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Original Articles

Exosomal transfer of miR-501 confers doxorubicin resistance and tumorigenesis via targeting of BLID in gastric cancer

Xu Liu[a,1], Ying Lu[d,1], Yunchao Xu[a], Sizhu Hou[a], Jinli Huang[a], Bo Wang[a], Jinyao Zhao[e], Shilin Xia[f], Shujun Fan[a], Xiaotang Yu[a], Yue Du[a], Li Hou[a], Zhiyue Li[g], Zijie Ding[h], Shuo An[h], Bo Huang[i], Lianhong Li[a], Jianwu Tang[a], Jingfang Ju[c,***], Hongwei Guan[b,**], Bo Song[a,*]

[a] Department of Pathology and Forensics, College of Basic Medical Sciences, Dalian Medical University, Dalian, 116044, China
[b] Department of Pathology, First Affiliated Hospital of Dalian Medical University, Dalian, 116011, China
[c] Translational Research Laboratory, Department of Pathology, Stony Brook University, Stony Brook, NY, 11794, USA
[d] Teaching Laboratory of Morphology, College of Basic Medical Sciences, Dalian Medical University, Dalian, 116044, China
[e] Institute of Cancer Stem Cells, Second Affiliated Hospital Collaborative Innovation Center of Oncology, Dalian Medical University, Dalian, 116044, China
[f] Clinical Laboratory of Integrative Medicine, First Affiliated Hospital of Dalian Medical University, Dalian, 116011, China
[g] Department of Clinical Medicine, Grade 2016, Dalian Medical University, Dalian, 116044, China
[h] Department of Clinical Medicine, Grade 2017, Dalian Medical University, Dalian, 116044, China
[i] Department of Pathology, Liaoning Cancer Hospital & Institute, Shenyang, 110042, China

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ABSTRACT

Exosomal transfer of oncogenic miRNAs can enhance recipient cell growth, metastasis and chemoresistance. Currently we found that microRNA-501-5p (miR-501) was overexpressed in doxorubicin-resistant gastric cancer (GC) SGC7901/ADR cell-secreted exosomes (ADR Exo) than that in SGC7901 cell-secreted exosomes (7901 Exo). ADR Exo was internalized by SGC7901, and a Cy3-miR-501 mimic was transferred from SGC7901/ADR to SGC7901 via exosomes. ADR Exo conferred doxorubicin resistance, proliferation, migration and invasion abilities to negative control miRNA inhibitor-expressing GC cells, whereas it inhibited apoptosis. MiR-501 knockdown or BH3-like motif-containing protein, cell death inducer (BLID) overexpression could reverse the effects of ADR Exo on recipient cells. SGC7901 cells cocultured with SGC7901/ADR prior to treatment with GW4869 or transfection of a miR-501 inhibitor were sensitive to doxorubicin and exhibited attenuated proliferation, migration and invasion and increased apoptosis. The intratumoral injection of ADR Exo into negative control miRNA inhibitor-expressing SGC7901 cells induced rapid subcutaneous tumor growth and resistance to doxorubicin compared to that of miR-501 knockdown or BLID-overexpressing cells. This effect is possibly achieved by exosomal miR-501-induced downregulation of BLID, subsequent inactivation of caspase-9/-3 and phosphorylation of Akt. Exosomal miR-501 might be a therapeutic target for GC.

1. Introduction

Gastric cancer (GC) is the fifth most common type of malignancy and the third leading cause of cancer-related death globally [1]. GC is commonly diagnosed at advanced stages due to a lack of molecular biomarkers [2,3] and results in an approximately 20–30% 5-year survival rate [4]. Multiple events, including genetics, epigenetics and the environment, contribute to the development and progression of GC [3,5]. Thus, a comprehensive understanding of the underlying molecular mechanisms is required and might be helpful for the development of novel diagnostic and therapeutic methods for GC. Although targeted therapies such as trastuzumab against human epidermal growth factor receptor 2 (HER2) and ramucirumab against vascular endothelial growth factor receptor (VEGFR) have achieved some benefits in advanced GC [6], traditional chemotherapy still represents the primary option for advanced GC. Doxorubicin (adriamycin, ADR), which is a member of the anthracycline family, has been used to treat numerous malignancies, including GC, in combination with

* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: Jingfang.Ju@stonybrookmedicine.edu (J. Ju), mudghw@aliyun.com (H. Guan), bosong@dmu.edu.cn (B. Song).
[1] Co-first authors.

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fluorouracil, cisplatin, paclitaxel and mitomycin [7,8]. However, resistance to doxorubicin remains an obstacle to GC treatment [9–11].

Exosomes are a class of 30–100 nm sized extracellular vesicles (EVs) with lipid bilayer membranes. When endosomal multivesicular bodies (MVBs) fuse with the cell membrane, exosomes carrying biomolecules including lipids, proteins and RNAs can be released into the extracellular environment [12–14]. Lipid membranes favor the uptake of exosomes by neighboring or distant recipient cells; after uptake, the exosomal contents exhibit biological activities such as immunomodulation [15], angiogenesis [16], autophagy [17], stem cell differentiation [18] and intercellular communication, which is known as the third method of cellular communication [19,20]. Almost all types of cells, including cancer cells, can generate exosomes [14]. RNA cargo in exosomes has garnered much attention from researchers, especially microRNAs (miRNAs). The lipid membranes of exosomes protect the internal miRNAs from being digested by RNases. Emerging evidence has demonstrated that tumor cell-secreted exosomal miRNAs (exo-miRNAs) play a crucial role in regulating tumor growth, metastasis, angiogenesis and chemoresistance [21–29].

MicroRNA-501-5p (miR-501) is a recently identified novel oncomiR. Several studies have reported that miR-501 is overexpressed in hepatocellular carcinoma (HCC) [30,31], lung adenocarcinoma [32], cervical cancer [33] and GC tissues [34,35]. Elevated miR-501 promotes the progression of lung adenocarcinoma [32] and cervical cancer [33] and predicts poor prognosis of GC [35]. Our previous study demonstrated that miR-501 enhances doxorubicin resistance and tumorigenesis in GC by targeting BH3-like motif-containing protein, cell death inducer (BLID). Hence, in this study, we sought to investigate whether miR-501 can be transmitted via exosomes and the roles exosomal miR-501 plays in the chemoresistance and tumorigenesis of GC *in vitro* and *in vivo*.

2. Materials and methods

2.1. Cell culture and reagents

Gastric cancer SGC7901 cells and doxorubicin-resistant SGC7901/ADR cells were gifted by the Department of Digestive Diseases of Xijing Hospital, Fourth Military Medical University (Xi'an, China). The BGC823 cell line was a kind gift from Professor Yan Li, Department of Anatomy of Dalian Medical University (Dalian, China). All three cell lines were cultured in RPMI 1640 media (Gibco, NY, USA) containing 10% fetal bovine serum (FBS) (ScienCell, CA, USA). SGC7901/ADR cells were maintained in medium with 0.5 μg/ml doxorubicin (Haizheng, Zhejiang, China) and were cultured in drug-free medium for at least 7 days before the experiments were initiated.

2.2. Exosome isolation

Exosome-free FBS was obtained by ultracentrifugation at 120,000 × *g* for 6 h at 4 °C [36]. SGC7901 and SGC7901/ADR cells (1×10^6) were cultured in medium containing 10% exosome-free FBS for 48 h. Then, 40 ml of the conditioned medium from each cell line was collected, and the exosomes were isolated according to the previously described ultracentrifugation method [37] or by the ExoQuick-TC Kit (System Bioscience, CA, USA) according to our previous study [38]. The concentration and number of exosomes was detected by Nanosight particle tracking analysis (NTA) as described below. The exosomal protein concentration was measured by the BCA method.

2.3. Transmission electron microscopy

The morphology and size of exosomes was observed under a transmission electron microscope (JEOL, Tokyo, Japan) according to our previous study [38].

2.4. Nanosight particle tracking analysis (NTA)

Exosomes were isolated from 40 ml of conditioned medium from SGC7901 and SGC7901/ADR cells by ultracentrifugation and re-suspended in 100 μl PBS. Prior to NTA, SGC7901 cell-secreted exosomes were diluted by a factor of 100 with PBS, and SGC7901/ADR cell-secreted exosomes were diluted by a factor of 1000 to obtain an approximate number of vesicles of no more than 1×10^7. The size and concentration of the exosomes was analyzed using the ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany) and its corresponding software (ZetaView 8.02.28) [39].

2.5. Exosome labeling and uptake

The PKH26 red fluorescent dye (Sigma-Aldrich, MO, USA) was used to label SGC7901/ADR cell-released exosomes according to our previous study [38].

2.6. Cy3-miRNA mimic, inhibitor and siRNA transfection

SGC7901/ADR cells (3×10^5/well) were transfected with 100 nM of the Cy3-miR-501 mimic (RiboBio, Guangzhou, China) by Lipofectamine 2000 (Invitrogen, CA, USA) [40] and then were co-cultured with SGC7901 cells in a coculture system.

