Life Sciences 221 (2019) 187–195

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Leukemia-derived exosomes induced IL-8 production in bone marrow stromal cells to protect the leukemia cells against chemotherapy

Tongtong Chen, Guozhen Zhang, Lingzhen Kong, Shujuan Xu, Yue Wang, Min Dong*

Department of Hematology, The Affiliated Hospital of Gulin Medical University, Guilin, China

ARTICLE INFO

Keywords:
BMSC
AML
Exosome
Etoposide
Apoptosis

ABSTRACT

Aims: The interplay between bone marrow stromal cells (BMSCs) and acute myeloid leukemia (AML) cells plays a critical role in AML drug resistance by secreting growth factors, cytokines, and extracellular vesicles. As kind of extracellular vesicles, exosomes consist of proteins and RNAs and regulate communication among cells.
Main methods: The BMSCs, HS5 cells, and AML cells were co-cultivated with transwell membranes, and treated with different doses of AML chemotherapy drug, etoposide.
Key findings: Findings of our research proved that co-cultivation of BMSCs with AML cells defended AML against cell death triggered via etoposide, without having an impact on cell growth. An increase in the expression of the 70 kDa heat shock proteins (HSP70) as well as lysosomal associated membrane protein 3 (CD63) was observed in the exosomes from BMSC and AML, co-cultivated in conditioned media. Exosome repression in BMSC and AML co-cultivating system rebuilt the sensitivity of the KG1A cells to apoptosis triggered via etoposide, indicating that exosome modulated drug resistance in AML. Our study proved that exosomes arising from KG1A cells could propel BMSCs to generate IL-8, which could regulate the effect of etoposide treatment. Furthermore, IL-8 inhibition by its antibody increased the sensitivity of AML cells to cell death triggered via etoposide.
Significance: Our results suggested that exosomes secreted by AML cells is an essential communicator for the interaction of BMSCs and AML, which can protect AML cells from chemotherapy drug induced apoptosis.

1. Introduction

Acute myeloid leukemia (AML) is a malignancy featuring differentiation blockage as well as clonal hematopoietic stem or progenitor cell (HSPC) proliferation that could bring about bone marrow (BM) failure quickly and terminal deaths without treatment [1]. Despite the appropriate treatments, such as aggressive chemotherapy and transplantation of HSC (allogeneic hematopoietic stem cells), only few patients recover from the disease [2]. Consequently, it is crucial to develop more effective treatment strategies to promote prognosis and to throw light upon pharmacologically target at the essential molecular mechanism. Chemotherapeutic agents, including mitoxatrone, cytarbine, and etoposide are commonly used for treating AML. Nevertheless, a high number of patients still confront recurrence and drug resistance [3]. It is widely accepted that the BM microenvironment, as well as the interaction between AML cells and bone marrow stromal cells (BMSCs) participate in resistance to drugs via intercellular adhesion and generation of soluble factors through BMSCs [4,5]. It has been recently proved that changing the crosstalk between AML cells and BMSCs could have an impact on leukemia cell viability as well as chemotherapy resistance [5,6]. Therefore, new researches are more focused on the effects of growth factors, cytokines, and adhesion agents generated via both malignant and non-malignant cells on the microenvironment, which offers an appropriate chance for malignant cell viability and growth. Exosomes have been recognized as innovative contributors to the malignant microenvironment [7].

As small vesicles (30–200 nm) generated from various normal and cancer cells [8], exosomes have been commonly regarded as crucial regulators of intercellular communication. Exosomes generated from tumors can promote tumor growth and disturb hemostasis of normal tissue [9,10]. Exosomes can activate target cells, transport membrane receptors, trigger epigenetic alterations in recipient cells and deliver proteins due to their capability to transport functional agents [11]. In addition to cell communication, they are related to immune reaction, cancer viability, cancer invasion, antigen presentation, cell migration, vessel generation, and differentiation [9,12,13]. It has been demonstrated that pathways, including Wnt, Notch, Fas, phosphatidylinositol 3-kinase/Akt, transforming growth factor (TGF)-β/Smad, and extracellular signal-regulated kinase 1/2 participate in physiological alteration triggered via exosome in recipient cells [13,14]. For instance,

* Corresponding author at: Department of Hematology, The Affiliated Hospital of Gulin Medical University, No. 15, Lequn Road, Guilin 541001, China.
E-mail address: mindongyx@163.com (M. Dong).

https://doi.org/10.1016/j.lfs.2019.02.003
Received 5 November 2018; Received in revised form 24 January 2019; Accepted 1 February 2019
Available online 02 February 2019
0024-3205/ © 2019 Elsevier Inc. All rights reserved.

exosomes generated via AML cells trigger BMSCs to generate IL-8, which promotes the development of leukemia [15]. Exosomes generated via AML cell lines as well as primary AML blasts can get into stromal cells and regulate their activity to promote the development of leukemia [7].

Despite numerous researches on the reinforcement of AML development and viability triggered via BMSC generated exosome or cytokines [2,5], the understanding of the direct impact of BMSCs on AML cells is insufficient. In order to understand the interaction between AML cells and BMSCs in BM microenvironment, we chose 3 AML cell lines and co-cultivated them with BMSCs without direct physical contact. Findings of our research suggested that exosomes generated from AML cells upregulated IL-8 in BMSCs, which regulated the chemotherapy resistance of AML cells in feedback. This research is the first to throw light upon an innovative mechanism in BMSC-triggered resistance to etoposide in AML via generation of exosomes as well as IL-8.

2. Material and methods

2.1. Cell lines, reagents and specimens

Acute myeloid leukemia cell lines (KG1A, NB4, and MV411; provided by ATCC) were cultured in Iscove's Modified Dulbecco's Medium (IMDM; Gibco, USA) containing 10% FBS (fetal bovine serum; Atlanta Biologicals, USA). The BMSC line, HS5, was acquired from ATCC (Manassas, USA) and cultivated in DMEM containing high glucose (Euroclone, UK) and 10% FBS. The normal human peripheral blood mononuclear cells (PBMC) were purchased from Lonza (Basel, Switzerland), and cultured in RPMI-1640 medium containing 10% FBS. All of the cell culture media were supplemented with 100 U/mL penicillin, 2 mM L-glutamine, and 100 μg/ml streptomycin (Euroclone, UK).

Recombinant IL 8 (R&D Systems, MN, USA) was processed in 10 ng/μl stock solution with the help of sterile phosphate-buffered saline (PBS), deliquated and preserved at −80 °C. The remaining materials, including IL-8 antibody, etoposide, GW4869, were bought from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise mentioned.

Specimens from 24 normal and 28 etoposide resistant AML patients (peripheral blood or BM) were acquired from the affiliated hospital of Guilin Medical University. The study was approved by the Ethics Committee of the affiliated hospital of Gulin Medical University. Fully informed consent was acquired from all the participants. Patient BMSCs were expanded as described [16]. Briefly, cells from bone marrow were seeded in complete media (α-minimal essential medium (α-MEM), 2 mM glutamine, 10 μg/ml gentamicin and 20% fetal bovine serum) for 24 h, and the non-adherent cells were removed. The adherent cells were expanded for future uses.

