Original Article
Colorectal cancer cell-derived exosomes promote proliferation and decrease apoptosis by activating the ERK pathway

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Abstract: Exosomes are small microvesicles released by various cells that play important roles in *cell-cell communication*. Numerous studies show that colorectal cancer (CRC) cell-derived exosomes are involved in the progression of CRC. However, the specific ways and mechanisms of action have not yet been fully clarified. In the present study, we found that, compared to normal colon epithelial cell (NCM460) derived exosomes, CRC cell-Lovo derived exosomes (Lovo-exo) could be more easily taken up by Lovo cells, most likely because of their cell tropism. In addition, Lovo-exo promoted Lovo cell proliferation and inhibited apoptosis, which is associated with an increased activation of the extracellular signal-regulated protein kinase (ERK). Lovo-exo also dramatically increased Lovo tumor cell growth *in vivo*. Moreover, the intratumoral injection of GW4869 (an exosomes inhibitor) suppressed tumor growth. These results indicate that CRC cell Lovo-derived exosomes may be a messenger for the proliferation requirements of the originating cancer cells, and targeting tumor-derived exosomes may be very promising for tumor treatment.

Keywords: Colorectal cancer, exosomes, ERK, tumor growth

Introduction

Colorectal cancer (CRC) is a common digestive system malignancy and has become the second leading cause of cancer deaths both in the United States and in Europe [1]. Regardless of recent advancements in multidisciplinary therapeutic strategies, CRC recurrence and metastasis still constantly occur [2, 3]. Determining the relevant factors involved in disease progression may aid in the development of novel strategies for effective therapies against CRC.

Exosomes are extracellular vesicles secreted by various cell types. These endogenous vehicles, ranging from 30 to 200 nm in diameter, are known to exist in many body fluids, such as plasma, cerebrospinal fluid and urine [4, 5]. Exosomes could fuse with the *plasma membranes* of recipient cells, thereby regulating the function of the target cells and affecting various biological functions [6, 7]. Some scientists even call exosomes "communicasomes"

because of their important roles in cell-cell communication [8]. Even more, exosomes have also been extensively studied as a novel cell-free drug or gene carrier [9]. Recently, an increasing number of studies have revealed that many tumor cells could secrete exosomes, including lung cancer [10], liver cancer [11], CRC, and so on, and these vehicles play a critical role in tumor progression.

CRC derived exosomes are able to increase the proliferation of endothelial cells, which could stimulate the development of tumor vasculature [12]. They can also induce a tumor-like behavior in colonic mesenchymal stem cells [13]. In addition, exosomes from CRC cells are able to regulate immune cells [14]. They carefully decorate the microenvironment for tumor growth and the effect of exosomes on cells in the tumor microenvironment has also become the focus of cancer research. Recently, some studies found that exosomes are more efficiently taken up by cells similar to the parent

cells than other cells [15, 16]. In addition, information exchange between tumor and tumor cells is also an important condition for cancer progression, and, as a communicator, cancer cell-derived exosomes may be involved in the communication between tumor and tumor cells [17]. Matsumoto et al. [18] found that after B16BL6-derived exosomes were intratumorally injected in mice, most exosomes were taken up by B16BL6 cells, and tumor growth significantly increased. Another study showed that incubation Hep3B cells with Hep3B-derived exosomes increased the number of Hep3B colonies in soft agar [19]. These studies indicated that cancer-derived exosomes may affect the behavior of the same kind of tumor cells and influence tumor progression. Consequently, we are curious to know whether CRC-derived exosomes could affect CRC itself.

In this study, we found that Lovo-derived exosomes (Lovo-exo) could be taken up by Lovo cells, and that these vesicles promoted Lovo cell proliferation and decreased apoptosis. In addition, animal experiments showed that Lovo-exo could promote Lovo tumor growth possibly by the activation of the extracellular signal-regulated protein kinase (ERK) pathway. Moreover, GW4869 inhibited Lovo growth in nude mice. CRC Lovo cell-derived exosomes may promote the growth of Lovo cells themselves, and targeting tumor cell-derived exosomes may provide a new direction for tumor treatment.

Materials and methods

Ethics statement

In present study, all procedures were approved by the ethics committee of the Nanjing Tongren Hospital (Nanjing, China).

Cell culture

The colon cancer cell lines Lovo and the normal colon epithelial cells NCM460 were purchased from ATCC. The cells were cultured in DMEM medium (Gibco, Grand Island, NY) and supplemented with 10% fetal calf serum and 1% penicillin-streptomycin (Gibco) at 37°C in 5% CO_2.

