CD244 maintains the proliferation ability of leukemia initiating cells through SHP-2/p27^{kip1} signaling

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Methods

Mice

C57BL/6 CD45.2 and NOD-SCID mice were purchased from the Shanghai SLAC Laboratory Animal Co. Ltd. The CD244 KO mice with a C57BL/6 background were kindly provided by Dr. Colin Stewart of the Frederick National Laboratory for Cancer Research. Animal experiments were approved by our institution and conducted according to the Guideline for Animal Care at Shanghai Jiao Tong University, School of Medicine.

Western blotting and co-immunoprecipitation

A combination of different plasmids of CMV-human CD244-Fc, MSCV-human c-Kit-IRES-GFP, PLVX-strepII-human p27-IRES-GFP, PLVX-human SHP-2-IRES-GFP, shRNA targeting human SHP-2 or c-Kit were transfected into 293T cells and followed by a co-immunoprecipitation (co-IP) process for further analysis of their interactions. In some cases, HEL cells, mouse or human primary AML cells were used for the co-immunoprecipitation assay with p27 or SHP-2 antibodies, followed by the western blotting analysis with indicated antibodies against endogenous proteins. The following antibodies were used: anti-human Fc, anti-human StrepII (GenScript), anti-SHP-2, anti-Phospho-SHP-2 (Tyr542), anti-c-Kit (CST), anti-CD244 (SAB), anti-p27 (Abcam), anti-Phospho-tyrosine (4G10) (Millipore), and anti-β-actin (Calbiochem).

Quantitative RT-PCR

Cells were sorted by flow cytometry for the isolation of total RNA. First strand cDNA was reverse transcribed using M-MLV reverse transcriptase (Promega Inc.). PCR reactions were performed according to the manufacturer's protocol. In brief, 20 μL reactions with 2×ABI SYBR® Green PCR master mix, primers, and cDNA were used for the analysis of expression levels. The experiments were conducted in triplicate

with Applied Biosystems 7900HT. The mRNA level was normalized to the level of β-actin RNA transcripts. The primer sequences used are shown in sTable 1.

Colony forming unit assays

The indicated numbers of cells from AML mice or AML patients were plated in methylcellulose (M3534 or H4436, Stem Cell Technologies) according to the manufacturer's protocols. The same numbers of 1^{st} plated leukemia cells were further used for 2^{nd} or 3^{rd} plating if necessary. The numbers of colonies were calculated 7–10 days after culture.

Bone marrow transplantation

Either 300,000 mouse CD45.2 WT or CD244-null donor BM cells were mixed with $3x10^5$ freshly isolated CD45.1 competitor BM cells followed by retro-orbital injection into 8-10-week-old CD45.1 mice preconditioned with lethal irradiation at a total dose of 10 Gy. For the analysis of mouse HSC reconstitution, peripheral blood cells of recipient CD45.1 mice were collected. After lysis of red blood cells, the remaining cells were stained with anti-CD45.2-FITC, anti-CD45.1-PE, anti-CD3e-APC (for T-lymphoid lineage), anti-B220-PE (for B-lymphoid lineage), anti-Mac-1-APC or anti-Gr-1-PE (cells costained with anti-Mac-1 and anti-Gr-1 were deemed to be of the myeloid lineage) monoclonal antibodies (eBioscience).

Statistical analyses

Data are expressed as mean ± SEM. Data were analyzed with a Student's t test, and statistical significance was set at $p < 0.05$. The survival rates of the two groups were analyzed using a log-rank test.

sTable 1. Primer Sequences for candidate genes and shRNAs

Gene	**Sequence for qPCR**
Human CD244-F	CTGCTTCTGTGTGTGGAGGA
Human CD244-R	AGCAGAAGACTGGGACTGGA
Mouse p16-F	CGTGAACATGTTGTTGAGGC
Mouse p16-R	TCGAATCTGCACCGTAGTTG
Mouse p19-F	ATCCTGACGCCCTGAACC
Mouse p19-R	TAGTACCGGAGGCATCTTGG
Mouse p21-F	CGGTGTCAGAGTCTAGGGGA
Mouse p21-R	AGAGACAACGGCACACTTTG
Mouse p27-F	GGGTCTCAGGCAAACTCTGA
Mouse p27-R	TCTGTTGGCCCTTTTGTTTT
Mouse p53-F	AGAGACCGCCGTACAGAAGA
Mouse p53-R	CTGTAGCATGGGCATCCTTT
Mouse Meis1-F	CAGAAAAAGCAGTTGGCACA
Mouse Meis1-R	TGCTGACCGTCCATTACAAA
Mouse Mef2c-F	GCCAGTTACCATCCCAGTGT
Mouse Mef2c-R	ATCAGACCGCCTGTGTTACC
Mouse Cbx5-F	GGTTAAGGGGCAAGTGGAAT
Mouse Cbx5-R	CCCTGGGCTTATTGTTTTCA
Mouse HoxA9-F	AAAACACCAGACGCTGGAAC
Mouse HoxA9-R	TCTTTTGCTCGGTCCTTGTT
Mouse Hmgb3-F	AAAGGTGACCCCAAGAAACC
Mouse Hmgb3-R	CGGACTTTATCTGCCTTTGC
shRNA for human CD244	**Target sequence**
ShCD244#1	GGGAGTGCCTCTTCAGTTA
ShCD244#2	GAGCAAGGCTGTTAAATAA
shRNA for human SHP-2	**Target sequence**
shSHP-2#1	AGAAGCACAGTACCGATTT
shSHP-2#2	AGGATTGAAGAAGAGCAGA
shSHP-2#3	CACCTATATTTATGGGTGT
shSHP-2#4	CGGATGGTGTTCCAAGAAA
shSHP-2#5	CGCTCATGACTATACGCTA
shRNA for human c-Kit	**Target sequence**
shKit#1	CACCCTGGTCATTACAGAA
shKit#2	AGTGGATGTGCAGACACTA
shKit#3	CGATTCTAAGTTCTACAAG
shKit#4	GGCTTAAGCAATTCCATTT
shKit#5	CCCTCATGTCTGAACTCAA

