Small extracellular vesicles derived from acute myeloid leukemia cells promote leukemogenesis by transferring miR-221-3p

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Supplementary Methods:

Cell culture

Primary murine AML cells were isolated from the bone marrow of MLL-AF9 AML mice as previously described.[1] AML cells were cultured in IMDM medium containing 10 ng/mL mouse interleukin (IL)-3 (Peprotech), 10 ng/mL mouse IL-6 (Peprotech), 50 ng/mL mouse stem cell factor (SCF) (Peprotech), 15% fetal bovine serum (FBS), and 1% penicillin and streptomycin (Gibco). HEK293T cells were cultured in high-glucose DMEM medium containing 10% FBS and 1% penicillin and streptomycin. THP-1, HL-60, Kasumi-1 and MOLM-13 cells were cultured in RPMI-1640 medium containing 10% FBS and 1% penicillin and streptomycin. All cells were maintained in an incubator with 5% CO_2 at 37 °C.

sEVs isolation, characterization, and quantification

To obtain sEVs-free fetal bovine serum (FBS), equal volumes of medium (IMDM or DMEM) and FBS were combined and the mixture was ultracentrifuged at 110 000 ×g at 4 °C overnight. To obtain the conditioned medium (CM), cells were washed with phosphate-buffered saline (PBS) three times and resuspended with sEVs-free medium. After 48 h, the CM was collected and sequentially centrifuged at 300 ×g for 10 min, 2 000 ×g for 10 min, and finally at 10 000 ×g for 30 min to remove the dead cells, cellular debris, and large vesicles, respectively. The supernatant was then filtered using a 0.22-μm filter followed by ultracentrifugation at 110 000 ×g for 70 min to obtain sEVs pellets. The

pellets were washed with PBS once and subjected to a final ultracentrifugation step at 110 000 ×g for 70 min to ensure the purification of sEVs. All centrifugation steps were conducted at 4 °C. After the sEVs were resuspended in PBS, protein concentration was detected using a BCA assay. The morphology and size of sEVs were evaluated using a transmission electron microscope (HT7700 Exalens, Tokyo, Japan). The quantity and size distribution of sEVs were assessed using the Zetasizer NanoSampler ZS90 (Malvern Panalytical, Malvern, UK). The sEVs-specific proteins CD9, CD63, and TSG101, and the endoplasmic-reticulum-derived protein Calnexin were detected by western blotting.

Human peripheral blood (PB) collection and plasma processing

This study was approved by the Ethics Committee according to the regulations of the institutional ethics review boards from the Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, informed consent was signed by all patients. Human PB samples from 25 healthy control and 41 AML patients were obtained from The First People's Hospital of Yunnan Province. PB were collected in EDTA blood tubes and centrifuged at 2 000 ×g for 10 min to separate the plasma fraction. Subsequently, add an equal volume of pre-cooled PBS to the plasma and the mixture was further ultracentrifuged to isolate sEVs.

AML cell proliferation assay

To examine the impact of sEVs on AML cells, AML cells were cultured in sEVs-free culture medium, supplemented with 10 μg/mL of sEVs, and co-cultured for 3 days prior to transplantation into mice or proliferation analysis. To investigate the effect of GW4869 (MCE) on sEVs and AML cells, it was used at a concentration of 5 μM to inhibit sEV release. Before co-culturing with sEVs, AML cells were pre-treated with GW4869 for 24 h. To investigate the effect of miR-221 or *Gbp2* on AML cells, AML cells were infected with lentivirus for 48 h, cell cycle was analyzed. Cell proliferation and cell apoptosis were measured for 6 consecutive days. Cell viability was assessed by the CCK-8 assay (DOJINDO) following the manufacturer's instructions.

HSPCs co-culture and PKH67 labeling

For the in vitro sEVs treatment, BM cells from WT mouse were enriched using CD117 Microbeads (Miltenyi) and 1×10^3 $cKit^+$ cells were cultured in a 48-well plate with 800 μL of StemSpan serum-free medium (STEMCELL Technologies), supplemented with 100 ng/mL mouse SCF (Peprotech), 100 ng/mL mouse TPO (Peprotech) along with 8 μg of AML-sEVs for 7 days. To visualize the internalization of sEVs by HSPCs, AML-sEVs were labeled with PKH67 according to manufacturer’s instructions. Briefly, sEVs pellet was resuspend with 1 mL of Diluent C, then 4 μL of the PKH67 dye solution was added and mixed thoroughly. After incubating the mixture for 5 minutes, 2 mL of 1% bovine

serum albumin serum was added to allow binding of excess dye. The sEVs were then centrifuged at 110 000 ×g for 70 minutes at 4°C, and sEVs pellet was resuspended for collection. The labeled sEVs were cultured with 1×10^5 $ckit^+$ cells. After 12 h, the sEV-treated HSPCs were cytospined and imaged using a laser-scanning confocal microscope.

In vitro erythroid differentiation assay

To assess the erythroid differentiation ability of HSPCs, 50 $cKit^+$ cells were sorted and cultured in a 96-well plate with 200 μL of Minimum Essential Medium (α-MEM, Gbico). The medium was supplemented with 10 ng/mL mouse SCF, 10 ng/mL mouse TPO (Peprotech) ,10 ng/mL mouse IL-3 (Peprotech), 1 IU/mL human EPO (Peprotech), 2 mM L-Glutamine, 50 U/mL penicillin-streptomycin solution and 15% sEVs-free fetal bovine serum, along with different concentration of AML-sEVs (10, 50 μg/mL) for 3 weeks. Erythroid differentiation ability was detected by Flow cytometry.

Colony formation assay

After 48 h of AML cells infection, GFP^+ or $mCherry^+$ cells were sorted into MethoCult M3231 (StemCell Technologies), a methylcellulose-based medium, supplemented with 50 ng/mL mouse stem cell factor (SCF), 10 ng/mL mouse IL-6, 10 ng/mL mouse IL-3, and 1% penicillin/streptomycin (all purchased from PeproTech). Colonies were observed and quantified after 7-10 days of culture. Murine BM cells or $cKit^+$ HSPCs were cultured in MethoCult M3434 medium (StemCell Technologies) for 7-10 days to generate CFU-G, CFU-M, CFU-GM,

and CFU-GEMM colonies. To form BFU-E colonies, the BM cells were cultured in MethoCult M3436 medium (StemCell Technologies) for 10-14 days.

RNA oligonucleotides, plasmids

RNA oligonucleotides were synthesized by RiboBio (Guangzhou, China), the oligos used in this study are listed in Supplementary Table 2. The anti-miR plasmid is custom made to specifically target miR-221-3p, which was generated by ligation of double stranded oligo's sense/antisense into the BamHI and EcoRI restriction sites of the miRZIP pGreen-Puro Lentiviral-based miRNA inhibition vector. The nucleotides for the specific anti-microRNA sequence targeting the miR-221-3p are shown: 5'-GAAACCCAGCAGACAATGTAGCT-3'. Green fluorescent protein (GFP) was used as the positive sorting reporter. The miR-221-overexpressing plasmid was constructed as previously described.[2] Oligos (synthesized by Tsingke) were annealed and ligated into the AgeI- and BamHI-digested GV298 plasmid, which was purchased from GeneChem (Shanghai, China). mCherry was used as the positive sorting reporter.

Lentivirus package and transfection

The lentiviral vectors, pSPAX2, and pMD2.G packaging plasmids were mixed at a 7:5:3 mass ratio and transfected into 293T cells using the Lipofectamine 2000 Transfection Reagent (Invitrogen). After 48-72 h, the lentivirus was collected and concentrated at 20 000 ×g for 2.5 h at 4 °C. For cell infection, 5×10^5 cells

were transferred to a vitronectin-coated 24-well plate and incubated with the lentivirus and 8 μg/mL polybrene (Sigma-Aldrich). The plate was centrifuged for 90 min at 1800 rpm and 37 °C, and the medium was replenished after 8-12 h.

