Title: Eutopic stromal cells of endometriosis promote neuroangiogenesis via exosome pathway

Running title: Endometriosis-exosomes promote neuroangiogenesis.

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Abstract

Endometriosis is a common multifactorial gynecological disorder defined as the proliferation of endometrial tissue outside of the uterine cavity. Neuroangiogenesis (co-recruitment of nerves and blood vessels) is believed to play an integral part in the establishment and growth of endometriotic lesions. We hypothesized that exosomes derived from abnormal endometrium may serve as the second identifier of

endometriosis and play an important role in the development of endometriosis by regulating neuroangiogenesis. Primary human endometrial stromal cells (ESCs) were isolated from eutopic endometrium (EmESC, n=22) with endometriosis and normal endometrium (CoESC, n=6). Exosomes were isolated from ESCs using “standard” ultracentrifugation method, and the characterization of exosomes were identified through transmission electron microscopy, nanoparticle tracking analysis, and western blot. The role of exosomes in regulating neuroangiogenesis was determined through in vitro tube formation assay, neurite outgrowth assay, and dorsal root ganglion (DRG) neuron apoptosis analysis. The data showed that EmESCs could secrete exosomes with a diameter of approximately 100 nm and a biconcave morphological feature; they were internalized by human umbilical vein endothelial cells (HUVECs) and DRG neurons and enhanced neuroangiogenic effects. We further validated the role of exosomes through blocking experiments. We found that when the exosome secretion was blocked, the pro-neuroangiogenesis effects were decreased. In conclusion, these data suggested that exosomes may play a key role in endometriosis by promoting neuroangiogenesis.

Summary Sentence

Exosomes may play a key role in endometriosis by promoting neuroangiogenesis.

Keywords: endometriosis, exosomes, neuroangiogenesis, GW4869

Introduction

Endometriosis is a multifactorial gynecological disorder characterized as the growth of

endometrial glands and stroma outside the uterine cavity, affecting 6%–10% of women of reproductive age [1, 2]. Chronic pelvic pain and infertility, as the most predominant clinical features of endometriosis, influence women's quality of life, work productivity, and sexual relationship, and cause heavy economic burden [3]. However, the mechanisms of endometriosis are poorly understood. Thus, specific non-invasive diagnostic markers and therapies for patients with endometriosis still need to be studied.

Neuroangiogenesis, the interaction of nerve fibers and blood vessels, has been proposed as a critical process in the establishment of endometriosis [4]. Increasing bodies of evidence point toward the idea that angiogenesis, the growth of blood vessels from pre-existing blood vessels, plays an integral part in the establishment and growth of endometriotic lesions, which must be vascularized to attach and survive [5, 6]. Importantly, blood vessels need innervation to control vasodilation or constriction; they are innervated by sensory and sympathetic fibers. Studies have found that the endometriotic lesions are highly vascularized; moreover, increasing densities of nerve fibers have been detected [7, 8]. Many factors, such as vascular endothelial growth factor (VEGF), nerve growth factor (NGF), and netrins, that act on blood vessels also act on nerve fibers. Among these factors, VEGF and NGF are the most important regulators of angiogenesis and neurogenesis. They are also highly expressed in the eutopic and ectopic endometria in women with endometriosis. Numerous other factors regulate neuroangiogenesis in endometriosis. Greaves, et al. [9] demonstrated that

estrogen receptor agonists can regulate neuroangiogenesis in peritoneal endometriosis via the repellent factor SLIT3. Overall, all these data suggest that neuroangiogenesis plays a vital role in endometriosis.

