P2X7 promotes the progression of *MLL-AF9*-induced acute myeloid leukemia by upregulation of Pbx3

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Supplementary Appendix

Methods

Gene expression datasets

Gene expression datasets of patients with hematopoietic malignancies were downloaded from NCBI GEO DataSets (GSE13204, GSE19577, GSE12417, and GSE10358) and the Cancer Genome Atlas (TCGA).

Antibodies

The antibodies against mouse c-Kit (PE-Cy7-conjugated, 2B8), CD11b (PerCp-Cy5.5-conjugated, M1/70), Gr-1 (PE-Cy7-conjugated, RB6-8C5), CD3 (PerCp-Cy5.5-conjugated, 17A2), B220 (APC-conjugated, RA3-6B2), F4/80 (APC-conjugated, BM8), CD115 (PE-conjugated, AFS98), Ki67 (PE-conjugated, 16A8), BrdU (APC-conjugated, Bu20a), Annexin V (Alexa Fluor®647), CD45.1 (APC-Cy7-conjugated, A20) and anti-rabbit IgG (Dylight™ 649-conjugated, Poly4053) were purchased from BioLegend. Monoclonal antibody against GAPDH (14C10, #2118) was from Cell Signaling Technology. Polyclonal antibodies against PBX3 were from proteintech (12571-1-AP). Polyclonal antibodies against P2X7 were from Alomone Labs (#APR-004) and Abcam (ab94717). The former was for western blot and the latter was for immunofluorescence.

Mice

C57BL/6J, C57B6.SJL congenic mice and nude mice were provided by the Animal Center of the Institute of Hematology and Blood Diseases Hospital, CAMS & PUMC. Female mice (6 to 8 weeks old, 18-20 g) were used and maintained in SPF-certified facility. Mice were randomly assigned to experimental groups.

Vectors

The retroviral vector for overexpressing P2X7 was constructed following standard protocols. Briefly, the blank retrovirus vector pMSCV-PGK-BFP was reconstructed from pMSCV-PGK-GFP, and the recombinant vector pMSCV-P2X7-PGK-BFP (pMSCV-P2X7) was constructed by insertion of P2X7 cDNA.

Validated small hairpin RNA (shRNA) sequences targeting P2X7 (P2X7sh1 and P2X7sh2), human Pbx3 (hPbx3sh1, hPbx3sh2) and mouse Pbx3 (mPbx3sh1) in knockdown (KD) experiments were obtained from Sigma-Aldrich. The shRNA sequences are listed below. Scramble (pLV-SC) and KD (pLV-P2X7sh1/sh2 for P2X7, pLV-hPbx3sh1/sh2 for human Pbx3 and pLV-mPbx3sh1 for mouse Pbx3) lentiviruses were constructed using the pLV-H1-EF1α-red vector following standard protocols.

Targets	Sequences
P2X7sh1	5'-AAAAGCATGAATTATGGCACCATTATTGGATCCAATAATGGTGCCATAATTCATGC-3'
P2X7sh2	5'-AAAACGGAATAATGGGCATTGAGATTTGGATCCAAATCTCAATGCCCATTATTCCG-3'
hPbx3sh1	5'-AAAAACTCGGATACCTCTAACTAATTTGGATCCAAATTAGTTAGAGGTATCCGAGT-3'
hPbx3sh2	5'-AAAAGGTTCTTCAGATAACTCTATTTTGGATCCAAAATAGAGTTATCTGAAGAACC-3'
mPbx3sh1	5'-AAAACGTGTGAAGCAGTTATGATTTTTGGATCCAAAAATCATAACTGCTTCACACG-3'

Verification of mouse AML models

The expression of P2X7 was verified by qRT-PCR, western blot and immunofluorescence as previously described.[1] The expression of Pbx3 was detected by qRT-PCR and western blot. P2X7 activity was assessed by measuring the intracellular free Ca^{2+} concentration upon BzATP stimulation.[2]

Human leukemia cells

THP1, HL60 and Kasumi cell lines were obtained from American Type Culture Collection (Manassas, VA). THP1 cells carry t(9;11)(q23;p22) MLL-MLLT3 (MLL-AF9) translocation.

THP1, HL60 or Kasumi cells were infected with blank or pMSCV-P2X7 retrovirus and sorted twice by flow cytometry. The GFP^+ stable transfected cell lines were named THP1-control, THP1-P2X7, HL60-control, HL60-P2X7, Kasumi-control and Kasumi-P2X7, respectively.

P2X7- or Pbx3-knockdown THP1 cells were established. Briefly, THP1 cells were infected with pLV-SC or the knockdown lentiviruses. After sorting by flow cytometry, the stable transfected cell lines were named THP1-SC, THP1-P2X7sh1, THP1-P2X7sh2, THP1-hPbx3sh1 and THP1-hPbx3sh2, respectively.

The xenotransplantation model in NOD/SCID mice with primary cells from an AML patient with *MLL* translocation and high levels of P2X7 expression were established. Mice were sacrificed when BM human leukemia cells exceeded 50%, and leukemia cells were sorted by flow cytometry. Pbx3-knockdown human leukemia cells were established as described above, and the stable transfected cells were named hMLL-SC, hMLL-hPbx3sh1, respectively.

All cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (Gibco) and antibiotics (Hyclone) in a humidified atmosphere of 5% CO_2 at 37°C.

Cell proliferation and apoptosis assay

For the BrdU incorporation assay, mice in the middle stage of leukemia (on approximately days 15) were intraperitoneally (*i.p.*) injected with 200 μl BrdU (10 mg/ml). Mice were sacrificed 16 hrs later. BM cells were harvested and further stained with BD Pharmingen™ APC BrdU Flow Kit (BD, San Jose, CA) according to the manufacturer's protocols. For the Ki67 staining assay, 2×10^6 GFP^+BFP^+ cells were fixed and permeabilized following the instructions of Cytofix/Cytoperm™

Fixation/Permeabilization Solution Kit (BD). After that, cells were stained with PE-conjugated anti-Ki67 and Hoechst 33342 before flow cytometry analysis.

Single cell suspension was prepared. Cells were first incubated with Annexin V-Alexa Fluor 647 antibody in 1 × binding buffer for 15 mins. Just before flow cytometry analysis, PI in 1 × binding buffer was added.

Cell viability assay

MTS assay was used for cell viability analysis. THP1 cells and stably transfected cells, *i.e.* THP1-control, THP1-P2X7, THP1-SC, THP1-P2X7sh1, THP1-P2X7sh2, THP1-hPbx3sh1 and THP1-hPbx3sh2, were seeded onto 96-well plates at the concentration of 3×10^4 cells/well and cultured for 4 hrs (designated as starting point, or 0 h point). Then cells were cultured for extended periods and MTS (20 μl/well) was added to culture systems 2 hrs prior to measurement of absorbance at 490 nm every 24 hrs. Cell viability was calculated as fold change of absorbance verses 0 hr.

Colony forming assay

Sorted GFP^+BFP^+ (500 cells/well) or $GFP^+BFP^+RFP^+$ (500 cells/well) leukemia cells were plated onto 24-well plates in triplicate in 500 μl MethoCult GF M3434 media (Stem Cell Technologies) following the manufacturer's instructions. After 10 days, the total number of colonies was counted. Colonies were stained with 0.05% crystal violet solution for 30 mins, rinsed 5-10 times with PBS and scanned by a scanner.