sTable 2. Clinical sample information

Sample	Blast cells% in PB	Blast cells% in BM	Subtype	Prognosis	Age	Sex
1	88%	79%	M2	Dead	62	Male
2	28%	None	M2	Alive	40	Male
3	85%	52%	M2	Alive	77	Male
4	None	13.8%	M3	Alive	50	Female
5	26%	32%	M4	Dead	29	Male
6	None	50%	M4	Alive	62	Male
7	None	72%	M4	Dead	22	Male
8	65%	64%	M4	Dead	54	Female
9	None	86%	M4	Dead	65	Female
10	None	46%	M4	Dead	52	Male
11	27%	52%	M4	Alive	50	Female
12	73%	95%	M5a	Dead	16	Male
13	65%	37%	M5	Dead	66	Female
14	None	28%	M5b	Alive	47	Male
15	None	20%	M5	Alive	71	Male
16	None	37%	M5	Alive	62	Male
17	45%	None	M5	Dead	56	Male
18	86%	None	M5b	Loss of contact	60	Male
19	71%	93.50%	M5	Alive	51	Female
20	70%	68%	M5	Alive	45	Female
21	83%	80%	B-ALL	Alive	37	Female
22	68%	74%	B-ALL	Alive	14	Female
23	42%	98%	B-ALL	Dead	18	Male
24	87%	92.50%	B-ALL	Alive	14	Female
25	81%	93.50%	B-ALL	Dead	11	Female
26	92%	89%	B-ALL	Dead	57	Male
27	68%	85%	B-ALL	Alive	13	Female
28	85%	77%	B-ALL	Alive	36	Male