SGC7901/ADR cells (3×10^5/well) were transfected with a miR-501 inhibitor (RiboBio, Guangzhou, China) or negative control miRNA inhibitor (RiboBio, Guangzhou, China) at 100 nM with Lipofectamine 2000. Cotransfection of 50 nM miR-501 inhibitor and 50 nM small interfering RNA (siRNA) specific for BLID (RiboBio, Guangdong, China) was also included as a negative control.

2.7. Stable transfection with lentiviral vectors

SGC7901 cells or BGC823 cells (2×10^5 per well) in 6-well plates were infected with 10 μl of lentiviral vectors containing the miR-501 inhibitor (501KD), negative control miRNA inhibitor (NCi) and BLID (BLID) (GeneChem, Shanghai, China) for 3 days. Then, 1.0 μg/ml puromycin (Sigma-Aldrich, MO, USA) was used to screen the infected cells for 4–6 weeks. The infection efficiency was confirmed by fluorescence microscopy, real-time quantitative RT-PCR (qRT-PCR) and Western blot analyses.

2.8. GW4869 treatment

GW4869 (Sigma-Aldrich, MO, USA) was utilized to block exosome formation and release as described previously [38,41].

2.9. Cell coculture assay

The Cy3-miR-501 mimic-transfected, miR-501 inhibitor-transfected or GW4869-treated SGC7901/ADR cells (5×10^5/well) were seeded in the upper chamber of a coculture system with a 0.4 μm pore membrane, and SGC7901 cells (3×10^5/well) were placed in the lower chamber. All cells were incubated in medium with 10% exosome-free FBS.

2.10. RNA extraction and real-time qRT-PCR analysis

Total RNA was extracted from exosomes, cocultured SGC7901 cells or BGC823 cells, and frozen xenograft tumor tissues using TRIzol reagent (Invitrogen, CA, USA). qRT-PCR analysis for miR-501 and BLID mRNA was performed according to a previous study [33]. The relative expression of miR-501 was normalized to U6, and BLID mRNA expression was normalized to GAPDH, and both were calculated by the $2^{(-\Delta\Delta CT)}$ method. The related primers were described in our previous study [35].

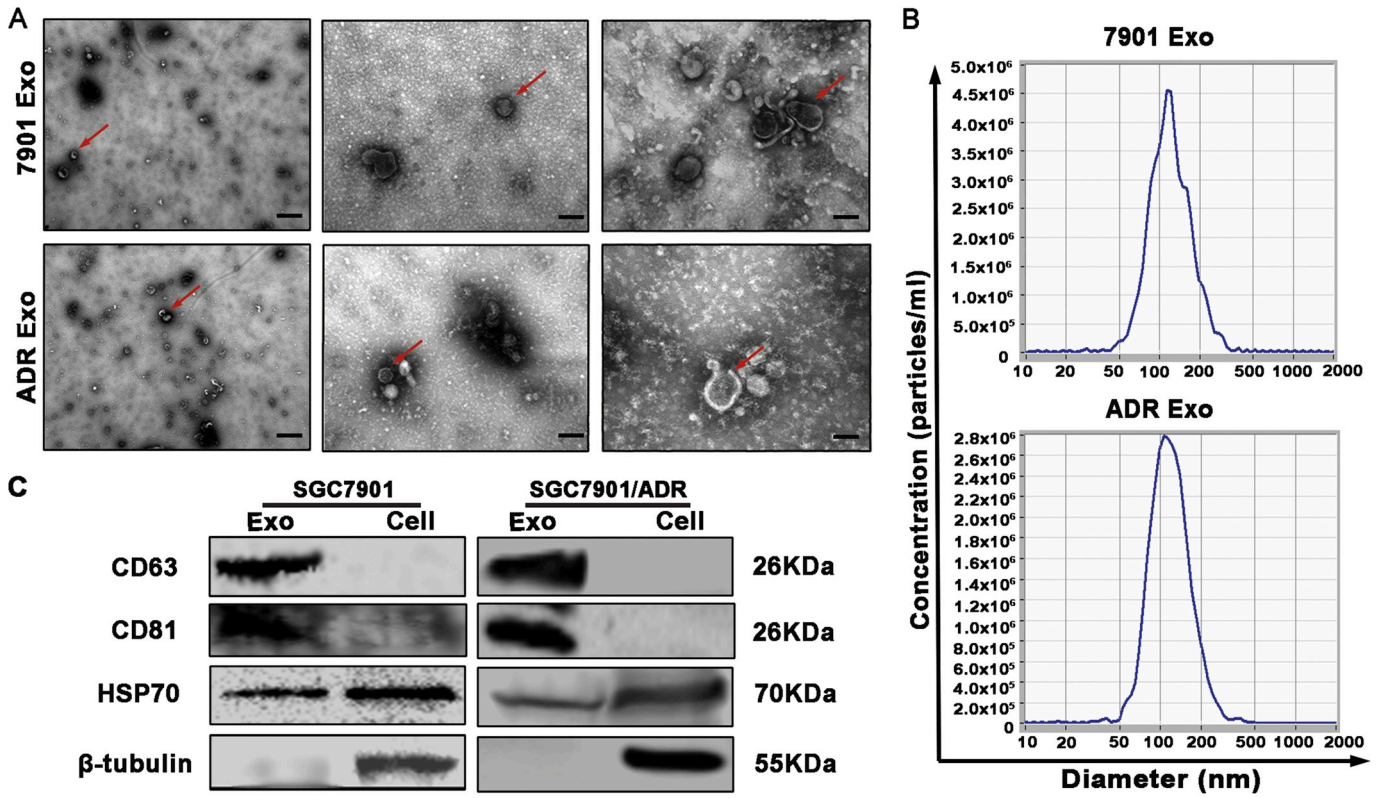

Fig. 1. Characteristics of exosomes derived from gastric cancer SGC7901 and SGC7901/ADR cells. (A) The transmission electron micrograph showed round-shaped vesicles with bilayered membranes ranging from 30 nm to 100 nm in diameter released by SGC7901 (7901 Exo) and SGC7901/ADR (ADR Exo) cells. Scale bar = 500 nm, 100 nm and 20 nm, respectively. **(B)** Nanosight particle tracking analysis (NTA) indicated that the dominant size of 7901 Exo and ADR Exo was approximately 100 nm, and the concentrations of the 100 nm particles were 4.6×10^8 particles/ml and 2.8×10^9 particles/ml, respectively, after calculation. **(C)** The positive markers of exosomes, CD63, CD81 and HSP70, were detected in 7901 Exo and ADR Exo by Western blot.

2.11. Western blot analysis

Protein extraction of cells, exosomes and xenograft tumor tissues as well as Western blot analysis was performed according to our previous study [35,38]. The antibodies against BLID (1:500), Akt (1:500), p-Akt Ser 473 (1:500), CD63 (1:500), and HSP70 (1:500) were purchased from Abcam (Cambridge, MA, USA). The antibodies against caspase-9 and -3 (1:500), cleaved caspase-9 and -3 (1:500), β-tubulin (1:1000) and GAPDH (1:1000) were obtained from Proteintech (Wuhan, China). The antibody against CD81 (1:200) was purchased from Boster (Wuhan, China).

2.12. Doxorubicin treatment

Cells (1×10^4/well) were seeded in 96-well plates for 24 h, and doxorubicin from 0.125 to 4 μg/ml was added and incubated for 48 h. A CCK-8 assay was performed as previously described [35].

2.13. Apoptosis assay

The flow cytometry analysis was performed by Annexin V-APC/PI Apoptosis Detection Kit (KeyGen BioTech, Jiangsu, China) for cell lines stably expressing GFP or the Annexin V-FITC/PI Apoptosis Detection Kit (KeyGen BioTech, Jiangsu, China) for cells without GFP. The protocols were described in our previous study [35].

2.14. Colony formation assay, migration and invasion assays

These assays were performed according to our published study [35].

2.15. Mouse xenograft tumor model

Twenty 6-week-old male BALB/c nude mice from Dalian Medical University were randomly divided into four groups ($n = 5$ each). Groups 1 and 2 were injected with 7901 NCi cells, group 3 was injected with 7901 501KD cells and group 4 was injected with 7901 BLID cells. The infected cells (1×10^6) in 100 μl PBS and Matrigel were subcutaneously injected into each nude mouse. The volume of the subcutaneous tumor was measured every 2 days [35]. When the volume of the xenografts reached approximately 50 mm^3, the mice in groups 2, 3 and 4 were intratumorally injected with 50 μg ADR Exo three times for a week. The negative control of group 1 was injected with PBS. When the xenograft volumes were approximately 100 mm^3, all groups were administered ADR (5 mg/kg) through the tail vein three times a week for 2 weeks and were sacrificed. The subcutaneous tumors were excised and frozen in liquid nitrogen. Animal experiments were approved by the Animal Care and Use Committee of Dalian Medical University.