Ara-C treatment

Mice were transplanted with MA9 or MA9-P2X7 cells to establish AML models, and PB leukemia cells were monitored. Intravenous administration of 500 mg/kg/day Ara-C was performed. In single-dose administration experiments, mice were treated

when the percentage of PB leukemia cells was approximately 4% in both groups. The percentage of PB leukemia cells and the apoptotic rate of BM leukemia cells was determined 24 hrs later. In multiple-dose administration experiments, mice were treated 4 times (every 24 hrs) when the percentage of PB leukemia cells was approximately 8% in both groups. The percentage of PB leukemia cells was monitored every 48 hrs, and the apoptotic rate of BM leukemia cells at 96 hrs was detected. In relapse experiments, mice were treated with Ara-C or PBS twice (days 12 and 13). The percentage of PB leukemia cells was monitored before (days 6 and 11) and after (days 14 and 19) Ara-C treatment. The survival of the mice was recorded.

THP1 xenograft mouse models

Nude mice were irradiated with 250 cGy, and 1.8×10^7 THP1 cells in 250 μl were subcutaneously inoculated in the right forelimbs. Mice were randomly divided into PBS and A740003 (P2X7 inhibitor) groups. After 10 days, tumors were palpable and measured with a manual caliber every two days. Tumor volume was calculated by the following equation: volume = (length × width × width/2). From days 12, intratumoral injection of 20 μl sterile PBS or 40 μM A740003 (Tocris Bioscience) was performed every two days. Mice were sacrificed on days 23, and the tumors were weighed.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Life Technologies, USA) and reverse transcribed using the Super Script First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) following the manufacturers' protocols. qRT-PCR was conducted on Stepone Plus or 7900 system (Applied Biosystems, Life Technologies) using SYBR Green PCR Master Mix. The expression level of target genes was analyzed by the relative quantity (RQ) value ($2^{-\Delta\Delta Ct}$) calculated using the ΔΔCt method [$\Delta\Delta Ct = (Ct_{TARGET} - Ct_{GAPDH})sample - (Ct_{TARGET} - Ct_{GAPDH})calibrator$]. For each gene, the RQ value of control group was designated as 1.00.

Western blot

Standard protocols were followed for sample preparation and experimental procedure. Briefly, GFP^+BFP^+ leukemia cells were sorted and lysed by sonication for ten cycles in lysis buffer containing RIRP and PMSF. BCA Protein Assay Kit (Thermo Scientific) was used for determination of protein concentration in supernatant. 10% SDS-polyacrylamide gel electrophoresis was undergone to separate proteins. Rabbit polyclonal antibodies against P2X7 and PBX3 or monoclonal GAPDH were used as primary antibodies and goat-anti-rabbit-HRP polyclonal antibodies were used as secondary antibodies. ImageQuant LAS-4010 (GE) was used for chemiluminescent visualization of proteins. Results were quantified by grey value analysis (Image J).

Flow cytometry analysis and cell sorting

BD Canto II or LSR II was used for FACS analysis and Aria III flow cytometer (BD Biosciences) was used for cell sorting, respectively. Standard protocols were followed for all experiments. Data analysis was carried out using FACS Diva (BD Biosciences) or FlowJo 7.6.1 (Tree Star) software.

References

1. Feng W, Yang F, Wang R, et al. High level P2X7-mediated signaling impairs function of hematopoietic stem/progenitor cells. Stem Cell Rev. 2016;12(3):305-314.
2. Chong JH, Zheng GG, Zhu XF, et al. Abnormal expression of P2X family receptors in Chinese pediatric acute leukemias. Biochem Biophys Res Commun. 2010;391(1):498-504.