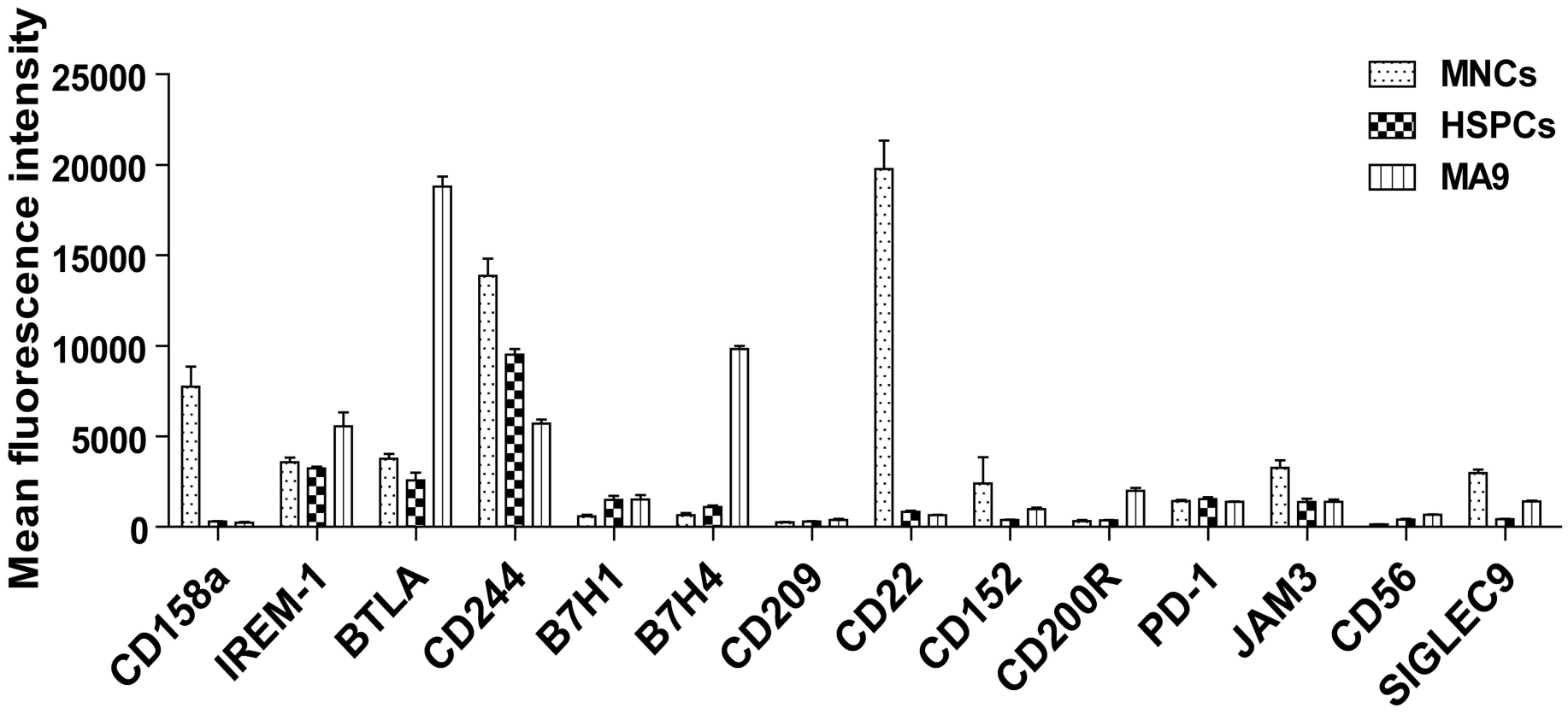
None: not examined

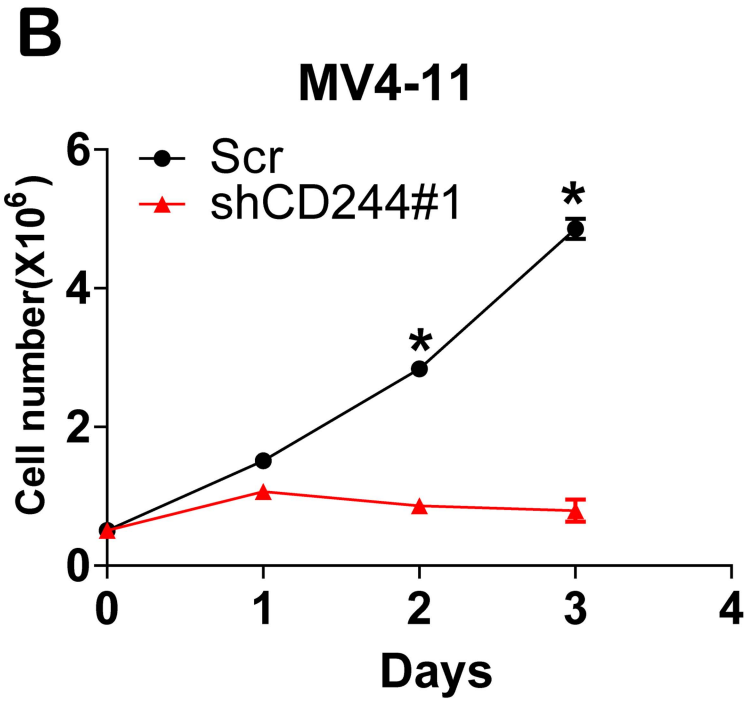
sTable 3. Leukemia initiating cell frequency

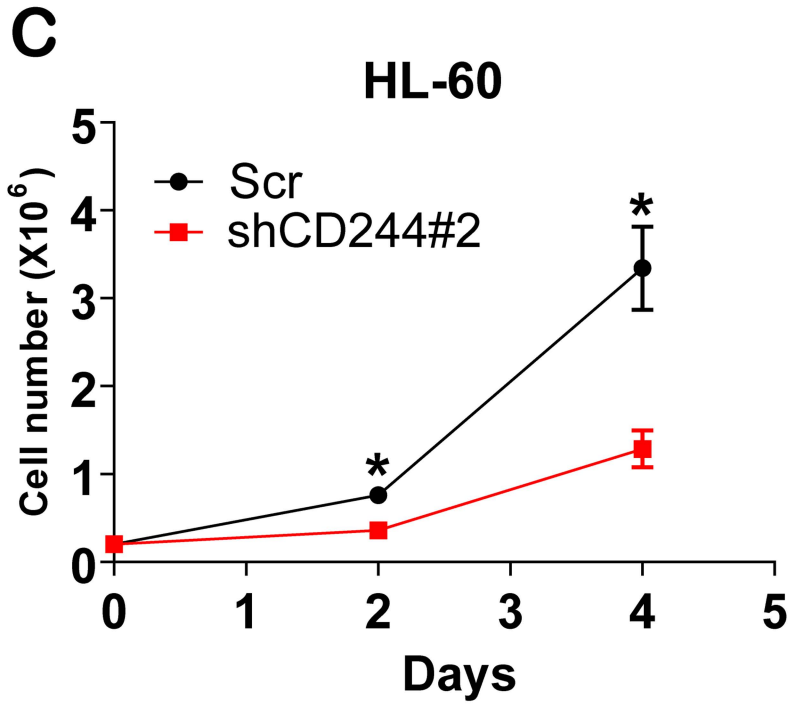
Cell #	WT	KO
100	5(5)	5(1)
300	5(5)	5(1)
1000	5(5)	5(4)
LIC Frequency	1 in 59	1 in 724
Range	36-97	473-1108

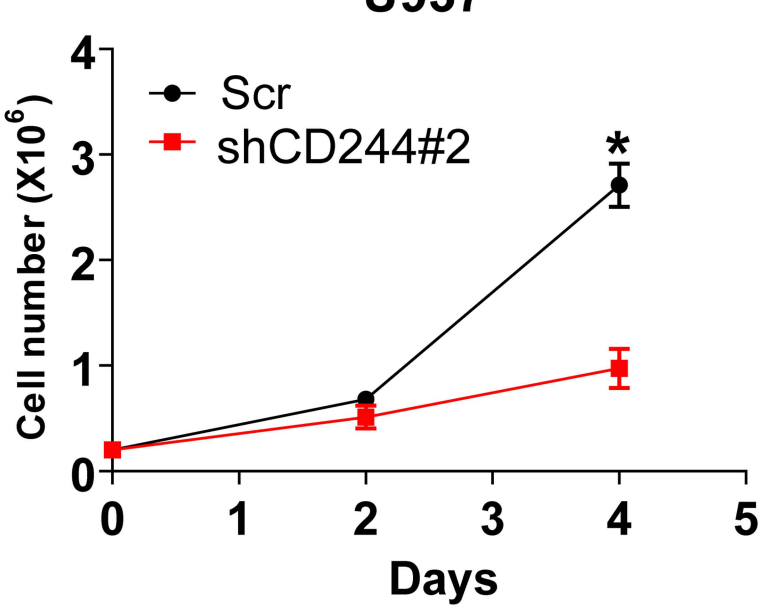
Indicated numbers of leukemia cells were co-transplanted with 2×10^5 competitor cells into each mouse. Numbers of mice in each dosage group are shown in each column. Numbers of mice that developed leukemia are shown in parentheses.