2.16. Statistical analysis

The statistical analysis was performed using SPSS software (version 17.0, NY, USA). The results are presented as the mean ± SD. Statistical significance between two groups was determined using a two-tailed Student's *t*-test. To compare multiple groups, one-way analysis of variance (ANOVA) followed by a Bonferroni-Dunn test was performed. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Exosomes derived from gastric cancer SGC7901 and SGC7901/ADR cells were isolated and confirmed

To investigate the roles of exosome transfer in doxorubicin resistance and tumorigenesis, we first isolated exosomes from the conditioned medium of SGC7901 and SGC7901/ADR cells. The vesicles exhibited a round shape with bilayered membranes, and the diameter ranged from 30 nm to 100 nm under the transmission electron microscope (Fig. 1A). The Nanosight particle tracking analysis (NTA) further identified that the predominant size of the vesicles was 100 nm, and the concentrations at the size of 100 nm were 4.6×10^8 particles/ml and 2.8×10^9 particles/ml generated from SGC7901 and SGC7901/ADR, respectively, after multiplying by the dilution factors (Fig. 1B). By Western blot analysis, the exosomal markers CD63, CD81 and HSP70 were detected in the exosomes, whereas β-tubulin was enriched in the whole cell lysates (Fig. 1C). These results indicate that the vesicles isolated from SGC7901 and SGC7901/ADR display typical characteristics of exosomes and that doxorubicin-resistant SGC7901/ADR cells secrete more exosomes than sensitive SGC7901 cells secrete.

3.2. MiR-501 is enriched in SGC7901/ADR cell-derived exosomes and enhances doxorubicin resistance of SGC7901 recipient cells via exosome transfer

Next, we compared the expression of miR-501 in the two cell-derived exosome populations. As shown in Fig. 2A, miR-501 expression in SGC7901/ADR secreted exosomes (ADR Exo) was 2.54 ± 0.31-fold greater than that in SGC7901 secreted exosomes (7901 Exo) by real-time qRT-PCR. Thus, we reasoned that miR-501 from ADR Exo may confer doxorubicin resistance to SGC7901 cells via exosome transfer. To visualize exosome transfer, we labeled ADR Exo with PKH26, which is a fluorescent tracer. After incubation of labeled ADR Exo with SGC7901, we observed strong red fluorescence in the cytoplasm of recipient cells under a confocal microscope (Fig. 2B), suggesting that SGC7901/ADR secreted exosomes were successfully taken up by SGC7901 recipient cells. We thereafter validated whether the delivery of miR-501 occurs via exosomes. SGC7901/ADR cells were transfected with the Cy3-miR-501 mimic and replated in the upper chamber of a coculture system with 0.4 μm pores, which inhibit direct contact with the cells and allow the transmission of exosomes but not larger particles. After 24 h of coculture, strong red fluorescence was observed in SGC7901 cells seeded in the lower chamber (Fig. 2C). Furthermore, miR-501 was dramatically upregulated in SGC7901 cells after co-incubation with ADR Exo (Fig. 2D). These data demonstrate that miR-501 might be directly transferred from SGC7901/ADR donor cells to SGC7901 recipient cells through exosomes.

Then, we utilized doxorubicin to treat SGC7901 cells cocultured with ADR Exo. The CCK-8 assay showed that the presence of ADR Exo induced doxorubicin resistance in SGC7901 cells in a dose-dependent manner (Fig. 2E). The half maximal inhibitory concentration (IC50) of doxorubicin was increased by 1.86 ± 0.41-fold (7901 + ADR Exo vs. 7901 + PBS). Taken together, these results indicate that miR-501 might be shuttled via exosomes from the resistant GC cells to the neighboring sensitive GC cells, thereby inducing resistance to doxorubicin.

3.3. SGC7901/ADR-secreted exosomal miR-501 confers doxorubicin resistance to recipient cells by targeting BLID

Above, we verified that SGC7901/ADR exosomes contain increased levels of miR-501 and that miR-501 can be horizontally transferred to recipient cells via exosomes, leading to doxorubicin resistance. We previously identified by luciferase reporter assay that miR-501 directly targets BLID to enhance doxorubicin resistance in GC [35]. We next investigated whether exosomal miR-501 targets BLID. We infected SGC7901 cells with lentiviral vectors expressing a miR-501 inhibitor (7901 501KD), negative control miRNA inhibitor (7901 NCi) or BLID (7901 BLID), selected for stably transfected cell lines by puromycin and confirmed them using fluorescence microscopy (Supplementary Fig. 1A, left panel), real-time qRT-PCR (Supplementary Figs. 1B and C, left panel) and Western blot (Supplementary Fig. 1D, upper panel).

Then, we directly added ADR Exo to the medium of 7901 NCi (7901 NCi + ADR Exo), 7901 501KD (7901 501KD + ADR Exo) and 7901 BLID cells (7901 BLID + ADR Exo) (Supplementary Fig. 2A). The 7901 NCi cells incubated with the equivalent amount of PBS (7901 NCi + PBS) were used as the negative control. Fig. 3A shows that miR-501 expression in 7901 NCi was dramatically increased in the presence of ADR Exo, whereas ADR Exo failed to increase miR-501 expression in 7901 501KD cells. In contrast, the expression of BLID at both the mRNA and protein level was decreased in 7901 NCi + ADR Exo compared with that of 7901 NCi + PBS; however, miR-501 knockdown or BLID overexpression abolished the ADR Exo-induced BLID decrease (Fig. 3B and C). Hence, our findings suggest that ADR Exo can induce the upregulation of miR-501 and the downregulation of BLID in recipient cells, supporting the hypothesis that miR-501 is transferred via exosomes and negatively regulates BLID expression.

To further determine whether the transmission of miR-501 to SGC7901 cells relies on exosomes, we used GW4869 to block exosome secretion by SGC7901/ADR cells (ADR GW4869) plated in the upper chamber of a coculture system with 0.4 μm pores, as shown in Supplementary Fig. 3A. SGC7901 cells were seeded in the lower chamber and harvested for subsequent experiments. We found that miR-501 expression was markedly decreased in 7901 + ADR GW4869-treated cells compared to that in 7901 + ADR DMSO-treated cells (Fig. 3D). Conversely, the BLID mRNA (Fig. 3E, left panel) and protein (Fig. 3F, left panel) expression levels were significantly increased. These results indicate that the delivery of miR-501 is dependent on exosomes and that exosomal miR-501 also downregulates BLID.

To determine whether miR-501 is present in exosomes, we transfected SGC7901/ADR cells with a miR-501 inhibitor (ADR 501i) and seeded ADR 501i in the upper transwell chamber. The cotransfection of the miR-501 inhibitor and siRNA specific for BLID (ADR 501i + siBLID) and negative control miRNA inhibitor (ADR NCi) was used for the negative controls. Similarly, SGC7901 cells were plated in the lower transwell chamber and harvested after coculturing for the following experiments (Supplementary Fig. 3B). BLID expression in 7901 + ADR 501i cells at both the mRNA (Fig. 3E, right panel) and protein (Fig. 3F, right panel) levels was higher than that in the negative controls. These results further confirm that ADR Exo contain miR-501 and that BLID is downregulated by exosomal miR-501.

Finally, we evaluated whether exosomal miR-501 induces doxorubicin resistance in SGC7901 cells by targeting BLID. As Fig. 3G shows, ADR Exo dramatically enhanced the doxorubicin resistance of 7901 NCi cells, whereas no significant difference was seen in miR-501-silenced or BLID-overexpressing SGC7901 cells. Thus, miR-501 knockdown and BLID overexpression can reverse the effects of ADR Exo on the doxorubicin resistance of SGC7901 cells. In contrast, SGC7901 cells were more sensitive to doxorubicin after coculturing with SGC7901/ADR cells treated with GW4869 than with SGC7901/ADR cells treated with DMSO (Fig. 3H, upper panel). In addition, the doxorubicin resistance of SGC7901 cells was reversed when they were cocultured with miR-501 knockdown SGC7901/ADR cells (Fig. 3H, bottom panel). Taken together, these results suggest that the acquisition of doxorubicin resistance can be achieved by exosomal transfer of miR-501 possibly via the inhibition of BLID in GC cells.