Figures

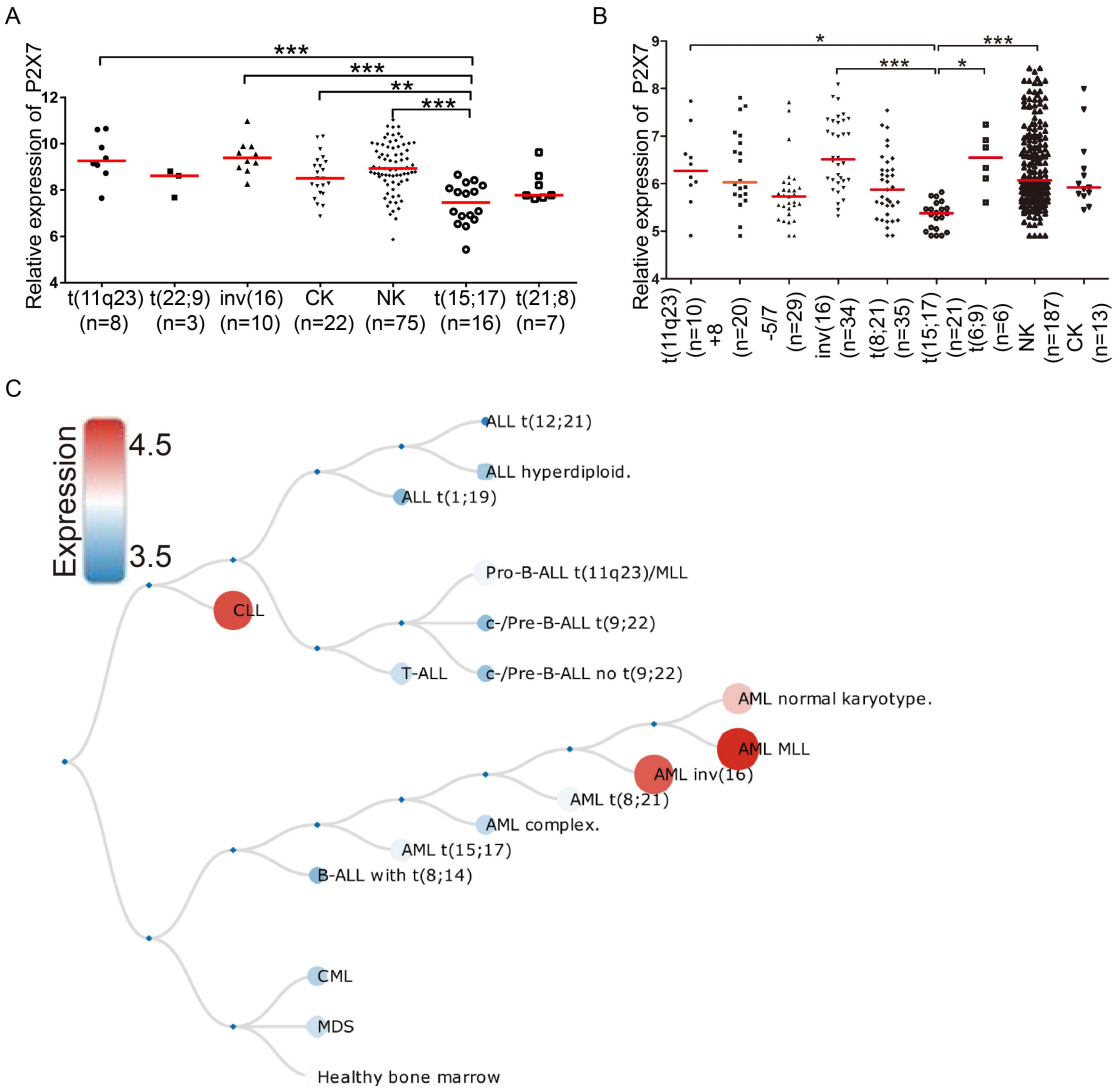

Figure 1

Expression of P2X7 in leukemia patients. (A) The relative expression of P2X7 in leukemia patients with t(11q23), t(22;9), inv(16), complex cytogenetics (CK), normal karyotype (NK), t(15;17) and t(21;8) was obtained from TCGA datasets. **(B)** The relative expression of P2X7 in leukemia patients with t(11q23), +8, -5/7(q), inv(16), t(8;21), t(15;17), t(6;9), NK, CK was obtained from GSE6891. **(C)** The tree map of P2X7 expression in different subtypes of leukemia was obtained from BloodSpot (http://servers.binf.ku.dk/bloodspot/?gene=P2RX7&dataset=allmile). Red and blue represent high and low expression levels, respectively. Bars represent mean ± SEM. *$p < 0.05$; **$p < 0.01$; *** $p < 0.001$.

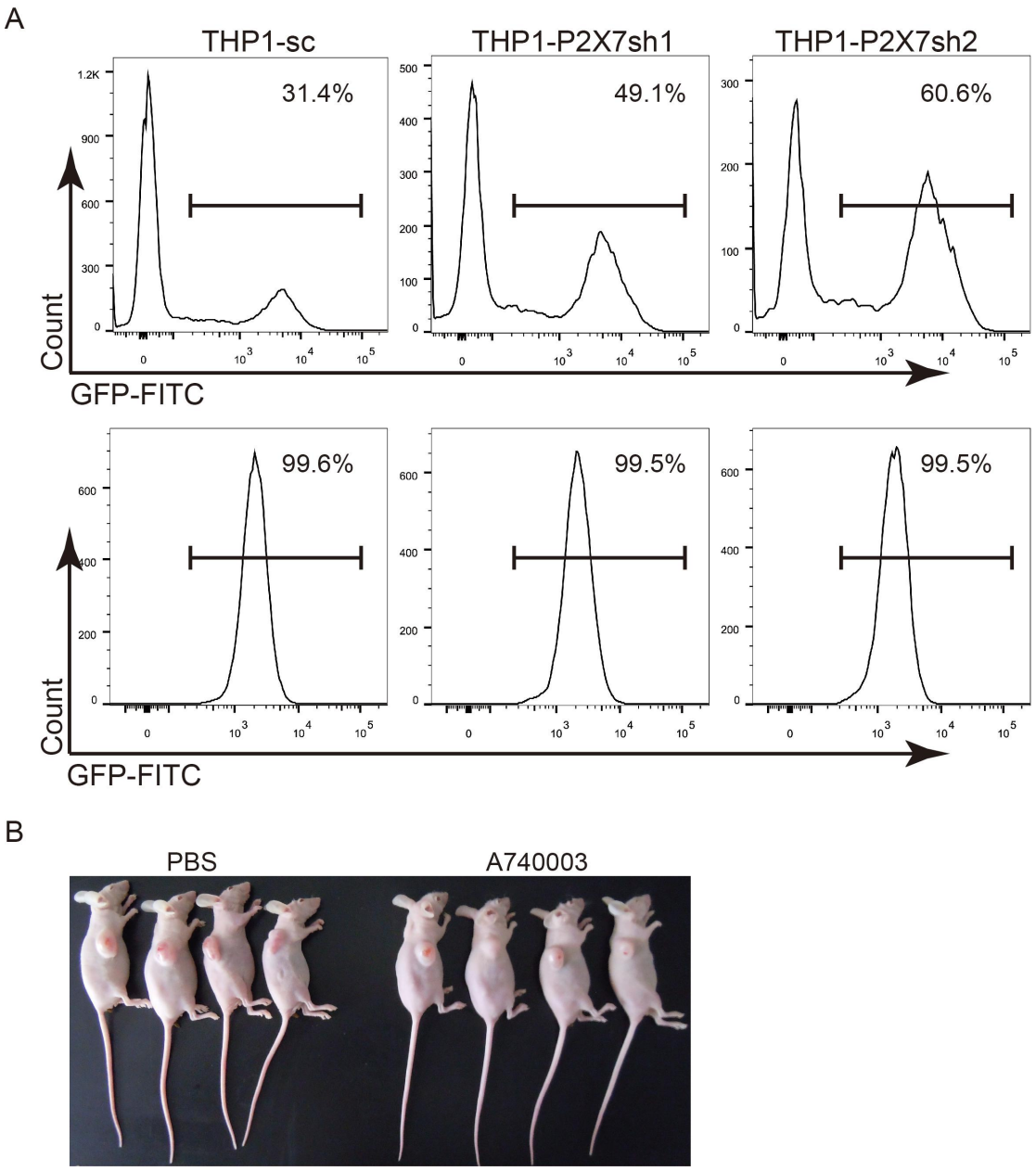

Figure 2

The expression and function of P2X7 are suppressed in THP1 cells. (A) THP1 cells were infected with pLV-sc, pLV-P2X7sh1 or pLV-P2X7sh2 lentivirus to establish THP1-sc, THP1-P2X7sh1 and THP1-P2X7sh2 cell lines, respectively. The percentage of GFP^+ cells before (upper) and after (lower) cell sorting is shown. **(B)** Nude mouse xenograft model was established by inoculation of THP1 cells. From days 12, intratumoral administration of PBS or A740003 was done every two days. Mice were sacrificed on days 23.

