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

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Abstract

Small extracellular vesicles (sEV) transfer cargos between cells and participate in various physiological and pathological processes through their autocrine and paracrine effects. However, the pathological mechanisms employed by sEV-encapsulated microRNA (miRNA) in acute myeloid leukemia (AML) are still obscure. In this study, we aimed to investigate the effects of AML cell-derived sEV (AML-sEV) on AML cells and delineate the underlying mechanisms. We initially used high-throughput sequencing to identify miR-221-3p as the miRNA prominently enriched in AML-sEV. Our findings revealed that miR-221-3p promoted AML cell proliferation and leukemogenesis by accelerating cell cycle entry and inhibiting apoptosis. Furthermore, *Gbp2* was confirmed as a target gene of miR-221-3p by dual luciferase reporter assays and rescue experiments. Additionally, AML-sEV impaired the clonogenicity, particularly the erythroid differentiation ability, of hematopoietic stem and progenitor cells. Taken together, our findings reveal how sEV-delivered miRNA contribute to AML pathogenesis, which can be exploited as a potential therapeutic target to attenuate AML progression.

Introduction

Acute myeloid leukemia (AML) is a malignant blood disease characterized by abnormal growth and differentiation of hematopoietic stem/progenitor cells (HSPC). The leukemic cells infiltrate the bone marrow (BM), peripheral blood, and extramedullary tissue, leading to cytopenia and death.[1-3] The BM is composed of hematopoietic and non-hematopoietic cells, which participate in multiple biological processes together with the extracellular matrix.[4] Under physiological conditions, the BM plays a critical role in supporting and regulating the self-renewal, multi-lineage differentiation, apoptosis, resting, and trafficking of HSPC.[5] Leukemic cells remodel the BM microenvironment into a leukemia-permissive one, impeding residual HSPC function and contributing to leukemia progression.[6] However, the mechanisms by which BM cells create and maintain the leukemic BM niche, as well as how this leukemic niche subsequently impacts leukemic cells and HSPC, remain to be explored.

The leukemic BM niche is intricate for extensive cell-cell interactions and biological processes, which are regulated by extracellular components such as cytokines, chemokines, and extracellular vesicles.[7,8] For instance, leukemic cells interact directly with mesenchymal stromal cells to inhibit

normal hematopoiesis and promote leukemic cell proliferation through the secretion of CCL3 and THPO.[9] Moreover, leukemic cells upregulate the expression of *CXCR4* and *VLA4*, leading to drug resistance.[10] Our previous research also demonstrate that the CCL3 secreted by leukemic cells inhibits erythropoiesis,[11] while BM endothelial cells inhibit the differentiation and maturation of megakaryocytes by expressing high level of interleukin (IL)-4 in MLL-AF9 mouse model.[12] By contrast to traditional forms of intercellular communication, extracellular vesicles (EV) act as messengers by transferring their contents to recipient cells. Small EV (sEV) have a 30-150 nm diameter and play pivotal roles in cellular communication by transporting cargos such as nucleic acids, proteins, and lipids between cells. Accumulating studies have demonstrated that sEV participate in various physiological and pathological processes by transferring cargos to adjacent or distant cells through paracrine or endocrine pathways, as well as acting on the same cell population through an autocrine effect.[13,14] Importantly, studies have shown that sEV derived from AML cells (AML-sEV) can transmit cargos to stromal cells and HSPC, thereby affecting their functions and, in turn, impacting leukemia progression.[8]

MicroRNA (miRNA) are a class of non-coding RNA, 20-24 nucleotides in length, typically bind to the 3' untranslated regions (3'UTR) of messenger RNA (mRNA) to regulate gene expression at the post-transcriptional level.[15] The miRNA contained in sEV have been extensively explored because of their abundance, diversity, and ability to regulate mRNA function. Emerging studies have confirmed that sEV-miRNA play a vital role in the occurrence, development, diagnosis, and treatment of various tumors.[16,17] However, the specific roles of sEV-miRNA in AML and the repertoire of miRNA within AML-sEV remain largely unknown. Therefore, the aims of the present study were to identify the miRNA prominently expressed in AML-sEV and explore the functions and molecular mechanisms of critical miRNA in AML development as well as normal hematopoiesis.

Methods

Mice and transplantation

C57BL/6J and B6.SJL mice were purchased from the animal facility of the State Key Laboratory of Experimental Hematology (SKLEH, Tianjin, China). All animal procedures were performed in compliance with the animal care guidelines approved by the Institutional Animal Care and Use Committees of the SKLEH and the Institute of Hematology. In order to establish the AML model, lineage- cells were isolated from B6.SJL mouse (CD45.1$^+$) bone marrow and transduced with a retrovirus expressing MLL-AF9 fusion gene.[18] After 72 hours, the cells were injected into lethally irradiated (9.5 Gy) C57BL/6J recipient mice (CD45.2$^+$) to induce AML development. Primary leukemic cells from bone marrow or spleen were harvested and transplanted into non-irradiated recipients (CD45.2$^+$) to establish MLL-AF9 AML model. For MLL-AF9 mouse model, 1×10^5 AML cells were intravenously injected into 6-8-week-old wild-type (WT) C57BL/6 recipient mice. For AML1-ETO9a mouse model, 1×10^5 established AML cells (a gift from Dr. Jianxiang Wang, Chinese Academy of Medical Sciences and Peking Union Medical College) were intravenously injected into 6-8-week-old WT C57BL/6 recipient mice after 5 Gy irradiation. For sEV *in vivo* treatment, 40 μg sEV was injected intravenously or 20 μg sEV was injected into one tibia of C57BL/6 mice.

Flow cytometry

Apoptosis of AML cells was measured by labeling the cells with Annexin V-APC for 15 minutes (min) in 1× binding buffer, according to the manufacturer's instructions (BD Bioscience). For Ki67 staining, AML cells were first fixed and permeabilized using the Intrasure Kit (BD Bioscience) and then stained with Ki67-APC antibody (BD Bioscience) for 30 min. For cKit$^+$ cells sorting, antibodies (all purchased from BD Bioscience) targeting the following molecules were used: CD3, CD4, CD8, B220, Gr-1, Mac-1, Ter-119, Sca1, cKit. For erythroid differentiation analysis, Ter-119 antibody was used. For the leukemic granulocyte macrophage progenitors (L-GMP) analysis, antibodies (all purchased from BD Bioscience) targeting the following molecules were used: CD3, CD4, CD8, B220, Gr-1, Ter-119, Sca1, cKit, CD34, CD16/32 (FcγR), CD127 (IL-7Rα), and CD45.1. An anti-CD45.1 antibody (BD Bioscience) was used in the analysis of peripheral blood cells. Finally, 4′,6 diamidino-2-phenylindole (DAPI, Sigma-Aldrich) was added to exclude the dead cells prior to analysis. The analysis was conducted using a BD LSRFortessa flow cytometer, and cell sorting was performed using a BD FACSAriaIII flow cytometer.

Statistical analysis

All experiments were repeated a minimum of three times. The data were analyzed using TreeStar FlowJo software. Quantitative values were presented as means ± standard deviation (SD). The unpaired, two-tailed Student's *t* test was used to compare two groups. The one-way ANOVA with Tukey's multiple comparison post-test or a two-way ANOVA with Sidak's multiple comparison test was used to compare more than two groups. Statistical analysis was performed using GraphPad Prism 9.0 and statistical significance was denoted by asterisks (*P<0.05, **P<0.01, ***P<0.001), whereby P values less than 0.05 were considered as a measure of statistical significance.