Limiting Dilution Assays (LDAs)

For in vivo LDAs, murine MLL-AF9 AML cells were transduced with either control or miR-221-overexpressed lentivirus. Recipients (n = 9 for each group) were irradiated with 5.0 Gy before being transplanted with four doses of leukemia cells for each group. The number of recipient mice that developed leukemia post-transplantation within 110 days were record in each group. The frequency of LSCs was analyzed by Extreme Limiting Dilution Analysis (ELDA) with online tool: http://bioinf.wehi.edu.au/software/elda/.[3]

Western blotting

Proteins from cells or sEVs were isolated using sodium dodecyl sulfate buffer, separated on a 10% polyacrylamide gel, and subsequently transferred onto PVDF membranes. After blocking the membranes with 5% bovine serum albumin for 1 h at room temperature, the membranes were incubated overnight with primary antibodies at 4 °C. The following day, the membranes were washed with PBST and then incubated with a horseradish-peroxidase-conjugated secondary antibody to detect the chemiluminescence signals. The primary antibodies used in this study are as

follows: CD63 (ab216130, Abcam), CD9 (ab92726, Abcam), TSG101 (ab125011, Proteintech), Calnexin (ab22595, Abcam), Gbp2 (11854-1-AP, Proteintech), β-actin (A1978, Sigma-Aldrich), BAX (A0207, ABclonal) and anti-AKT (9272), anti-phospho-AKT (4058), anti-SAPK/JNK (9252), anti-phospho-SAPK/JNK (9251) antibodies were purchased from Cell Signaling Technology.

RNA extraction and qRT-PCR

RNA was extracted from sEVs using the miRNeasy Kit (217004, QIAGEN) and from cells using the Trizol reagent (Invitrogen). For the miRNA analysis, the miRNA was reverse-transcribed using the miRNA First-Strand Synthesis Kit (Takara, 638313) and subjected to qRT-PCR analysis using the miRNA TB Green Kit (Takara, 638314). Meanwhile, mRNA was reverse-transcribed into cDNA using the HiScriptII 1st strand cDNA synthesis kit (Vazyme, R211) and subjected to qRT-PCR analysis using the SYBR qPCR Master Mix (Vazyme, Q711). The qRT-qPCR was conducted on a QuantStudio5 system (Applied Biosystems). *U6* or *GAPDH* were used as internal reference for detecting miRNA or mRNA, respectively, in cells and sEVs. The primers used for qRT-PCR can be found in Supplementary Table 1.

Dual-luciferase reporter assay

To generate the dual-luciferase reporter vectors, the full-length 3′UTR

sequences from the selected AML-associated genes were PCR-amplified and inserted between the SacI and XhoI restriction sites of the pmirGLO dual-luciferase miRNA target expression vector (Promega) using the Basic Seamless Cloning and Assembly Kit (TransGen Biotech). A mutated *Gbp2* 3′UTR sequence with a mutation at positions of the miRNA seed sequence, as described in the main text, was synthesized by GENEWIZ. The pmirGLO vector expressed firefly luciferase, with *Renilla* luciferase serving as the internal reference. 293T cells (1.2×10^5 cells) were seeded in 24-well plates and co-transfected with reporter plasmids containing the 3'UTR of the target gene and either a negative control miRNA (miR NC) or an miR-221 mimic using Lipofectamine 3000 and the Lipofectamine RNAiMAX Transfection Reagent (Invitrogen), respectively. Cell lysates were collected after 36 h, and luciferase activity was measured using a microplate reader according to the manufacturer's instructions (Promega).

Small RNA sequencing and analyses

Each experimental group comprised two or three technical replicates. Once collected, the sEVs were sent to RiboBio Co., Ltd. (Guangzhou, China) for high-throughput miRNA sequencing. The RNA was extracted by trizol and the libraries of small RNAs were constructed using TruSeq Small RNA Library Preparation Kits (Illumina) according to the manufacturers' instructions, followed by sequencing by Illumina HiSeq 2500 platform. Raw data (raw reads)

underwent initial filtration, involving the removal of joints at both ends of reads. Additionally, the reads with fragment length less than 17nt and low-quality reads are removed to obtain high-quality data (clean reads). Clean reads were mapped to the mouse genome (GRCm38) using Bowite 2.2.5. The differential miRNA expression analysis was carried out using DESeq2. For miRNA with significant differences, the target genes of miRNA will be further predicted, and the target genes were enriched by gene ontology (GO) and KEGG biological pathway analyses.

RNA sequencing and analyses

For transcriptome sequencing, MLL-AF9 AML cells were infected with control or miR-221 knockdown/overexpressing lentivirus. After 48 h, cells were sorted by flow cytometry and lysed in Trizol reagent to extract RNA. The cDNA libraries were prepared using an Illumina RNA library preparation kit and sequenced by the Illumina NovaSeq 6000 in Novogene Co. (Tianjin, China). Differential gene expression analyses were conducted using the DESeq2 R package with Padj < 0.05 and Foldchange > 1.2 were set as the threshold for significant differential expression.

Supplementary Method References:

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2. Wang XW, Hao J, Guo WT, et al. A DGCR8-Independent Stable MicroRNA Expression Strategy Reveals Important Functions of miR-290 and miR-183-182 Families in Mouse Embryonic Stem Cells. Stem cell reports. 2017;9(5):1618-1629.

3. Hu Y, Smyth GK. ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. Journal of immunological methods. 2009;347(1-2):70-78.

Supplementary Figure Legends:

Figure S1. Identification of acute myeloid leukemia cells-derived small extracellular vesicles (AML-sEVs) and their role on AML cells. (A) Transmission electron microscopy image of sEVs isolated from AML cells. (B) Nanoparticle tracking analysis was used to determine the size and number of sEVs isolated from AML cells. (C) Western blotting showing the protein expression level of Calnexin, TSG101, CD63, and CD9 in AML-sEVs and AML cells. Calnexin is an endoplasmic reticulum membrane marker. TSG101, CD63, and CD9 are sEV-specific markers. (D) AML cells were quantified after incubation with or without AML-sEVs for 3 days. (E-F) Flow cytometric analysis of Ki67-APC and Annexin-V-APC staining in AML cells treated with GW4869 for 3 days, in the presence or absence of AML-sEVs. Data are presented as the mean ± S.D., ***$p < 0.001$.

Figure S2. miRNA quantification in sEVs and AML cells. (A-C) Quantitative real-time PCR (qRT-PCR) analysis of differentially expressed miRNAs in sEVs and AML cells. (D) Conservation analysis of the miR-221 sequence across various species. Data are presented as the mean ± S.D., *$p < 0.05$, **$p < 0.01$.

Figure S3. Impact of miR-221-sEVs on AML cells. (A-B) qRT-PCR analysis of miR-221 in miR-221-ovexpressing 293T cells and sEVs. (C-D) Evaluation of AML cell number and viability after exposure to the indicated treatment

conditions. (E-F) Analysis of AML cell cycle and apoptosis after a 3-day co-culture with miR-221-sEVs. (G-H) Flow cytometric analysis of Ki67-APC and Annexin-V-APC staining in AML cells treated with GW4869 for 3 days, in the presence or absence of Ctrl-sEVs or miR-221-sEVs. Data are presented as the mean ± S.D., ***$p < 0.001$.