2.2. Co-culture assay of exosomes derived from AML cells and stromal cells

5×10^4 cells/well BMSCs (HS5) were seeded in the 6-well plates in DMEM plus 10% FBS on day −1. At day 0, 1×10^6 KG1A cells were seeded into the Transwell system. Mono-cultures of HS5, KG1A cells were seeded at the same above mentioned conditions as controls. Cells were harvested after 24 h, 48 h and 72 h, and were stored at −80 °C until use. Supernatants collected after 72 h were stored immediately at −80 °C.

2.3. Exosome separation, characterization and labeling

For the separation of exosomes from AML, BMSC, and healthy PBMC, cells were directly cultivated with the help of centrifuged media, which was supplemented with human TPO (2 ng/μl) as well as KITL (Peprotech). The supernatant was harvested after 48 h and centrifuged at 800 ×*g* for 10 min at 4 °C to remove whole cells. The supernatant was again centrifuged at 10,000 ×*g* for 10 min at 4 °C to eliminate the cellular debris. A 40 μm membrane was used to filter the admixture. Exosomes were subsequently prepared by centrifuging at 100,000 ×*g* for 1 h. The exosome pellets were washed twice with PBS.

2.4. Fluorescent labeling and confocal microscopy

KG1A or HS5 cells were incubated with the exosomes, generated from co-cultivating media, at 37 °C for 20 min. The media were used to wash the cells 3 times. The 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine (DID [red]) cell-labeling solution was used to label the exosome suspension, which was then re-pelleted by using the ExoQuick-TC exosome precipitation solution 3 times. The nuclei were stained with 4,6 diamidino-2-phenylindole. A confocal laser scanning microscope LSM 710 was used to observe and record exosome uptake with the help of ZEN software (Carl Zeiss).

2.5. Cell death analysis

The apoptosis of AML cells was analyzed by Hoechst 33258 staining (3.7% formaldehyde, 0.5% Nonidet P-40, and 10 μg/ml Hoechst 33258 (Invitrogen)), followed microscopic visualization of condensed chromatin and micronucleation as described [17]. The cell viability was determined by MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Promega) as described by manufacturer.

2.6. ELISA assay

HS5-conditioned medium (CM) was collected from cells stimulated with or without AML co-culture or AML exosomes (20–50 μg/ml). CM aliquots were centrifuged to remove cellular debris and used to quantify IL8 with ELISA kits according to the manufacturer's protocol (R&D Systems, Abingdon, UK).

2.7. Western blot

Cells were lysed in RIPA Lysis buffer (Santa Cruz Biotech) and mixed with 4X Blue loading buffer (Biorad, Shanghai, China). Protein lysates (25–100 μg) were loaded on SDS-PAGE and transferred onto nitrocellulose membranes using a standard western blot protocol. Membranes were probed with primary antibodies against Hsp70, CD63, Calreticulin (CRT) (abcam, Cambridge, UK), Caspase-3, Bax, actin, p-STAT1, STAT1 (cell signaling, Danver, MA, USA), Snail, p-p65, p65 (Santa Cruz (Dallas, TX, USA). Signal was detected and quantified by the Chemidoc gel imaging system (Biorad).

2.8. Quantitative real-time PCR

The RNeasy RNA isolation kit (Qiagen, USA) was utilized for RNA isolation from cells, which was then reverse transcribed into cDNA with the help of Sensiscript RT kit (Qiagen). The amplification of the specific targets was carried out using TaqMan assays with the help of ViiA 7 Real-Time PCR System (Applied Biosystems) using 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Primer sequences were used as follows: GAPDH (5′ATGGGGAAGGTGAAGGTCG3′, 5′GGGTCATTGATGGCAACAATAT3′) and IL8 (5′GAATGGGTTTGCTAGAATGTGATA3′, 5′CAGACTAGGGTTGCCAGATTTAAC3′).

2.9. RNA interference and plasmid transfection

Small-interfering RNAs (siRNA) targeting Snail, STAT1, p65 or scramble siRNA were purchased from Dharmacon (ON-TARGET plus SMART pool, Human, Dharmacon Inc., Lafayette, CO, USA). The eGFP-STAT1 and eGFP control plasmids were purchased from Addgene (Watertown, MA, USA). The siRNA and plasmid transfection were processed by Lipofectamine 2000 Reagent (Invitrogen, Shanghai, China) according to manufacturer's instruction.

2.10. Statistical analysis

Statistical analysis was carried out with Prism software v.7 (GraphPad, La Jolla, CA, USA). The results were represented as mean ± SD. Two-tailed Student's *t*-test was applied for the comparison between 2 groups. A $p < 0.05$ was regarded as statistically significant. Statistical test was not applied to examine sample size.

3. Results

3.1. BMSCs induce etoposide resistance in AML cells

The HS5 cells were co-cultivated with KG1A cells in transwells with no physical contact, aiming to explore the interaction between BMSCs and leukemia cells. We found that co-culture of BMSCs and AML cells did not affect the growth of AML cells and BMSCs (Fig. 1A). We next tested whether co-culture of BMSCs and AMLs has any effects on the chemotherapy drug response. We found that the presence of BMSCs suppressed cell death in AML cells in response to etoposide treatment