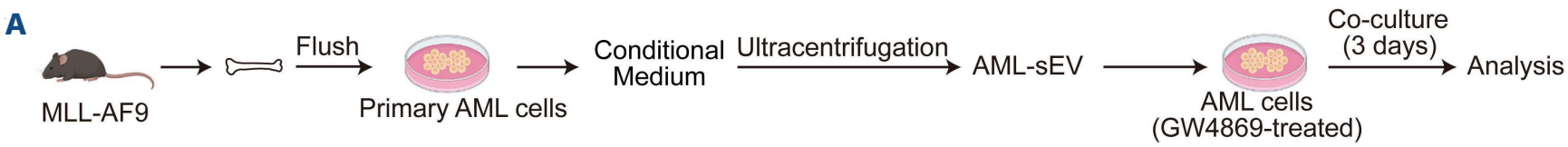
Results

Acute myeloid leukemia extracellular vesicles promote acute myeloid leukemia cell growth through autocrine effects

Previous studies have shown that sEV derived from leukemic cells participate in leukemia development.[19] In order to

evaluate the impact of AML-sEV on the growth of AML cells, we isolated sEV from the culture supernatant of AML cells, previously isolating from the BM of MLL-AF9 AML mouse, by ultracentrifugation according to established protocol (Figure 1A).[20] The structure of sEV was assessed using transmission electron microscopy, which revealed that they had a typical saucer-like shape (*Online Supplementary Figure S1A*). Moreover, nanoparticle tracking analysis determined that the peak particle diameter ranged from 93 to 242 nm, with a mean diameter of 130 nm (*Online Supplementary Figure S1B*). Western blotting also confirmed that the isolated sEV expressed high level of sEV-specific protein markers (e.g., CD63, CD9, and TSG101) but exhibited negligible expression of the sEV exclusion marker Calnexin (*Online Supplementary Figure S1C*).

Co-culturing AML cells with the isolated AML-sEV for 3 days significantly increased their proliferation versus that of untreated AML cells (*Online Supplementary Figure S1D*). Treating AML cells with GW4869, an inhibitor of sEV secretion, significantly inhibited AML cell growth and viability *in vitro*. Notably, this inhibitory effect was counteracted by treating the AML cells with AML-sEV (Figure 1B, C). In order to assess whether these treated cells affect leukemogenic progression *in vivo*, we transplanted the treated AML cells into non-irradiated mice. We found the survival of mice transplanted with the GW4869-treated AML cells was prolonged compared with that of mice in the untreated group. Meanwhile, the survival of mice transplanted with AML-sEV-treated AML cells was shorter than that of the mice transplanted with GW4869-treated AML cells (Figure 1D). In order to determine why AML-sEV promoted tumor growth, we investigated the effects of GW4869 treatment on the cell cycle and apoptosis of AML cells. The results showed that GW4869 treatment caused cell cycle arrest, whereas AML-sEV treatment restored cell cycle entry (Figure 1E; *Online Supplementary Figure S1E*). We also observed an increase in AML cell apoptosis following GW4869 treatment, which was significantly inhibited upon AML-sEV treatment (Figure 1F; *Online Supplementary Figure S1F*). Collectively, these results indicate that AML-sEV promote the growth of



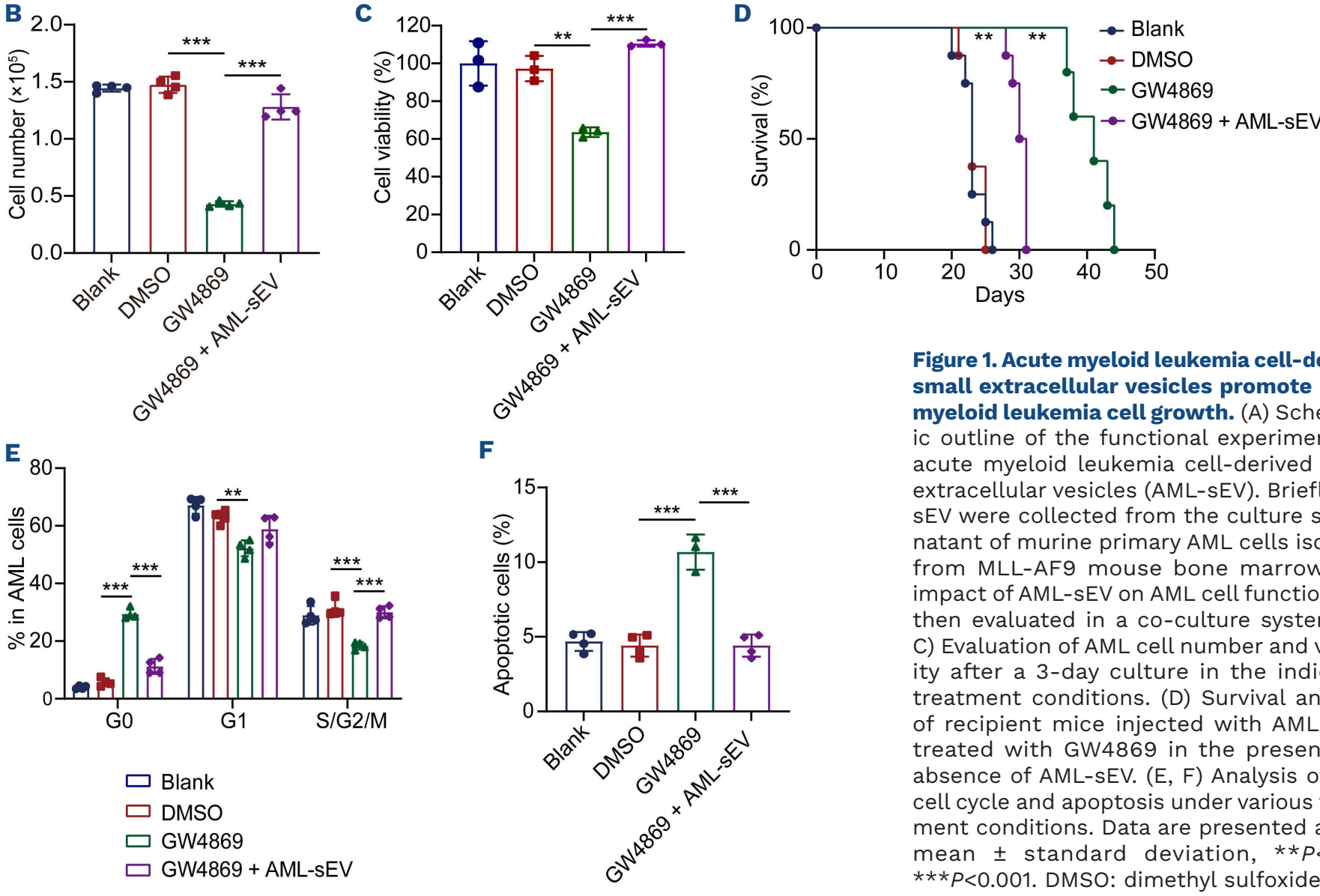


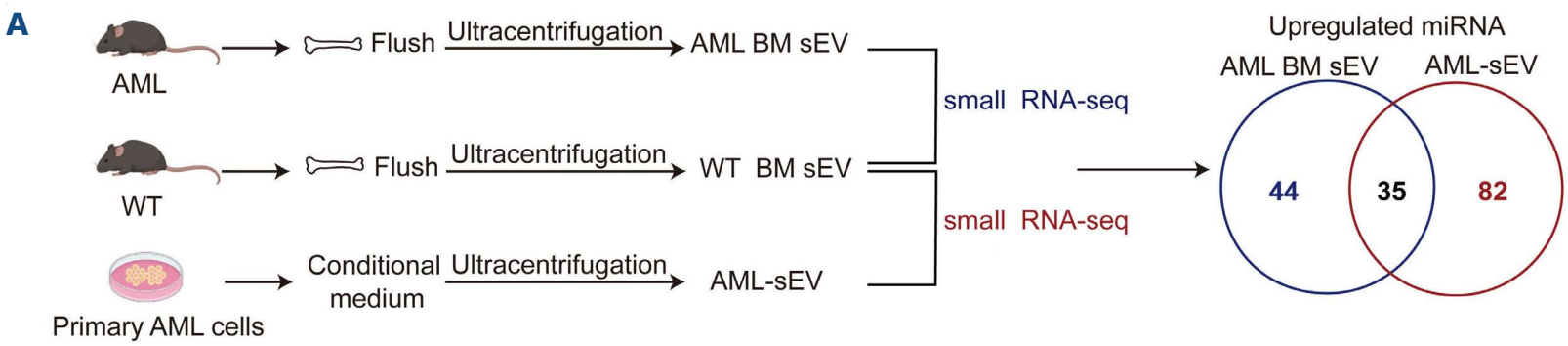
Figure 1. Acute myeloid leukemia cell-derived small extracellular vesicles promote acute myeloid leukemia cell growth. (A) Schematic outline of the functional experiments of acute myeloid leukemia cell-derived small extracellular vesicles (AML-sEV). Briefly, the sEV were collected from the culture supernatant of murine primary AML cells isolated from MLL-AF9 mouse bone marrow. The impact of AML-sEV on AML cell function was then evaluated in a co-culture system. (B, C) Evaluation of AML cell number and viability after a 3-day culture in the indicated treatment conditions. (D) Survival analysis of recipient mice injected with AML cells treated with GW4869 in the presence or absence of AML-sEV. (E, F) Analysis of AML cell cycle and apoptosis under various treatment conditions. Data are presented as the mean ± standard deviation, $^{**}P<0.01$, $^{***}P<0.001$. DMSO: dimethyl sulfoxide.