Figure S4. Impact of miR-221 overexpression on leukemogenesis. (A) Colony numbers were determined after miR-221 overexpression (OE). (B) Analysis of the percentage of CD45.1$^+$ AML cells in the peripheral blood (PB) of recipient mice injected with wild-type (WT) or miR-221 OE AML cells (n = 5-8). (C-E) Representative photographs, liver to body weight ratios, and H&E staining images of control and miR-221 OE mouse livers. (F-G) Bar graph and flow cytometric analysis of the frequency of leukemic granulocyte macrophage progenitors (L-GMPs) in the BM of recipients injected with WT or miR-221 OE AML cells (n = 5-8). (H) The in vivo limiting dilution assay was conducted using leukemia cells transduced with either control or miR-221-overexpressed lentivirus. Left (up): Table showing the doses of leukemia cells and the corresponding number of recipients that developed leukemia. Left (down): the estimated leukemia stem cells (LSCs) frequency was analyzed. Right: Log fraction plot showing the LSCs frequency. Recipients (n = 9 for each group) were irradiated with 5.0 Gy before transplanted with different doses of leukemia cells and the estimated LSCs frequency was analyzed by ELDA. Data are

presented as the mean ± S.D., $^{**}p < 0.01$. ns, no significant differences.

Figure S5. miR-221 transfer by AML-sEVs. (A-B) qRT-PCR analysis of miR-221 in miR-221 KD AML cells and their sEVs. (C-D) qRT-PCR analysis of miR-221 in miR-221 OE AML cells and their sEVs. (E-F) Evaluation of the number or apoptosis of control and miR-221 KD AML cells cultured with or without miR-221-sEVs. (G) Representative flow cytometry contour plots of Annexin-V staining. Data are presented as the mean ± S.D., $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Figure S6. Impact of miR-221 knockdown on Human leukemia cells. (A) Analysis of number, viability, apoptosis, and cell cycle of THP-1 cells after miR-221 knockdown (KD). (B) Analysis of number, viability, apoptosis, and cell cycle of Kasumi-1 cells after miR-221 KD. (C) Analysis of number, viability, apoptosis, and cell cycle of MOLM-13 cells after miR-221 KD. Data are presented as the mean ± S.D., $^{**}p < 0.01$, $^{***}p < 0.001$.

Figure S7. Gbp2 promotes the proliferation of AML cells. (A) Heatmap of differentially expressed genes between the control and miR-221 KD AML cells. (B) Heatmap of differentially expressed genes between the control and miR-221 OE AML cells. (C) Geno Ontology (GO) enrichment analyses of miR-221 target genes predicted by TargetScan, miRTarBase, miRWalk, and miRDB. (D) The

luciferase activity of miR NC or miR-221 mimic co-transfected with gene-3' untranslated regions (3'UTR)-expressing vector into 293T cells. (E) qRT-PCR analysis to evaluate the expression level of *Gbp2*. (F) qRT-PCR analysis to evaluate the knockdown efficiency of *Gbp2*. (G-H) Evaluation of the proliferation and apoptosis after Gbp2 KD in AML cells. (I) Western blotting was conducted to examine the indicate protein expression level in control- or Gbp2-overexpressed AML cells. Data are presented as the mean ± S.D., *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure S8. The effect of AML-sEVs on hematopoietic stem/progenitor cells (HSPCs). (A) Confocal microscopy for GFP^+ sEVs in the cytosol of HSPCs after co-culture with PKH67-labeled sEVs for 12 h. DAPI, 4′,6-diamidino-2-phenylindole. (B) Number of HSPCs was assessed after a 7-day co-culture with AML-sEVs. (C) Evaluating the clonogenicity of $cKit^+$ HSPCs isolated from WT mice intravenously injected with AML-sEVs or PBS. (D) Analysis of the percentage of $Ter119^+$ cells. (E) qRT-PCR analysis of miR-221 expression in HSPCs, mesenchymal stem cells (MSCs) and endothelial cells (ECs). (F) Cell sorting strategy of Long-term hematopoietic stem cells (LT-HSCs), short-term hematopoietic stem cells (ST-HSCs), multipotent progenitors (MPPs), common myeloid progenitors (CMPs), common lymphoid progenitors (CLPs), granulocyte/monocyte progenitors (GMPs), megakaryocyte/erythroid progenitors (MEPs). Three independent experiments.

Data are represented as mean ± SD. ** $P < 0.01$, *** $P < 0.001$.

Supplementary Table 1. The primers used in the qRT-PCR analysis

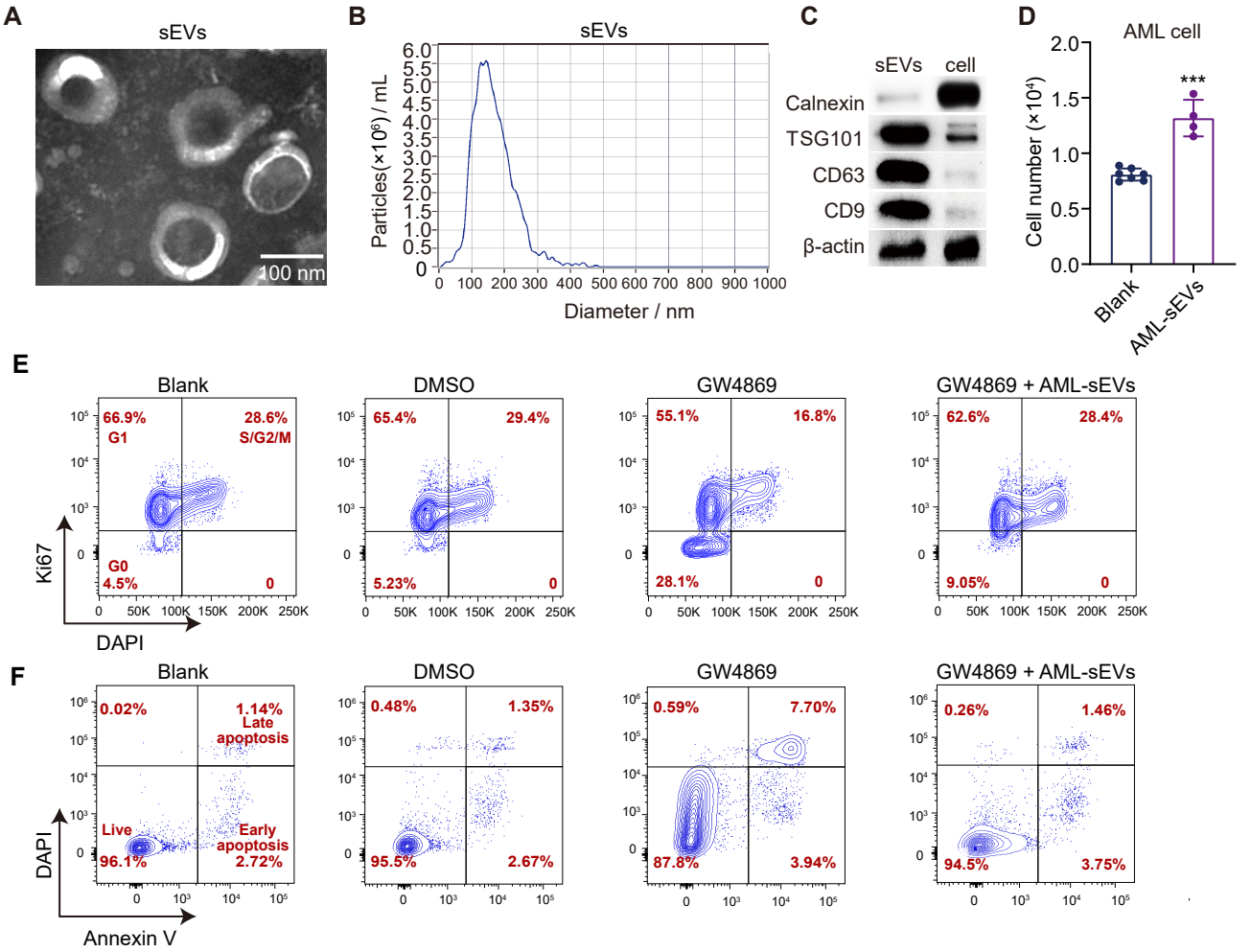
Name	Primer (5'→3')
U6-F	GGAACGATACAGAGAAGATTAGC
U6-R	TGGAACGCTTCACGAATTTGCG
mmu-miR-221-3p	AGCTACATTGTCTGCTGGGTTTC
mmu-miR-181b-5p	AACATTCATTGCTGTCGGTGGGTT
mmu-miR-181d-5p	AACATTCATTGTTGTCGGTGGGT
mmu-miR-27a-5p	AGGGCTTAGCTGCTTGTGAGCA
mmu-miR-129-5p	CTTTTTGCGGTCTGGGCTTGC
mmu-miR-6538	CGCGGGCTCCGGGGCGGCG
mmu-miR-23a-5p	GGGGTTCCTGGGGATGGGATTT
mmu-miR-5100	TCGAATCCCAGCGGTGCCTCT
mmu-miR-222-3p	CTCAGTAGCCAGTGTAGATCC
mmu-miR-331-3p	CTAGGTATGGTCCCAGGGATCC
mmu-miR-877-5p	GTAGAGGAGATGGCGCAGGG
mmu-miR-3968	CGAATCCCACTCCAGACACCA
mmu-miR-219a-1-3p	TGATTGTCCAAACGCAATTCT
mmu-miR-3473f	CAAATAGGACTGGAGAGATG
mmu-miR-298-5p	GGCAGAGGAGGGCTGTTCTTCCC
mmu-miR-3963	TGTATCCCACTTCTGACAC
mmu-miR-34c-3p	AATCACTAACCACACAGCCAGG
mmu-miR-351-3p	GGTCAAGAGGCGCCTGGGAAC
mmu-miR-130b-5p	ACTCTTTCCCTGTTGCACTACT
mmu-miR-5099	TTAGATCGATGTGGTGCTCC
mmu-miR-709	GGAGGCAGAGGCAGGAGGA
mmu-miR-3473a	TGGAGAGATGGCTCAGCA
mmu-miR-3473b	GGGCTGGAGAGATGGCTCAG
mmu-miR-1931	ATGCAAGGGCTGGTGCGATGGC