Exosomes are membrane vesicles secreted by a multitude of cell types and derived from multivesicular bodies (MVBs) and are released into the extracellular environment after MVBs fuse with the plasma membrane [10]. As new players in cell–cell communication, exosomes can transfer proteins, lipids, and RNAs to recipient cells and facilitate diverse intracellular or intercellular signaling mechanisms to influence physiological pathways in recipient cells. Emerging bodies of evidence have shown the importance of exosomes in regulating neurogenesis and angiogenesis. Oh et al. [11] found that miR-193a facilitates neurogenesis in F11 cells by blocking proliferation-related target genes; studies conducted by Yang et al. [12] showed that miR-124-loaded exosomes ameliorate brain injury by promoting neurogenesis; moreover, a previous study [13] indicated that exosomes released by chronic myeloid leukemia cells promote angiogenesis in a src-dependent fashion. Another study [14] demonstrated that exosomes released by retinal pigment epithelium cells promote angiogenesis in endothelial cells, and oxidative stress can accelerate this process. All these data suggest that exosomes play a pivotal role in promoting neurogenesis and angiogenesis. However, their roles in endometriosis are unclear.

The pathogenesis of endometriosis is likely to be multifactorial and involve several or sequential cellular and biological interactions. Among the theories about endometriosis,

retrograde menstruation is the most accepted. Most women have some degree of retrograde menstruation, and some of them who have endometriosis are thought to have abnormal endometria, which is predisposed to establishment of ectopic disease. Hence, we speculate that abnormal exosomes that come from eutopic stromal cells of patients with endometriosis may serve as the second identification for endometriosis and play an important role in the endometriosis progression, especially in regulating the effect of neuroangiogenesis. Harp, et al. [15] demonstrated that exosomes derived from endometriotic stromal cells have enhanced angiogenic effects in vitro. In our study, we used the standard ultracentrifugation method, which is the most commonly used conventional approach for exosome isolation [16, 17] and further expand the characterization of exosomes secreted by eutopic stromal cell of endometriosis. We have made further progress in expanding the role of exosomes in pro-angiogenesis by doing blocking experiments. Likewise, the role of exosomes in pro-neurogenesis was also confirmed in our study.

Materials and methods

Human sample collection

This study was approved by the institutional review board of Qilu Hospital of Shandong University. Twenty-two women with ovarian endometriosis, as diagnosed by clinical pathologists at the Qilu Hospital of Shandong University, were recruited in this study (age range, 31–39 years; n=18, proliferative phase; n=4, secretory phase). The

stage of endometriosis was determined according to the revised American Society for Reproductive Medicine classification system. All patients in this study were in stage III or IV. Six women without endometriosis (age range, 29–36 years; n=6, proliferative phase) were included in our study; these six patients exhibited no uterine abnormalities, as confirmed via laparoscopic examination during tubal recanalization. All women recruited in this study have not undergone hormonal treatments or used intra-uterine contraceptive devices for at least 6 months. All women provided informed written consent before they were recruited. The endometrium tissues were collected at the time of laparoscopy and transferred to the laboratory on ice immediately.

Primary cell culture

We used a previously reported method to isolate endometrial stromal cells (ESCs) from the endometrium [18]. ESCs were isolated from all endometriosis patients and control patients. EmESCs were isolated from eutopic endometrium and CoESCs were isolated from normal endometrium. The samples were washed with phosphate-buffered saline (PBS) twice and dissected to small pieces. After the samples were dissected into 1 mm minces, they were digested with 0.25% (w/v) collagenase II mixed with 0.25% (w/v) collagenase IV (Worthington, Lakewood, USA) for 60 min at 37 °C. The samples digested with collagenase were passed through 100-μm aperture sieves to remove the debris and 40-μm aperture sieves in sequence to remove the epithelial cells. The isolated stromal cells resuspended in Dulbecco's modified Eagle's medium/F12 (Gibco, Beijing, China), which contained

10% (v/v) fetal bovine serum (FBS; Gibco), plated into a 150 cm^2 cell culture flask (Corning, USA), and cultured at 37 °C at 5% CO_2. The purity of ESCs was examined via immunostaining and flow cytometry (FCM) using the stromal marker vimentin (ab92547, Abcam). Only cultures that contained more than 95% vimentin-positive cells were included in our study.