In addition to assessing the effects in SGC7901 cells, we also showed that SGC7901/ADR-secreted exosomal miR-501 induces doxorubicin resistance by targeting BLID in the BGC823 gastric cancer cell line. After stable transfection of lentiviral vectors expressing the miR-501 inhibitor (823 501KD), negative control miRNA inhibitor (823 NCi) or

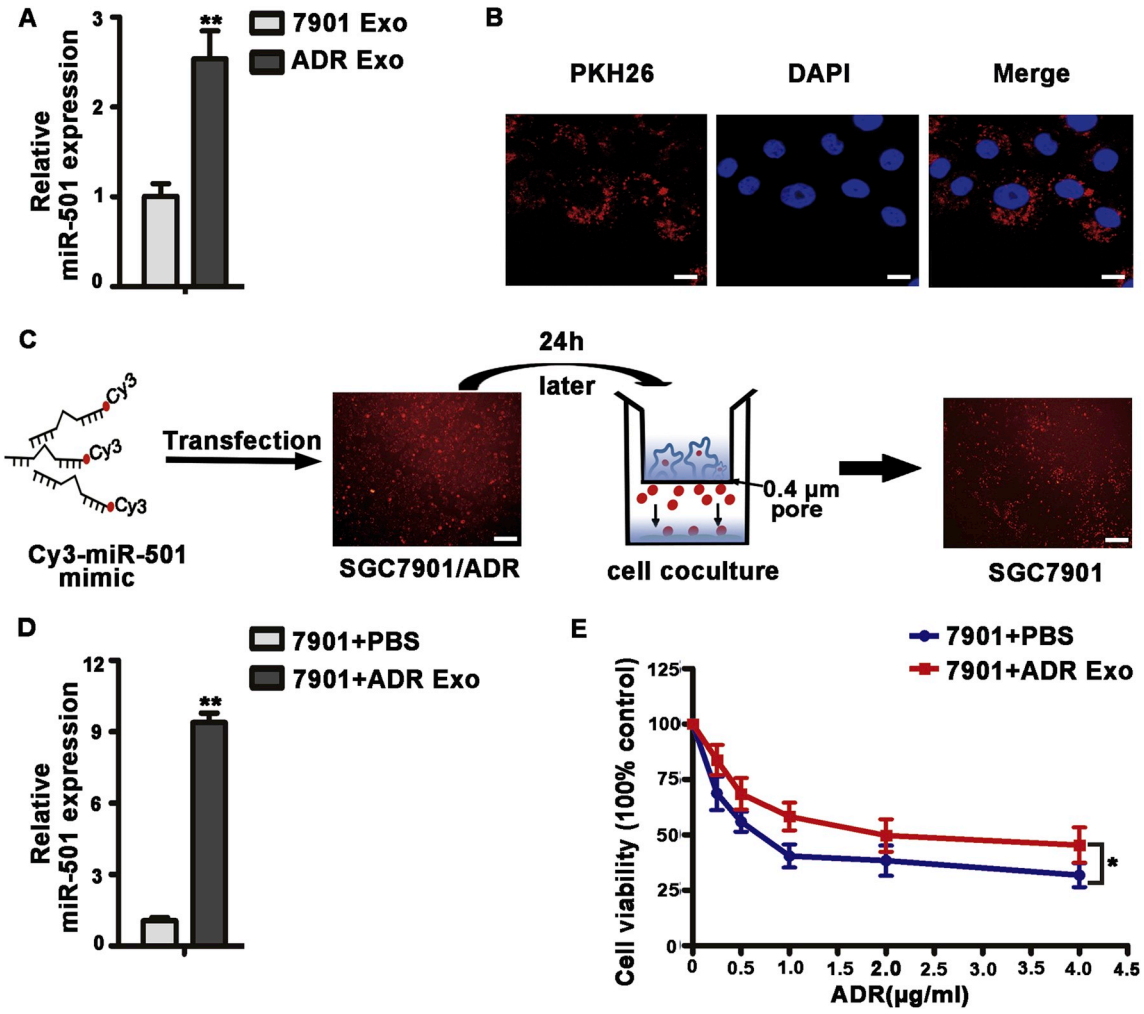

Fig. 2. ADR Exo has a higher level of miR-501 than 7901 Exo, and exosomal miR-501 transfer enhances the doxorubicin resistance of SGC7901 recipient cells. (A) Real-time qRT-PCR revealed that the level of miR-501 was higher in ADR Exo than in 7901 Exo. **(B)** Confocal microscopy showed exosome internalization by SGC7901 recipient cells after co-incubation with PKH26-labeled (red fluorescence) ADR Exo. DAPI was used to stain the nuclei of SGC7901 recipient cells with blue fluorescence. Scale bar = 25 μm. **(C)** SGC7901/ADR cells transfected with the Cy3-miR-501 mimic (red fluorescence) were placed in the upper chamber and co-incubated with SGC7901 cells seeded in the lower chamber in a coculture system with a 0.4 μm pore membrane. Red fluorescence was observed in the SGC7901 recipient cells under the fluorescence microscope. Scale bar = 100 μm. (D) After co-incubation with ADR Exo for 24 h, the level of miR-501 in the SGC7901 cells was significantly increased as assessed by real-time qRT-PCR. **(E)** SGC7901 cells cocultured with ADR Exo were treated with a series of concentrations of doxorubicin for 48 h. The CCK-8 assay showed enhanced doxorubicin resistance of SGC7901 cells. Experiments were performed three times, and the results are represented as the mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$.

BLID (823 BLID) (Supplementary Figs. 1A–C, right panel and D, bottom panel), we added ADR Exo to the cell culture medium (Supplementary Fig. 2B). As we expected, resistant cell-derived exosomes (ADR Exo) increased the expression of miR-501 but decreased the expression of BLID both at the mRNA and protein level in 823 NCi (Supplementary Figs. 4A–C). The presence of ADR Exo increased the number of 823 NCi cells that were resistant to doxorubicin (Supplementary Fig. 4D). However, knockdown of miR-501 or overexpression of BLID failed to lead to changes in miR-501 and BLID expression and the resistant phenotype (Supplementary Figs. 4A–D). These data further verify that resistant cell-derived exosomal miR-501 confers doxorubicin resistance to target cells via BLID targeting.

3.4. Exosomal transfer of miR-501 suppresses apoptosis to enhance doxorubicin resistance in recipient cells by downregulating BLID and inactivating the downstream caspase pathway

A fundamental mechanism of doxorubicin resistance is the evasion of apoptosis [42,43]. We therefore incubated 7901 NCi, 7901 501KD and 7901 BLID cells with ADR Exo and detected the rate of apoptosis using flow cytometry. PBS-treated 7901 NCi cells were used as the negative control. The 7901 NCi + ADR Exo group showed a significant decline (35.57% ± 1.46%) in apoptosis compared to that of the 7901 NCi + PBS group, especially in the early apoptosis phase (Fig. 4A and B). The 7901 501KD + ADR Exo group and 7901 BLID + ADR Exo group did not show any obvious difference to the 7901 NCi + PBS group (Fig. 4A and B). Parallel results were obtained in stably transfected BGC823 cells (Supplementary Figs. 5A and B). In addition, 7901 + ADR GW4869 and 7901 + ADR 501i cells exhibited higher rates of apoptosis than the corresponding negative controls (Fig. 4C and D). These results reveal that miR-501 inhibits apoptosis by downregulating BLID in GC cells via exosomal transfer.

A study has reported that BLID-mediated activation of the caspase pathway induces apoptosis [44]. Our results showed that cleavage of caspase-9 and caspase-3 was inhibited in 7901 NCi + ADR Exo and 823 NCi + ADR Exo compared to that in 7901 NCi + PBS and 823 NCi + PBS, respectively. No notable difference was observed in miR-501 knockdown (7901 501KD + ADR Exo and 823 501KD + ADR Exo)