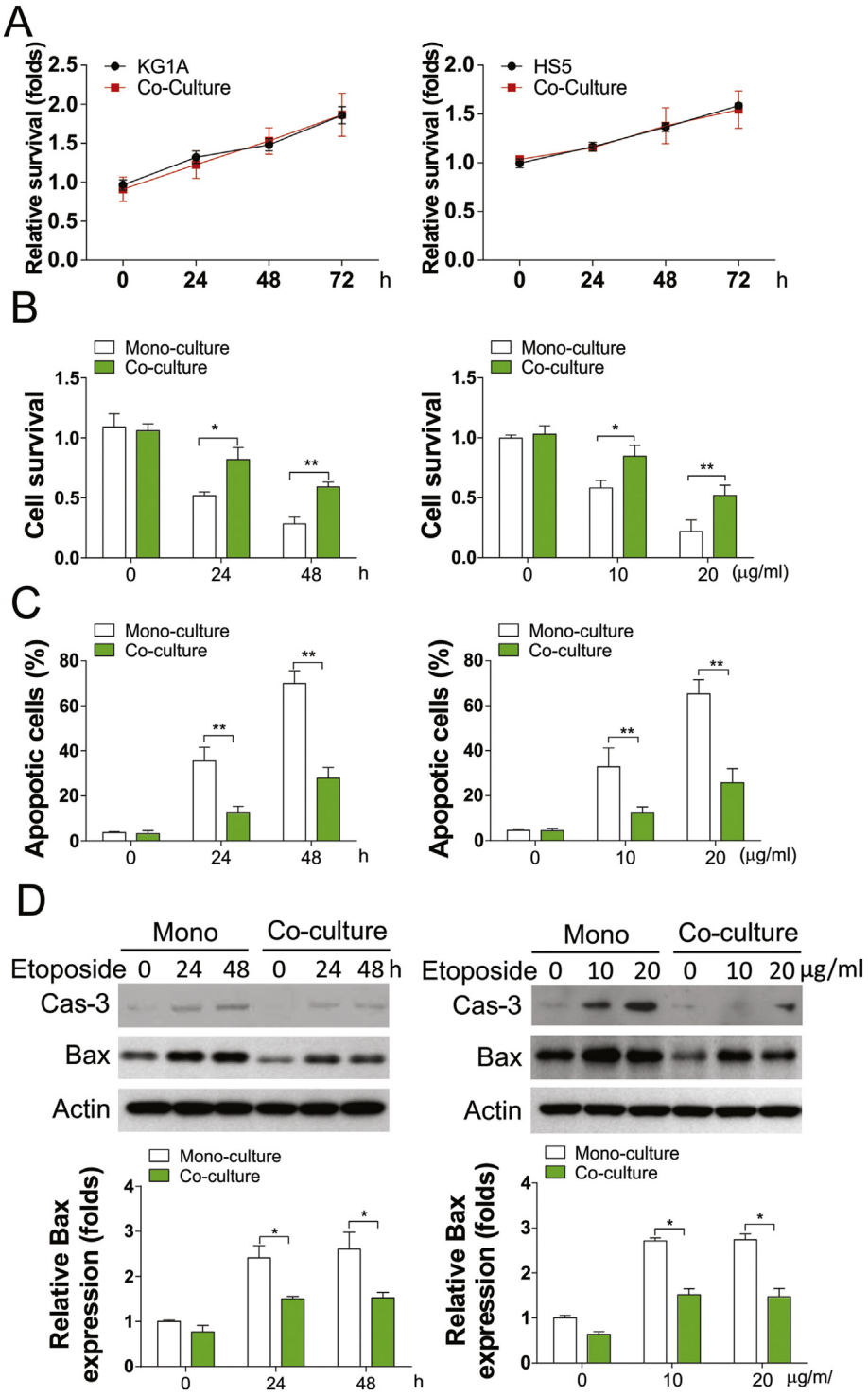

Fig. 1. Co-culture of BMSCs and AML cells resistant to etoposide induced apoptosis. (A) The growth of HS5 BMSCs and KG1A AML cells in mono-culture or co-culture was analyzed by MTT assay. (B) KG1A cells were culture alone or co-cultured with HS5 cells for 24 h, subsequently treated with 20 μg/ml etoposide for indicated time (left), or indicated dosages (right) of etoposide for 48 h. The survival of KG1A was analyzed by MTT assay. (C) KG1A cells were treated as in (B), the apoptosis was analyzed by nuclear staining with Hoechst-33342. (D) KG1A cells were treated as in (B), the expression of caspase-3 and Bax in KG1A cells were analyzed by western blot. Upper, the representative pictures were shown; lower, the relative expression level of Bax was calculated by Imaje J and plotted. Each experiment was triplicated. *, $p < 0.05$; **, $p < 0.01$.

with different doses and time points (Fig. 1B), suggesting AML cells co-cultured with BMSCs rendered chemotherapy resistance. Since apoptosis is induced by etoposide, we investigated the apoptotic cell death in AML with or without BMSC co-culture. As predicted, BMSC co-culture significantly suppressed the apoptosis, induced by etoposide, in the AML cells (Fig. 1C). Furthermore, BMSC co-culture also suppressed the caspase-3 cleavage in AML cells in response to etoposide treatment (Fig. 1D). As Bax is an important pro-apoptotic gene in response to chemotherapy drug treatment [18], we also investigated its expression level. Accordingly, BMSC co-culture reduced the endogenous Bax level in KG1A AML cells, and thereafter suppressed its induction by etoposide (Fig. 1D). Collectively To further confirm the chemotherapy suppression effect of BMSC co-culture, we also investigated the effects of HS5 co-culture with other AML cells and normal PBMCs. We found that HS5 co-cultured with AML cells, MV411 or NB4, significantly suppressed the cell death induced by etoposide (Fig. S1A, B). However, HS5 co-culture with normal PBMCs did not affect its response to etoposide treatment (Fig. S1C). Collectively, our results indicated that BMSCs selectively contribute to drugs resistance in AML cells.

3.2. Secretion of exosome mediates the etoposide resistance in AML cells

Exosomes have been widely accepted as crucial regulators of intercellular communication and their abnormal reinforcement has been identified in several kinds of malignancies [19,20]. Our study investigated whether exosomes were generated from BMSCs as well as AML cells. Some markers of exosomes, such as Hsp90 as well as CD63, were identified in exosomes generated via HS5 as well as KG1A cells (Fig. 2A). Calreticulin, which served as a contaminant inside the cells, was not detected (Fig. 2A). The HS5 and KG1A co-culture enhanced the secretion of exosomes (Fig. 2A). In contrast, HS5 and PBMCs co-culture did not showed any obvious induction of exosome secretion (Fig. 2A), suggesting that exosome secretion might contribute to etoposide resistance in AML. Furthermore, the real-time PCR for exosome marker, EGFR, also confirmed that the secretion of exosomes in HS5 and KG1A co-culture was much higher than that in single cell culture, which was not observed in HS5 and PBMCs co-culture system (Fig. 2B). Our research subsequently explored whether exosomes generated from AML cells as well as BMSCs were absorbed via each other. Consequently, exosomes labeled with DID were added to co-cultivation with HS5 and KG1A cells. Exosomes absorbed by BMSCs or AML cells were discovered (Fig. 2C). Furthermore, inhibition of exosome secretion by GW4869 re-sensitized the AML cells to etoposide-induced apoptosis (Fig. 2D, E). Therefore, our results suggested that secretion of exosome contributes to the resistance of AML cells to etoposide treatment.

3.3. BMSCs promote AML drug resistance by secretion of exosome and IL-8

It has been widely accepted that BMSCs participate in resistance to drugs in AML cells via intercellular contact or cytokine generation. Our research explored whether exosomes generated via BMSC participated in resistance. BMSC-derived exosomes slightly but not significantly increased AML cells viability or suppressed the apoptosis in the presence of etoposide (Fig. 3A), suggesting that there is other mechanism for BMSCs promoting AML drug resistance. Since IL-8 secretion is another factor [21], which also contributes to AML drug resistance, we therefore tested the IL-8 secretion in BMSCs. Our results showed that co-culture of AML and BMSC significantly enhanced the secretion and mRNA expression of IL-8 in BMSCs (Fig. 3B, C). Inhibition of IL-8 by its antibody re-sensitizes the AML cells to etoposide treatment when co-cultured with BMSCs (Fig. 3D, E). However, anti-IL-8 did not have any effect on AML single culture in response to different etoposide treatment (Fig. 3D, E), suggesting the recovered sensitivity to treatment is dependent on the presence of BMSCs. Furthermore, supplementation of IL-8 in AML cells significantly suppressed the cell death induced by etoposide (Fig. 3F, G), which is similar to the effects of BMSCs co-culture. Our results suggested that secretion of IL-8 and exosome by BMSCs contributes to the AML drug resistance in response to etoposide treatment.

3.4. BMSCs IL-8 secretion was stimulated by AML exosome

In the next step, we tried to figure out the mechanism of IL-8 secretion in BMSCs. Since IL-8 secretion in BMSCs was dramatically enhanced when cultured with AML cells, it was inferred that AML cells were able to remodel BMSC microenvironment via exosome generation. Inhibition of exosome by GW4869 significantly suppressed the IL-8 secretion and mRNA expression in BMSC, when co-cultured with AML cells (Fig. 4A, B). However, treatment of GW4869 did not affect IL-8 secretion in BMSCs in the absence of co-culture with AML cells (Fig. 4A, B), suggesting that IL-8 secretion by BMSCs was modulated by AML exosome. Exosomes were produced from supernatants of AML cell cultures via filtration as well as centrifugation aiming to verify this hypothesis. As predicted, treatment with AML exosome significantly induced IL-8 secretion and transcription in HS5 cells (Fig. 4C, D). These results collectively suggested that IL-8 secretion by BMSCs was induced by the exosomes secreted by AML cells.