Exosomes collection

Lovo and NCM460 cells were cultured until reaching approximately 80% confluence in the culture dishes, and then they were washed twice with PBS and reincubated with a serum-free culture medium. After 24 h, the cell culture supernatants were collected and centrifuged at 500 g for 5 min, 2000 g for 20 min, followed by ultracentrifugation at 100,000 g in an Optima L-100XP ultracentrifuge (Beckman, Fullerton, CA) for 90 min at 4°C. Then, the pellets were gathered and washed in PBS, subjected to a second ultracentrifugation, and resuspended in PBS. The particle size distribution of Lovo-exo was measured using Nanosight NS 300 (Malvern Instruments Ltd., Worcestershire, UK). The quantification of Lovo-exo protein content was measured using a bicinchoninic acid protein assay (BCA) kit (Beyotime, Nantong, China). The Lovo-exo aliquots were stored at -80°C until needed. The "μg/mL" in this study means the protein concentration of Lovo-exo.

Transmission electron microscopy

The purified exosomes were fixed with 4% paraformaldehyde (PFA) and 4% glutaraldehyde in PBS at 25°C. Then, the fixed exosomes were dropped onto a carboncoated copper grid and immersed in a 2% phosphotungstic acid solution for 40 s. The grid was detected using a transmission electron microscope (JEM-1010; JEOL Ltd., Tokyo, Japan).

Uptake of exosomes by Lovo cells

The NCM460-exo and Lovo-exo were labeled with DIO (green), and the excess dye was removed using ultracentrifugation, and then washed. After the DIO-labeled exosomes were incubated with Lovo or NCM460 cells for 2 h, they were fixed by 4% PFA and then permeabilized with Triton-X 100. The cell nuclei were stained using DAPI. All reagents were purchased from Invitrogen (Carlsbad, CA). Images of the Lovo-exo uptake were then made using a Nikon Eclipse Ti confocal laser scanning microscope.

Cell proliferation assay

The Lovo cells were seeded in 96-well plates (5 × 10^3 cells per well) and cultured overnight. Then the medium was replaced with 100 μL of serum-free medium in the absence or presence of various concentrations of the Lovo-exo for 24 h [6]. Next, 10 μL of CCK-8 was added to each well, and the cells were incubated for 1.5 h. Finally, the absorbance of the cells in each

well was measured at 450 nm using a micro-plate reader (Synergy HT, BioTek, Biotek Wino-ooski, VT). A culture medium without cells was used as a blank control.

The Lovo cells were seeded in 96-well plates (5 × 10^3 cells per well) and cultured overnight. Then, the medium was replaced with 100 μL of serum-free medium in the absence or presence of various concentrations of the Lovo-exo plus with/without U0126 (10 μM; Sigma-Aldrich, St. Louis, MO) for 24 h. Cell proliferation was measured using a Brdu cell proliferation assay kit (BioVision, Milpitas, CA), according to the manufacturer's instructions.

Cell apoptosis assay

The Lovo cells were seeded in 6-well plates overnight, washed, and then stimulated by Lovo-exo plus with/without U0126 (10 μM) for 24 h in a serum-free medium [6, 18]. Cell apoptosis was evaluated by staining with fluorescein isothiocyanate-labeled Annexin V and 7-amino-actinomycin D (BD Biosciences, San Jose, CA) according to the manufacturer's manual. The percentage of apoptotic cells was measured using a FACSCalibur flow cytometer.

Western blotting

Lovo cells treated by Lovo-exo were collected in a RIPA buffer containing PMSF and quantified using a BCA protein Assay kit (Beyotime). Protein extracts (50 μg) were electrophoresed and transferred to polyvinylidene fluoride membrane. Immuno-blotting was performed by incubating each membrane with an anti-human HSP70, TSG101, Flotillin-1, Calreticulin, phosphorylated-MEK (p-MEK), GAPDH, T-ERK, p-ERK at 4°C. Then, the membranes were washed in PBS/T, and each membrane was incubated for 1 h with a secondary antibody conjugated by peroxidase at room temperature. The antibodies were all purchased from Abcam (Cambridge, MA). The band was detected by the use of enhanced chemi-luminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

Inhibition of exosomes secretion

5 × 10^6 Lovo cells were seed in a 10-cm dish and were incubated for 24. Then, the cells were incubated with or without 10 μM GW4869 (dis-solved in 0.5% DMSO; Sigma) for 24 h. Exosomes were collected, and the amount of the generated exosomes was determined by measuring the protein concentration. The levels of HSP70 and TSG101 were measured by performing western blotting, as described previously.

Animal studies

Four-week old female BALB/c nude mice (Laboratory Animal Center of Nanjing Medical University, Nanjing, China) were housed under specific-pathogen-free conditions.

The mice received subcutaneous injections of 0.2 mL PBS per mouse containing 3 × 10^6 Lovo cells. After 3 days, the mice were randomly divided into 2 groups (n = 3 each), which were intratumorally injected with 100 ul of either Lovo-exo (10 μg exosomes protein) or PBS every three days, a process described in previous studies [18]. Macroscopic tumor formation was determined at day 15 after the Lovo-exo injection.