mmu-miR-6240	CCAAAGCATCGCGAAGGCCCACGGCG
mmu-miR-296-3p	GAGGGTTGGGTGGAGGCTCTCC
mmu-miR-6238	TTATTAGTCAGTGGAGGAAATG
mmu-miR-3470a	TCACTTTGTAGACCAGGCTGG
mmu-miR-3470b	TCACTCTGTAGACCAGGCTGG
mmu-miR-690	AAAGGCTAGGCTCACAACCAAA
mmu-miR-1195	TGAGTTCGAGGCCAGCCTGCTCA
mmu-miR-196b-3p	TCGACAGCACGACACTGCCTTC
mmu-miR-221-5p	ACCTGGCATACAATGTAGATTTCTGT
mmu-miR-222-5p	TCAGTAGCCAGTGTAGATCCT
mmu-miR-223-5p	CGTGTATTTGACAAGCTGAGTTG
Gapdh-F	GTCTCCTCTGACTTCAACAGCG
Gapdh-R	ACCACCCTGTTGCTGTAGCCAA
Gbp2-F	TCACAGAGGCAGCAAAGGAG
Gbp2-R	TGCTGCATCATCAGCTCGAA

Supplementary Table 2. The oligonucleotides used in this study

Name	Sequence (5'→3')
Oligos-F for miR-221 overexpression	GATCCGCCAGCAGACAATGTAGCTCTCCTGCTACCCTGACCCAGTAGCCCAAGAGCTACATTGTCTGCTGGCTTTTTTG
Oligos-R for miR-221 overexpression	AATTCAAAAAAGCCAGCAGACAATGTAGCTCTTGGGCTACTGGGTCAGGGTAGCAGGAGAGCTACATTGTCTGCTGGCG
Gbp2-shRNA1-F	CCGGGATGTTGTTGAAACACTTCTACTCGAGTAGAAGTGTTTCAACAACATCTTTTT
Gbp2-shRNA1-R	GATCAAAAAGATGTTGTTGAAACACTTCTACTCGAGTAGAAGTGTTTCAACAACATC
Gbp2-shRNA2-F	CCGGGCGACTGTGCATCAGGAAATTCTCGAGAATTTCCTGATGCACAGTCGCTTTTT
Gbp2-shRNA2-R	GATCAAAAAGCGACTGTGCATCAGGAAATTCTCGAGAATTTCCTGATGCACAGTCGC
miRNA negative control (miRNA NC)	UUCUCCGAACGUGUCACGUTT
miR-221 mimic	UAACACUGUCUGGUAAAGAUGG

Figure S1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TSG101
CD63
CD9
β-actin
D
AML cell
Cell number (×10⁴)
***
Blank
AML-sEVs
E
Blank
DMSO
GW4869
GW4869 + AML-sEVs
66.9%
G1
28.6%
S/G2/M
G0
4.5%
0
65.4%
29.4%
5.23%
55.1%
16.8%
28.1%
62.6%
28.4%
9.05%
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2.67%
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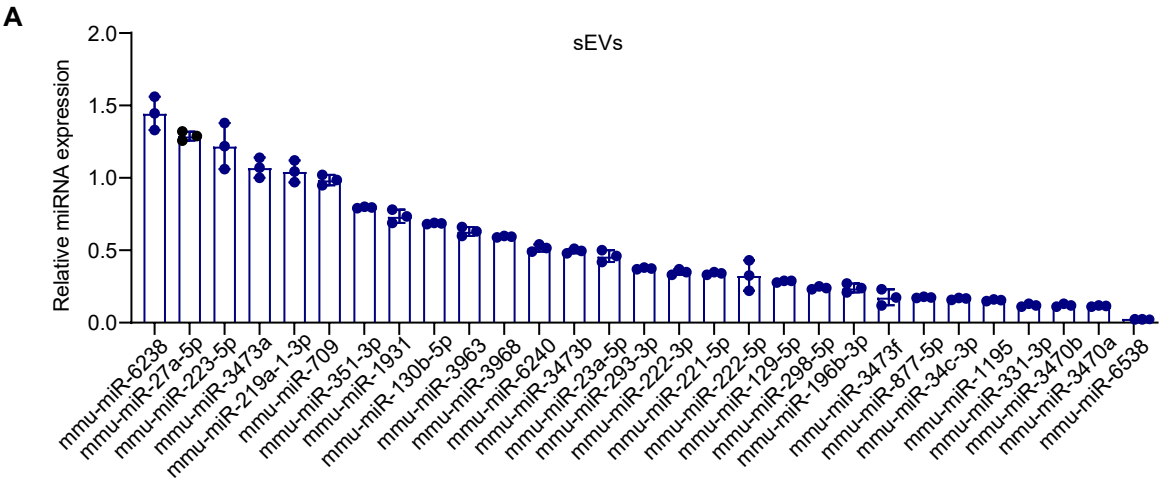
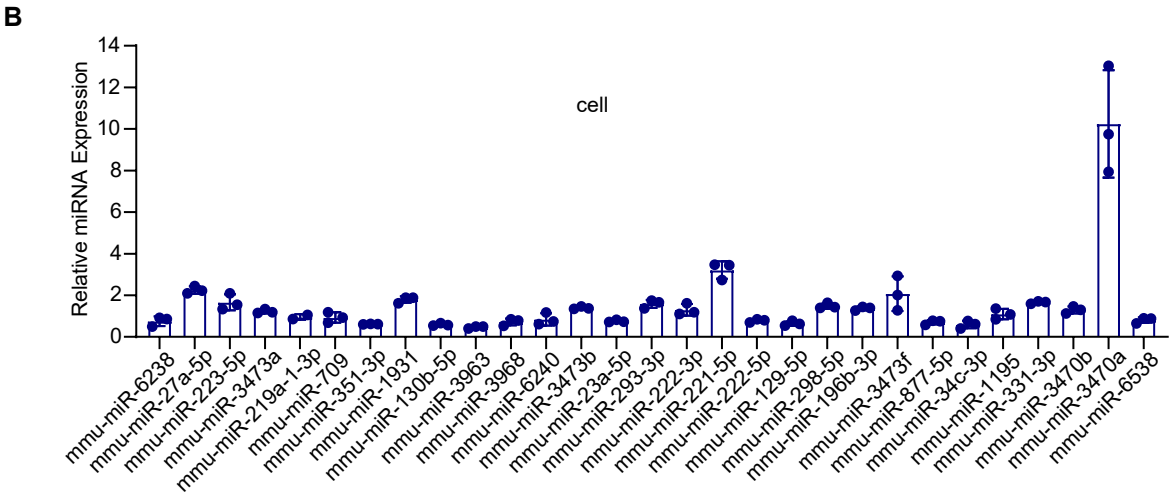
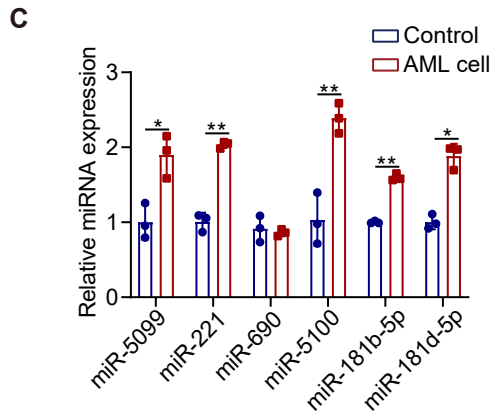
Annexin V