3.5. AML exosome modulated BMSCs IL-8 generation by Snail

To understand the mechanism of IL-8 secretion by BMSCs, we investigated the regulation of several IL-8 upstream regulators, including Snail, NF-κB, and STAT1. It was revealed that Snail was noticeably upregulated in BMSCs treated with AML exosome (Fig. 5A). In contrast, there is no significantly change in phosphorylation or expression of NF-κB, and mild suppression of STAT1 phosphorylation (Fig. 5A). These results suggested that Snail might be the upstream regulator of IL-8 secretion in BMSCs in response to AML exosome. To further confirm the hypothesis, we used different siRNA targeting Snail, NF-κB, and STAT1, and found that only depletion of Snail suppressed the IL-8 secretion and mRNA expression in BMSCs in response to AML exosome stimulation (Fig. 5B, C). Since STAT1 phosphorylation was reduced by AML exosome, we tested whether STAT1 overexpression has any effect on IL-8 secretion. We found that enhanced STAT1 expression actually promoted the IL-8 secretion and mRNA expression (Fig. S2A–B). However, supplement of AML exosome did not further enrich the induction of IL-8 (Fig. S2A–B), which further rule out the function of STAT1 in IL-8 transcription upon exosome exposure. In contrast, depletion of Snail compromised the IL-8 secretion and mRNA expression in BMSCs during co-cultivation with AML cells (Fig. 5D). Depletion of Snail in BMSCs also abolished the drug resistance effect in AML cells caused by BMSC co-culture (Fig. 5E). The findings above verified the essential impact of Snail on the regulation of IL-8 expression.

3.6. Exosome and IL-8 expression is correlative to the drug resistance in AML patients

Plasma samples from 24 normal and 28 etoposide resistant AML patients were examined in order to investigate whether exosomes in blood and IL-8 generation were promoted in patients suffering from AML. With the help of nanoparticle tracking assay, it was discovered that exosomes in blood were remarkably reinforced in AML patients resistant to etoposide (Fig. 6A) [13]. Consistently, the IL-8 secretion in the AML patients was also significantly higher than that in the normal AML patients (Fig. 6B). To further confirm the contribution of BMSCs to the AML drug resistance, we co-cultured BMSC from AML normal or etoposide resistant patients with three different AML cell lines, KG1A, MV411 and NB4. Our results also indicated that AML cells co-cultured with BMSCs from etoposide resistant patients were more resistant to etoposide-induced cell death (Fig. 6C). Therefore, our results further suggested that BMSCs contribute to the AML chemotherapy drug resistance.

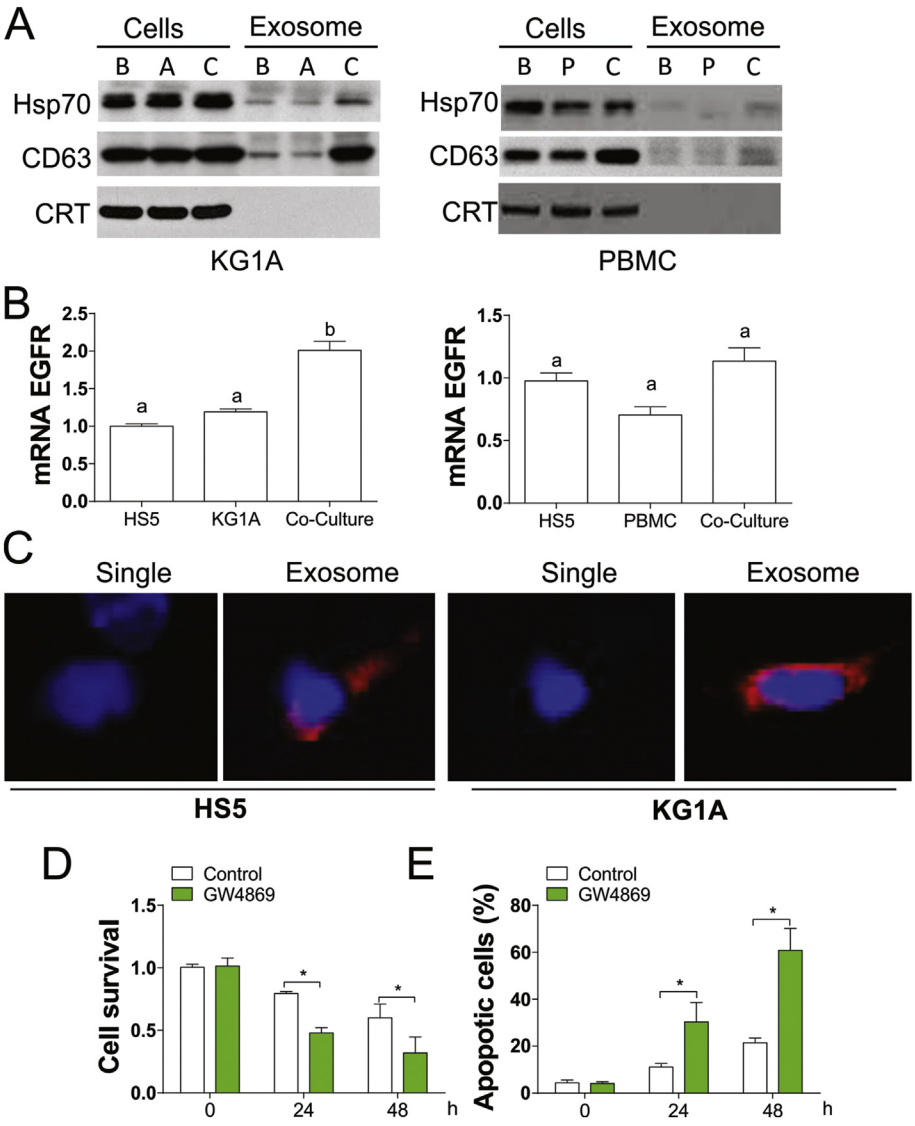

Fig. 2. Secretion of exosome mediated the etoposide resistance in AML cells. (A) Exosomal positive markers, Hsp90 and CD63 were detected in medium from HS5, KG1A mono-culture and co-culture (left) or HS5, PBMC mono-culture and co-culture (right). Expression of calreticulin was used as negative marker. B: BMSC cells, HS5; A, AML cells, KG1A; P, PBMC; C: co-culture. (B) The expression of exosome marker, EGFR, in mRNA level in HS5, KG1A mono-culture and co-culture (left) or HS5, PBMC mono-culture and co-culture (right). (C) DID-labeled esoxome (red) were incubated with HS5 and KG1A for 2 h, and the exosome uptake by BMSCs and AML cells was detected. Representative pictures were shown. (D) KG1A co-cultured with HS5 with or without pretreatment of 10 μM GW4869 were treated with 20 μg/ml etoposide for indicated time. The cell survival was analyzed by MTT assay. (E) KG1A cells were treated as in (D), the apoptosis was analyzed by Hoechst-33342 staining. Each experiment was triplicated. *, $p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Previous research has proved that malignant microenvironment could change the sensitivity of malignant cells to cytotoxic agents as well as radiation [22,23]. It has been found that BMSCs could prohibit drug induced cell death in AML, through the generation of soluble factors [24]. Conditioned medium from BMSCs, which received cultivation independently, did not affect myeloma cells. Soluble factors generated via BMSCs and linked with myeloma could trigger cell death counteraction abilities, indicating a dynamic reaction between myeloma and BMSCs [5]. However, the communication between BMSCs and AML cells is incomplete, and our research aimed at exploring the influence of AML cells on leukemia microenvironment, which might feedback to influence the chemotherapy response on AML cells upon drug treatment. It has been proved in this research that the presence of BMSCs noticeably prohibits death of AML cells triggered by etoposide. In contrast to the effects of physical contact, we found that the exosomes from AMLs promote IL-8 secretion in HS-5 BMSCs, which contributes to the etoposide resistance in AML cells. Mechanically, our results suggested that AML exosomes modulated IL-8 secretion in BMSCs via Snail. Furthermore, inhibition of exosome, IL-8, or depletion of Snail in BMSCs can sensitize the AML cells to etoposide-induced apoptosis, which provides a novel rationale to overcome the chemo-resistance in AML therapy.

