2.G (4:3:1), while retroviral construct MSCV-MLL-AF9-IRES-YFP was mixed with PCL-ECO (2:1), followed by transfecting into 293T cells using Lipofectamine 2000 (Invitrogen, USA). Virus-containing supernatants were collected at 48-72 hour post-transfection and used for infection.

Cell lines, infection and reagents

NB4, U937, and THP1 cells were grown in RPMI-1640 medium (Life Technologies, USA) supplemented with 10% FBS (Sigma, USA) and 1%

penicillin/streptomycin (New Cell & Molecular Biotech CO. Ltd, China). Three cell lines were obtained from Shanghai Institute of Hematology. MLL-AF9 primary bone marrow cells were grown in StemSpan Serum-Free Expansion Medium (StemCell Technologies, Canadian) with 2% FBS, 1% penicillin/streptomycin, 20 ng/ml SCF, 20 ng/ml IL-3, 10 ng/ml IL-6 (all cytokines from PeproTech, USA). These cells were grown in a humidified incubator at 37 ℃ and 5% CO_2/95% air (v/v). NB4, U937 and THP1 cells were respectively infected with virus supernatants (1×10^5 cells/ml) with 6 μg/ml polybrene (TR-1003, Sigma, USA) and centrifuged at 1200 g for 2 hours at 32 ℃, followed by replacing with fresh culture medium for incubating another 4 hours. After 48 hours of infection, GFP^+ cells expressing shRNAs were flow-sorted for subsequent experiments. PINDUCER20-transduced NB4 cells were induced by doxycycline (1μg/ml, D9891, Sigma, USA) for efficient expression. Okadaic acid was purchased from Sigma-Aldrich (459616). Cycloheximide was purchased from CST (2112) and MG132 was purchased from Calbiochem (474790). PBMCs (peripheral blood mononuclear cells) were bought from Shanghai Miao-tong Biological Technology (PB010C, TPCS), with informed consents obtained from all patients in accordance with the Declaration of Helsinki. Puromycin (540411, MERCK, Germany) and G418 (345810, Sigma, USA) were purchased.

$CD34^+$ HSPCs' isolation and lentiviral transduction

Total mononuclear cells were isolated from cord bloods of healthy donors from Obstetrics and Gynecology Department of Shanghai Ren-Ji Hospital using Ficoll (p9011, Solarbio, Beijing, China), and HSPCs were enriched using human CD34 positive selection kit (18056, Stem Cell Technologies, Vancouver, Canada). The $CD34^+$ HSPCs were cultured in StemSpan SFEM medium (09655, StemCell

Technologies, Vancouver, Canada) supplemented with 100 ng/ml human SCF, 100 ng/ml human FLT3-L, 50 ng/ml human TPO, and 1% penicillin/streptomycin. All these cytokines were purchased from Novoprotein (Shanghai, China). The experiments related to human cord bloods were approved by the Ethics Committee for Medical Research (IRB) at SJTU-SM.

$CD34^+$ HSPCs were transfected with lentiviruses carrying FAM122A pLKO.1-GFP-shRNAs as used in AML cells. Briefly, 12-well plates were pre-coated with 40 μg/ml fibronectin (CA82, Novoprotein, Shanghai, China), followed by added with freshly collected virus supernatants and centrifuged at 2000 g for 2 hours at 32°C. Afterwards, supernatants in plates were removed and added $CD34^+$ HSPCs with culture medium for 18 hours. After that, the transduction cells were washed and cultured *in vitro* for assessing the cell growth in $CD34^+$ HSPCs with FAM122A silencing. For this purpose, the percentages of GFP^+ cells and total cell numbers were monitored at days 2, 5 and 8 by flow cytometry (CytoFLEX S, Beckman Coulter, USA), and GFP^+ cells were sorted for confirming FAM122A silencing effects with qRT-PCR.

Cell growth assay

FAM122A shRNA GFP^+ cells were sorted by flow cytometry one day post-infection, and subsequently 1×10^5 (NB4, U937) or 2×10^5 (THP1) cells were seeded in 12-well plates. Cell numbers were calculated by a cellular counter (Beckman Coulter Vi-CELL XR Automated Cell Viability Analyzer, USA) on culturing days 2, 4 and 6 with triplicate wells.