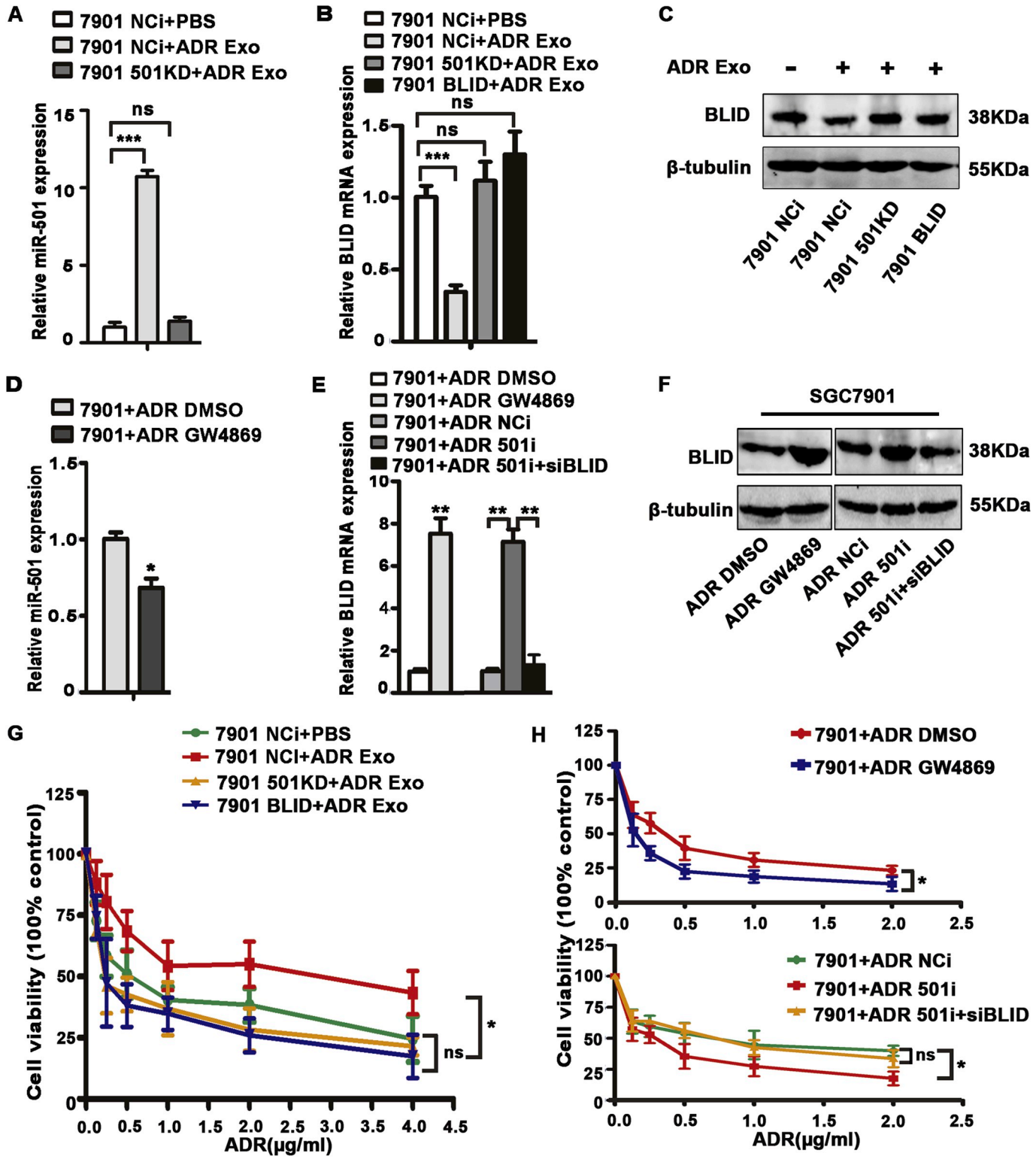

Fig. 3. SGC7901/ADR-secreted exosomal miR-501 confers doxorubicin resistance to SGC7901 recipient cells by inhibiting BLID. (A) SGC7901 cells infected with lentiviral vectors expressing a negative control miRNA inhibitor (7901 NCi), miR-501 inhibitor (7901 501KD) or BLID (7901 BLID) were cocultured with ADR Exo for 24 h. Coculture of 7901 NCi with PBS (7901 NCi + PBS) was used as a negative control. The level of miR-501 was markedly increased in the ADR Exo-treated 7901 NCi cells; however, ADR Exo treatment did not increase miR-501 in 7901 501KD cells. **(B–C)** The mRNA and protein levels of BLID were reduced in 7901 NCi + ADR Exo cells compared with those of 7901 NCi + PBS cells, whereas miR-501 knockdown or BLID overexpression abolished the ADR Exo-induced BLID reduction. **(D)** qRT-PCR showed a significant decrease in miR-501 expression in SGC7901 cells after coculturing with SGC7901/ADR cells treated with 10 μM GW4869 compared to cells cocultured with SGC7901/ADR cells treated with DMSO. **(E–F)** The upregulation of BLID mRNA and protein was detected by qRT-PCR and Western blot in SGC7901 cells cocultured with GW4869-treated SGC7901/ADR cells (left panel) or with SGC7901/ADR cells transfected with a miR-501 inhibitor (right panel). **(G)** After co-incubation with ADR Exo, the lentiviral vector-infected SGC7901 cells were treated with different concentrations of doxorubicin for 48 h. Doxorubicin resistance was enhanced in 7901 NCi + ADR Exo cells compared to that of 7901 NCi + PBS cells, whereas no significant difference was induced in 501KD + ADR Exo or BLID + ADR Exo cells. **(H)** Inhibition of exosome release or miR-501 knockdown in SGC7901/ADR cells reversed the doxorubicin resistance of SGC7901 cells. All data are presented as the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

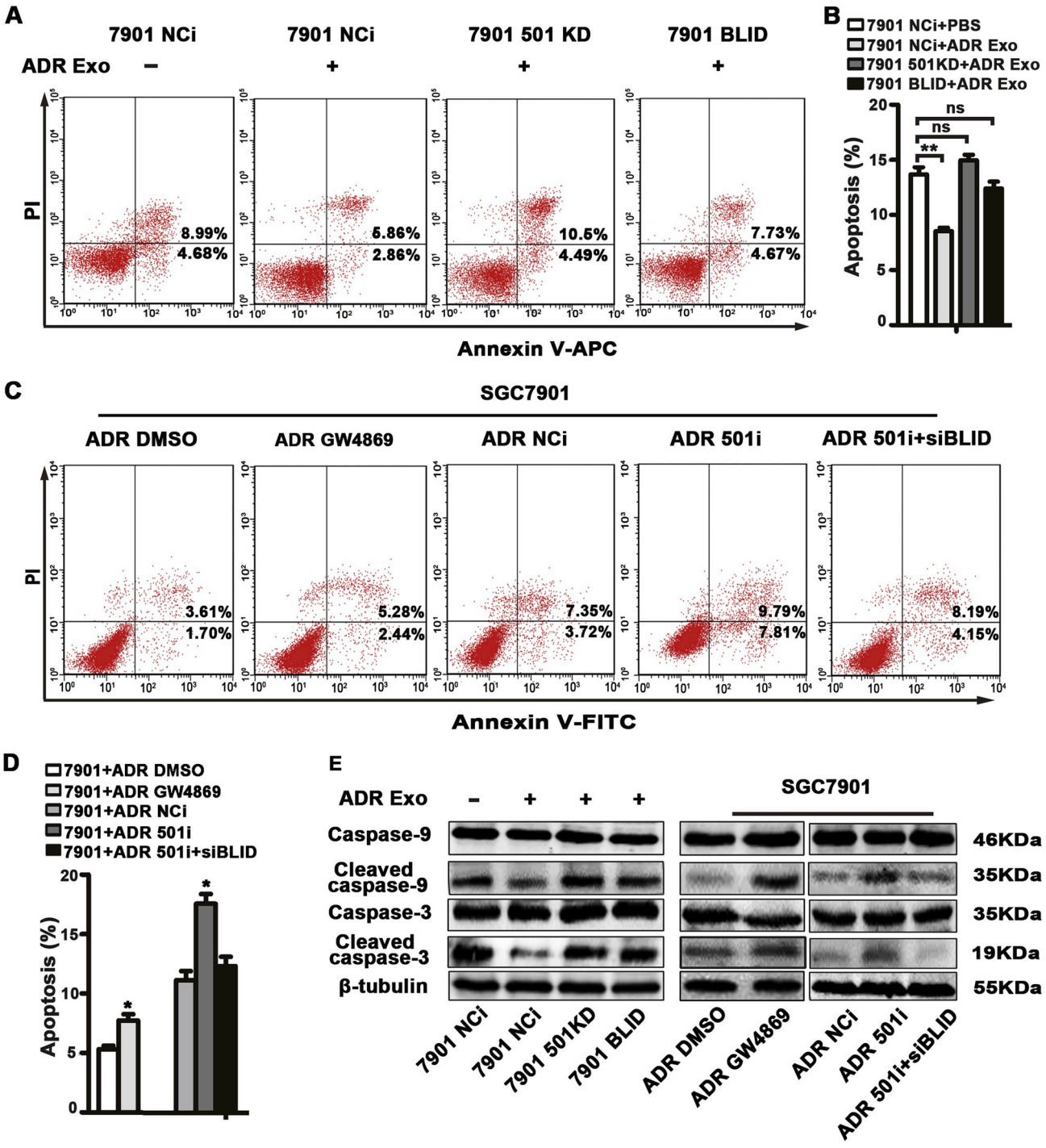

Fig. 4. Exosomal miR-501 induces doxorubicin resistance in SGC7901 recipient cells via downregulation of BLID and subsequent evasion of apoptosis mediated by inactivation of caspase-9 and -3. (A–B) 7901 NCi, 7901 501KD and 7901 BLID cells were cocultured with ADR Exo for 24 h, and the rates of apoptosis were measured by flow cytometry assay. The 7901 NCi cells cocultured with PBS (7901 NCi + PBS) were used as the negative control. The Annexin V-APC/PI assay showed that the rate of apoptotsis was reduced in 7901 NCi + ADR Exo cells compared to that in 7901 NCi + PBS cells, whereas no notable difference was found among 7901 501KD + ADR Exo, 7901 BLID + ADR Exo and 7901 NCi + PBS cells (A). Quantitative analysis of the rate of apoptosis is shown (B). **(C–D)** SGC7901 cells were cocultured with SGC7901/ADR cells treated with GW4869 or transfected with a miR-501 inhibitor. The rate of apoptosis was determined by the Annexin V-FITC/PI assay (C). Quantitative analysis of the rate of apoptosis is shown (D). **(E)** Western blot showing that the protein levels of cleaved caspase-9 and caspase-3 in 7901 NCi + ADR Exo cells were reduced (left panel). There was no obvious difference in the 7901 501KD + ADR Exo and 7901 BLID + ADR Exo cells compared with the 7901 NCi + PBS cells. The cleaved caspase-9 and caspase-3 protein levels were increased in SGC7901 cells cocultured with SGC7901/ADR cells treated with GW4869 or transfected with miR-501 inhibitor (right panel). All results represent three independent experiments, and each value is the mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$.