The protective effect between AML cells and BMSCs is not restrained to adhesion. Emerging evidence has suggested that exosomes modulate intercellular communication and have verified the essential activities of exosomes in hematological tumors [25]. Our research demonstrated that exosomes defend AML cells against cell death, triggered via chemotherapy, with the help of cell cultivation. Various studies suggest that BMSCs promote leukemia cell viability and prohibit the death of these cells via chemotherapy [24,26]. Increasing evidence has indicated that not only exosomes generated via BMSCs but also leukemia cells themselves assist leukemia cells in resistance to chemotherapy [27]. Exosomes were generated via not only healthy BMSCs but also AML-BMSCs attenuated cytotoxic influence of nucleoside analogue cytarabine on MOLM-14 cells in FLT3-ITD (internal tandem duplication) mutation. Only exosomes generated from AML-BMSCs defended the cells against AC220 (FLT3 kinase suppressor) therapy [28]. In this study, we also found that the exosomes secreted by BMSCs slightly but not significantly contributed to the chemo-resistance in AML cells (Fig. 3A). Except exosomes, the secretion of IL-8 by BMSCs also mediated the etoposide resistance in AML cells when co-cultured with BMSCs.

Previous research revealed that BMSCs in the BM microenvironment had an effect on AML cells via cytokine generation that promotes malignant cell growth, instead of intercellular contact; however, the understanding of the role of exosomes in the changes is insufficient. This

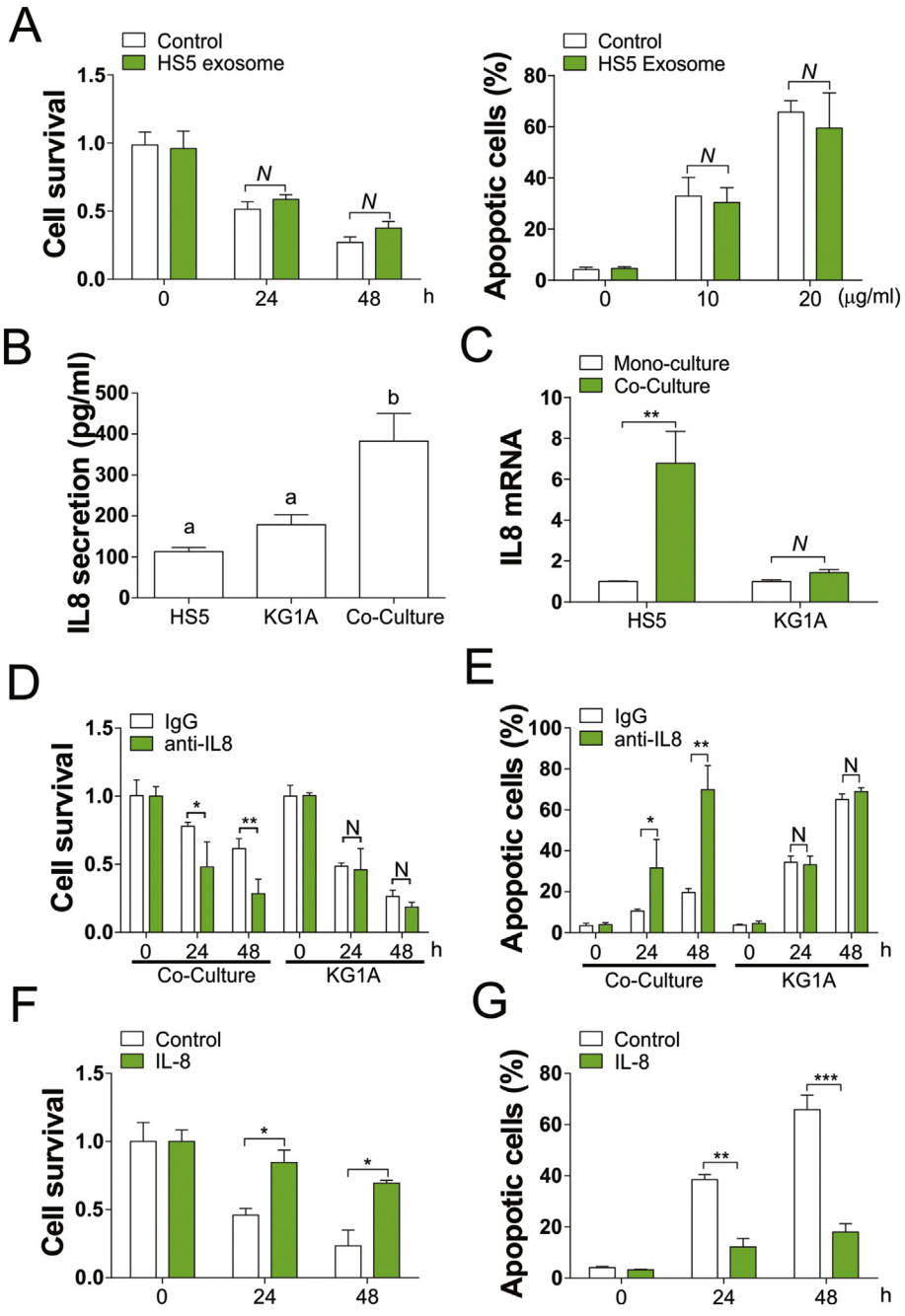

Fig. 3. Drug resistance of AML is controlled by both BMSCs secreted exosome and IL-8. (A) KG1A cells were incubated with 20 μg/ml HS5 drived exosome, and treated with 20 μg/ml etoposide as indicated time. The cell survival (left) and apopotisis (right) were analyzed. (B) The IL-8 secretion in HS5 and KG1A cells under mono-culture or co-culture condition. (C) The IL-8 mRNA expression in HS5 and KG1A cells under mono-culture or co-culture condition. (D) KG1A cells co-cultured with HS5 or single culture were treated with 20 μg/ml etoposide with or without pretreatment of anti-IL8 (10 ng/ml) for indicated time. The cell survival was analyzed by MTT assay. (E) KG1A cells were treated as in (D), the apoptosis was analyzed by Hoechst-33342 staining. (F) KG1A cells were treated with 20 μg/ml etoposide with or without pretreatment of IL8 (10 ng/ml) for indicated time. The cell survival was analyzed by MTT assay. (G) KG1A cells 0bwere treated as in (F), the apoptosis was analyzed by Hoechst-33342 staining. Each experiment was triplicated. N, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