Cell apoptosis and cell cycle assays

Cell apoptosis assay were performed according to the manufacturer's instructions (56547, BD, USA). Briefly, cells were infected with FAM122A shRNAs for 48 hours. Subsequently, the cells were cultured for another 1 day for NB4 or another 4 days for U937 and THP1 cells. Then, the cells were collected and washed twice with cold PBS followed by resuspending in 100 μl of 1 × Binding Buffer with 5 μl of FITC AnnexinV and 5 μl PI. After incubation for 15 minute at RT (25℃) in the dark, each sample was added with 200 μl of 1 × Binding Buffer and analyzed by flow cytometry (BD FACSCalibur, USA) within 1 hour. For cell cycle analysis, NB4 cells were collected after virus infection for 48 hours, and U937 or THP1 cells were collected at 4 days after infection, followed by fixation in 70% ethanol. The fixed cells were washed and resuspended in 0.5 ml of FxCycle™ PI/RNAse Solution (1985233, Invitrogen, USA). After incubation for 15-30 minutes at room temperature in the dark, samples were analyzed by flow cytometry (BD FACSCalibur, USA).

MLL-AF9 mouse leukemia model study

Leukemia mice were generated by infection with recombinant retroviruses expressing MLL-AF9. Specifically, Lin^- fetal liver cells were isolated and infected with MLL-AF9 retroviruses with 4 μg/ml polybrene and centrifuged at 1200 g for 2h at 32℃. Cells were cultured overnight, followed by another round of spin infection. Infected cells were transplanted into lethally irradiated (10 Gy) C57BL/6 mice by retro-orbital injection to generate MLL-AF9 mice. Bone marrow cells from leukemia mice were further infected with pLKO.1-GFP lentiviruses expressing FAM122A shRNAs with 4μg/ml polybrene and centrifuged at 1200 g for 2 h at 32 ℃. For transplantation assays, FACS sorting GFP^+ cells (2000 or 5000 cells/mouse) together with 2×10^5 normal mouse bone marrow cells were transplanted into lethally irradiated

recipient mice at 24 hours post-infection. For colony formation assays, 500 purified GFP^+ leukemia cells were cultured in 1 ml of methylcellulose supplemented with cytokines (M3434, StemCell Technologies, Canadian) for 5-7 days. For HE staining, liver and spleen tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were stained with HE for the analysis of the infiltration of leukemia cells. C57BL/6 mice (Shanghai SLAC Laboratory Animal Co. Ltd., China) were housed in the SPF facility of Rui-Jin Hospital affiliated to SJTU-SM.

Western blot

Whole cell lysates were extracted in PBS plus 2×SDS, equally loaded on 10-15% SDS-polyacrylamide gels, and subsequently transferred to nitrocellulose membrane (162-0115, Bio-Rad, USA). After blocking in 5% skim milk in TBST at room temperature for 1 hour, the membranes were incubated with indicated primary antibodies overnight at 4℃, followed by HRP-linked secondary antibodies (7074, CST, USA) at room temperature for 1 hour. The signals were detected by reacting with chemiluminescent HRP substrate (WBKLS0500, Millipore, USA) and visualized using a chemiluminescent detector (LAS-4000, FUJIFILM, Japan). The antibodies include as following: anti-FAM122A (customized by Abclone, China), anti-phospho-Akt (Thr308) (13038, CST, USA), anti-phospho-Akt (Ser473) (4060, CST, USA), anti-Akt (10176-2-AP, proteintech, China), anti-MYC (10828-1-AP, proteintech, China), anti-phospho-MYC (ser62) (11311, Signalway Antibody, USA), anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (4370, CST, USA), anti-p44/42 MAPK (Erk1/2) (4695, CST, USA), anti-phospho-Bad (Ser112) (5284, CST, USA), anti-Bcl-2 (60178-1-lg, proteintech, China), anti-phospho-Bcl-2 (Ser70) (2827, CST, USA), anti-phospho-Jak2 (Tyr1007/1008) (3771, CST, USA) and

anti-HRP-conjugated alpha-tubulin (HRP-66031, proteintech, China).

Real-time quantitative reverse transcription polymerase chain reaction

Total RNA was extracted by TRIzol reagent (molecular research center, USA), followed by treatment with FastQuant RT kit (TIANGEN, China) to wipe genomic DNA and complete reverse transcription of RNA to cDNA. RT-PCR was performed with SYBR select master mix (Applied Biosystem, USA) following the manufacturer's instruction. Gene abundance was normalized against Ct of β-actin with the ΔΔCt method. The specific primers for real-time qPCR were given as following:

Genes	Forward primers	Reverse primers
FAM122A	CCGCTTGCACCAGATCAAAC	CGGATTTCTCCACGTCGTTG
MYC	GTCAAGAGGCGAACACACAAC	TTGGACGGACAGGATGTATGC
β-actin	CATCCTCACCCTGAAGTACCC	AGCCTGGATAGCAACGTACATG