Figure S2







D

miR-221

seed sequence

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Rattus norvegicus	AGCUACAUUGUCUGCUGGGUUUC
Macaca mulatta	AGCUACAUUGUCUGCUGGGUUUC
Sus scrofa	AGCUACAUUGUCUGCUGGGUUUC
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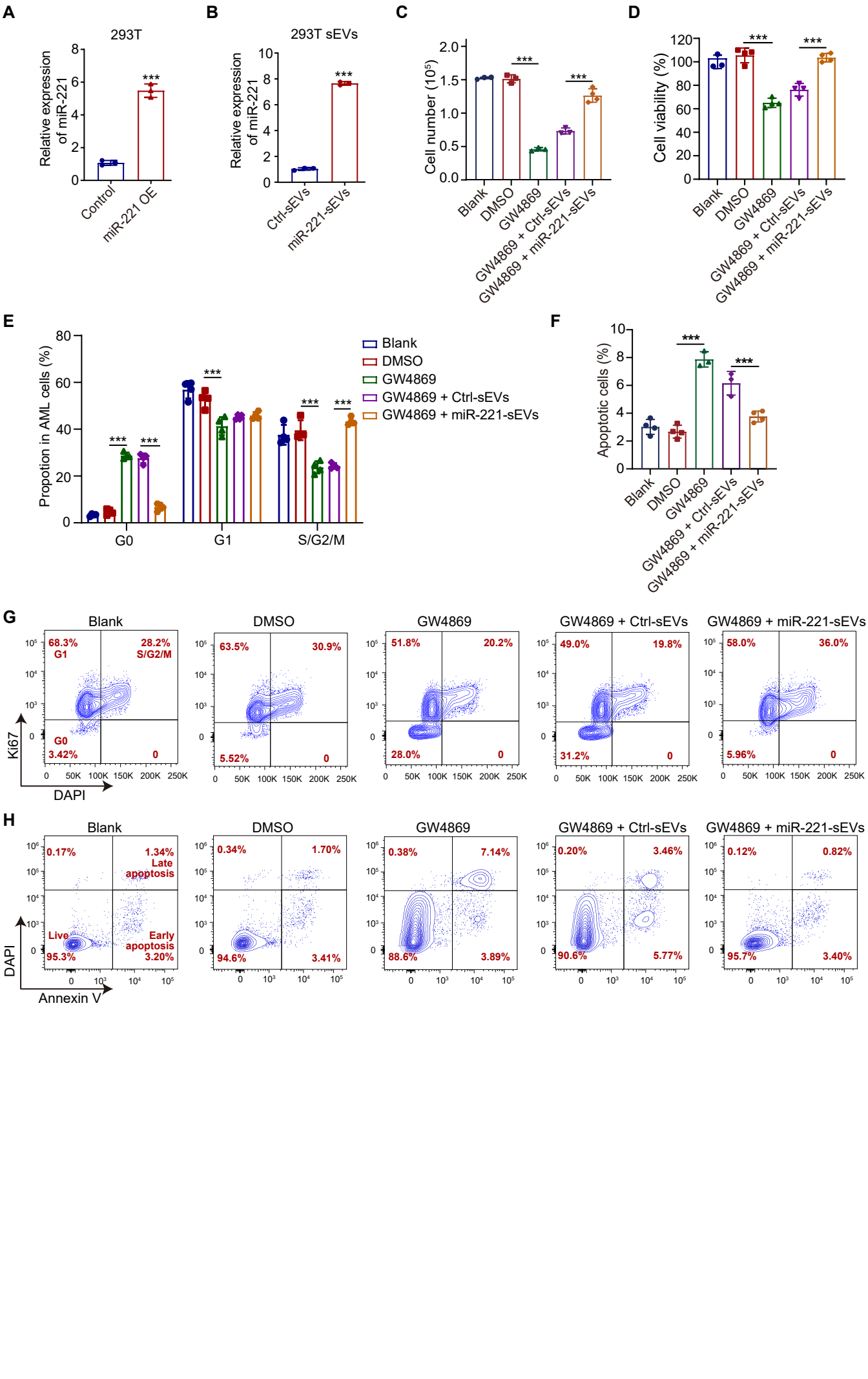
Danio rerio	AGCUACAUUGUCUGCUGGGUUUC