research offers an explicit and innovative proof of participation of not only exosomes generated from BMSCs but also from AML in resistance to chemotherapy, which is triggered via BMSC and throws light upon the interactions between AML cells and exosomes of BM microenvironment. The BMSCs generate growth factors to stimulate the proliferation and viability of multiple myeloma (MM) cells by triggering various growth and cell death counteracting pathways [29]. According to a previous report, chronic myelogenous leukemia (CML) exosomes reinforce adhesion between leukemia cells and HS5 stromal cells via IL-8 enhancement [15]. Our research has proved that AML exosomes reinforce IL-8 generation of BMSCs, which enhances resistance to etoposide in reaction to AML cells. Our research also proved that blocking of the IL-8 pathway with neutralizing antibody helps to rebuild the sensitivity of malignant cells to etoposide. This finding was extended to the capability of IL-8 inhibition to recover drug resistance, which is dominant in cells resistant to etoposide. Some studies have suggested the influence of IL-8 signaling on malignant resistance to chemotherapy. For instance, silencing of the CXCR2 receptor in murine breast cancer cells promotes malignancy, counteracting the ability of paclitaxel in vivo [30]. Inhibition of the CXCR2-regulated pathway in the human colon malignant lines suppressed cell migration as well as invasion and reinforced malignant reaction to oxaliplatin [31]. Our research indicated that BMSCs reinforced the drug resistance in AML through IL-8 generation.

As to the mechanism of IL-8 secretion in BMSCs in response to AML exosomes exposure, we found that Snail expression in BMSCs is responsible for the IL-8 secretion. In our study, we investigated several possible transcription factor of IL-8, including STAT1 [32], p65, and Snail [33]. However, we found that only Snail is necessary for IL-8 secretion by AML exosome, suggesting the mechanism of IL-8 secretion might be stimuli dependent. It is well known that IL8 is an important target of Snail, which plays a critical role in colorectal cancer stemness

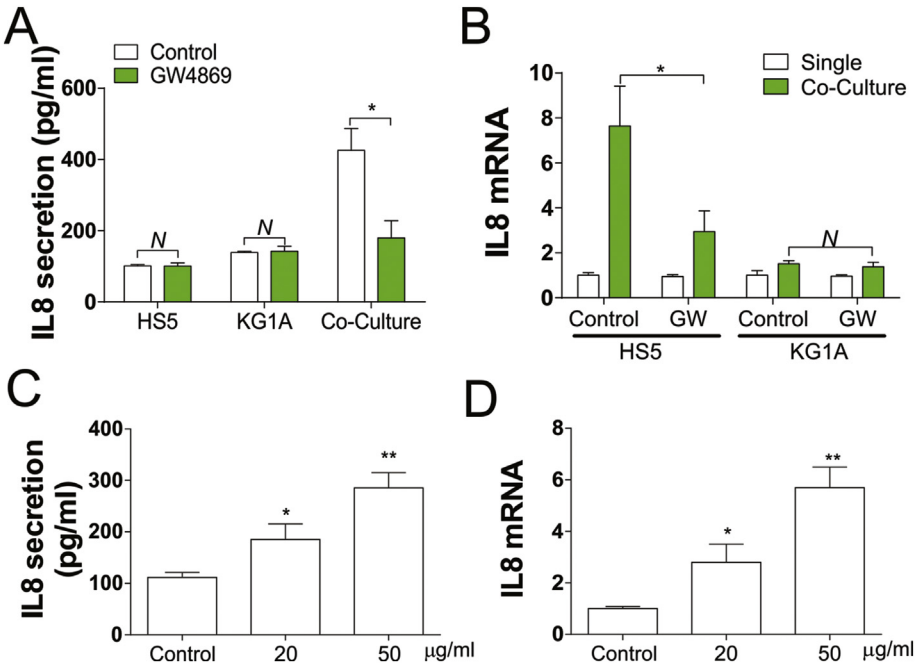

Fig. 4. Exosomes from AML cells stimulates the secretion of IL-8 in BMSCs. (A) KG1A co-cultured with HS5 were treated with 10 μM GW4869. The secretion of IL-8 was analyzed by ELISA. (B) The IL-8 mRNA expression in KG1A and HS5 in mono-culture or co-culture system with or without pretreatment of GS4869. (C) The secretion of IL-8 in HS5 treated with exosome from KG1A cells. (D) The IL-8 mRNA level in HS5 treated with exosome from KG1A cells. Each experiment was triplicated. N, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$.