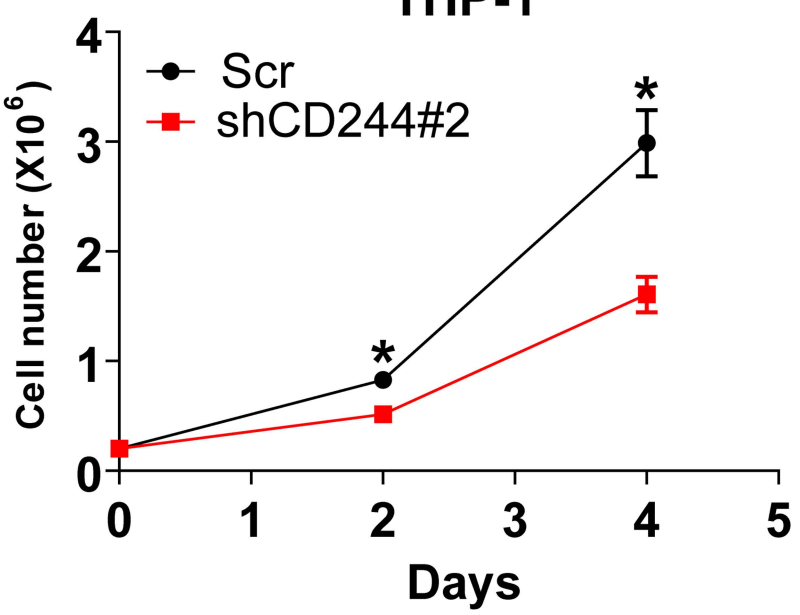
sFigure 1


A
Mean fluorescence intensity
25000
20000
15000
10000
5000
0
MNCs
HSPCs
MA9
CD158a
IREM-1
BTLA
CD244
B7H1
B7H4
CD209
CD22
CD152
CD200R
PD-1
JAM3
CD56
SIGLEC9