and BLID-overexpressing (7901 BLID + ADR Exo and 823 BLID + ADR Exo) cells compared with 7901 NCi + PBS and 823 NCi + PBS cells (Fig. 4E, left panel and Supplementary Fig. 5C), respectively. In contrast, cleaved caspase-9 and caspase-3 levels were increased in 7901 + ADR GW4869 (Fig. 4E, right panel) and 7901 + ADR 501i cells, respectively (Fig. 4E, right panel). These data suggest that the

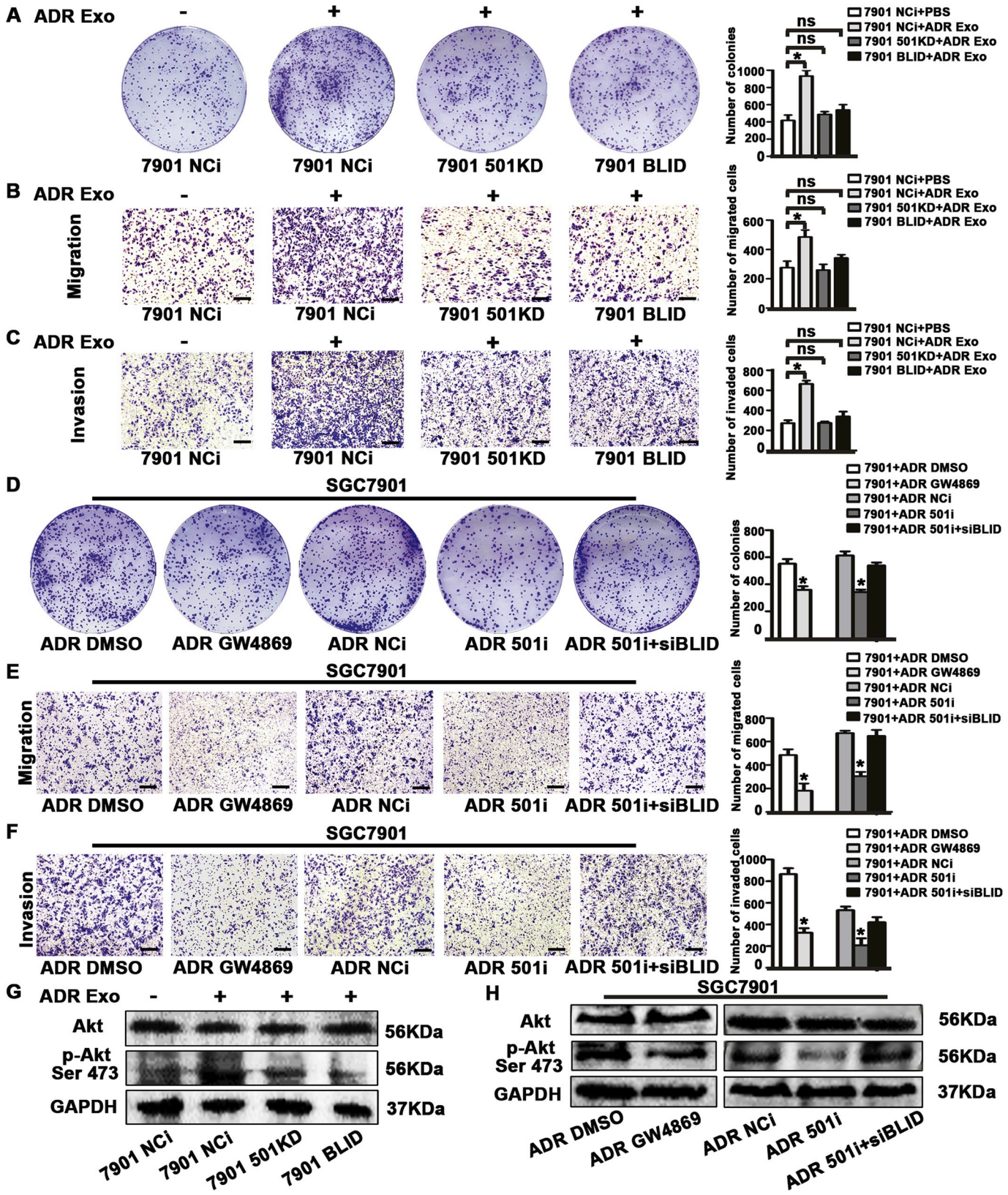

Fig. 5. Exosomal miR-501 promotes SGC7901 recipient cell proliferation, migration, and invasion by downregulating BLID and the subsequent phosphorylation of Akt. (A–C) Infected SGC7901 cells were cocultured with ADR Exo for 24 h. Colony formation, transwell migration and invasion assays showed that exosomal miR-501 promoted cell proliferation, migration and invasion abilities in 7901 NCi + ADR Exo cells compared to those in 7901 NCi + PBS cells. No obvious alteration was found in 7901 501KD + ADR Exo or 7901 BLID + ADR Exo cells relative to 7901 NCi + PBS cells. Scale bar = 100 μm. **(D–F)** SGC7901 cells exhibited decreased proliferation, migration and invasion capacities when cocultured with ADR GW4869 or ADR 501i cells. Scale bar = 100 μm. **(G)** Western blot showing that the phosphorylation of Akt at Ser 473 (p-Akt Ser 473) was dramatically increased in 7901 NCi + ADR Exo cells compared to that in 7901 NCi + PBS cells. The 7901 501KD + ADR Exo or 7901 BLID + ADR Exo cells showed no significant difference compared to 7901 NCi + PBS cells. **(H)** SGC7901 cells were cocultured with ADR GW4869 or ADR 501i cells, and the protein level of p-Akt Ser 473 was reduced. All data represent three experiments and are displayed as the mean ± SD.

exosomal miR-501/BLID axis enhances doxorubicin resistance by inhibiting apoptosis, possibly through caspase pathway inactivation.

3.5. Exosomal miR-501 targeting of BLID promotes recipient cell proliferation, migration, and invasion via Akt phosphorylation

We investigated the impact of the exosomal miR-501/BLID axis on GC cell proliferation, migration and invasion (Supplementary Fig. 6A). Our results showed that 7901 NCi cells treated with ADR Exo exhibited increased colony formation, migration and invasion capacities compared to those of cells treated with PBS; however, there was no obvious alteration when 7901 501KD or 7901 BLID cells were cocultured with ADR Exo compared to 7901 NCi cells cocultured with PBS (Fig. 5A–C). Similar results were obtained in BGC823 recipient cells (Supplementary Figs. 7A–C). In contrast, the proliferation, migration and invasion abilities of SGC7901 cells after coculturing with ADR GW4869 (Fig. 5D–F, left panel) or with ADR 501i cells were attenuated (Fig. 5D–F, right panel) compared to those of the corresponding negative controls (Supplementary Fig. 6B). Our results reveal that exosomal miR-501 promotes GC cell proliferation, migration, and invasion via BLID downregulation.

A study reported that BLID inhibits cell growth and metastasis by inhibiting the Akt pathway in breast cancer [45]. Thus, we conducted Western blotting to measure the phosphorylation of Akt at Ser 473 (p-Akt Ser 473). As shown in Fig. 5G and Supplementary Fig. 7D, p-Akt Ser 473 was significantly increased in 7901 NCi + ADR Exo and 823 NCi + ADR Exo cells. The 7901 501KD, 823 501KD, 7901 BLID, or 823 BLID cells cocultured with ADR Exo showed no dramatic difference compared to 7901 NCi + PBS or 823 NCi + PBS cells. Conversely, inhibition of exosome secretion (Fig. 5H, left panel) or miR-501 knockdown (Fig. 5H, right panel) in SGC7901/ADR cells inhibited the phosphorylation of Akt at Ser 473. Our findings suggest that exosomal miR-501 enhances GC cell proliferation, migration, and invasion by downregulating BLID and the subsequent phosphorylation of Akt.