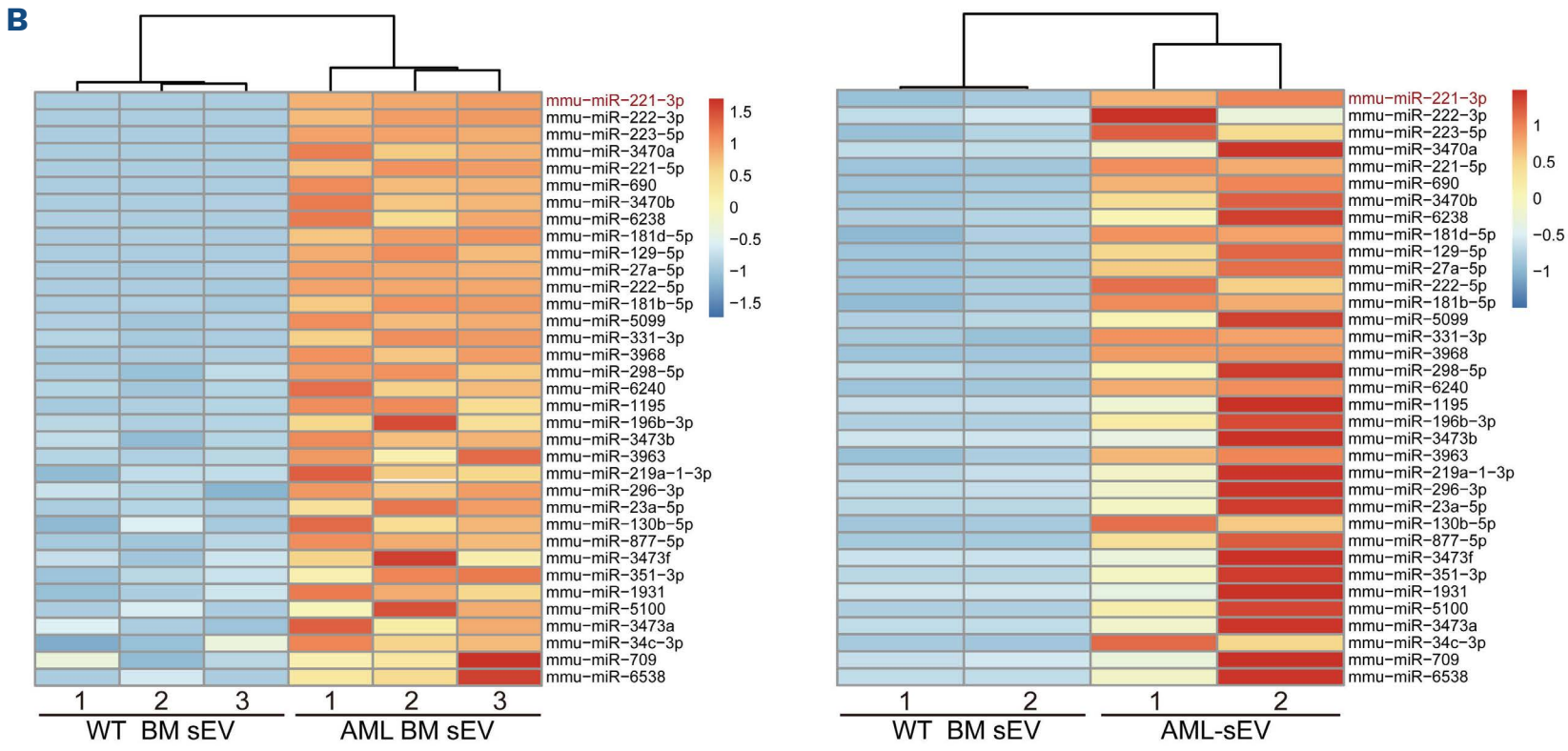
AML cells by facilitating cell cycle entry and inhibiting cell apoptosis through their autocrine effects.

Acute myeloid leukemia extracellular vesicles promote acute myeloid leukemia cell growth by delivering miR-221-3p to acute myeloid leukemia cells

In order to screen differentially expressed miRNA present in AML-sEV, small RNA sequencing was conducted on the sEV isolated from the BM of WT mice and MLL-AF9 AML mice. Considering that the BM supernatant of AML mice contains sEV from various cellular sources, small RNA sequencing was also conducted using cultured AML-sEV. We identified 79 upregulated miRNA that were differentially enriched in BM-derived AML-sEV relative to WT-sEV. In addition, we obtained 117 upregulated miRNA between WT mouse BM sEV and cultured AML-sEV (Figure 2A). By intersecting the miRNA upregulated in AML identified by both analyses, we obtained 35 miRNA that were highly expressed in AML-sEV, as shown in the heatmap (Figure 2B).

In order to validate the sequencing results, we detected the expression level of candidate miRNA by quantitative real-time polymerase chain reaction (qRT-PCR) and identified six highly enriched miRNA (miR-5099, miR-221, miR-690, miR-5100, miR-181b-5p, and miR-181d-5p) in both AML-sEV and AML cells (Figure 2C; *Online Supplementary Figure S2A-C*; *Online Supplementary Table S1*). Among these miRNA, miR-5099, miR-690, and miR-5100 were not conserved across various species. By contrast, miR-221-3p (hereafter referred to as





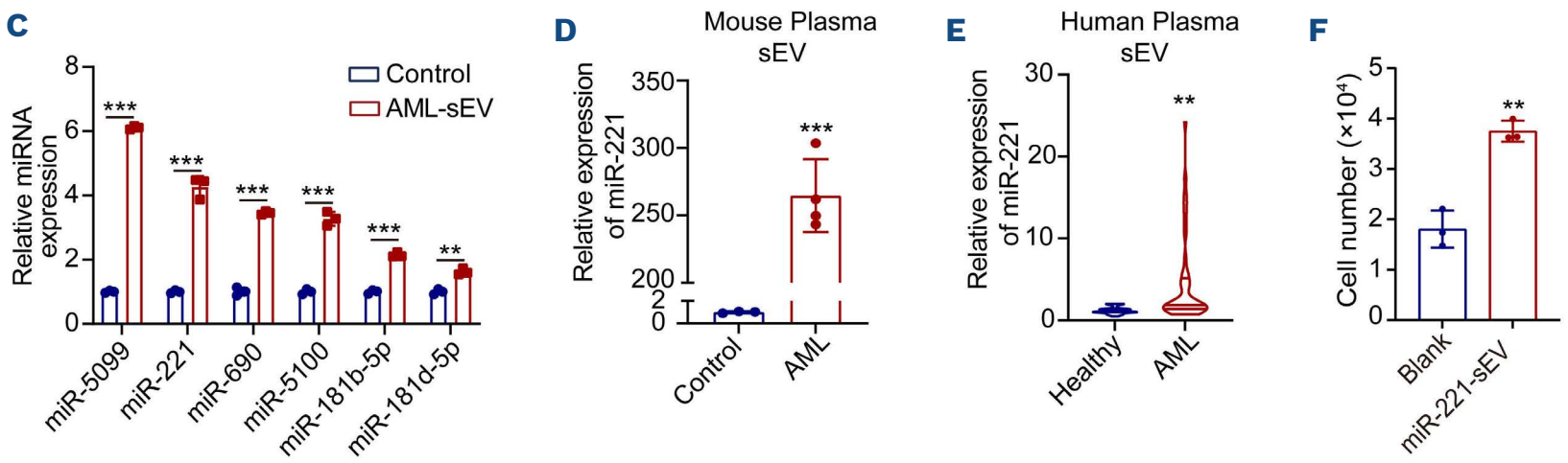


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Figure 2. Acute myeloid leukemia cell-derived small extracellular vesicles promote acute myeloid leukemia cell growth by transferring miR-221 to acute myeloid leukemia cells. (A) Schematic outline of the acute myeloid leukemia cell-derived small extracellular vesicles (AML-sEV) microRNA (miRNA) profiling experiments. sEV collected from the bone marrow (BM) of wild-type (WT) or AML mice and AML-sEV were subjected to small RNA sequencing (RNA-seq). Venn diagram showing the overlapping microRNA (miRNA) upregulated in AML from the two small RNA-seq experiments. (B) Heatmap of differentially expressed miRNA between the WT and AML BM-derived sEV (left panel). Heatmap of differentially expressed miRNA between WT BM-derived sEV and AML-sEV (right panel). (C) Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of differentially expressed miRNA in sEV. (D, E) qRT-PCR analysis of miR-221 in plasma sEV from mice and human samples. (F) Assessment of 2×10^3 AML cell number after a 3-day incubation with miR-221-sEV. Data are presented as the mean ± standard deviation, $^{**}P<0.01$, $^{***}P<0.001$.