Figure S3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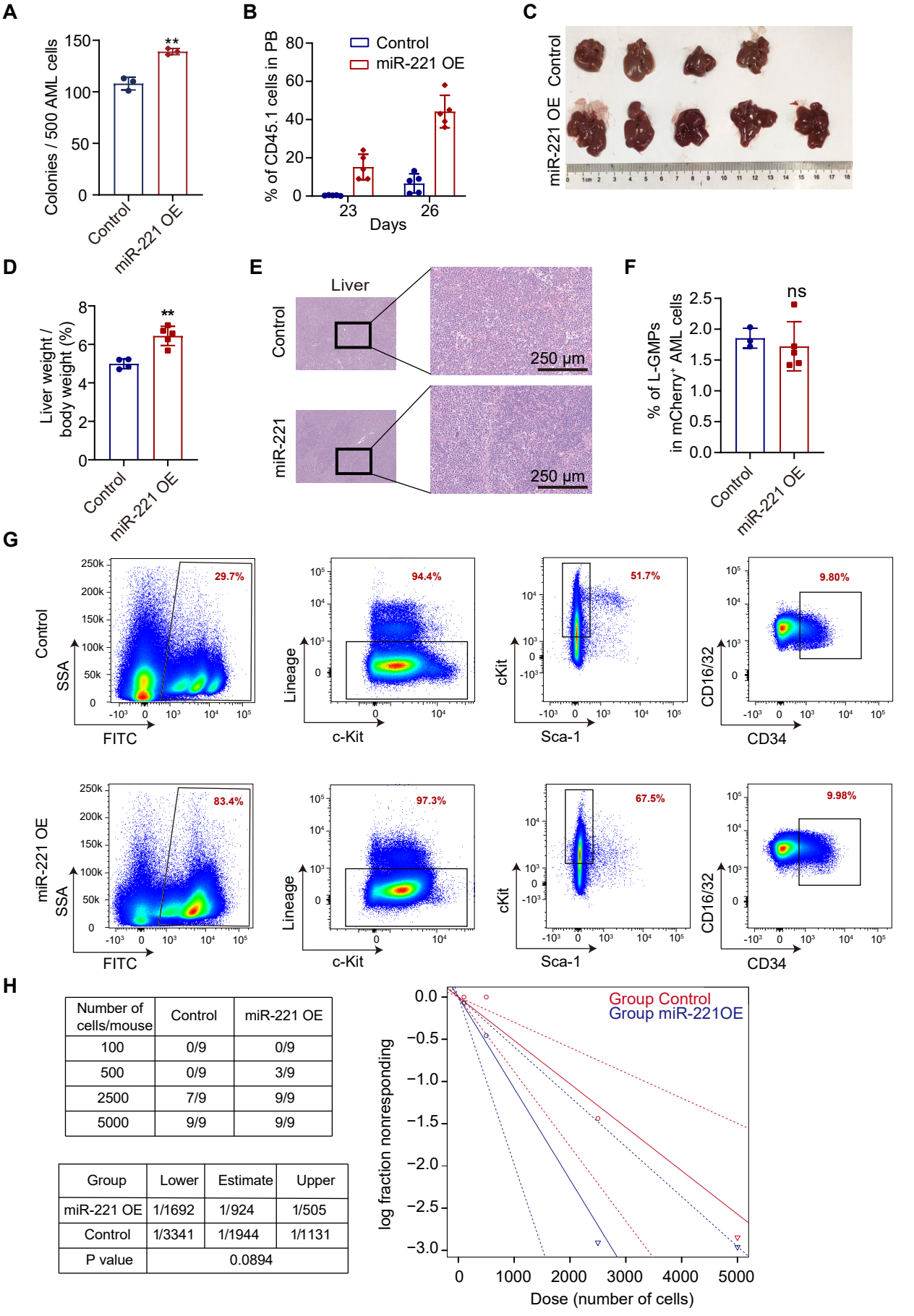
293T
Relative expression of miR-221
Control
miR-221 OE
B
293T sEVs
Relative expression of miR-221
Ctrl-sEVs
miR-221-sEVs
C
Cell number (10^5)
Blank
DMSO
GW4869
GW4869 + Ctrl-sEVs
GW4869 + miR-221-sEVs
D
Cell viability (%)
E
Propotion in AML cells (%)
G0
G1
S/G2/M
F
Apoptotic cells (%)
G
Blank
DMSO
GW4869
GW4869 + Ctrl-sEVs
GW4869 + miR-221-sEVs
68.3%
G1
28.2%
S/G2/M
G0
3.42%
0
63.5%
30.9%
5.52%
51.8%
20.2%
28.0%
49.0%
19.8%
31.2%
58.0%
36.0%
5.96%
Ki67
DAPI
H
0.17%
1.34%
Late apoptosis
Live
95.3%
Early apoptosis
3.20%
0.34%
1.70%
94.6%
3.41%
0.38%
7.14%
88.6%
3.89%
0.20%
3.46%
90.6%
5.77%
0.12%
0.82%
95.7%
3.40%
DAPI
Annexin V

Figure S4



H

Number of cells/mouse	Control	miR-221 OE
100	0/9	0/9
500	0/9	3/9
2500	7/9	9/9
5000	9/9	9/9

Group	Lower	Estimate	Upper
miR-221 OE	1/1692	1/924	1/505
Control	1/3341	1/1944	1/1131
P value	0.0894		

Figure S5

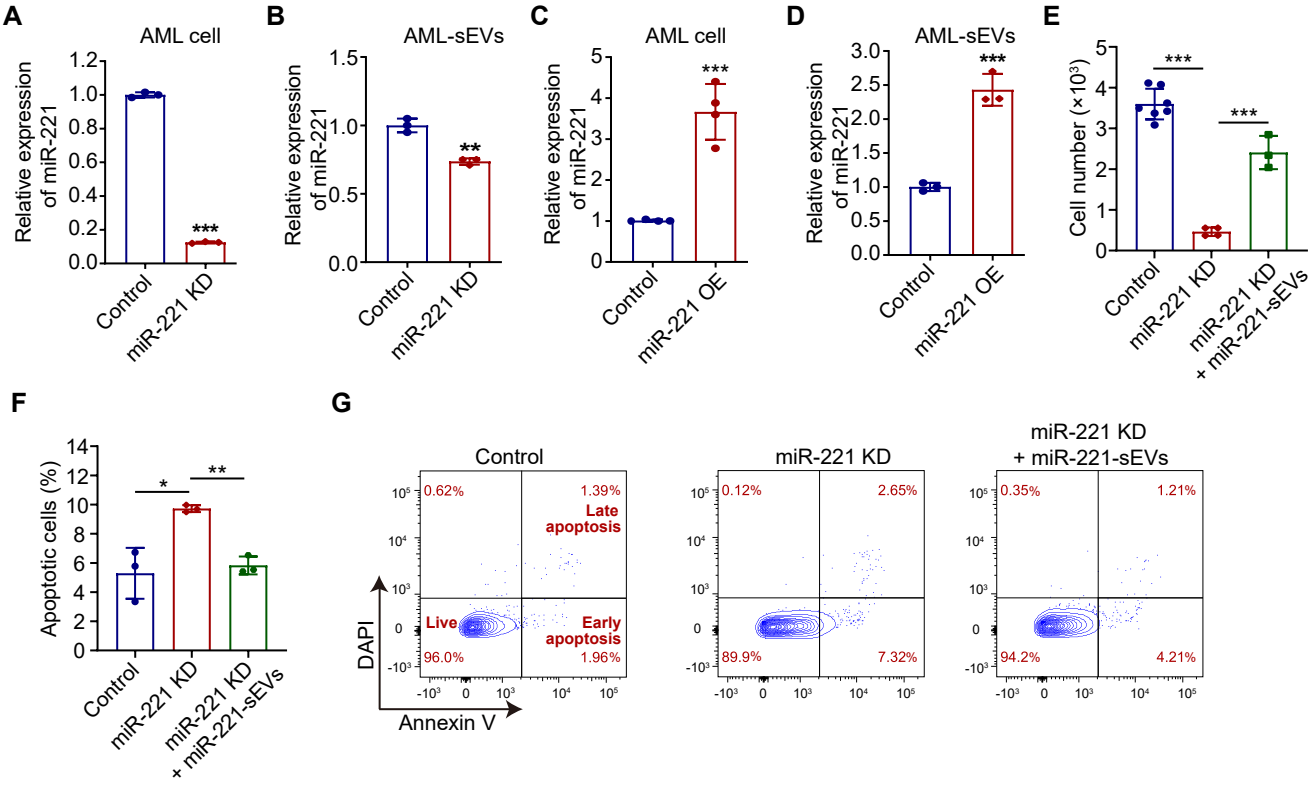

A
AML cell
Relative expression of miR-221
Control
miR-221 KD
B
AML-sEVs
Relative expression of miR-221
Control
miR-221 KD
C
AML cell
Relative expression of miR-221
Control
miR-221 OE
D
AML-sEVs
Relative expression of miR-221
Control
miR-221 OE
E
Cell number (×10³)
Control
miR-221 KD
miR-221 KD + miR-221-sEVs
F
Apoptotic cells (%)
Control
miR-221 KD
miR-221 KD + miR-221-sEVs
G
Control
miR-221 KD
miR-221 KD + miR-221-sEVs
0.62%
1.39%
Late apoptosis
Live
Early apoptosis
96.0%
1.96%
0.12%
2.65%
89.9%
7.32%
0.35%
1.21%
94.2%
4.21%
DAPI
Annexin V