B
MV4-11
Scr
shCD244#1
Cell number(X10^6)
Days
*

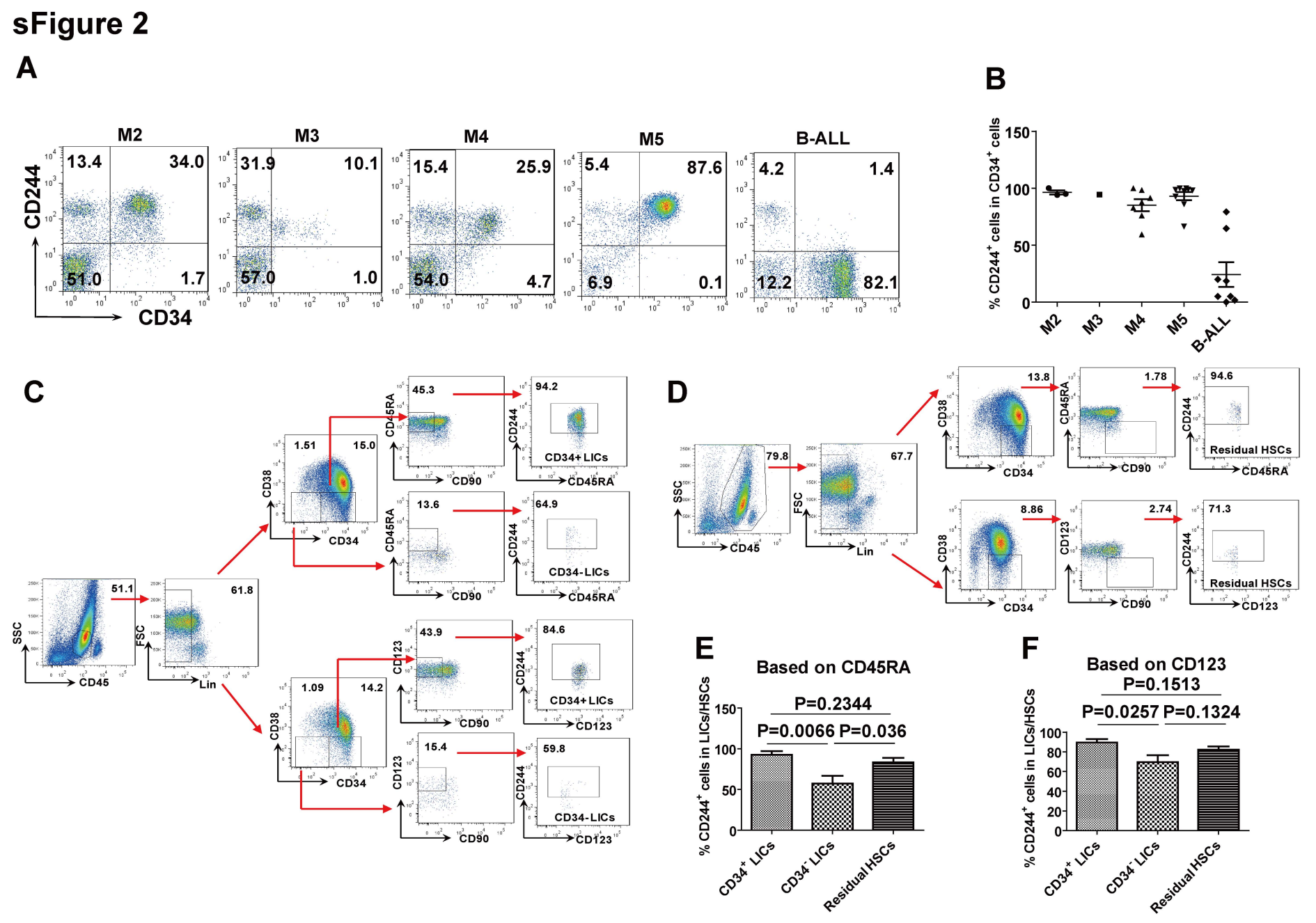

C
HL-60
Scr
shCD244#2
Cell number (X10^6)
Days
*


D
U937
Scr
shCD244#2
Cell number (X10^6)
Days
*


E
THP-1
Scr
shCD244#2
Cell number (X10^6)
Days
*

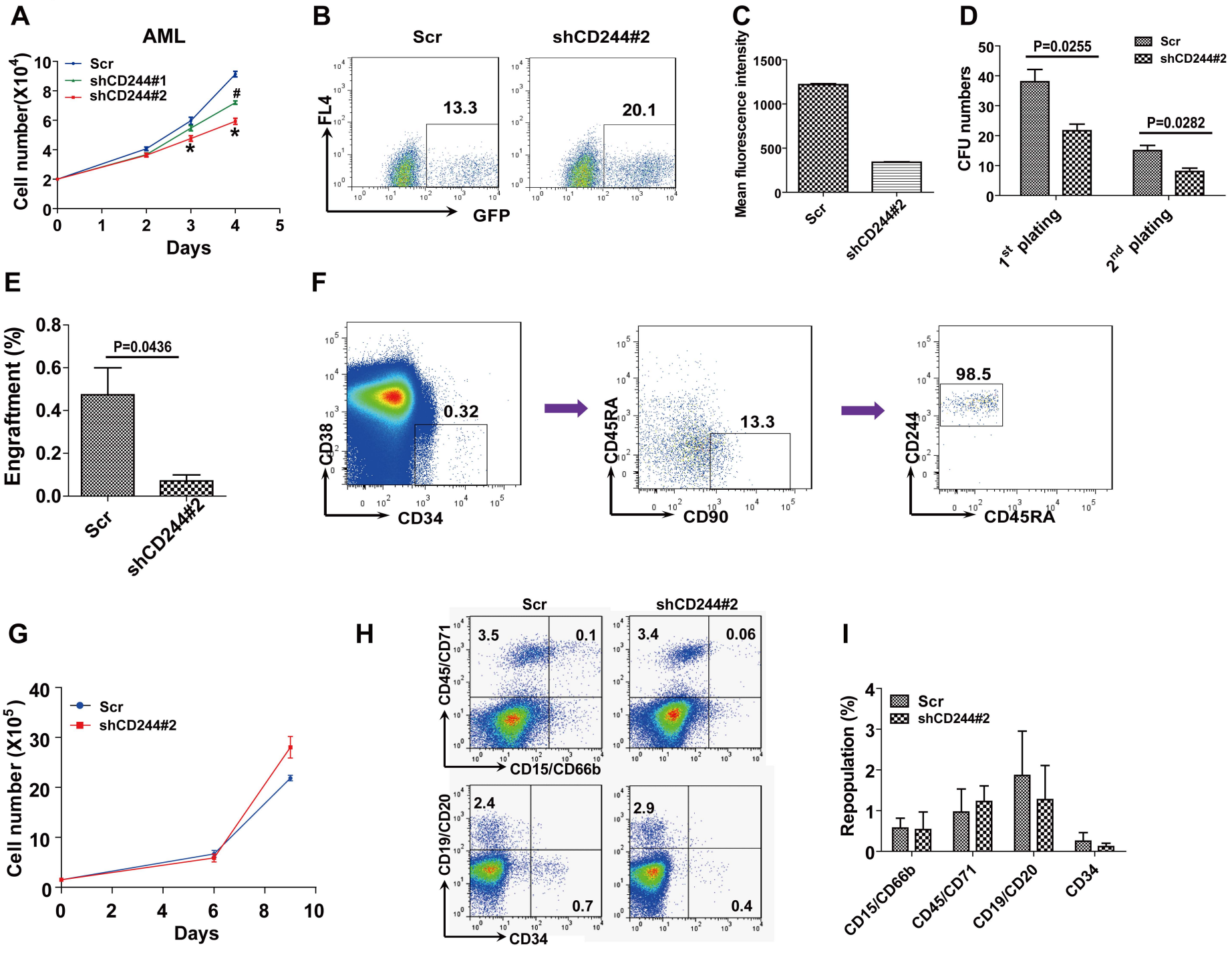
sFigure 1.CD244 is required for the proliferation of human leukemia cell lines. A) Mean fluorescence intensity of representative surface immune molecules on human cord blood HSCs and human MA9 cells were measured by flow cytometric analysis. Cord blood mononuclear cells (MNCs) were used for serving as the control of total cell population. B) Cell numbers at indicated days after infection with CD244-targeting shCD244#1 or scrambled shRNA in MV4-11 cells. C-E) Cell numbers at indicated days after infection with CD244-targeting shCD244#2 or scrambled shRNA in HL-60 (C),U937 (D), and THP-1 cells (E). (n=3, *, $p<0.05$)