miR-221) is conserved in various species and was highly expressed in sEV isolated from the peripheral blood plasma of AML mice or patients compared with control or healthy individuals, suggesting its important role in AML (Figure 2D, E; *Online Supplementary Figure S2D*). Next, we investigated the impact of miR-221-containing sEV on the growth of AML cells. 293T cells were infected with lentiviruses overexpressing either a scrambled control miRNA (Ctrl-miR) or miR-221. We then isolated sEV from the conditioned medium to obtain Ctrl-sEV and miR-221-sEV, respectively. Subsequent qRT-PCR analysis confirmed the effective overexpression of miRNA-221 in both 293T cells and the resulting sEV (*Online Supplementary Figure S3A, B*). Co-culturing AML cells with miR-221-sEV significantly increased their proliferation (Figure 2F). Moreover, miR-221-sEV also rescued the proliferation defect in AML cells caused by GW4869 treatment (*Online Supplementary Figure S3C-H*). These findings underscore the crucial role of miR-221 in the autocrine effect of AML-sEV on AML cell growth.

miR-221 facilitates leukemogenesis by promoting acute myeloid leukemia cell cycle entry and inhibiting apoptosis

In order to understand how miR-221 affected leukemic cell function, we knocked down miR-221 in AML cells and found that miR-221 depletion inhibited the growth and colony formation ability of AML cells (Figure 3A, B). Moreover, miR-221 knockdown (KD) in AML cells significantly delayed the onset of AML and prolonged the survival of recipient mice (Figure 3C, D). Furthermore, the downregulation of miR-221 was associated with AML cell cycle arrest and increase in AML cell apoptosis (Figure 3E, F). These data indicate that miR-221 is important in AML cell proliferation and survival. In order to clarify the oncogenic potential of miR-221, AML cells were infected with a miR-221-overexpressing lentivirus. Overexpression of miR-221 promoted the growth and colony formation ability of AML cells, exacerbating leukemogenesis (Figure 4A, B; *Online Supplementary Figure S4A, B*). Consistent with earlier findings, miR-221 promoted the cell cycle entry and inhibited the apoptosis of AML cells (Figure 4C, D). Compared with control group, the ratio of spleen or liver to body weight in overexpressed group was higher (Figure 4E; *Online Supplementary Figure S4C, D*). Histological examination of the spleen and liver revealed an increased infiltration of leukemic cells in the mice of miR-221-overexpressing group, in comparison with that of the control group (Figure 4F; *Online Supplementary Figure S4E*). Remarkably, while miR-221 overexpression impacted the whole leukemic cell population, it did not appear to affect the frequency of phenotypically defined leukemia stem cells (LSC) and functional defined LSC determined by limiting dilution analysis (*Online Supplementary Figure S4F-H*). These results implies that miR-221 predominantly impacts the bulk leukemic cell population rather than specifically targeting LSC.

Given that AML cells can exert their effects on tumor growth through the sEV-mediated secretion of miR-221, we next investigated whether the expression of miR-221 in sEV was influenced by the KD or overexpression of miR-221 in AML cells. In order to achieve this, we collected conditioned medium from miR-221-depleted or -overexpressing AML cells after 48 hours of culture and isolated sEV by ultracentrifugation. qRT-PCR revealed that the expression level of miR-221 significantly decreased or increased in miR-221-depleted or -overexpressing AML cells and their corresponding sEV respectively, relative to that of the control group (*Online Supplementary Figure S5A-D*). The attenuated proliferation and increased apoptosis of AML cells caused by miR-221 KD were rescued by co-culturing with miR-221-sEV for 3 days (*Online Supplementary Figure S5E-G*). These results further confirm that AML-sEV promote the proliferation of AML cells via miR-221 transfer.

miR-221 promotes the proliferation of AML1-ETO9a cells and human leukemic cell lines

In order to further explore the role of miR-221 in primary leukemogenesis, we employed another AML model driven by the AML1-ETO9a fusion.[21] miR-221 was either knocked down or overexpressed in AML1-ETO9a cells, and the transfected cells were subsequently transplanted into recipients. The results elucidated that miR-221 KD in AML cells increased apoptosis and prolonged the survival of recipient mice (Figure 5A). Conversely, the overexpression of miR-221 inhibited the apoptosis of AML cells and shortened the survival of recipient mice, thereby exacerbating leukemogenesis (Figure 5B). Given that the seed sequence of miR-221 is conserved across species (*Online Supplementary Figure S2D*), our investigation extended to various human leukemic cells. We explored the role of miR-221 in a MLL-AF9 cell line (THP-1) and other cell lines with diverse gene mutations (HL-60, Kasumi-1, MOLM-13). Leukemia cells were infected with miR-221-KD or a control lentivirus followed by cell proliferation analysis. The