The mice were subcutaneously inoculated with 3 × 10^6 Lovo cells. GW4869 (1 μg) or a vehicle (0.5% DMSO) was intraturmorally injected in into the nude mice (n = 3 each group) respectively every three days (day 6, day 9, and day 12). The tumor sizes were determined with calipers at 15 days.

The tumor volumes were examined with the following formula: (length × width^2)/2.

Immunohistochemistry

Briefly, the tumor tissue slides were incubated with an antibody to human Ki-67 (Abcam), followed by an HPR-conjugated secondary antibody, and DAB was used as the substrate. A negative control was performed by replacing the primary antibodies. The nuclei were counterstained with Harris's hematoxylin.

Cell apoptosis was investigated using a terminal TUNEL assay and using an In Situ Cell Death Detection Kit (Roche, Basel, Switzerland) according to the manufacturer's manual.

Statistical analysis

The statistical analyses were performed with GraphPad Prism (Version 5.0; La Jolla, CA). The
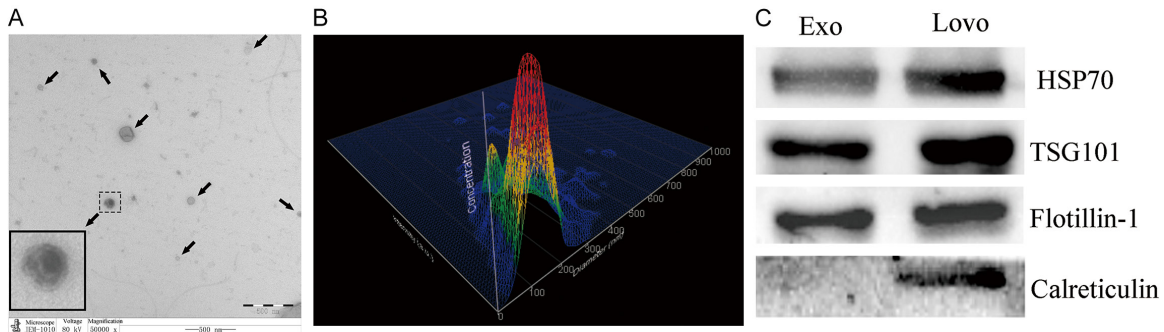
Figure 1. Identification of Lovo cell-derived exosomes. A. Transmission electron microscopic images of Lovo-exosomes. The scale bars indicate 500 nm, and the arrows indicate typical Lovo-exo. B. The size distribution of the Lovo-exosomes was examined using nanoparticle trafficking analysis. C. The positive marker for exosomes, HSP70, TSG101, Flotillin-1, were detected in Lovo-exo using western blotting, but the negative marker calreticulin was not detected.

data are expressed as the means ± SD. Statistical significance was determined using the Mann-Whitney test or a one-way analysis of variance, and *P* < 0.05 was considered statistically significant.

Results

Isolation and characterization of CRC cancer Lovo cell-derived exosomes

We first extracted exosomes from the culture supernatant of Lovo cells by ultrahigh-speed centrifugation. Transmission electron microscopy (TEM) revealed that the Lovo-exo displayed a cup-like vesicular structure (**Figure 1A**). Nanoparticle tracking analysis (NTA) confirmed that the Lovo-exo were approximately 170 nm in size (**Figure 1B**). Western blotting showed that the exosomes expressed the *70-kilodalton heat shock protein* (HSP70), tumor susceptibility gene 101 (TSG101) and flotillin-1. However, no calreticulin expression was detected (**Figure 1C**).

Lovo-exo promoted the growth of colon cancer cells in vitro and in vivo

Compared to NCM460 derived exsomes, Lovo-exo could be taken up more easily by the Lovo cells. In contrast, NCM460-exo is easier to be taken up by the NCM460 cells than Lovo (**Figure 2A**). In addition, compared with NCM460-exo, Lovo-exo significantly promoted the cell viability of the Lovo cells (**Figure 2B**).

The proliferation and apoptosis of tumor cells play important roles in cancer progression. Therefore, we next examined the effect of Lovo-

exo on the proliferation and apoptosis of CRC cells. *BrdU* assays showed that Lovo-exo significantly promoted the proliferation of Lovo cells in a dose-dependent manner (**Figure 2C**). In addition, our results indicated that the Lovo-exo dramatically inhibited Lovo cell apoptosis (**Figure 2D**, **2E**).

To further evaluate the role of Lovo-exo in CRC growth, we injected Lovo cells into BALB/c nude mice to establish subcutaneous xenograft tumor models and treated the mice with Lovo-exo or phosphate-buffered saline (PBS), as done in previous studies [18]. Compared with the PBS group, Lovo-exo significantly promoted tumor growth (**Figure 3A-C**). In addition, an immunohistochemical examination showed that the expression of ki-67 was significantly increased in the Lovo-exo group (**Figure 3D**, **3F**). Meanwhile, fewer terminal transferase-mediated dUTP nick-end labeling (TUNEL)-positive cells were observed in the tumor sections from the Lovo treated group (**Figure 3E**, **3G**).