PP2A-Cα knockdown rescue study

PP2A-Cα shRNA was generated and tested for the role of PP2A in FAM122A silencing-triggered cellular apoptosis. PP2A-Cα shRNA Oligos were cloned into PLKO.1-GFP vector and the target sequence is 5'-CCCATGTTGTTCTTTGTTATT-3'. FAM122A shRNA Oligos were cloned into PLKO.1-puro vector. The PP2A-Cα- and FAM122A-shRNA viruses were packaged in the same batch, and then the virus supernatants were mixed to infect NB4 cells. Six experimental cell groups were generated as indicated. Three days after infection, the apoptosis of transduced NB4 cells were detected by flow cytometry and knockdown effects were examined by western blot.

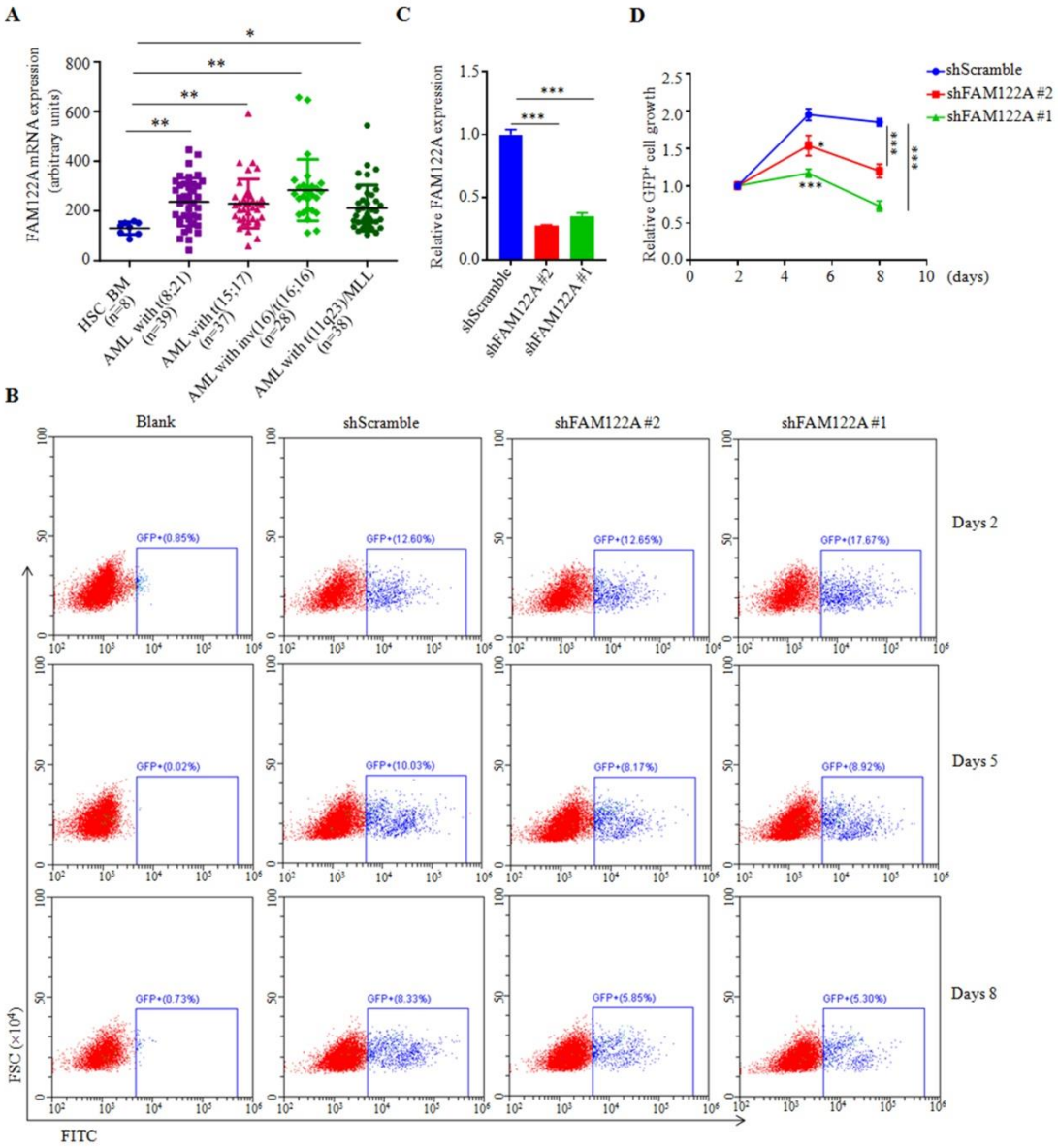
Statistical analyses

Data are expressed as mean ± SD and analyzed by Student's t test, with $p < 0.05$ indicating significant difference. The Kaplan-Meier method was used to analyze the association between overall survival and *FAM122A* gene expression levels in AML patients, and the survival comparison was performed by log-rank test, $p < 0.05$ indicating significant difference. Data were analyzed using Graphpad Prism 7.0 software. All experiments were repeated at least three times.