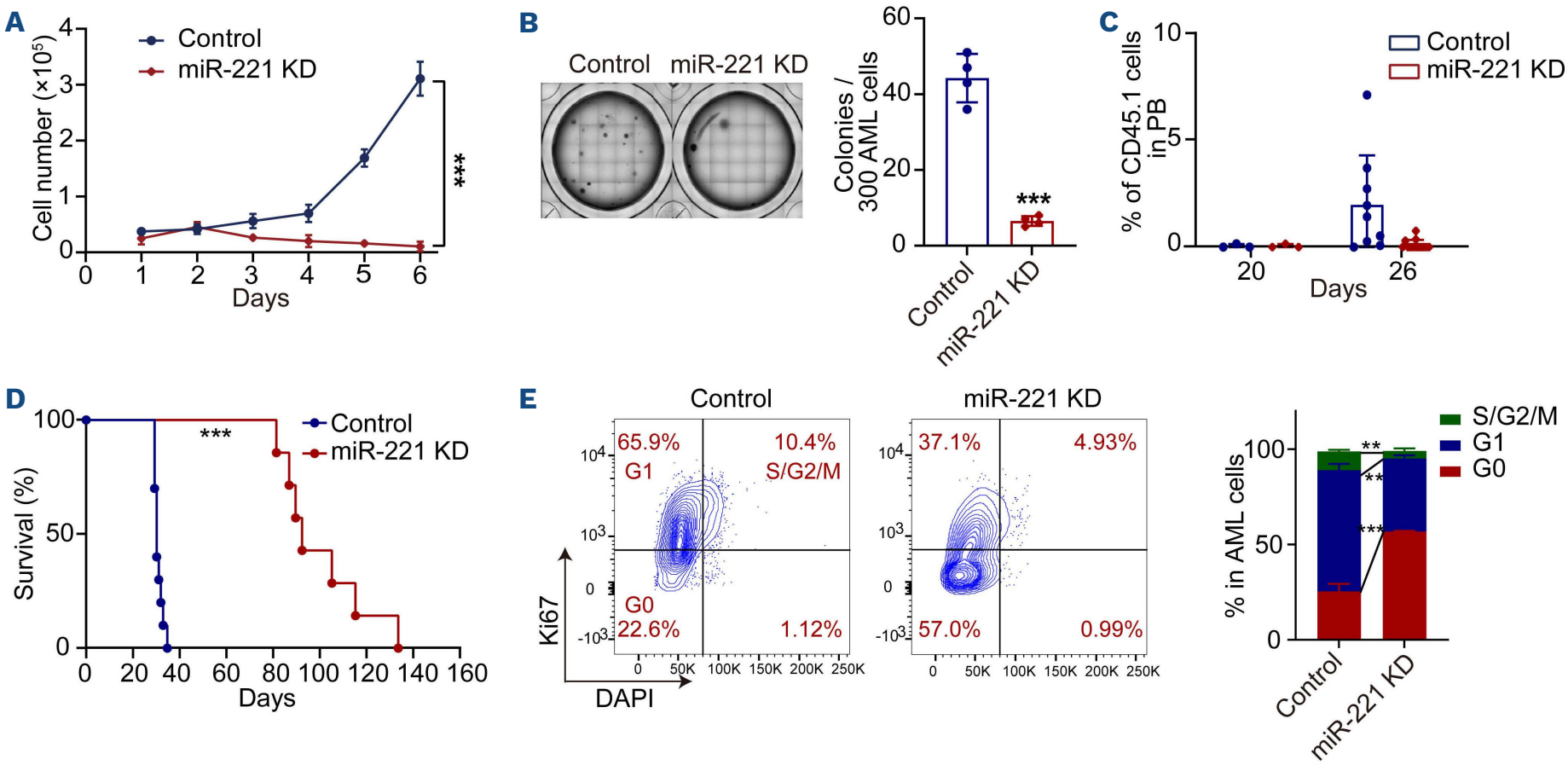

Figure 3. miR-221 knockdown inhibits leukemia development. (A) Acute myeloid leukemia (AML) cell number was calculated after miR-221 knockdown (KD). (B) Representative images showing the impact on colony formation after miR-221 KD in AML cells (left panel). Impact of miR-221 KD on AML cell colony number (right panel). (C) 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 KD AML cells (N=7-10). (D) Survival analysis of recipients injected with WT or miR-221 KD AML cells (N=7-10). (E) Representative flow cytometry contour plots (left panel) and corresponding bar graph showing the impact of miR-221 KD on AML cell cycle (right panel). (F) Representative flow cytometry contour plots (left panel) and corresponding bar graph showing the impact of miR-221 KD on AML cell apoptosis over 6 days (right panel). ***P*<0.01, ****P*<0.001.

results revealed that miR-221 KD reduced cell proliferation and cell viability, potentially by promoting apoptosis and cell cycle arrest (Figure 5C-F; *Online Supplementary Figure S6A-C*). These findings illustrate the pivotal role of miR-221 in diverse leukemic cell types, thereby expanding its relevance in the intricate landscape of leukemia pathogenesis.

miR-221 increases acute myeloid leukemia cell proliferation by targeting *Gbp2*

The seed sequence of miRNA recognizes complementary sequences in the 3' UTR of downstream target genes, leading to translational inhibition.[22,23] In order to identify the potential target genes of miR-221, we sorted miR-221-depleted and -overexpressed AML cells for transcriptome sequencing. We identified 130 upregulated genes and 93 downregulated genes in the miR-221 KD group *versus* the scramble group. A further 770 upregulated genes and 781 downregulated genes were identified by comparing the miR-221 overexpressing group with the scramble group (Figure 6A).

In most cases, miRNA negatively regulates the expression of its target gene.[24] By overlapping the upregulated genes from the miR-221 KD group with the downregulated genes from the miR-221-overexpressing group, we identified a set of seven genes as potential targets of miR-221: *Gbp2*, *Mylip*, *Pcdh7*, *Angpt1*, *Tent5a*, *Ammecr1*, *Ddx4* (Figure 6A, B; *Online Supplementary Figure S7A, B*). Notably, the gene ontology (GO) functional enrichment analyses revealed that predicted target genes of miR-221 were enriched in terms related to cell

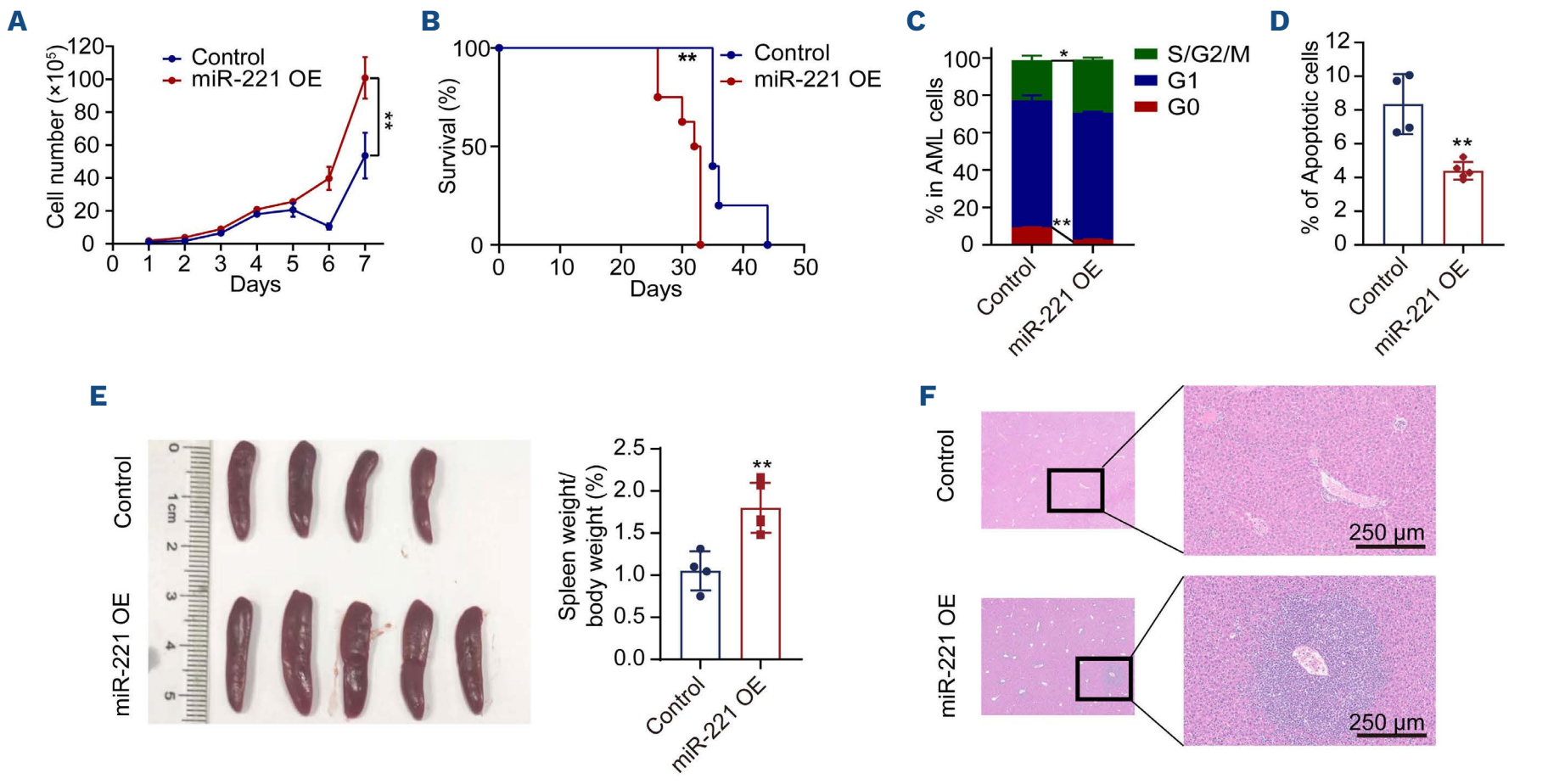

Figure 4. miR-221 overexpression accelerates leukemia development. (A) Acute myeloid leukemia (AML) cell number was calculated after miR-221 overexpression (OE). (B) Survival analysis of recipient mice injected with wild-type (WT) or miR-221 OE AML cells (N=5-8). (C-D) Graphs showing the impact of miR-221 OE on AML cell cycle and apoptosis. (E) Representative photographs of spleens from the control and miR-221 OE groups (left panel) and comparison of spleen to body weight ratios (expressed as %) in the control and miR-221 OE groups (right panel). (F) Representative hematoxilin and eosin staining images of spleens from the control and miR-221 OE groups. Scale bars=250 μm. Data are presented as the mean ± standard deviation, **P*<0.05, ***P*<0.01.

growth and death processes (*Online Supplementary Figure S7C*). In order to further validate the target genes of miR-221, we inserted the 3'UTR sequence of the candidate genes into a dual luciferase reporter gene vector and co-transfected it with miRNA negative control (miR NC) or miR-221 mimic into 293T cells. The activity level of luciferase in the group co-transfected with *Gbp2* 3'UTR and miR-221 mimic was significantly lower than that co-transfected with *Gbp2* 3'UTR and miR NC (*Online Supplementary Figure S7D*). Notably, this reduction in luciferase activity was restored when 293T cells were co-transfected with a vector carrying a mutated 3'UTR sequence of *Gbp2*, indicating that miR-221 interacted with *Gpb2* (Figure 6C). Additionally, we observed that the mRNA and protein expression level of *Gbp2* is significantly higher in miR-221-depleted AML cells than that in control cells, supporting the notion that miR-221 regulated *Gpb2* expression (Figure 6D; *Online Supplementary Figure S7E*).