Figure S6

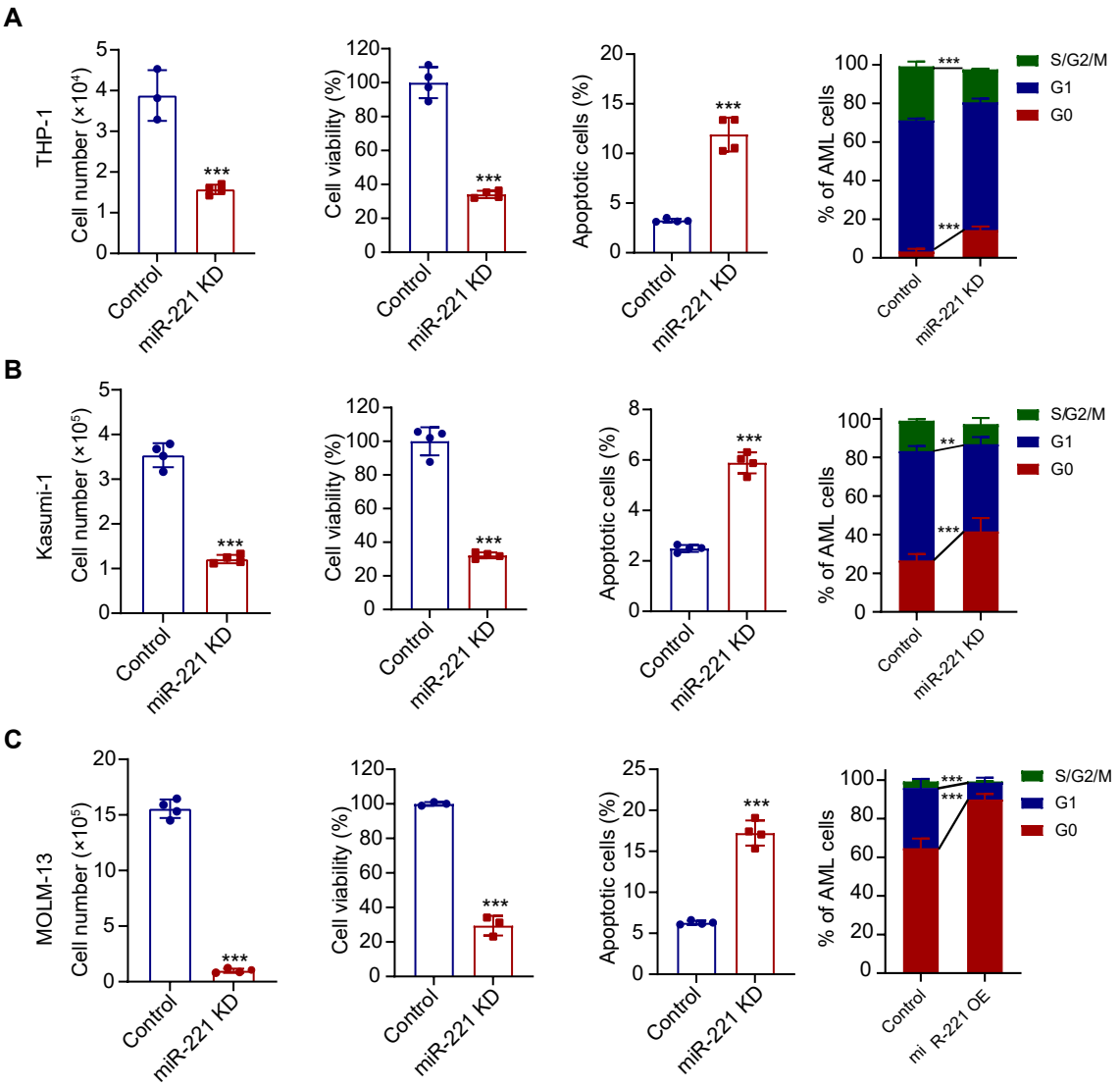

A
THP-1
Cell number (×10⁴)
Cell viability (%)
Apoptotic cells (%)
% of AML cells
S/G2/M
G1
G0
Control
miR-221 KD
B
Kasumi-1
Cell number (×10⁵)
C
MOLM-13
miR-221 OE