Supplemental Figure legends

Supplemental Figure 1. The effect of FAM122A silencing on the growth of $CD34^+$ cells. (A) The expression of *FAM122A* gene was compared among several subtypes of AML cells and HSCs in database from HemaExplorer (http://servers.binf.ku.dk/hemaexplorer). Each dot in the plot corresponded to the expression of *FAM122A* in a microarray, with horizontal lines representing the median expression for each class of cells. Symbols ** and * were presented $p < 0.01$ or $p < 0.05$, compared with HSCs. (B) Representative FACS plots were shown for GFP^+ cell percentages in $CD34^+$ HSPCs (cultured *in vitro*) after lentiviral transduction for 2, 5 and 8 days. (C) *FAM122A* knockdown efficiency was confirmed by qRT-PCR in GFP^+ cells by sorting $CD34^+$ HSPCs with lentiviral transduction for 5 days. (D) The relative growth rates of indicated cells were shown by absolute GFP^+ cells after transduction for indicated days, which were calculated by the percentages of GFP^+ cells multiplied with the total cell numbers against that of shScramble cells. Symbol * indicated $p<0.05$, and symbols ** and *** were respectively presented $p<0.01$ or $p<0.001$.

Supplemental Figure 2. The role of PP2A in FAM122A silencing-induced apoptosis. (A) Effects of FAM122A and/or PP2A Cα knockdown on cell apoptosis were examined in NB4 cells after lentiviral transduction for 3 days. Quantification of apoptosis was analyzed by $AnnexinV^+/7AAD^+$ cells. Symbol *** indicated $p<0.001$. (B) Phosphorylation states of several PP2A substrates were examined in FAM122A and/or PP2A-Cα knockdown cells by western blot.



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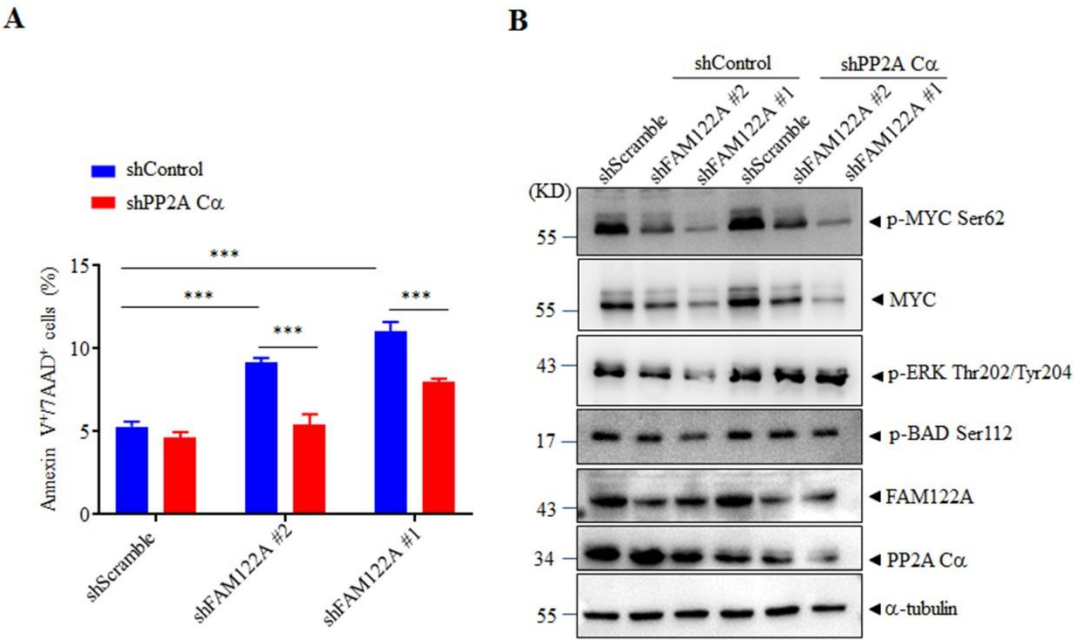

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