sFigure 2


A
M2
M3
M4
M5
B-ALL
CD244
CD34
13.4
34.0
51.0
1.7
31.9
10.1
57.0
1.0
15.4
25.9
54.0
4.7
5.4
87.6
6.9
0.1
4.2
1.4
12.2
82.1
B
% CD244+ cells in CD34+ cells
150
100
50
0
M2
M3
M4
M5
B-ALL
C
SSC
CD45
51.1
FSC
Lin
61.8
CD38
CD34
1.51
15.0
CD45RA
CD90
45.3
CD244
CD45RA
94.2
CD34+ LICs
13.6
64.9
CD34- LICs
CD123
1.09
14.2
43.9
84.6
15.4
59.8
D
79.8
67.7
13.8
1.78
94.6
Residual HSCs
8.86
2.74
71.3
E
Based on CD45RA
P=0.2344
P=0.0066
P=0.036
% CD244+ cells in LICs/HSCs
CD34+ LICs
CD34- LICs
Residual HSCs
F
Based on CD123
P=0.1513
P=0.0257
P=0.1324

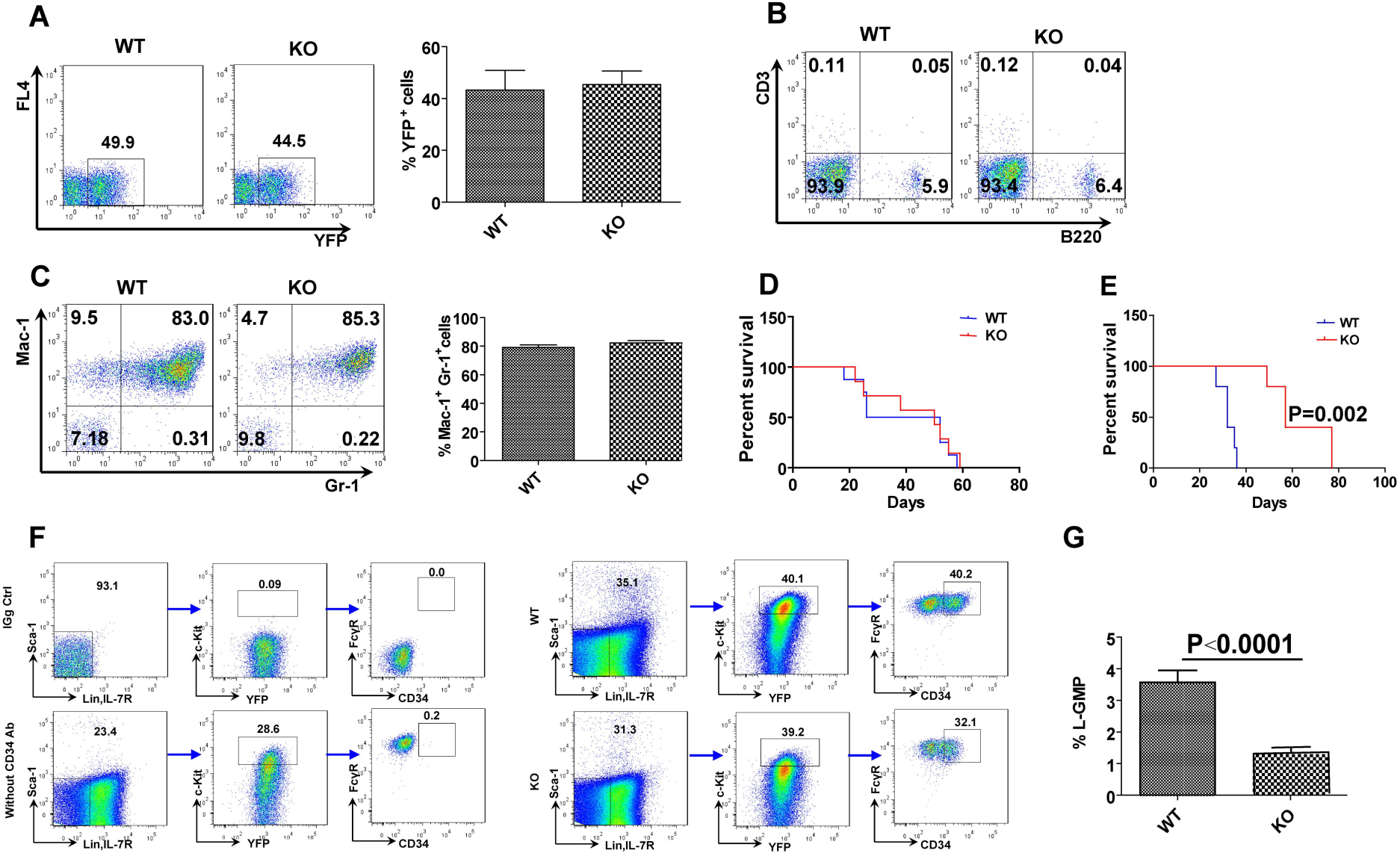
sFigure 2. CD244 is expressed on both human AML LICs and residual HSPCs. A) Representative flow cytometric analysis of CD244 expression in primary patients' samples including AML and B-cell ALL. B) Quantification of data from different human samples (n=3, 1, 7, 9, 8 for M2-M5 and B-cell ALL samples, respectively). C) Representative flow cytometric analysis of CD244 expression on the immunophenotypic $CD45^{dim}SSC^{low}$ $CD34^{+}CD38^{-}CD90^{-}CD45RA^{+}$, $CD45^{dim}SSC^{low}$ $CD34^{+}CD38^{-}CD90^{-}CD123^{+}$ blasts ($CD34^{+}$ LICs), $CD45^{dim}SSC^{low}$ $CD34^{-}CD38^{-}CD90^{-}CD45RA^{+}$, $CD45^{dim}SSC^{low}$ $CD34^{-}CD38^{-}CD90^{-}CD123^{+}$ blasts ($CD34^{-}$ LICs). D) Representative flow cytometric analysis of CD244 expression on the immunophenotypic $CD34^{+}CD38^{-}CD90^{+}CD45RA^{-}$ or $CD34^{+}CD38^{-}CD90^{+}CD123^{-}$ residual HSCs (residual HSCs). E-F) Quantification of data for CD244 expression in $CD34^{+}$ LICs, $CD34^{-}$ LICs or residual HSCs in Panel C-D (n=8).