ERK signaling was partly involved in the Lovo-exo induced Lovo cells proliferation and apoptosis

ERK signaling is related to CRC proliferation and apoptosis [20-22], and the exosomes are able to modify the cancer cells by regulating ERK signaling [23]. Lovo cells, exposed up to 24 h to exosomes showed a dose-dependent increased proliferation and decreased apoptosis compared with the controls. Therefore, we wanted to know whether the ERK signaling was changed in the Lovo-exo treatment group at 24
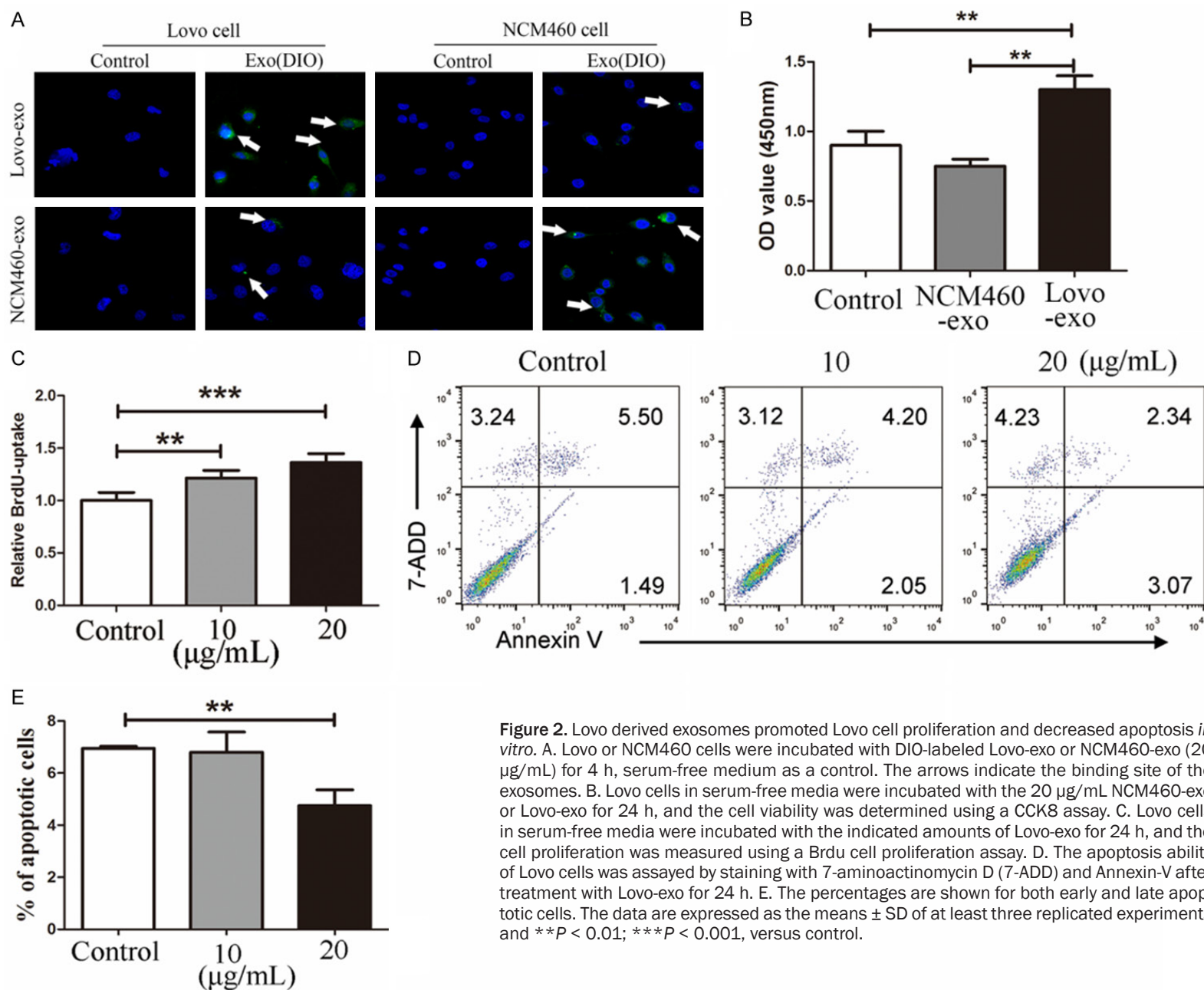
Figure 2. Lovo derived exosomes promoted Lovo cell proliferation and decreased apoptosis *in vitro*. A. Lovo or NCM460 cells were incubated with DIO-labeled Lovo-exo or NCM460-exo (20 μg/mL) for 4 h, serum-free medium as a control. The arrows indicate the binding site of the exosomes. B. Lovo cells in serum-free media were incubated with the 20 μg/mL NCM460-exo or Lovo-exo for 24 h, and the cell viability was determined using a CCK8 assay. C. Lovo cells in serum-free media were incubated with the indicated amounts of Lovo-exo for 24 h, and the cell proliferation was measured using a Brdu cell proliferation assay. D. The apoptosis ability of Lovo cells was assayed by staining with 7-aminoactinomycin D (7-ADD) and Annexin-V after treatment with Lovo-exo for 24 h. E. The percentages are shown for both early and late apoptotic cells. The data are expressed as the means ± SD of at least three replicated experiments and **$P < 0.01$; ***$P < 0.001$, versus control.