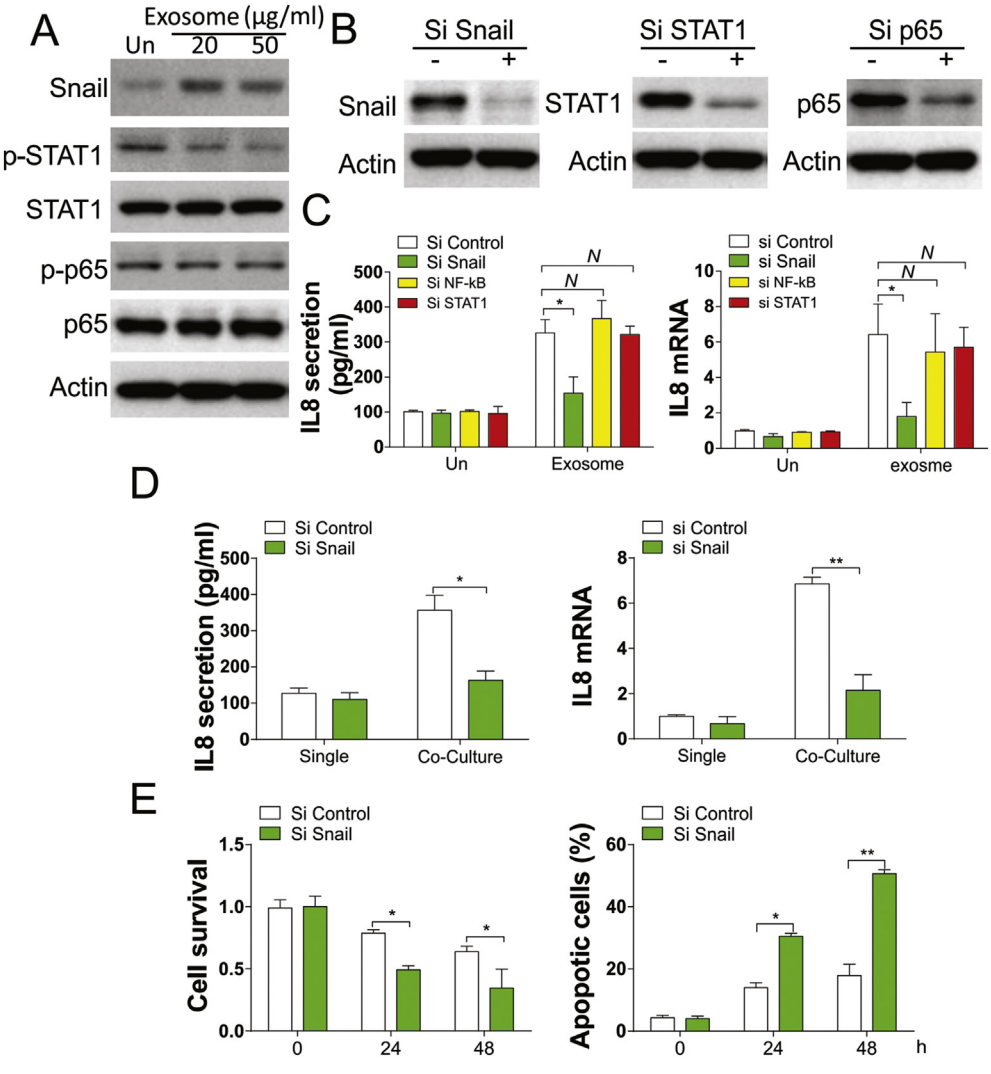

Fig. 5. IL-8 secretion in BMSCs is modulated by Snail. (A) The expression of indicated proteins in HS5 cells treated with exosome from KG1A cells. (B) The expression of indicated proteins in HS5 cells transfected with indicated siRNAs. (C) The IL-8 secretion (left) and mRNA expression (right) in HS5 cells transfected with indicated siRNA and treated with exosomes from KG1A cells. (D) HS5 cells transfected with control or Snail siRNA and co-culture with KG1A. The secretion (left) and mRNA expression (right) of IL-8 was analyzed. (E) HS5 cells transfected with control or Snail siRNA and co-culture with KG1A. The survival (left) and apoptosis (right) of KG1A cells treated with 20 μg/ml etoposide was analyzed. Each experiment was triplicated. N, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$.

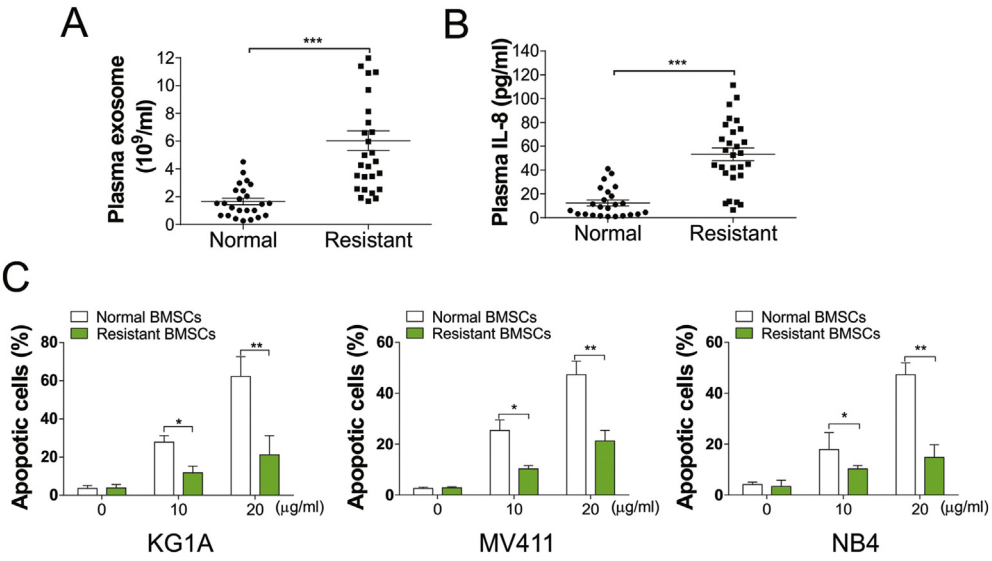

Fig. 6. Etoposide resistant AML patient showed higher exosome and IL-8 secretion. (A) The exosome level in the plasma from regular (n = 24) and etoposide resistant (n = 28) AML patients. (B) The IL-8 secretion in the plasma from regular (n = 24) and etoposide resistant (n = 28) AML patients. (C) KG1A, MV411, and NB4 cells were co-cultured with BMSCs drived from regular and etoposide resistant AML patients. The apoptosis of these 3 cells in response to indicated etoposide treatment was analyzed. Each experiment was triplicated. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

and malignancy [34], and erlotinib resistance in lung cancer [35]. Therefore, IL8 may function as a significant regulatory factor within the tumor microenvironment. Recently, it was reported that chronic myeloid leukemia (CML) exosomes activated IL-8 secretion in stromal cells by the expression of Snail, and eventually promotes the growth and invasiveness of leukaemic cells [36]. Consistently, we also found that the IL-8 secretion in stromal cells is also modulated by Snail, and contribute to the AML chemotherapy resistance. However, the expression of Snail is not linear response to dosage increase of exosome (Fig. 5A), suggesting there should other underlying mechanism for the IL-8 secretion in extreme condition of exosome exposure.

Collectively, findings of our research showed the essential impact of BMSCs on the death of AML cells. Two possible mechanisms have been proposed in the interaction between AML cells and BMSCs. Firstly, direct intercellular contact brings about partial inhibition of AML cell proliferation. This could offer first-line defense against the cell death triggered by drugs. Secondly, the interactions between BMSCs and AML cells generate soluble factors, which prohibit the death of myeloma cells, especially in mitochondria. Our study has proved the complicated effect of BMSCs and AML cells on each other, which participates in resistance to drugs of AML cells de novo. Our research offers a model for better exploration of independent mechanisms. Moreover, the future investigation into the effects of different contents in exosome might be interesting and crucial to identify innovative strategies to deal with initial resistance to drugs based on current data.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.02.003.

Acknowledgements

None.

Conflict of interests

None.

Funding

This work was supported by The National Natural Science Fund (regional fund); (grant number 81560032) and Beijing Medical and Health Foundation (grant number YWJKJJHKYJJ-B17485).