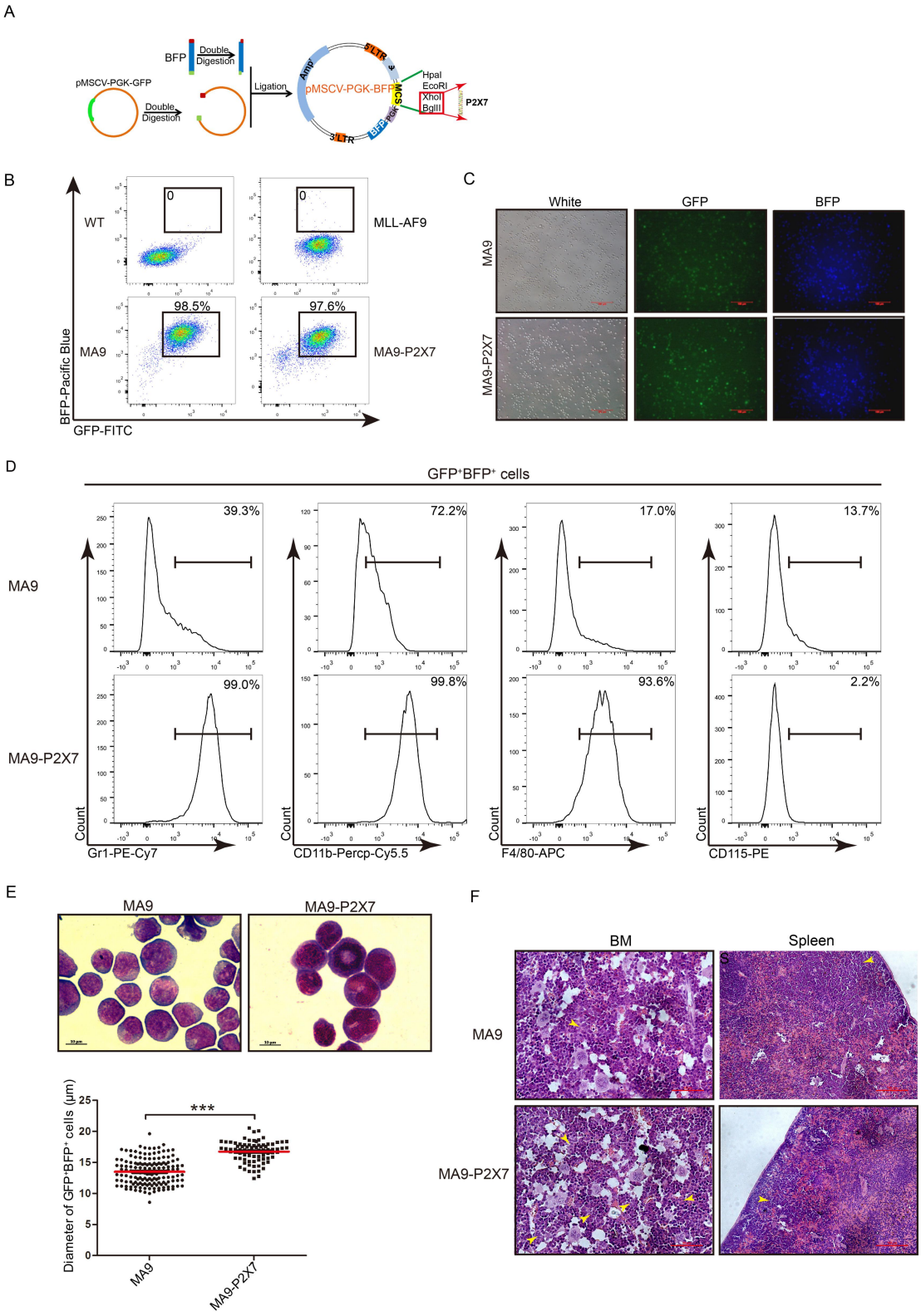

Figure 3

Establishment of mouse AML model caused by MLL-AF9-induced AML cells overexpressing P2X7. (A) Experimental design for the construction of pMSCV-PGK-BFP vector and recombinant plasmid carrying P2X7 cDNA. **(B)** Mice

were sacrificed and GFP^+BFP^+ cells were analysis by flow cytometry. The double positive rate of WT, MLL-AF9, MA9 and MA9-P2X7 cells in GFP^+ cells is shown. **(C)** GFP^+BFP^+ leukemia cells were detected under fluorescence microscope. **(D)** The surface markers (Gr1, CD11b, F4/80 and CD115) of MA9 and MA9-P2X7 cells were studied by flow cytometry. **(E)** Morphological analysis of sorted MA9 and MA9-P2X7 AML cells by Wright-Giemsa staining. Scale bars, 20 μm. Diameter of cells was measured under light microscope. **(F)** Typical HE stained BM and spleen tissue sections from MA9 and MA9-P2X7 mice are shown. Scale bars, 100 μm. Bars represent mean ± SEM. *** $p < 0.001$.

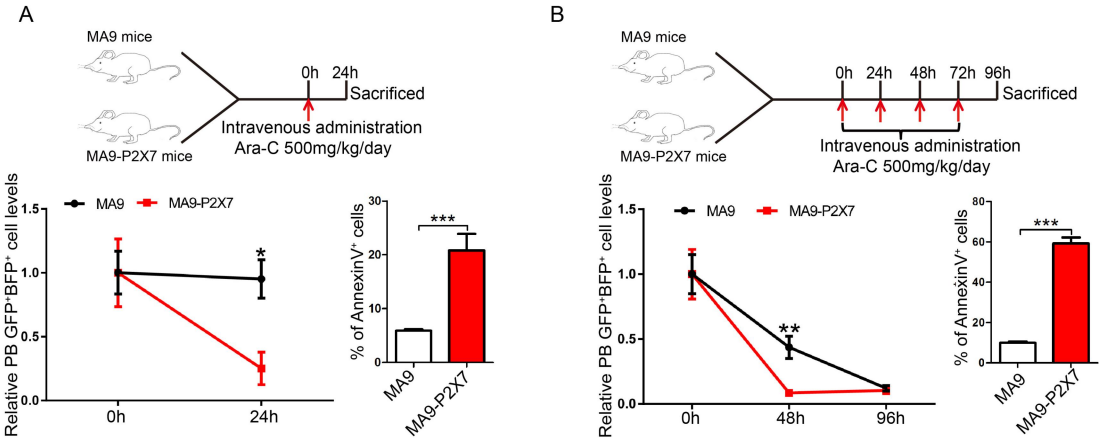

Figure 4

Effects of Ara-C treatment on MA9 and MA9-P2X7 mice. (A) The schedule for single-dose administration of Ara-C to is shown (*upper*). The relative GFP^+BFP^+ leukemia cells levels in PB was monitored (*lower left*). The apoptotic rate of BM leukemia cells at 24 hrs was detected (*lower right*) (n=5). **(B)** The schedule for multiple-dose administrations of Ara-C to leukemic mice is shown (upper). The relative GFP^+BFP^+ leukemia cells levels in PB was monitored (*lower left*). The apoptotic rate of BM leukemia cells at 96 hrs was detected (*lower right*) (n=5). The proportion of leukemia cells at 24 hrs, 48 hrs and 96 hrs was normalized to that at 0 h. Bars represent mean ± SEM. *$p < 0.05$; **$p < 0.01$; *** $p < 0.001$.