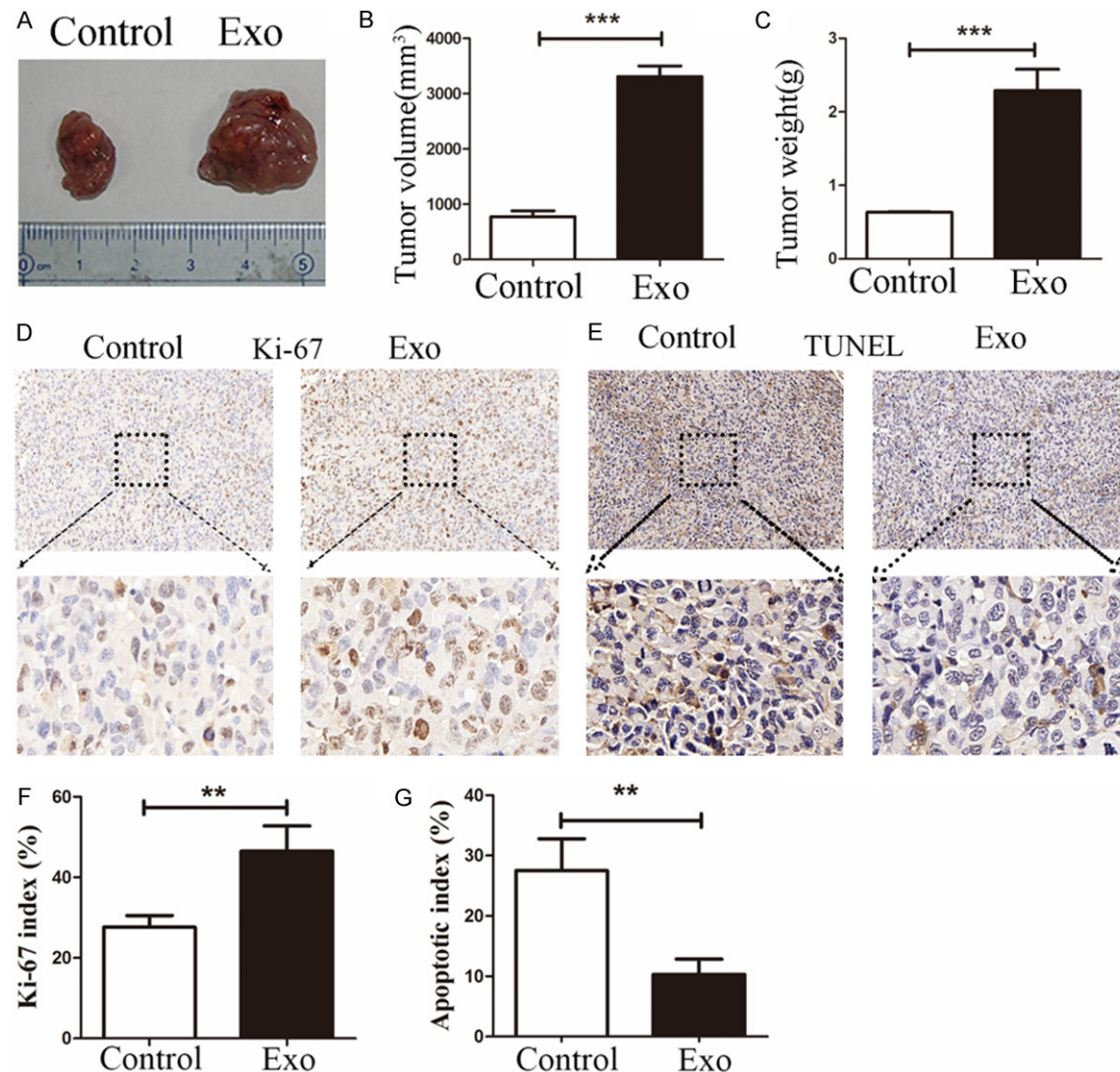**Figure 3.** Lovo-exo promoted the growth of colon cancer cells *in vivo. (*A) Nude mice were subcutaneously inoculated with Lovo cells (3×10^6). The figure shows tumors obtained from the sacrificed mice at day 15 after the subcutaneous injection of Lovo-exo or PBS. (B) Measurements of tumor volumes and (C) tumor weights (n = 3). (D) Ki-67 and (E) TUNEL immunohistochemical staining on tumor sections from the control or Lovo-exo stimulated group. The images are shown at 100 × (above panel) and 400 × (below panel). (F) The percentage of the Ki-67 index and (G) the apoptotic index were measured. The data are expressed as the means ± SD of three mice and $**P < 0.01$; $***P < 0.001$, versus control.