In order to explore the effect of *Gbp2* on AML cell, AML cells were infected with *Gbp2*-shRNA or control lentiviruses. The results indicated that Gbp2 KD significantly increased the proliferation and reduced the apoptosis of AML cells (*Online Supplementary Figure S7F-H*; *Online Supplementary Table S2*). In order to confirm the regulatory role of miR-221, the *Gbp2*-depleted AML cells were infected with miR-221 KD or control lentiviruses. The results demonstrated that KD of *Gbp2* attenuated the effect of miR-221 KD on proliferation and apoptosis of AML cells (Figure 6E, F). As previous study showed the role of *Gbp2* in modulating cell proliferation through PI3K/AKT pathway,[25] we assessed the protein expression level of AKT, p-AKT, JNK, p-JNK and BAX in *Gbp2*-overexpressed AML cells and control cells. The results revealed that *Gbp2* overexpression led to a decrease in the protein expression level of AKT, p-AKT, JNK and p-JNK, while increased the expression of BAX (*Online Supplementary Figure S7I*). Collectively, these results confirmed that miR-221 promoted AML cell proliferation, in part by targeting *Gbp2*, through regulating PI3K/AKT pathway activation.

Acute myeloid leukemia small extracellular vesicles impair hemtopoietic stem cell function through a paracrine effect

In addition to exhibiting autocrine effects, sEV possess the ability to exert paracrine effects by transferring their cargos to neighboring cells.[26] In order to explore whether AML-sEV could be delivered to and internalized by HSPC, AML-sEV were labeled with PKH67 and co-cultured with HSPC for 12 hours. Subsequently, the GFP$^+$ sEV signal was detected in the cytosol of HSPC using confocal imaging, indicating that AML-sEV were delivered to HSPC (*Online Supplementary Figure S8A*). In order to investigate the impact of AML-sEV on HSPC function, we co-cultured AML-sEV with HSPC for 3 days and conducted colony formation assays (Figure 7A). AML-sEV significantly compromised the colony forming ability and proliferation of HSPC *in vitro* (Figure 7B; *Online Supplementary Figure S8B*). In order to investigate the effect of AML-sEV *in*

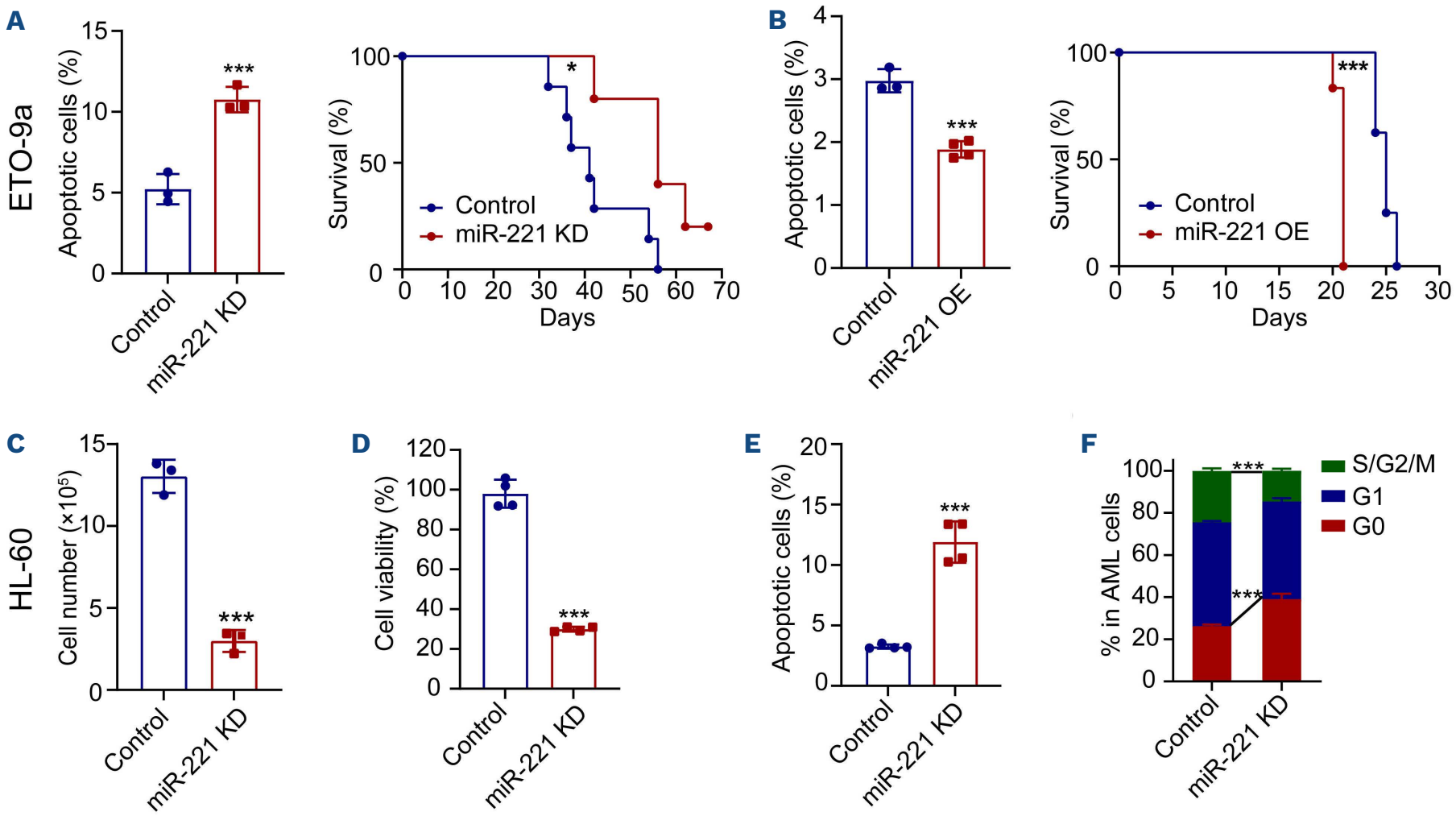

Figure 5. The impact of miR-221 on other types of leukemia cells. (A) Graphs showing the impact of miR-221 KD on acute myeloid leukemia (AML) cell apoptosis (left panel) and survival analysis of recipients injected with wild-type (WT) or miR-221 knockdown (KD) AML-ETO9a cells (N=6-7) (right panel). (B) Graphs illustrating the impact of miR-221 overexpression (OE) on AML cell apoptosis (left panel) and survival analysis of recipients injected with WT or miR-221 OE AML-ETO9a cells (N=6-7) (right panel). (C-F) Analysis of the number, viability, apoptosis, and cell cycle of HL-60 cells after miR-221 KD. Data are presented as the mean ± standard deviation, **P*<0.05, ****P*<0.001.

vivo, we injected AML-sEV into WT mice, either via the tail vein or intratibially, and then harvested cKit$^+$ HSPC from sEV-treated mice for colony formation assays. Consistent with the *in vitro* co-culture results, the colony-forming ability of HSPC was impaired following sEV treatment (*Online Supplementary Figure S8C*). Coincidentally, there was a decrease in the number of BFU-E, suggesting that AML-sEV preferentially impaired the erythroid differentiation of HSPC (Figure 7C). In order to further investigate this phenomenon, we cultured cKit$^+$ cells with AML-sEV in an erythroid differentiation culture system and assessed their erythroid differentiation ability. The results revealed a significant reduction in the proportion of Ter119$^+$ cells compared to the control group (*Online Supplementary Figure S8D*). These findings indicated that AML-sEV exerted an inhibitory effect on the erythroid differentiation of normal HSPC.