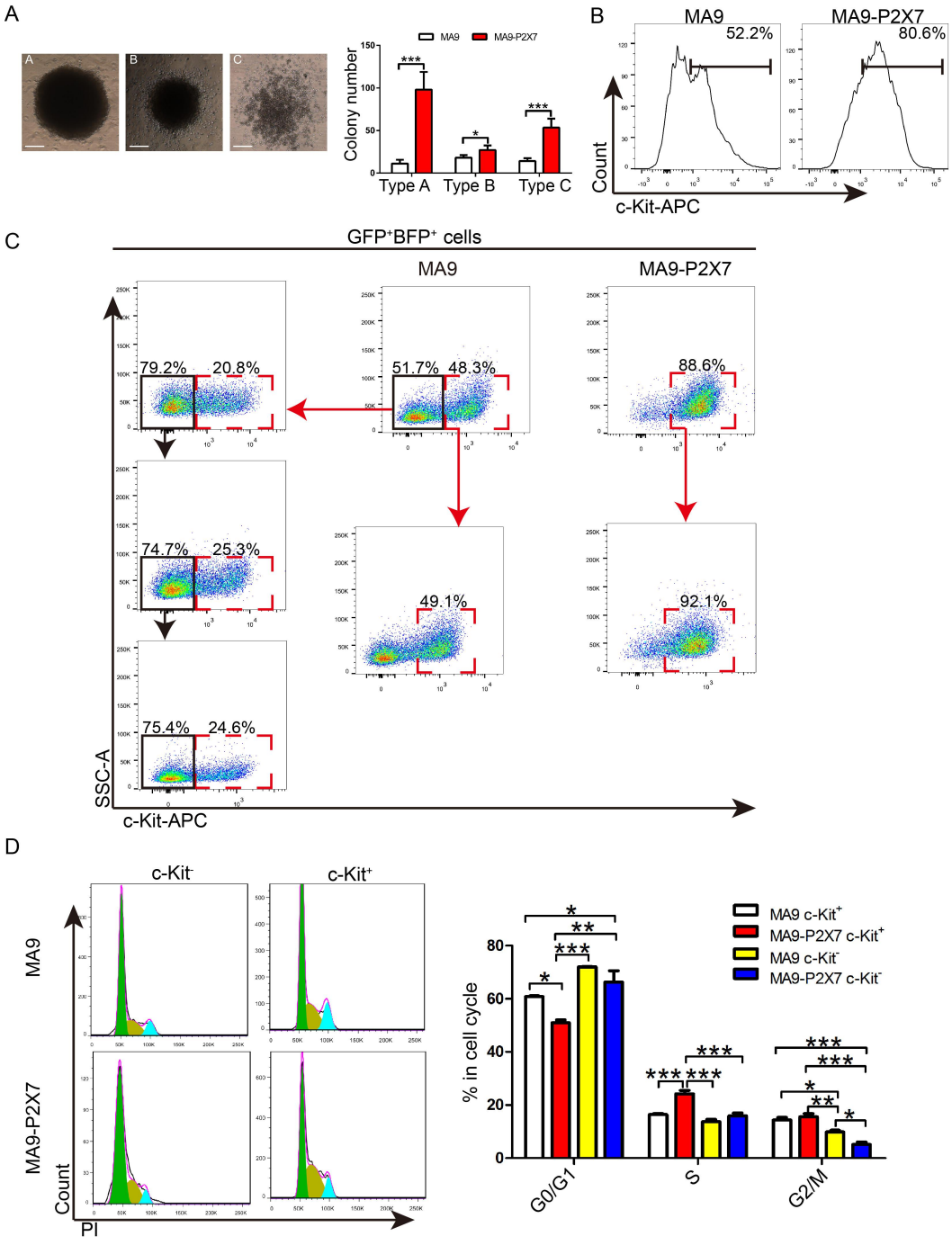

Figure 5

MA9-P2X7 cells have more LSCs. (A) Typical type A, B and C colonies are shown (*left*). Colony numbers of different types are plotted (*right*). **(B)** Colony cells of MA9 and MA9-P2X7 were stained with c-Kit. Percentage of c-Kit$^+$ cells is shown. **(C)** C-Kit$^-$ (black solid box) population was sorted from MA9 cells while c-Kit$^+$ (red dotted box) populations were sorted from MA9 and MA9-P2X7 cells. MA9-P2X7 c-Kit$^+$ (*right*) and MA9 c-Kit$^+$ (*middle*) cells were undergone transplantation while MA9 c-Kit$^-$ cells (*left*)

were undergone serial transplantations. Typical flow cytometry results showed the frequencies of c-Kit$^-$ and c-Kit$^+$ populations. **(D)** Leukemia cells were sorted and stained with PI. Gating strategy for G0/G1 (green), S (khaki), and G2/M (blue) phase cells was shown. The percentage of them is plotted. Bars represent mean ± SEM. *$p < 0.05$; **$p < 0.01$; *** $p < 0.001$.

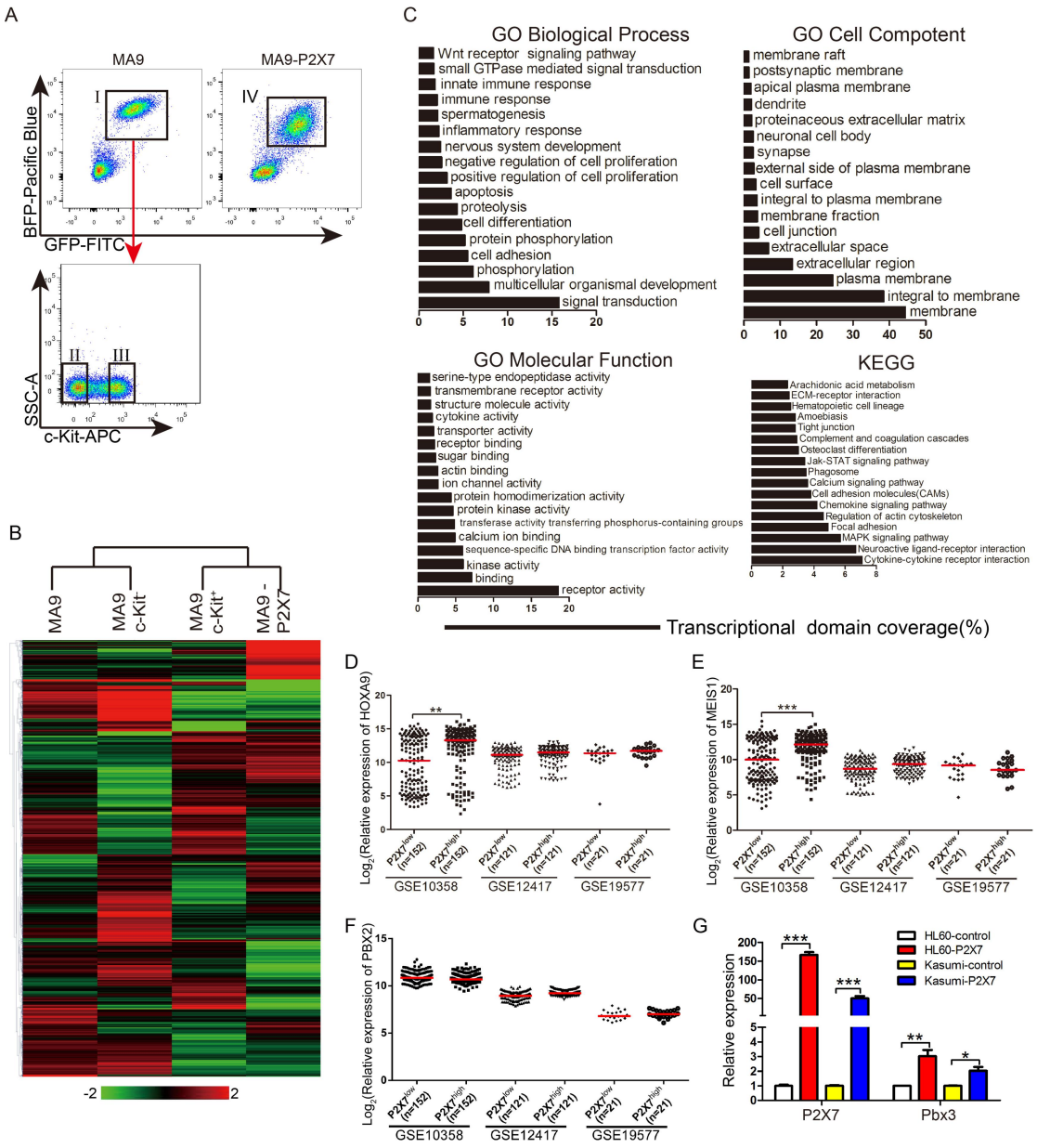

Figure 6

Microarray analysis to identify key molecules mediating pro-leukemic effects of P2X7 in MLL-AF9-induced AML cells. (A) Gating strategy for sorting MA9 (gate I) , MA9 c-Kit$^-$ (gate II), MA9 c-Kit$^+$ (gate III), and MA9-P2X7 (gate IV) cells, which were sent for microarray analysis. **(B)** Hierarchical clustering analysis of gene expression profiles among four populations, which were normalized to a quantile algorithm. **(C)** GO and KEGG analyses of DEGs between MA9-P2X7 and MA9. **(D-F)** Correlation between the expression of P2X7 and HOXA9, MEIS1, PBX2 was studied from GSE10358 (n=304), GSE12417 (n=242) and GSE19577 (n=42). For each dataset,