Figure S7

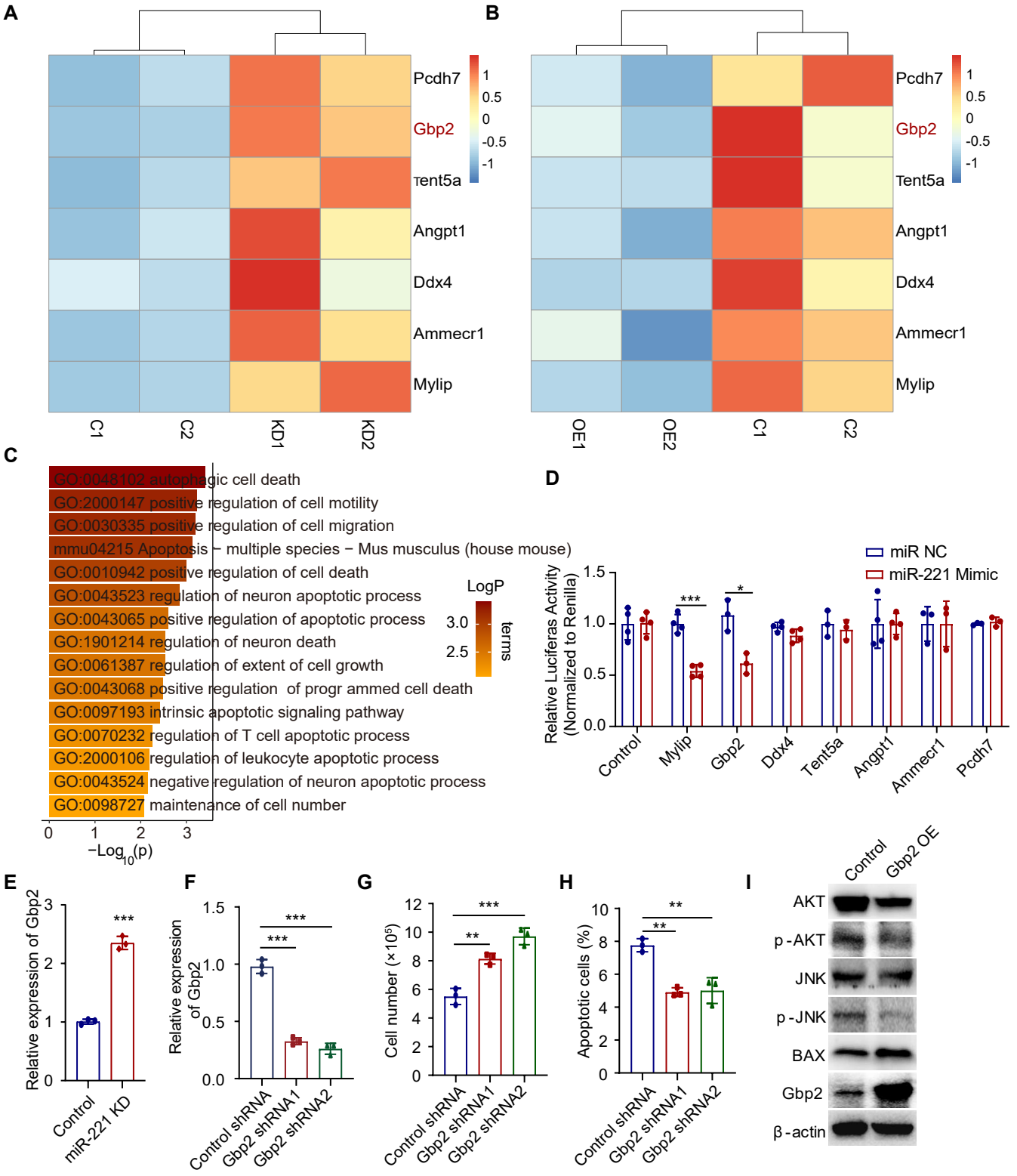
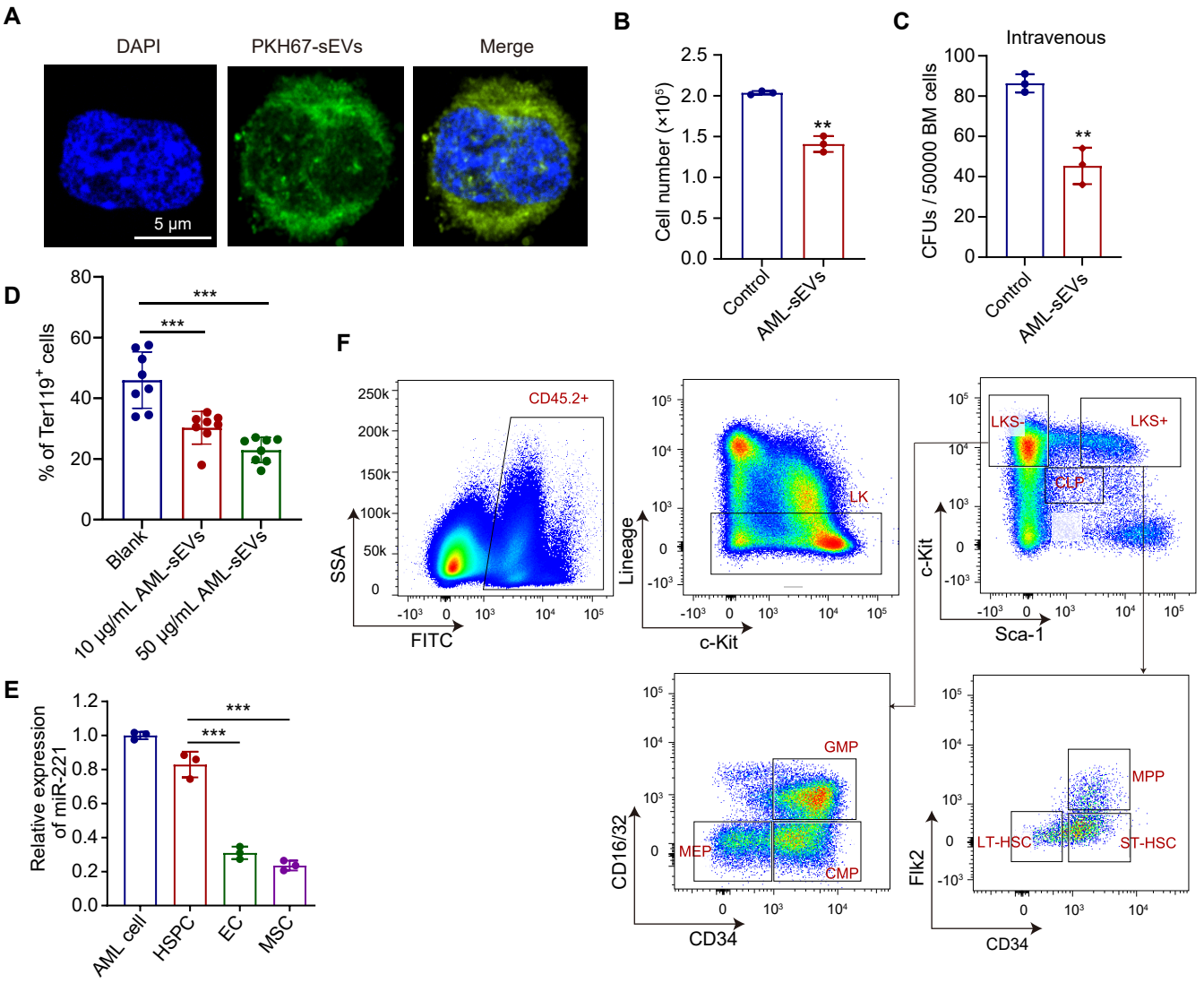

A
Pcdh7
Gbp2
Tent5a
Angpt1
Ddx4
Ammecr1
Mylip
C1
C2
KD1
KD2
1
0.5
0
-0.5
-1
B
Pcdh7
Gbp2
Tent5a
Angpt1
Ddx4
Ammecr1
Mylip
OE1
OE2
C1
C2
C
GO:0048102 autophagic cell death
GO:2000147 positive regulation of cell motility
GO:0030335 positive regulation of cell migration
mmu04215 Apoptosis − multiple species − Mus musculus (house mouse)
GO:0010942 positive regulation of cell death
GO:0043523 regulation of neuron apoptotic process
GO:0043065 positive regulation of apoptotic process
GO:1901214 regulation of neuron death
GO:0061387 regulation of extent of cell growth
GO:0043068 positive regulation of progr ammed cell death
GO:0097193 intrinsic apoptotic signaling pathway
GO:0070232 regulation of T cell apoptotic process
GO:2000106 regulation of leukocyte apoptotic process
GO:0043524 negative regulation of neuron apoptotic process
GO:0098727 maintenance of cell number
LogP
3.0
2.5
terms
0
1
2
3
−Log10(p)
D
miR NC
miR-221 Mimic
Relative Luciferas Activity (Normalized to Renilla)
1.5
1.0
0.5
0.0
***
*
Control
Mylip
Gbp2
Ddx4
Tent5a
Angpt1
Ammecr1
Pcdh7
E
Relative expression of Gbp2
3
2
1
0
***
Control
miR-221 KD
F
Relative expression of Gbp2
1.5
1.0
0.5
0.0
***
***
Control shRNA
Gbp2 shRNA1
Gbp2 shRNA2
G
Cell number (×10⁵)
12
10
8
6
4
2
0
***
**
Control shRNA
Gbp2 shRNA1
Gbp2 shRNA2
H
Apoptotic cells (%)
10
8
6
4
2
0
**
**
Control shRNA
Gbp2 shRNA1
Gbp2 shRNA2
I
Control
Gbp2 OE
AKT
p-AKT
JNK
p-JNK
BAX
Gbp2
β-actin

Figure S8


A
DAPI
PKH67-sEVs
Merge
5 μm
B
Cell number (×10⁵)
Control
AML-sEVs
C
Intravenous
CFUs / 50000 BM cells
Control
AML-sEVs
D
% of Ter119⁺ cells
Blank
10 μg/mL AML-sEVs
50 μg/mL AML-sEVs
E
Relative expression of miR-221
AML cell
HSPC
EC
MSC
F
CD45.2+
SSA
FITC
Lineage
LK
c-Kit
LKS-
LKS+
CLP
Sca-1
GMP
MEP
CMP
CD16/32
CD34
MPP
LT-HSC
ST-HSC
Flk2
CD34