3.6. Exosomal miR-501 induces doxorubicin resistance and promotes the tumorigenesis of GC in vivo

Given the observed effects of exosomal miR-501 on GC cells *in vitro*, we subsequently confirmed the aforementioned results *in vivo*. We subcutaneously injected the stably transfected 7901 NCi, 7901 501KD and 7901 BLID cells into nude mice and found that the tumors of 7901 NCi grew faster than those of 7901 501KD and 7901 BLID, while no significant difference was observed between 7901 501KD and 7901 BLID tumors (Fig. 6A). Importantly, the mice of the 7901 NCi group intratumorally injected with ADR Exo had greater tumor growth than the same group injected with PBS. No significant difference was found among 7901 501KD + ADR Exo, 7901 BLID + ADR Exo and 7901 NCi + PBS groups (Fig. 6B). Additionally, the tumor volumes of the 7901 NCi + ADR Exo group were significantly greater than those of the 7901 NCi + PBS group after doxorubicin treatment, and no obvious difference was seen among the 7901 NCi + PBS + ADR, 7901 501KD + ADR Exo + ADR and 7901 BLID + ADR Exo + ADR groups (Fig. 6C and D). These results indicate that ADR Exo can promote the growth and induce the doxorubicin resistance of SGC7901 cells *in vivo*.

We then examined the level of miR-501 and the mRNA and protein expression level of its target, BLID, in mouse xenograft tumor tissues. As shown in Fig. 6E and F, the 7901 NCi + ADR Exo + ADR group had a higher level of miR-501 and decreased expression of BLID mRNA and protein compared to that of the 7901 NCi + PBS + ADR group. Correspondingly, the cleavage of caspase-9 and caspase-3 was inhibited, and the p-Akt Ser 473 level was elevated in the tumor tissues of the 7901 NCi + ADR Exo + ADR group (Fig. 6G). These data suggest that exosomal transfer of miR-501 can enhance the doxorubicin resistance and growth of SGC7901 recipient cells *in vivo*, possibly through the downregulation of BLID and subsequent inactivation of caspase-9/-3 and phosphorylation of Akt.

According to the results above, we propose a model for the potential functions of exosomal miR-501 in GC (Fig. 7). SGC7901/ADR cell-secreted exosomes containing miR-501 can be taken up by the recipient cells and therefore enhance the malignant phenotype, including doxorubicin resistance, proliferation, migration and invasion capacities. This enhancement is achieved possibly via the suppression of BLID and subsequent inactivation of caspase-9/-3 and phosphorylation of Akt.

4. Discussion

Exosomes, as novel intracellular communication tools, have been reported to play an important role in cancer progression and chemoresistance [19–21]. Recently, miRNAs have been shown to be encapsulated in exosomes to avoid degradation. These exo-miRNAs can be shuttled to recipient cells and modify their phenotypes via changes in gene expression [13,14,21]. Drug-resistant cancer cells may release exo-miRNAs into the microenvironment and confer drug resistance to recipient cells [22–24,27–29]. Herein, we found that miR-501 is directly transferred from resistant cells to sensitive cells via exosomes to enhance doxorubicin resistance and tumorigenesis by targeting BLID. For the first time, to the best of our knowledge, we clarified that donor cell-secreted exosomal miR-501 can be taken up by recipient cells and retain its function in both *in vitro* and *in vivo* experiments.

A number of studies have identified that horizontal transfer of exo-miRNAs induces chemoresistance and malignant phenotypic traits [22–25,28,29]. Exosomal miR-126a and miR-222 have been reported to be involved in the doxorubicin resistance of breast tumors [23,25]. Wei et al. [24] demonstrated that exosomal miR-222-3p enhances recipient lung cancer cell resistance to gemcitabine and malignant phenotypes by activating the suppressor of cytokine signaling 3 (SOCS3)/Stat3 signaling pathway. MiR-32-5p is delivered from HCC multidrug-resistant cells to sensitive cells via exosomes and activates the PI3K/Akt pathway to induce multidrug resistance [28]. Exosomal delivery of miR-155-5p from paclitaxel-resistant GC cells promotes epithelial-mesenchymal transition (EMT) and chemoresistance phenotypes in sensitive cells [29]. Our group previously demonstrated that highly metastatic lymph node mouse hepatocarcinoma Hca-F cell-secreted exosomes accelerate the migration and invasion of minimally metastatic Hca-P cells and stimulate lymphatic endothelial cell (LEC) proliferation and lymphangiogenesis via horizontal transfer of CXC chemokine receptor-4 (CXCR4) [38].

MiR-501 was first shown to be upregulated in the HCC HepG2 cell line and HCC tissues [30]. Later, a series of studies, including those from our lab, showed that miR-501 functions as an oncogene in gastric, liver, lung, and cervical cancer [30–35]. However, a recently published study found that high miR-501 levels predict a better prognosis in early-stage pancreatic ductal adenocarcinoma (PDAC). The authors of the study suggest that the effect may be due to the small sample size [46]. Nevertheless, the functions of exosomal miR-501 in drug resistance and tumor progression have yet to be revealed.

In this study, we show that SGC7901/ADR-secreted exosomal miR-501 is taken up by recipient cells. MiR-501 knockdown or BLID overexpression in recipient cells neutralizes the effects of ADR Exo on doxorubicin resistance, cell growth and invasion *in vitro* and *in vivo*, and exosomal miR-501 functions possibly through targeting BLID. The BLID gene is located at 11q24.1 [44,47]. Chromosome 11q has been found to be related to multiple diseases and developmental abnormalities [48]. Importantly, loss of heterozygosity (LOH) of 11q has been shown to be involved in the development and progression of GC [49–51]. Climent et al. [52] also reported that chromosome 11q deletion can predict the response to anthracycline-based chemotherapy in early breast cancer. The BLID protein, which contains a BH3-like domain, directly binds to Bcl-X_L and subsequently activates Bax to increase cytosolic cytochrome *c*, eventually leading to caspase-dependent mitochondrial cell death [44,53]. Li et al. [45] revealed that BLID inhibits the migration and