AML cases were divided into $P2X7^{low}$ and $P2X7^{high}$ groups according to median value of P2X7, and the expression of HOXA9, MEIS1 and PBX2 is plotted. **(G)** HL60 and Kasumi cells were infected with blank (control) or pMSCV-P2X7 (P2X7). GFP^{+} cells were sorted and the expression of P2X7 and Pbx3 was detected by qRT-PCR. Bars represent mean ± SEM. *$p < 0.05$*; **$p < 0.01$*; ***$p < 0.001$*.

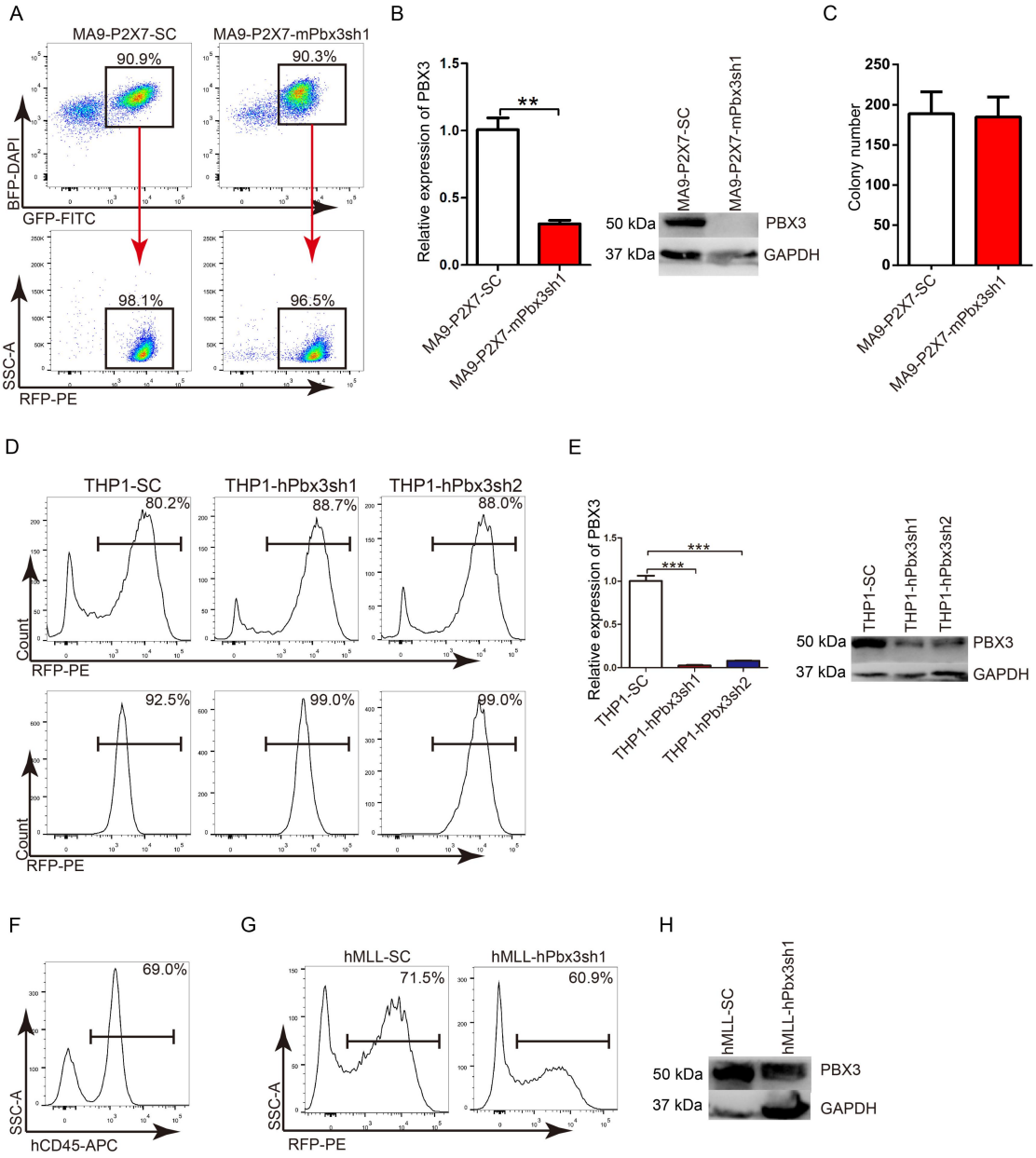

Figure 7

Knockdown of Pbx3 expression in mouse and human AML cells with *MLL* translocation. (A,B) Pbx3 was suppressed in MA9-P2X7 cells by shRNA against Pbx3. **(A)** MA9-P2X7-SC and MA9-P2X7-mPbx3sh1 cell lines ($GFP^+BFP^+RFP^+$) were verified by flow cytometry. **(B)** KD efficiency of Pbx3 in MA9-P2X7 was identified by qRT-PCR (left), western blot (right). **(C)** $GFP^+BFP^+RFP^+$ leukemia cells were sorted and seeded onto 24-well plates (500 cells/well) for colony forming assay. Colony numbers are plotted. **(D)** THP1 cells were infected with pLV-SC, pLV-hPbx3sh1 and pLV-hPbx3sh2. Typical flow cytometry results before (*upper panel*) and after (*lower*

panel) cell sorting are shown. **(E)** KD efficiency of Pbx3 in THP1 was identified by qRT-PCR (*left*), western blot (*right*). **(F)** The patient derived xenograft model by an AML patient with *MLL* translocation and high levels of P2X7 expression were established. Human leukemia cells were sorted for lentivirus infection. **(G)** Leukemia cells were infected with pLV-SC or pLV-hPbx3sh1. Typical flow cytometry results are shown. **(H)** KD efficiency of Pbx3 was identified by western blot. Bars represent mean ± SEM. *** $p < 0.001$.