h. As shown in **Figure 4A**, Lovo-exo treatment triggered a robust increase in the phosphorylation levels of MEK (p-MEK) and ERK (p-ERK) in a dose dependent manner, but the expression of total ERK (T-ERK) did not change. In addition, an increase in p-MEK and p-ERK could be observed in the tumor biopsies from the mice treated with exosomes compared with the control mice (**Figure 4A**).

Next, we used the ERK inhibitor U0126 (10 µM) to specifically block the MEK/ERK signal in the Lovo cells. As shown in **Figure 4B**, the Lovo-exo-

mediated high expression of p-MEK, p-ERK was impaired after being treating with U0126. In addition, the Lovo-Exo-induced apoptosis suppression effect also significantly diminished (**Figure 4C**, **4D**). In addition, our results also showed that the Lovo-exo induced cell proliferation was dramatically diminished but not totally abolished by treatment with U0126 (**Figure 4E**).

Effects of GW4869 on tumor growth

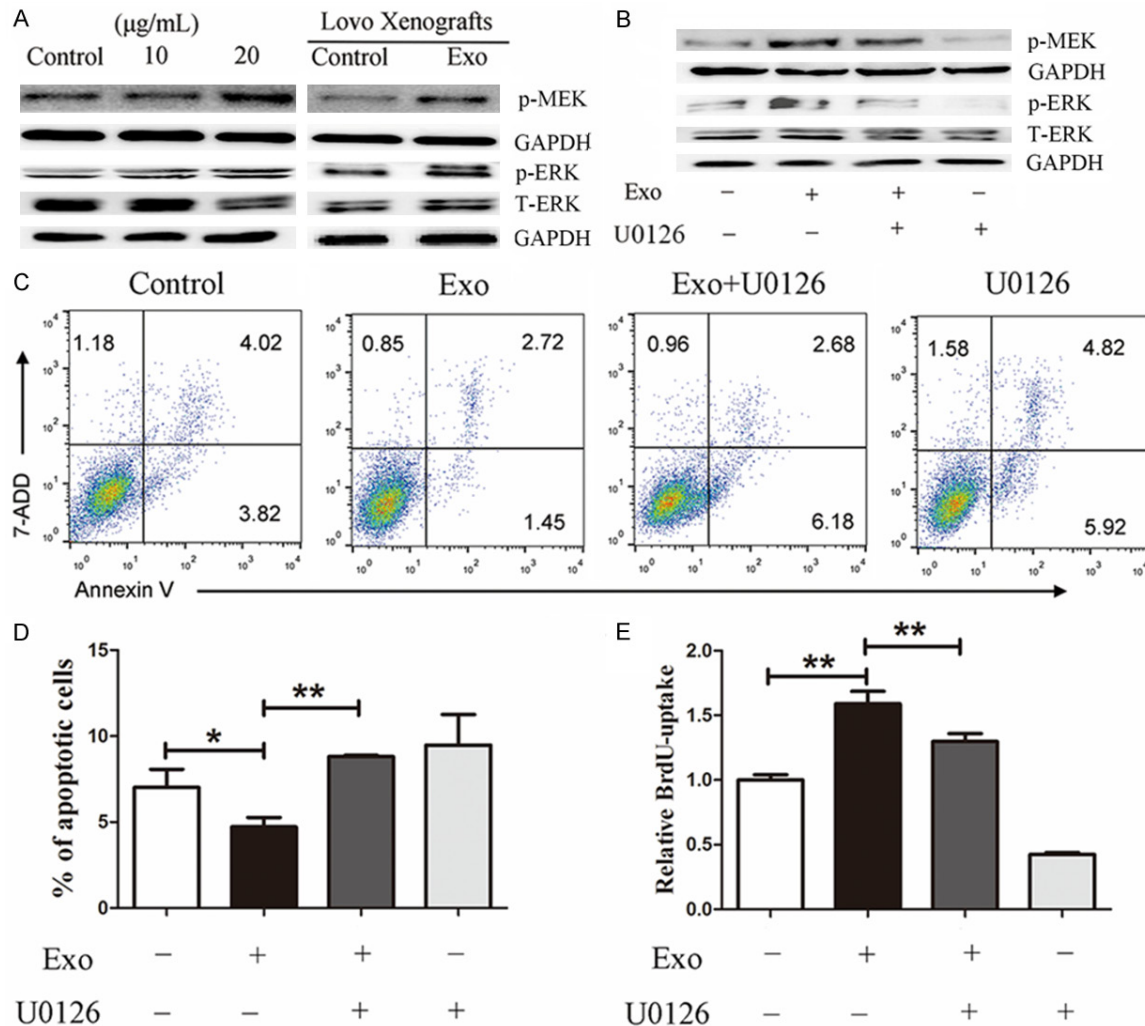We further investigated whether inhibiting exosomes release from tumor cells would sup-