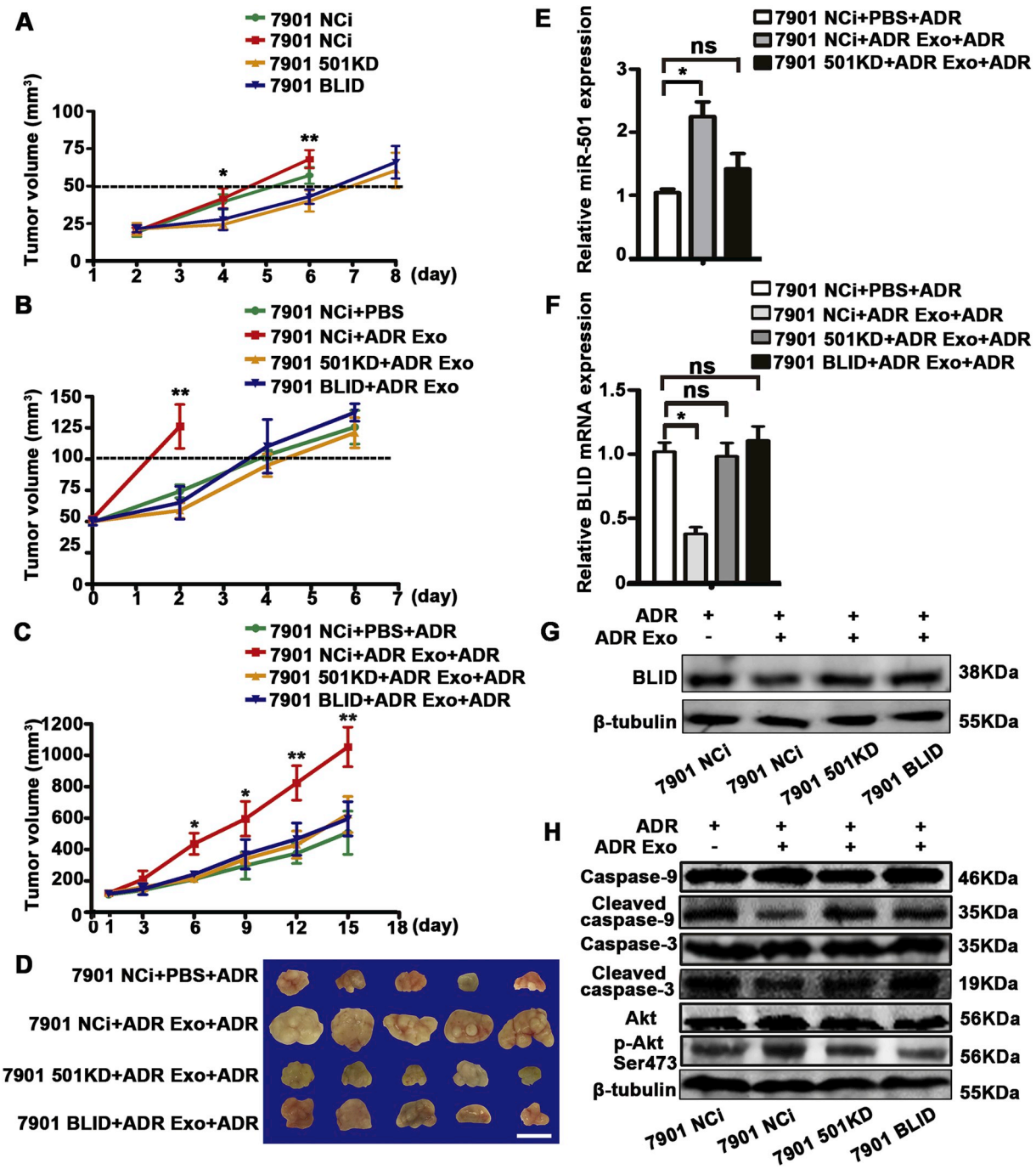

Fig. 6. Exosomal miR-501 induces resistance to doxorubicin and enhances the carcinogenesis of gastric cancer cells *in vivo*. **(A)** 7901 NCi, 7901 501KD and 7901 BLID cells were injected subcutaneously into nude mice. The tumor growth of the 7901 NCi group was faster than that of the 7901 501KD and 7901 BLID groups. **(B)** When the tumor sizes reached approximately 50 mm^3, 50 μg of ADR Exo was intratumorally injected. The 7901 NCi + ADR Exo group tumors grew faster than the 7901 NCi + PBS group tumors. No marked difference was seen among the 7901 NCi + PBS, 7901 501KD + ADR Exo and 7901 BLID + ADR Exo groups. **(C–D)** When the size of xenograft tumors was approximately 100 mm^3, ADR was injected through the tail vein. The tumor volumes were increased in the 7901 NCi + ADR Exo group, and no obvious difference was seen among the 7901 NCi + PBS, 7901 501KD + ADR Exo and 7901 BLID + ADR Exo groups. Images of xenograft tumors are shown. Scale bar = 10 mm. **(E)** The expression of miR-501 was higher in 7901 NCi + ADR Exo + ADR cells than in 7901 NCi + PBS + ADR cells. There was no significant difference between 7901 501KD + ADR Exo + ADR and 7901 NCi + PBS + ADR cells. **(F–G)** The levels of BLID mRNA and protein were downregulated in the 7901 NCi + ADR Exo + ADR group as assessed by qRT-PCR (F) and Western blot (G) in mouse xenograft tumor tissues compared to those in the 7901 NCi + PBS + ADR group. No significant difference was found in the 7901 501KD + ADR Exo + ADR and 7901 BLID + ADR Exo + ADR groups compared with the 7901 NCi + PBS + ADR group. **(H)** Western blot analysis of cleaved caspase-9, caspase-3, and p-Akt Ser 473 in xenograft tumor tissues. The protein levels of cleaved caspase-9 and caspase-3 were notably decreased, and the phosphorylation of Akt was significantly increased in the 7901 NCi + ADR Exo + ADR group. No difference was displayed among 7901 NCi + PBS + ADR, 7901 501KD + ADR Exo + ADR and 7901 BLID + ADR Exo + ADR groups. The experimental results are presented as the mean ± SD. $^*P < 0.05$ and $^{**}P < 0.01$.

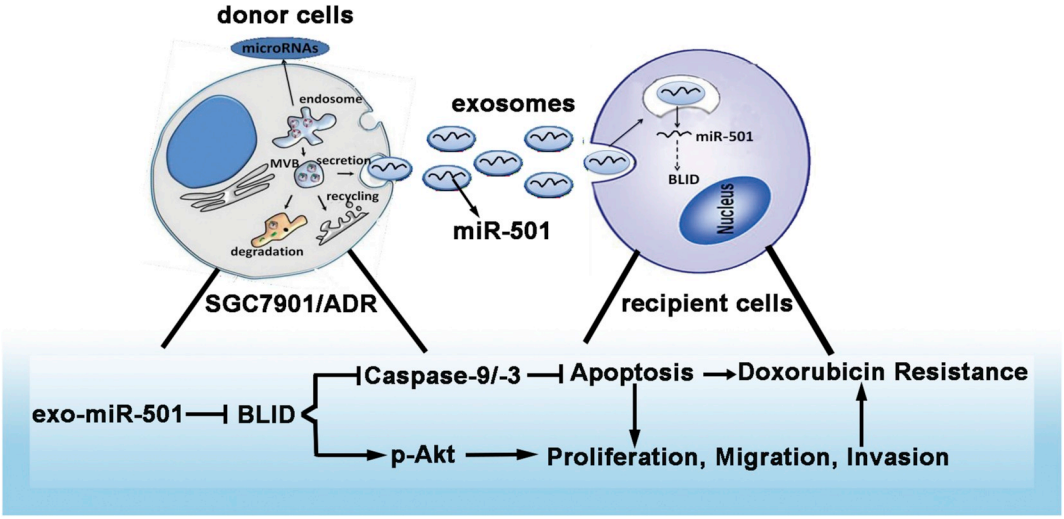

Fig. 7. Schematic diagram of the potential roles of exosomal miR-501 in gastric cancer. The doxorubicin-resistant SGC7901/ADR cell-secreted exosomes containing miR-501 can be taken up by the sensitive cells. Exosomal miR-501 (exo-miR-501) suppresses apoptosis by downregulating BLID (an apoptosis inducer) and subsequent inactivation of caspase-9/-3 and phosphorylation of Akt, resulting in enhanced proliferation, migration, invasion and doxorubicin resistance of the recipient cells.

invasion of breast cancer cells through phosphorylation of Akt-mediated MMP-2 and MMP-9 signaling. A BLID decease has been verified in breast and lung cancer tissues, and its downregulation is related to poor disease-free and overall survival in breast cancer [53]. Hence, the BLID gene may be a novel tumor suppressor [54]. Herein, we confirmed that SGC7901/ADR cell-released exosomal miR-501 suppresses apoptosis to enhance doxorubicin resistance in recipient cells by downregulating BLID and inactivating downstream caspase-9/-3. The enhanced proliferation and invasion of recipient cells caused by the exosomal transfer of miR-501 is related to the phosphorylation of Akt after downregulation of BLID. Akt has been reported to be dephosphorylated at Thr 308 and Ser 473 sites by protein phosphatase 2A (PP2A) and PH domain leucine-rich repeat phosphatase (PHLPP), respectively [55–57]. BLID binding to molecules, including PP2A and PHLPP, may result in the dephosphorylation of Akt; however, this hypothesis still needs to be verified.

Santos et al. [58] reported that exosomes are secreted from breast cancer stem cells and doxorubicin and paclitaxel-resistant breast cells at higher rates than from parental cells. Fang et al. [59] found that more exosomes are secreted from liver cancer cells with high metastatic potential than from those with low metastatic potential. Our results are consistent with the above findings that SGC7901/ADR cells release more exosomes than SGC7901 cells release. We therefore expect that highly malignant cancer cells with drug resistance and metastatic abilities may have the potential to produce increased numbers of exosomes and may facilitate the progression of cancer cells that were previously sensitive and non-aggressive through exosomal transfer. Dorayappan et al. [60] demonstrated that hypoxic conditions induce STAT upregulation of Rab27a, a regulator of vesicle exocytosis, and downregulation of Rab7, a mediator of lysosomal endocytic degradation, to promote exosome release in ovarian cancer cells, in combination with a more secretory lysosomal phenotype. The increased exosome release in drug-resistant cancer cells identified herein might be correlated with changes in Rab proteins and the lysosomal phenotype under the stress of chemotherapy. In addition to being vehicles for biomolecules, exosomes have been found to be responsible for drug efflux, resulting in decreased cytotoxicity of chemotherapeutic drugs [60–62]. Whether the above mechanisms is relevant to exosomal miR-501-induced doxorubicin resistance remains to be confirmed.

We found that both SGC7901/ADR cells and their corresponding exosomes are enriched in endogenous miR-501 relative to SGC7901 cells and their corresponding exosomes, respectively. SGC7901/ADR cell-secreted exosomes can be internalized by recipient cells and retain their functions, suggesting that miR-501 is possibly suitable for packaging into exosomes to maintain stability and plays an important role in drug resistance and tumorigenesis [25]. Given the notion that exosomes exist in a variety of bodily fluids [63–65], and recent studies have demonstrated that cancer cell-secreted exo-miRNAs are found in blood, urine, breast milk and saliva [66,67], exosomal miR-501 in body fluids such as serum may have the potential to be used as a diagnostic biomarker for the detection of GC. Our group is collecting the serum of gastric cancer patients to detect the exosomal miR-501 expression levels and determine its clinicopathological significance.

Taken together, our current study results demonstrate that doxorubicin resistance and carcinogenesis can be induced by the horizontal transfer of exosomal miR-501 via the suppression of BLID in GC. Exosomal miR-501 might serve as a potential diagnostic biomarker and a novel therapeutic target for GC.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.05.035.

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