Tables

Table 1. Main characteristics of newly diagnosed AML patients obtained from Blood Diseases Hospital, CAMS & PUMC

Patient ID	Gender	Age	FAB Subtype	Karyotype	Fusion gene	WBC	HGB	PLT
1	Male	11	M3	46,XY,t(15;17)(q22;q21) [7]	PML/RARa	27.65	83	16
2	Male	4	M5	46,XY,6p+,11q-	TCR/IGK	5.45	79	8
3	Male	15	M3	46,XY[1]	-	2.18	93	40
4	Female	4	M2b	46,XX,t(8;21)(q22;q22)[15]	AML1/ETO	5.42	51	13
5	Male	6	M6	46,XY,t(3;5,?),13q-,15q-[5]	-	31.14	52	16
6	Female	11	M2b	45,X,-Y,t(8;21;10)(q22;q22;q26),del(9)(q11q22)[11]	AML1/ETO	23.03	70	42
7	Female	10	M5a	46,XX,t(9;22)(q34;q11)[5]	BCR/ABL190	108.57	123	133
8	Female	18	M3	46,XX	PML/RARa	1.39	88	40
9	Male	18	AML	47-50,XY,+X,+7,+13,+15,-16,+18[CP4]/46,XY[4]	-	255.93	112	80
10	Male	9	M2b	46,XY,t(8;21)(q22;q22)[7]/46,XY[3]	AML1/ETO	10.59	59	25
11	Female	7	M3	42,XX,-8,-16,-19,-21[1]	PML/RARa	1.5	69	7
12	Male	3	M3	46,XY,t(15;17)(q22;q12)[6]	PML/RARa	226.03	59	43
13	Male	16	M3	46,XY,t(15;17)(q2	PML/RARa	1.65	71	2

				2;q12)[15]				
14	Male	10	M2b	46-47,XY,1p+.2p-,+4,t(8;21)(q22;q22),-15,+mark[cp3]	AML1/ETO	35.74	118	107
15	Male	6	M2b	50,XX,YY,t(8;21)(22,q22)x2[3]/45,X,-Y,t(8;21)(q22;q22)[7]/46,XY[2]	AML1/ETO	5.17	62	58
16	Female	9	M5	46,XX[8]	-	193.15	70	53
17	Male	3	M2a	46,XY	-	110	68	19
18	Female	11	M5	47,XX,+8[3]	-	103.01	57	81
19*	Male	6	M5	46,XY	MLL	426	85	20
20	Female	1	M5	47,XX,+8	MLL	47.59	113	133

NOTE: FAB, French-American-British criteria for subtypes. The clinical data were collected at initial diagnosis. WBC, white blood cell count ($\times 10^9$/L); HGB, hemoglobin (×g/L); PLT, platelet ($\times 10^9$/L); -, unknown or undetected; *This patient has *MLL* translocation and high levels of P2X7 expression. The xenotransplantation model in NOD/SCID mice with AML primary cells from this patient was established.

Table 2. Websites to download detailed characteristics of AML patients from public database

Public Database	Websites	Patient Characteristics Provided	Reference
TCGA	https://portal.gdc.cancer.gov/repository?facetTab=cases&filters=%7B%22op%22%3A%22and%22%2C%22content%22%3A%5B%7B%22op%22%3A%22in%22%2C%22content%22%3A%7B%22field%22%3A%22cases.primary_site%22%2C%22value%22%3A%5B%22hematopoietic%20and%20reticuloendothelial%20systems%22%5D%7D%7D%5D%7D&searchTableTab=cases	Gender, age, race, bone marrow blast percentage, PB blast percentage, WBC, AML in skin percentage, subclones, cytogenetics, genomic rearrangement, cytogenetic code, histological subtype, risk (Cyto), risk (Molecular), structural variants from WGS, DFS months, DFS status, OS months, OS status, induction, type of transplant (sib Allo, MUD, Auto, etc) , dark zone stat at transplant, disease type, cancer type detailed	
GEO DataSets			
GSE13204	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13204	Sample type, sample source name, sample organism, sample characteristics with disease type and genomic rearrangement, sample treatment protocol, sample growth protocol, sample extract protocol	PMID: 18573112 20406941 20065290
GSE19577	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE19577	Sample type, sample source name, sample organism, sample characteristics with disease type, sample description with genomic rearrangement, sample extract protocol	PMID: 21331072
GSE12417	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE12417	Sample type, sample source name, sample organism, sample description with disease type, genomic rearrangement,	PMID: 18716133

		FAB subtype, age, OS months, OS status, sample treatment protocol, sample growth protocol, sample extract protocol	
GSE10358	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE10358	Sample type, sample source name, sample organism, sample characteristics with FAB subtype, age, gender, race, cytogenetics, genomic rearrangement, sample growth protocol, sample extract protocol	PMID: 18270328 19651600

Table 3. Main characteristics of AML patients and treatment types received in P2X7^{high} and P2X7^{low} groups from TCGA database

	No. of cases	
	P2X7^{low} group (n=87)	P2X7^{high} group (n=86)
Gender		
Male	43 (49.4%)	38 (44.2%)
Female	44 (50.6%)	48 (55.8%)
Age at diagnosis, median	56 (IQR, 22-82)	60 (IQR, 18-88)
FAB subtype		
M0	8 (9.2%)	8 (9.3%)
M1	29 (33.3%)	15 (17.4%)
M2	25 (28.7%)	13 (15.1%)
M3	16 (18.4%)	0 (0)
M4	3 (3.5%)	31 (36.1%)
M5	3 (3.5%)	15 (17.4%)
M6	1 (1.1%)	1 (1.2%)
M7	2 (2.3%)	1 (1.2%)
NC	0 (0)	2 (2.3%)
BM blasts at diagnosis, median percentage	71 (IQR, 30-100)	73.5 (IQR, 30-98)
PB blasts at diagnosis, median percentage	44 (IQR, 0-98)	28.5 (IQR, 0-97)
WBC at diagnosis, median count (×10^9/L)	10.1 (IQR, 0.4-297.4)	26.85 (IQR, 0.8-137.2)
Cytogenetic code		
BCR-ABL1	2 (2.3%)	1 (1.2%)
CBFB-MYH11	1 (1.1%)	9 (10.5%)
Complex Cytogenetics	12 (13.8%)	10 (11.6%)
Intermediate Risk Cytogenetic	10 (11.5%)	10 (11.6%)

Abnormality		
MLL translocation	0 (0)	4 (4.7%)
Normal Karyotype	33 (37.9%)	47 (54.6%)
PML-RARA	15 (17.3%)	0 (0)
Poor Risk Cytogenetic Abnormality	7 (8.1%)	2 (2.3%)
RUNX1-RUNX1T1	6 (6.9%)	1 (1.2%)
N.D.	1 (1.1%)	2 (2.3%)
Histological subtype		
BCR-ABL1	2 (2.3%)	1 (1.2%)
CBFB-MYH11	1 (1.1%)	9 (10.5%)
Complex Cytogenetics	12 (13.8%)	10 (11.6%)
Intermediate Risk Cytogenetic	9 (10.4%)	8 (9.3%)
Abnormality		
MLL translocation	2 (2.3%)	6 (7.0%)
Normal Karyotype	31 (35.6%)	44 (51.1%)
PML-RARA	16 (18.4%)	0 (0)
NUP98 translocation	0 (0)	3 (3.5%)
Poor Risk Cytogenetic Abnormality	7 (8.1%)	2 (2.3%)
RUNX1-RUNX1T1	6 (6.9%)	1 (1.2%)
N.D.	1 (1.1%)	2 (2.3%)
Risk (Molecular)		
Good	23 (26.4%)	10 (11.6%)
Intermediate	40 (46.0%)	52 (60.5%)
Poor	23 (26.5%)	22 (25.6%)
N.D.	1 (1.1%)	2 (2.3%)
Risk (Cyto)		
Good	22 (25.3%)	10 (11.6%)
Intermediate	43 (49.4%)	58 (67.5%)
Poor	21 (24.2%)	16 (18.6%)