Figure 4. U0126 impaired Lovo-exo-induced cell proliferation and anit-apopotisis. A. p-MEK, p-ERK, T-ERK, and GAPDH proteins in the Lovo cells and Lovo xenografts were analyzed by western blotting. B-E. Lovo cells were treated with/without 10 μM U0126 plus exosomes for 24 h; B. p-MEK, p-ERK T-ERK and GAPDH protein were analyzed by western blotting. C. FACS analysis of Lovo cell apoptosis. D. The percentages are shown for both early and late apoptotic cells. E. Lovo cell proliferation was evaluated by Brdu assay. The data are expressed as the means ± SD of three replicates and *$P < 0.05$; **$P < 0.01$, versus control group.

press tumor growth. GW4869 is a well-recognized exosome inhibitor [24, 25]. Our result confirmed that the expressions of the exosome marker proteins HSP70 and TSG101 in exosomes derived from Lovo cells pre-stimulated by GW4869 were dramatically decreased (**Figure 5A**). Moreover, we found that, compared with the DMSO group, GW4869 remarkably inhibited Lovo growth (**Figure 5B-D**), suggesting that inhibiting the release of tumor exosomes may suppress tumor growth.

Discussion

Exosomes were first discovered in 1980 and were once considered waste products excreted by cells [26]. At present, an increasing number of studies demonstrate that exosomes are a class of extremely important substances for cellular information delivery [4]. It has been discovered that exosomes take part in multiple biological processes, including signal transduction, immune modulation and so on. Moreover, some scholars have pointed out that tumor-derived exosomes are involved in tumor progression [17, 27]. We support this view, as we found that Lovo derived exosomes (Lovo-exo) dramatically increased Lovo tumor cell growth.

In recent years, the exosomes of colorectal cancer (CRC), to some extent, have been studied. They have certain regulatory effects on
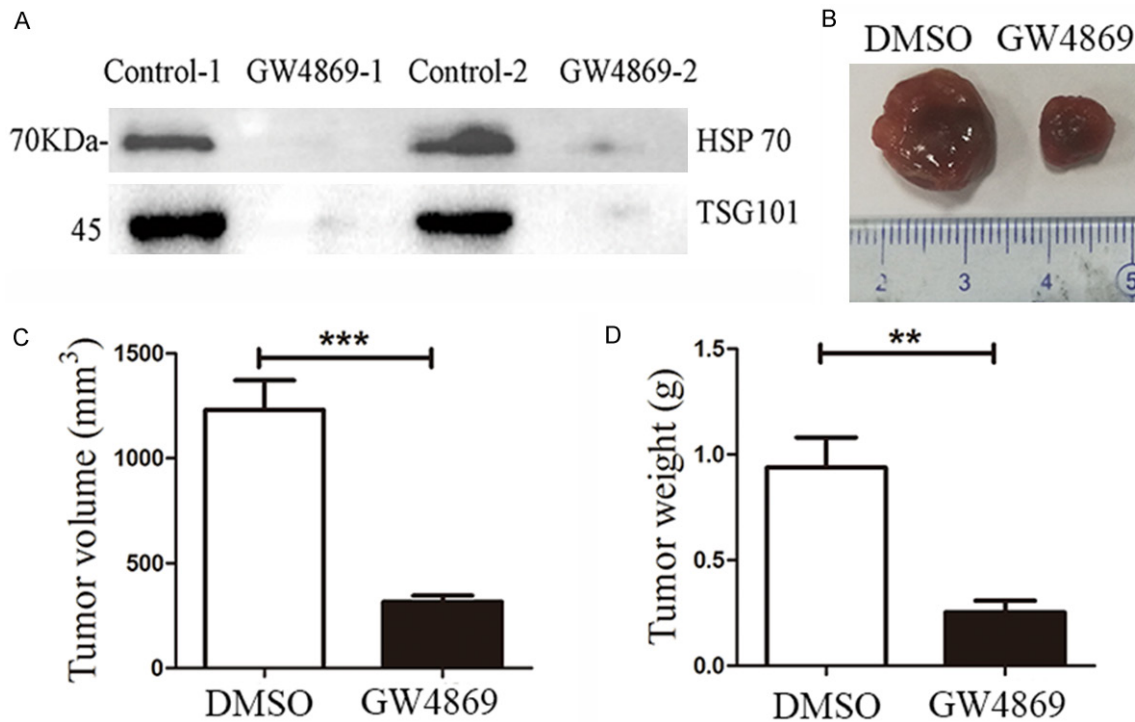
Figure 5. Effect of GW4869 on tumor growth. (A) HSP70 and TSG101 proteins in exosomes from Lovo cell pre-stimulated with/without GW4869 were analyzed by western blotting. (B) Imaging of tumors from the GW4869 and control groups at 15 days. (C) The tumor volumes were measured, and (D) the tumor weights were determined at 15 days (n = 3). The data are expressed as the means ± SD of three mice and **$P < 0.01$; ***$P < 0.001$, versus DMSO group.