In order to ascertain the main target cells of AML-sEV within the leukemic BM microenvironment, we sorted immunophenotypically-defined HSPC, mesenchymal stem cells (MSC), and endothelial cells (EC) from the BM of WT and AML mice. The qRT-PCR results showed that miR-221 exhibited the highest fold change in HSPC, compared with that in MSC and EC (*Online Supplementary Figure S8E*), indicating that miR-221 may be preferentially transferred to HSPC. Moreover, co-culturing HSPC with miR-221-sEV markedly inhibited their proliferation (Figure 7D). Among the distinct HSPC compartments, miR-221 displayed the most significant increase in expression fold change in the megakaryocytic-erythroid progenitors (MEP) and megakaryocyte progenitors (MkP) of AML mice *versus* control mice (Figure 7E; *Online Supplementary Figure S8F*). In order to investigate whether the transfer of miR-221 contributes to the AML-sEV-mediated regulation of HSPC, we conducted miR-221 KD in AML cells and collected conditional medium to isolate control- or miR-221KD-sEV. Subsequently, recipients were intratibially injected with either control- or miR-221 KD-sEV. After 24 hours, Lin$^-$cKit$^+$ cells were sorted from the tibia and cultured in complete methylcellulose-based medium for 7-10 days. The results indicated that miR-221-contained sEV mainly affect the BFU-E formation of HSPC (Figure 7F). Taken together, these data indicate that AML-sEV preferentially affect the clonogenicity of HSPC via the transfer of miR-221, thus perturbing their erythroid differentiation ability.

Discussion

Evidence suggests that tumor-derived sEV contribute to tumor progression, metastasis and therapeutic resistance.[27]

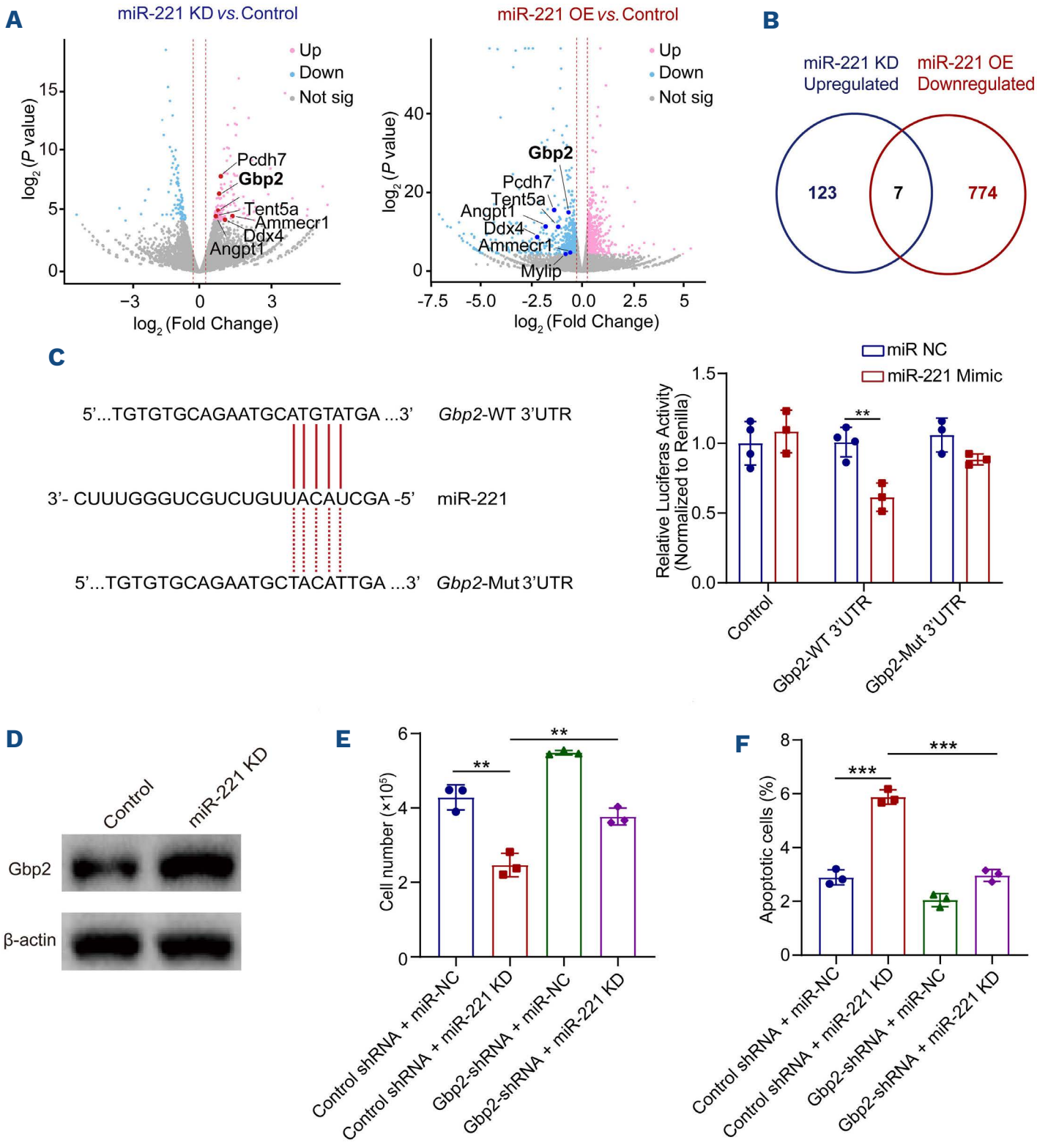

Figure 6. miR-221 promotes growth of acute myeloid leukemia cell by targeting *Gbp2*. (A) Volcano plot showing significant differentially expressed genes between control and miR-221 knockdown (KD) acute myeloid leukemia (AML) cells (left panel). Volcano plot showing significant differentially expressed genes between control and miR-221 overexpression (OE) AML cells (right panel). (B) Venn diagram showing the overlapping genes of upregulated genes in miR-221 KD AML cells and downregulated genes in miR-221 OE AML cells. (C) A schematic diagram showing the miR-221 binding site on the 3' untranslated regions (3'UTR) of mouse *Gbp2*, which is not present in the mutated *Gbp2* 3'UTR (left panel). The luciferase activity of cells co-transfected with microRNA (miRNA) negative control (NC) or miR-221 mimic and the wild-type (WT) or mutated (mut) *Gbp2* 3'UTR was detected (right panel). (D) Western blotting analysis was conducted to examine the protein expression level of *Gbp2* after miR-221 KD in AML cells. (E, F) Impact of *Gbp2* KD on the proliferation and apoptosis of miR-221 KD in AML cells. Data are presented as the mean ± standard deviation, ***P*<0.01, ****P*<0.001. shRNA: short hairpin RNA.

In the present study, we demonstrated the pivotal role of AML-sEV in promoting leukemogenesis, by facilitating the proliferation and inhibiting the apoptosis of AML cells, which are consistent with previous study.[28] Our data demonstrated that the isolated sEV were of relative purity, but we cannot entirely exclude the possibility that some other factors were co-purified with sEV. The noteworthy observation that sEV treatment abolished the effect of GW4869 strongly suggests that sEV were a major contributor. Since miRNA is an important cargo of sEV, we performed small RNA sequencing, which revealed that miR-221 was enriched in AML-sEV. Notably, sEV with a high miR-221 content had a similar effect on AML cells as AML-sEV. We also demonstrated that miR-221 accelerated leukemia progression, indicating that AML-sEV exerted autocrine effects on AML cells, mainly via the transfer of miR-221. miR-221 has been linked to various human cancers, whereby