N.D.	1 (1.1%)	2 (2.3%)
DFS, median months	11.40 (IQR, 0-100.5)	8.45 (IQR, 0.1-99.9)
DFS status		
Disease Free	49 (56.4%)	40 (46.5%)
Recurred/Progressed	37 (42.5%)	45 (52.3%)
NA	1 (1.1%)	1 (1.2%)
OS, median months	26.30 (IQR, 0-118.1)	11.15 (IQR, 0.1-99.9)
OS status		
Living	35 (40.2%)	24 (27.9%)
Deceased	52 (59.8%)	62 (72.1%)
Induction		
7+3	25 (28.7%)	35 (40.7%)
7+3+3	23 (26.5%)	22 (25.6%)
7+3+3, gleevec	1 (1.1%)	0 (0)
7+3+3+PSC	3 (3.5%)	1 (1.2%)
7+3+AMD	1 (1.1%)	1 (1.2%)
7+3+ATRA	12 (13.8%)	0 (0)
7+3+Genasense	1 (1.1%)	2 (2.3%)
7+3+study drug	1 (1.1%)	2 (2.3%)
7+3+dauno	1 (1.1%)	2 (2.3%)
7+4+ATRA	2 (2.3%)	0 (0)
Decitabine	7 (8.1%)	7 (8.1%)
Revlimid	3 (3.5%)	6 (7.0%)
Others	7 (8.1%)	8 (9.3%)

Table 4. The list of the genes satisfy the condition (FC ≥ 2.0) in all populations

Gene	MA9	MA9 c-Kit^{+}	MA9 c-Kit^{-}	MA9-P2X7
Hnf4a	4.315762	4.981476	5.12598	14.53355
Six1	5.042897	5.583961	5.224246	13.77709
Gprasp2	5.278139	5.244366	5.24583	13.07547
Six4	4.76146	4.516208	1.897203	9.44581
Xlr	6.787568	5.374322	5.014948	11.02067
Gm14625	6.690696	5.434371	5.113621	10.80462
Gm2030	6.754176	5.446716	5.209487	10.8959
Pdgfrl	7.868452	7.972529	6.872536	12.4145
Zdhhc2	5.381557	5.658747	4.633071	9.921941
Abcb9	9.355733	10.57929	8.721681	13.94806
Paqr5	6.197612	5.911735	6.263482	11.45583
LOC102638793	5.9447	4.969644	5.068087	10.0132
Zfp820	5.062906	4.194351	4.036269	8.742249
Pbx3	10.21993	9.215693	10.47261	15.08317
Hkdc1	5.428403	5.033161	5.795447	10.1967
Abcg2	6.265685	7.351389	5.952943	10.3353
Akr1c13	7.754127	7.503615	7.520504	11.88503
Slc39a8	8.216914	7.643287	6.501695	10.71311
Stab2	5.48605	5.734866	4.660516	8.778222
Gm13298	5.843244	5.938307	4.905674	8.885473
Usp51	5.520485	5.676248	4.122709	7.93472
Rbp4	6.814951	6.71742	8.039157	11.84367
Farp1	6.593319	6.48847	5.654502	9.309437
Dlk1	6.1247	5.729072	6.24702	9.85371
Hdc	11.49419	10.31877	11.31893	14.91544
Rtp4	6.37367	5.597343	6.752101	10.33988
Serpina11	9.596564	9.95379	8.698203	12.14983

Hoxb13	6.660536	5.360974	6.643797	10.02119
4933409K07Rik	5.312283	5.184112	4.069137	7.180141
Gm13298	5.583842	5.552136	5.412778	8.467941
Prg3	8.010638	8.60393	8.509789	11.5526
Ffar4	4.766295	5.22853	5.83119	8.820567
Atp6v1g3	5.398016	4.255027	5.289241	8.24122
4930500A05Rik	7.556674	6.9017	6.829518	9.660049
LOC102636053	7.145287	6.402517	5.977464	8.781041
Dusp18	4.474358	5.535305	5.439974	8.135218
Vstm5	4.229132	5.317394	4.815373	7.498062
Nefh	6.334524	6.405229	6.010756	8.578825
Alas2	5.475668	4.775726	4.155989	6.677557
Angpt1	9.128366	8.286065	9.404198	11.92548
Tnfsf13b	5.821169	6.305813	5.956012	8.449716
Mical3	6.552386	5.548242	6.494635	8.972683
Serpina11	9.111115	9.452453	8.803384	11.01234
Car13	4.81504	4.926811	4.999743	7.188988
Rspo1	8.381624	8.885473	8.507838	10.52745
Zscan18	5.547385	5.027604	4.721982	6.669403
Dclk2	5.724476	5.487093	5.889796	7.836155
Slc23a2	5.495347	5.55005	5.186237	6.88388
Alpi	4.050382	4.44265	3.896716	5.508654
Cttn	5.763858	5.567925	5.770294	7.134366

Table 5. List of the primers used in qRT-PCR in this study

Genes	Forward	Reverse
hGAPDH	5'-GAAGGTGAAGGTCGGAGTC-3'	5'-GAAGATGGTGATGGGATTTC-3'
mGapdh	5'-CACTTGAAGGGTGGAGC-3'	5'-GGGCTAAGCAGTTGGTG-3'
hP2X7	5'-TTGACACCGCAGACTACACCTT-3'	5'-GGCACCAGGCAGAGACTTCA-3'
mHoxa9	5'-GTGTTTGGTGCCTCGTGGAA-3'	5'-CGCCTTCGCTGGGTTGTTT-3'
hHOXA9	5'-ACCACCACCATCACCACCA-3'	5'-GGAGGAGAACCACAAGCATAGTCA-3'
mMeis1	5'-CAGCACAGGTGACGATGATGAC-3'	5'-GCTCGGTTGGACTGGTCTATCA-3'
hMEIS1	5'-CAGCAGTGAGCAAGGTGATGG-3'	5'-GGATGGTGAGTCCCGTGTCT-3'
mAbcb9	5'-TGCCGAGAGCGAATACCTGATT-3'	5'-TCCTTGTGGCTGGCTGTGTA-3'
mDusp18	5'-TCCAGCAATCAGATCACCACAGT-3'	5'-ATGAGGTAGGCGAGGCACAG-3'
mHoxb13	5'-GGCTACCTACCCTTCGGAAACT-3'	5'-TTCACCTTGGCAACACATCTGG-3'
hPBX3	5'-AGCATCACAGTGTCACAGGTATCC-3'	5'-GGCGAATTGGTCTGGTTGTTCTG-3'
mPbx3	5'-GGTTCTTCAGATAACTCTATTG-3'	5'-GGAGAAGGTTCATCACAT-3'
mRbp4	5'-CGAGTCCGTCTTCTGAGCAACT-3'	5'-CACCAGCCTCCGTGTCTCT-3'
mRhbdl3	5'-CAATCACGCTGCTGGAGGTT-3'	5'-CAAGGAGCCTGCCACAACAC-3'
mRspo1	5'-TCTGCTGGAGAGGAACGACATC-3'	5'-GGACCACTCGCTCATTTCACATTG-3'
mPbx2	5'-TGCCCTGTTTAGTGTCCTGTGT-3'	5'-CTTGCTGCGATAGTCTGAGTGTTC-3'