sFigure 3


A
AML
Scr
shCD244#1
shCD244#2
Cell number(X10^4)
Days
B
Scr
shCD244#2
FL4
GFP
13.3
20.1
C
Mean fluorescence intensity
Scr
shCD244#2
D
P=0.0255
P=0.0282
Scr
shCD244#2
CFU numbers
1st plating
2nd plating
E
P=0.0436
Engraftment (%)
Scr
shCD244#2
F
CD38
CD34
0.32
CD45RA
CD90
13.3
CD244
CD45RA
98.5
G
Scr
shCD244#2
Cell number (X10^5)
Days
H
Scr
shCD244#2
CD45/CD71
CD15/CD66b
3.5
0.1
3.4
0.06
CD19/CD20
CD34
2.4
0.7
2.9
0.4
I
Scr
shCD244#2
Repopulation (%)
CD15/CD66b
CD45/CD71
CD19/CD20
CD34

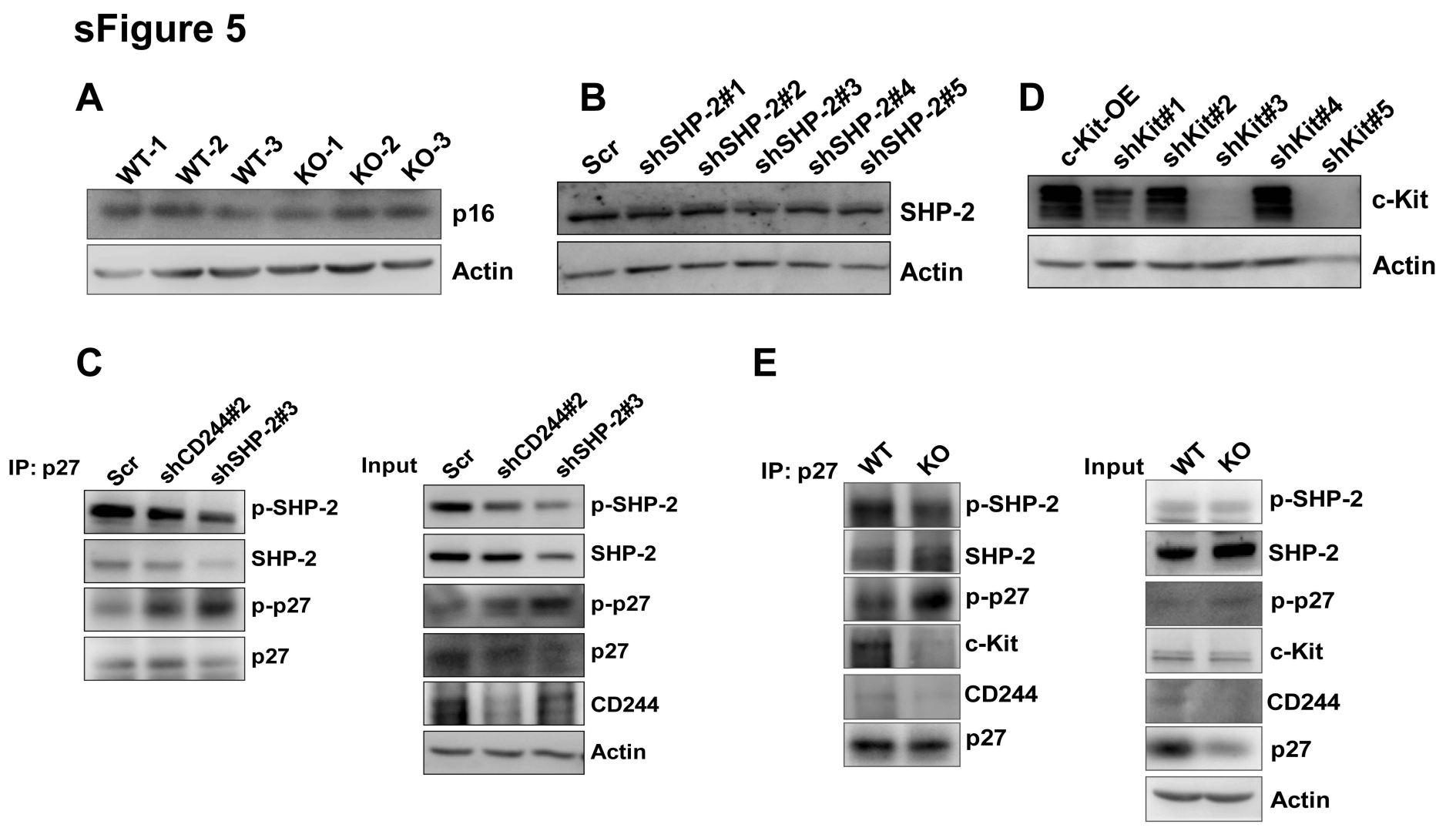
sFigure 3. CD244 is required for the proliferation of human AML LICs, but not HSCs. A) Cell numbers of patients' $CD34^+$ AML cells were assessed at indicated days after infection with CD244-targeting shCD244#1, shCD244#2 or scrambled shRNA in a representative experiment (n=3). B) Representative FACS plot for human AML cells infected with CD244-targeting shCD244#2 or scrambled shRNA. C) Knockdown efficiency was evaluated in human AML cells infected with shCD244#2 or scrambled shRNA by flow cytometric analysis. D) Colony numbers of patients' $CD34^+$ AML cells were examined upon CD244-knockdown after the serial replating (n=3). E) Engraftment for human primary leukemia cells upon CD244-knockdown by CD244-targeting shCD244#2 and scrambled shRNA in another representative experiment (n=4-7). F) Representative flow cytometric analysis of CD244 levels on human cord blood $CD34^+CD38^-CD90^+CD45RA^-$ HSCs. G) Cell numbers at indicated days after transfection with shCD244#2 or scrambled shRNA (n=3). H-I) Representative flow cytometric analysis and quantification data for lineage analysis of human HSCs upon CD244-knockdown 12 weeks after transplantation (n=7-9).