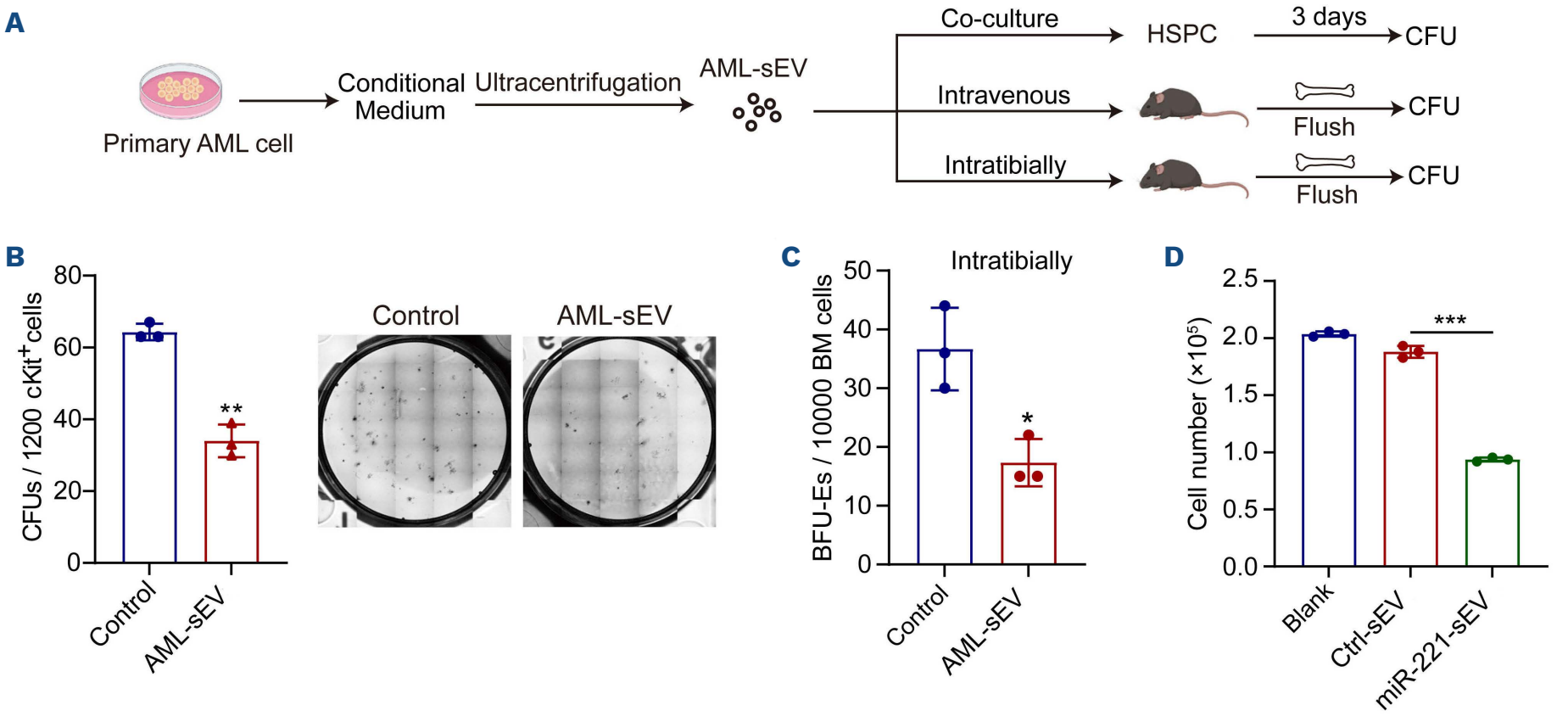

Figure 7. Acute myeloid leukemia cell-derived small extracellular vesicles impair erythroid differentiation of hematopoietic stem/progenitor cells. (A) Schematic outline of the experimental procedure used to investigate the paracrine effect of acute myeloid leukemia cell-derived small extracellular vesicles (AML-sEV) on hematopoietic stem/progenitor cells (HSPC). (B) The clonogenicity of HSPC isolated from wild-type (WT) mice exposed to AML-sEV or not was assessed by culturing in methylcellulose (left panel). Representative images showing the colony formation of untreated and AML-sEV-treated HSPC (right panel). (C) Bone marrow cells were harvested from WT mice after the intratibial injection of AML-sEV for 24 hours. The cells were cultured in erythroid specific methylcellulose-based medium M3436 (STEM CELL) for 10-14 days. (D) Assessment of HSPC number after a 7-day incubation with miR-221-sEV or control-sEV (Ctrl-sEV). (E) Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of miR-221 expression in mouse hematopoietic cell populations: long-term hematopoietic stem cells (LT-HSC), short-term hematopoietic stem cell (ST-HSC), multipotent progenitors (MPP), common myeloid progenitors (CMP), common lymphoid progenitors (CLP), granulocyte-macrophage progenitors (GMP), megakaryocyte-erythroid progenitors (MEP), and megakaryocyte progenitors (MkP) from the WT and AML mouse bone marrow. (F) $Lin^{-}cKit^{+}$ cells were harvested from WT mice after the intratibial injection of phosphate-buffered saline (PBS), ctrl-sEV or miR-221KD-sEV for 24 hours. The cells were cultured in methylcellulose-based medium M3434 (STEM CELL) for 7-10 days. Data are presented as the mean ± standard deviation, **P*<0.05, ***P*<0.01, ****P*<0.001. CFU: colony-forming unit.

it typically functions as an oncomiR by promoting tumor growth,[29] lymphangiogenesis, lymphatic metastasis,[30] and angiogenesis[31,32] following its sEV-mediated transfer. In the context of AML progression, miR-221 has emerged as a diagnostic and prognostic biomarker, while also conferring drug resistance.[33] Consistent with previous reports that miR-221 drives the expansion of leukemic cells,[34,35] the present study further demonstrated the significance of miR-221 in accelerating AML cell growth *in vitro* and leukemogenesis *in vivo*. Although numerous investigations have focused on the paracrine effects of sEV, their autocrine effects are less explored. Some studies have implied that tumor-derived sEV promote tumor metastasis and chemoresistance by acting on the same cell population.[28,36] To the best of our knowledge, sEV prevent the RNase-mediated degradation of RNA within it,[37] further highlighting their potential to exert autocrine effects.

Although the precise mechanism through which sEV exert their autocrine effects is not well understood, it is an important pathway that should not be overlooked when exploring the function of sEV. Previous evidence has shown that proteins and lipids transmitted by sEV can drive the progression of leukemia.[28,38,39] Our research provides new insights into the key miRNA found in AML-sEV and underscores the role of sEV-encapsulated miRNA in leukemia.

Our mechanistic explorations showed that miR-221 regulates *Gbp2* expression and that the proapoptotic effect of miR-221 KD could be rescued through *Gbp2* knockdown. Additionally, luciferase reporter analysis confirmed that *Gbp2* is targeted by miR-221, thereby substantiating the role of miR-221 in promoting leukemogenesis through *Gbp2* modulation. As a member of the guanylate-binding protein (Gbp) family, *Gbp2* plays a role in host defense against viral and bacterial pathogens.[40-42] Although *Gbp2* is downregulated during normal erythropoiesis, it has been shown to regulate the proliferation and erythroid differentiation of TF-1 cells.[43] Moreover, *Gbp2* regulates MCL-1 and BAK to induce caspase-dependent apoptosis by inhibiting the activation of PI3K/AKT pathway.[25] sEV participate in paracrine communication by delivering cargos from one cell population to another, thus regulating the functions of these cells. For instance, AML-sEV can transfer miRNA to HSPC to suppress their clonogenicity[44,45] or protein synthesis.[46] Simultaneously, they facilitate the formation of a tumor-permissive BM niche by modulating the proliferation, differentiation,[47-49] and angiogenesis of MSC.[50,51] Our study also delved into the paracrine effect of AML-sEV and found that they inhibited the colony formation ability of HSPC, particularly their erythroid differentiation potential. miR-221-sEV also suppressed the proliferation of HSPC, with the highest level of miR-221 expression being detected in MEP among all the AML mouse BM hematopoietic cells subsets. A previous study has demonstrated that miR-221 impeded the growth of erythroid cells, suggesting that it inhibits erythroid differentiation.[34]

In conclusion, our study provided evidence of the autocrine function of AML-sEV. Moreover, we have demonstrated that AML-sEV can transmit miR-221 to leukemic cells to promote proliferation and leukemogenesis. This newly identified regulatory loop involving miR-221 and AML-sEV holds great promise as a potential therapeutic target to suppress AML progression.

Disclosures

No conflicts of interest to disclose.

Contributions

MYL, GHS, and JLZ designed the study and performed the experiments. YLL helped with the bioinformatics analyses. SSP, YFW, YPL and XNZ helped with the mouse experiments. YJW, SDY, TC, and HC conceptualized the study, designed the experiments, interpreted the results, wrote the paper, and oversaw the study.

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Data-sharing statement

All data are available upon request sent to the corresponding authors.

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