a variety of cells in the tumor microenvironment, including vascular endothelial cells [12], immune cells [28], and mesenchymal stem cells (MSCs) [13]. As in previous studies [18, 19, 29], we found that Lovo-exo significantly increased the proliferation and decreased the apoptosis of Lovo cells, suggesting that tumor exosomes may affect their originating cancer cells, which might be helpful or essential for tumor progression. However, Mulvey et al. [30] found that there was no significant change in the number of HCT116 colonies formed after HCT116-derived extracellular vesicles were cocultured with HCT116 cells; in other words, HCT1116-derived extracellular vesicles may have no effect on HCT116's growth. The specificity of CRC cells themselves, the dosage of the exosomes or the different types of "vesicle" may have caused this difference. For example, Lopez-Verrilli et al. [31] found that the exosomes and microvesicles derived from MSC had different effects on neuronal growth. For all this, the specific reasons for these differences still need attention. Meanwhile, the roles of more kinds of CRC cell source exosomes on CRC itself needs further exploration.

Exosomes are more efficiently taken up by cells similar to the producing cells than other cells [15, 16]. Indeed, we found that, compared to normal colon epithelial cell (NCM460) derived exsomes, Lovo-exo could be taken up more easily by Lovo cells. However, additional studies on the uptake mechanisms of cancer cell-derived exosomes by their producing cells are required. In addition, some studies indicated that modified tumor exosomes might be employed as biological "Trojan horses" to suppress tumor cells [9, 32, 33]. However, the data obtained in our studies raised concerns regarding the use of Lovo exosomes as future therapeutic interventions for CRC.

The extracellular signal-regulated kinase (ERK) signaling pathway is a major determinant in the control of diverse tumor processes, such as proliferation and apoptosis [20-22]. In addition, increasing evidence has shown that exosomes are able to modify the cancer microenvironment by regulating the ERK signaling pathways in target cells [23]. In this study, we found Lovo-exo could dramatically increase p-MEK and p-ERK expression *in vitro* and *in vivo*, which

confirms what Raimondo and collaborators previously described in exosome-treated chronic myeloid leukemia cancer cell lines [34]. In addition, we further demonstrated that the ERK pathway indeed plays an important role in Lovo-exo-induced Lovo cell proliferation and anti-apoptosis by using the mek/ERK signaling inhibitor U0126. Kogure et al. [19] found that Hep3B-exosomes could modulate TAK1 signaling in recipient cells (Hep3B cells), but the exosome-mediated intercellular signaling may serve a maladaptive role to promote tumor growth. They thought TAK1 signal activation may be achieved by exosomes transferring miRNA to recipient cells. Unfortunately, in our study, it was not clear why the activation of the ERK signal pathway is affected. Meanwhile, our present results could not exclude the possibility that other substances in Lovo-exo, such as miRNAs, some proteins or lipids, may also play roles to support Lovo growth [35]. More evidence is needed to address this concern.

GW4869 is a potent, cell-permeable, specific, and non-competitive inhibitor of neutral sphingomyelinases. Exosome secretion is modulated by neutral sphingomyelinases and is inhibited by GW4869 [24, 25]. We treated cells with GW4869 (10 μM), which is considered to have no cytotoxic effect. Interestingly, we found that the GW4869 significantly inhibited Lovo growth. It can be seen that directly inhibiting the release of tumor-derived exosomes may bring some hope for the treatment of cancer. However, the routes of administration and the safety of GW4869 still need to be further evaluated. In addition, it is necessary to compare the effects of other exosome inhibitors (such as RAB27a) to determine which one is more effective in inhibiting cancer growth. Furthermore, it is also necessary to explore the secretion mechanism of tumor exosomes and find specific targets, perhaps bringing a new dawn for tumor treatment.

In summary, the present study lends support to the hypothesis of tumor-derived exosomes serving as a messenger for the growth requirements of the originating cancer cells. ERK signaling sustained activation plays a role in exosome-induced tumor cell growth. Inhibition of the release of CRC-lovo-derived exosomes significantly suppresses lovo tumor growth, suggesting that targeting the tumor-derived exo-somes may be conducive to the treatment of cancers.

Disclosure of conflict of interest

None.

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