sFigure 4


A
WT
KO
FL4
49.9
44.5
YFP
% YFP+ cells
60
40
20
0
WT
KO
B
WT
KO
CD3
0.11
0.05
0.12
0.04
93.9
5.9
93.4
6.4
B220
C
WT
KO
Mac-1
9.5
83.0
4.7
85.3
7.18
0.31
9.8
0.22
Gr-1
% Mac-1+ Gr-1+ cells
100
80
60
40
20
0
WT
KO
D
Percent survival
150
100
50
0
0
20
40
60
80
Days
WT
KO
E
Percent survival
150
100
50
0
0
20
40
60
80
100
Days
P=0.002
WT
KO
F
IGg Ctrl
Without CD34 Ab
Sca-1
Lin,IL-7R
93.1
23.4
c-Kit
YFP
0.09
28.6
FcγR
CD34
0.0
0.2
WT
KO
35.1
31.3
40.1
39.2
40.2
32.1
G
% L-GMP
5
4
3
2
1
0
P<0.0001
WT
KO

sFigure 4. CD244 is required for the leukemia development in a transplantable AML model. A) Representative flow cytometric analysis for detection of YFP^+ leukemia cells in peripheral blood of the recipients receiving transplants of MLL-AF9-transduced WT or CD244-null hematopoietic stem/progenitors upon primary transplantation (left). Quantification of the percentages of leukemia cells (n=5, right). B-C) Lineage analysis with lymphoid cell markers (CD3/B220, B) and myeloid cell markers (Mac-1/Gr-1, C, left) were conducted in WT and CD244-null leukemia cells. Quantification of the percentages of $Mac\text{-}1^+/Gr\text{-}1^+$ cells was shown (n=3, C, right). D) Mice transplanted with MLL-AF9-infected CD244-null hematopoietic stem/progenitors had no change of survival upon primary transplantation (n=8, log-rank test). E) Another experiment for secondary transplantation with CD244-null leukemia cells revealed a markedly extended survival compared to WT cells (n= 5, log-rank test). F-G) Representative FACS plot and quantification data for $Lin^-IL7\text{-}R^-c\text{-}Kit^+CD34^+Fc\gamma R^+$ L-GMPs in WT and CD244-null leukemia cells. Both the isotype control and the control stained with all the L-GMP markers except for CD34 were used for the threshold cutoff.

sFigure 5


A
WT-1
WT-2
WT-3
KO-1
KO-2
KO-3
p16
Actin
B
Scr
shSHP-2#1
shSHP-2#2
shSHP-2#3
shSHP-2#4
shSHP-2#5
SHP-2
Actin
D
c-Kit-OE
shKit#1
shKit#2
shKit#3
shKit#4
shKit#5
c-Kit
Actin
C
IP: p27
Scr
shCD244#2
shSHP-2#3
p-SHP-2
SHP-2
p-p27
p27
Input
Scr
shCD244#2
shSHP-2#3
p-SHP-2
SHP-2
p-p27
p27
CD244
Actin
E
IP: p27
WT
KO
p-SHP-2
SHP-2
p-p27
c-Kit
CD244
p27
Input
WT
KO
p-SHP-2
SHP-2
p-p27
c-Kit
CD244
p27
Actin

sFigure 5. CD244 interacts with c-Kit to maintain the stability of p27 through SHP-2 pathway. A) p16 expression levels in WT and CD244-null LICs were measured by western blotting analysis. B) SHP-2 knockdown efficiencies by shRNAs were evaluated by western blotting analysis. C) Human primary leukemia cells were infected with CD244-targeting shCD244#2, SHP-2-targeting shSHP-2#3 and scrambled shRNA. And their lysates were immunoprecipitated by p27 antibody, followed by western blotting analysis for the levels of endogenous p-SHP-2, SHP-2, 4G10 (for p-p27), p27 and CD244. D) c-Kit knockdown efficiencies by shRNAs were evaluated by western blotting analysis. E) The lysates of WT and CD244-null primary AML cells were immunoprecipitated by p27 antibodies, followed by western blotting analysis for the levels of endogenous p-SHP-2, SHP-2, p-p27, c-Kit and CD244.