References

[1] C.T. Jordan, Unique molecular and cellular features of acute myelogenous leukemia stem cells, Leukemia 16 (2002) 559–562.
[2] B. Zhang, Y.W. Ho, Q. Huang, T. Maeda, A. Lin, S.U. Lee, et al., Altered microenvironmental regulation of leukemic and normal stem cells in chronic myelogenous leukemia, Cancer Cell 21 (2012) 577–592.
[3] M. Lemaire, S. Deleu, E. De Bruyne, E. Van Valckenborgh, E. Menu, K. Vanderkerken, The microenvironment and molecular biology of the multiple myeloma tumor, Adv. Cancer Res. 110 (2011) 19–42.
[4] Y. Nefedova, P. Cheng, M. Alsina, W.S. Dalton, D.I. Gabrilovich, Involvement of Notch-1 signaling in bone marrow stroma-mediated de novo drug resistance of myeloma and other malignant lymphoid cell lines, Blood 103 (2004) 3503–3510.
[5] Y. Nefedova, T.H. Landowski, W.S. Dalton, Bone marrow stromal-derived soluble factors and direct cell contact contribute to de novo drug resistance of myeloma cells by distinct mechanisms, Leukemia 17 (2003) 1175–1182.
[6] S.M. Garrido, F.R. Appelbaum, C.L. Willman, D.E. Banker, Acute myeloid leukemia cells are protected from spontaneous and drug-induced apoptosis by direct contact with a human bone marrow stromal cell line (HS-5), Exp. Hematol. 29 (2001) 448–457.
[7] J. Huan, N.I. Hornick, M.J. Shurtleff, A.M. Skinner, N.A. Goloviznina, C.T. Roberts Jr.et al., RNA trafficking by acute myelogenous leukemia exosomes, Cancer Res. 73 (2013) 918–929.
[8] H. Valadi, K. Ekstrom, A. Bossios, M. Sjostrand, J.J. Lee, J.O. Lotvall, Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells, Nat. Cell Biol. 9 (2007) 654–659.
[9] S.A. Melo, H. Sugimoto, J.T. O'Connell, N. Kato, A. Villanueva, A. Vidal, et al., Cancer exosomes perform cell-independent microRNA biogenesis and promote tumorigenesis, Cancer Cell 26 (2014) 707–721.
[10] J. Ratajczak, K. Miekus, M. Kucia, J. Zhang, R. Reca, P. Dvorak, et al., Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery, Leukemia 20 (2006) 847–856.
[11] G. Camussi, M.C. Deregibus, S. Bruno, C. Grange, V. Fonsato, C. Tetta, Exosome/microvesicle-mediated epigenetic reprogramming of cells, Am. J. Cancer Res. 1 (2011) 98–110.
[12] A. Bobrie, M. Colombo, G. Raposo, C. Thery, Exosome secretion: molecular mechanisms and roles in immune responses, Traffic 12 (2011) 1659–1668.
[13] H. Sheldon, E. Heikamp, H. Turley, R. Dragovic, P. Thomas, C.E. Oon, et al., New mechanism for Notch signaling to endothelium at a distance by Delta-like 4 incorporation into exosomes, Blood 116 (2010) 2385–2394.
[14] J. Gu, H. Qian, L. Shen, X. Zhang, W. Zhu, L. Huang, et al., Gastric cancer exosomes trigger differentiation of umbilical cord derived mesenchymal stem cells to carcinoma-associated fibroblasts through TGF-beta/Smad pathway, PLoS One 7 (2012) e52465.
[15] C. Corrado, S. Raimondo, L. Saieva, A.M. Flugy, G. De Leo, R. Alessandro, Exosome-mediated crosstalk between chronic myelogenous leukemia cells and human bone marrow stromal cells triggers an interleukin 8-dependent survival of leukemia cells, Cancer Lett. 348 (2014) 71–76.
[16] M. Sabatino, J. Ren, V. David-Ocampo, L. England, M. McGann, M. Tran, et al., The establishment of a bank of stored clinical bone marrow stromal cell products, J. Transl. Med. 10 (2012) 23.
[17] D. Chen, L. Ming, F. Zou, Y. Peng, B. Van Houten, J. Yu, et al., TAp73 promotes cell survival upon genotoxic stress by inhibiting p53 activity, Oncotarget 5 (2014) 8107–8122.
[18] F. Reichenbach, C. Wiedenmann, E. Schalk, D. Becker, K. Funk, P. Scholz-Kreisel, et al., Mitochondrial BAX determines the predisposition to apoptosis in human AML, Clin. Cancer Res. 23 (2017) 4805–4816.

[19] J. Kowal, M. Tkach, C. Thery, Biogenesis and secretion of exosomes, Curr. Opin. Cell Biol. 29 (2014) 116–125.
[20] D.D. Taylor, C. Gercel-Taylor, Exosomes/microvesicles: mediators of cancer-associated immunosuppressive microenvironments, Semin. Immunopathol. 33 (2011) 441–454.
[21] A. Kuett, C. Rieger, D. Perathoner, T. Herold, M. Wagner, S. Sironi, et al., IL-8 as mediator in the microenvironment-leukaemia network in acute myeloid leukaemia, Sci. Rep. 5 (2015) 18411.
[22] J.S. Damiano, A.E. Cress, L.A. Hazlehurst, A.A. Shtil, W.S. Dalton, Cell adhesion mediated drug resistance (CAM-DR): role of integrins and resistance to apoptosis in human myeloma cell lines, Blood 93 (1999) 1658–1667.
[23] L.A. Hazlehurst, N. Valkov, L. Wisner, J.A. Storey, D. Boulware, D.M. Sullivan, et al., Reduction in drug-induced DNA double-strand breaks associated with beta1 integrin-mediated adhesion correlates with drug resistance in U937 cells, Blood 98 (2001) 1897–1903.
[24] M. Konopleva, S. Konoplev, W. Hu, A.Y. Zaritskey, B.V. Afanasiev, M. Andreeff, Stromal cells prevent apoptosis of AML cells by up-regulation of anti-apoptotic proteins, Leukemia 16 (2002) 1713–1724.
[25] J. Zhou, S. Wang, K. Sun, W.J. Chng, The emerging roles of exosomes in leukemogeneis, Oncotarget 7 (2016) 50698–50707.
[26] A. Manabe, E. Coustan-Smith, F.G. Behm, S.C. Raimondi, D. Campana, Bone marrow-derived stromal cells prevent apoptotic cell death in B-lineage acute lymphoblastic leukemia, Blood 79 (1992) 2370–2377.
[27] J. Wang, A. Hendrix, S. Hernot, M. Lemaire, E. De Bruyne, E. Van Valckenborgh, et al., Bone marrow stromal cell-derived exosomes as communicators in drug resistance in multiple myeloma cells, Blood 124 (2014) 555–566.
[28] S. Viola, E. Traer, J. Huan, N.I. Hornick, J.W. Tyner, A. Agarwal, et al., Alterations in acute myeloid leukaemia bone marrow stromal cell exosome content coincide with gains in tyrosine kinase inhibitor resistance, Br. J. Haematol. 172 (2016) 983–986.
[29] K.H. Shain, T.H. Landowski, W.S. Dalton, The tumor microenvironment as a determinant of cancer cell survival: a possible mechanism for de novo drug resistance, Curr. Opin. Oncol. 12 (2000) 557–563.
[30] B. Sharma, D.M. Nawandar, K.C. Nannuru, M.L. Varney, R.K. Singh, Targeting CXCR2 enhances chemotherapeutic response, inhibits mammary tumor growth, angiogenesis, and lung metastasis, Mol. Cancer Ther. 12 (2013) 799–808.
[31] Y. Ning, M.J. Labonte, W. Zhang, P.O. Bohanes, A. Gerger, D. Yang, et al., The CXCR2 antagonist, SCH-527123, shows antitumor activity and sensitizes cells to oxaliplatin in preclinical colon cancer models, Mol. Cancer Ther. 11 (2012) 1353–1364.
[32] E.V. Efimova, H. Liang, S.P. Pitroda, E. Labay, T.E. Darga, V. Levina, et al., Radioresistance of Stat1 over-expressing tumour cells is associated with suppressed apoptotic response to cytotoxic agents and increased IL6-IL8 signalling, Int. J. Radiat. Biol. 85 (2009) 421–431.
[33] Y. Wu, J. Deng, P.G. Rychahou, S. Qiu, B.M. Evers, B.P. Zhou, Stabilization of snail by NF-kappaB is required for inflammation-induced cell migration and invasion, Cancer Cell 15 (2009) 416–428.
[34] W.L. Hwang, M.H. Yang, M.L. Tsai, H.Y. Lan, S.H. Su, S.C. Chang, et al., SNAIL regulates interleukin-8 expression, stem cell-like activity, and tumorigenicity of human colorectal carcinoma cells, Gastroenterology 141 (91) (2011) 279–291 (e1-5).
[35] R.I. Fernando, D.H. Hamilton, C. Dominguez, J.M. David, K.K. McCampbell, C. Palena, IL-8 signaling is involved in resistance of lung carcinoma cells to erlotinib, Oncotarget 7 (2016) 42031–42044.
[36] C. Corrado, L. Saieva, S. Raimondo, A. Santoro, G. De Leo, R. Alessandro, Chronic myelogenous leukaemia exosomes modulate bone marrow microenvironment through activation of epidermal growth factor receptor, J. Cell. Mol. Med. 20 (2016) 1829–1839.