Human umbilical vein endothelial cells (HUVECs) can be isolated from normal human umbilical veins. Approximately 10 cm normal human umbilical cord were put on a culture dish; after the largest vessel of the three blood vessel was found, two syringes were inserted to the vessel holes (Fig 2A). The veins were washed with warm PBS to remove the blood, and 6 ml of 0.25% (w/v) collagenase II mixed with 0.25% (w/v) collagenase IV (Worthington, Lakewood, USA) were pumped into the vein and incubated in a humidified atmosphere containing 5% CO_2 at 37 °C for 60 min. Collagenase solutions were centrifuged to obtain HUVECs. The cells were cultured in complete endothelial cell medium (ScienCell, USA) with 10% (v/v) FBS and endothelial cell growth supplement (ScienCell, USA). HUVECs were identified through CD31 immunofluorescent staining and flow cytometry (FCM) analysis.

Dorsal root ganglions (DRGs) were removed from 3–4 week old C57BL/6 female mice. The mice were killed via cervical dislocation, and the skin was incised at the dorsal medial line with the standard scissors and deflected laterally. Two long cuts were made closely left and right to the spinal column, and the tail and skull were removed. Lastly, the remaining paravertebral muscles were largely removed to expose

the spinal column. The cleaned spinal column was cut through the middle, and the spinal marrow is removed from the column. DRG is round in shape, has hyaline appearance. Thus, removing the neurofilaments in the DRGs taking process was very important. DRGs were placed into a 1.5-ml microcentrifuge tube containing 0.125% (w/v) collagenase IV and incubated for 1 h at 37 °C. The DRGs were removed from the incubator, and the sample was centrifuged for 5 min. The primary neurons were cultured in in a cover dish coated with poly-L-lysine hydrobromide (P1274, Sigma) and Laminin Mouse Protein (23017-015, Invitrogen) under 5% CO_2 atmosphere at 37 °C. The neurons were identified via the immunofluorescent staining of MAP2 and FCM analysis.

Isolation of exosomes from cell culture supernatants

Once 70% confluency was attained, ESCs were washed twice with PBS and then cultured in Dulbecco's modified Eagle medium/F12 (Gibco, Beijing, China) with 10% exosome-free FBS (EXO-FBS-250A-1, System Biosciences, USA) for 24 h. Exosomes were isolated according to previous studies with minor modifications [19]. First, the culture supernatants were centrifuged at 300 ×g for 10 min to remove the cells. Second, the supernatants were centrifuged at 2000 ×g for 10 min to remove the dead cells. Then, the supernatants were refrigerated at 4 °C overnight. The mixture was centrifuged at 10 000 ×g for 30 min to remove cell debris and at 100 000 ×g for 70 min to obtain the exosomes. Subsequently, exosomes were washed in PBS by centrifuging at 100 000 ×g at 4 °C for 70 min. The supernatant was poured, and the pellet was resuspended by

adding 100 µl PBS. The exosomes were stored at -80 °C. On average, 20 µg of purified exosomes was obtained from approximately 10 million cells.

Transmission electron microscopy (TEM)

Morphology of exosomes from three independent sample of EmESCs or CoESCs were detected using TEM. Fresh exosomes were placed on the surface of a carbon-coated electron microscopy grid. The sample was fixed with 3% glutaraldehyde for 5 min and washed in distilled water 10 times. The exosomes were stained with 4% uranyl acetate for 10 min, resuspended in 1% methyl cellulose aqueous solution for 5 min, and dried naturally for more than 30 min. The transmission electron microscopy samples were observed by using a JEOL 1200EX instrument.

Nanoparticle tracking analysis (NTA)

The exosome particle size and quantification were measured by using NTA with ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany) and the corresponding software ZetaView 8.04.02. Exosomes from three independent samples of EmESCs or CoESCs were detected. The isolated exosome samples were appropriately diluted with 100 µl PBS buffer to measure particle size and concentration. NTA measurement was recorded and analyzed at 11 positions. The ZetaView system was calibrated using 110 nm polystyrene particles. The temperature was maintained at approximately 23 °C and 37 °C.

Western blot

The proteins in the exosome preparations were examined using western blot analysis. Exosomes from three independent samples from EmESCs or CoESCs were detected. Exosomes (40 μg) were scraped and extracted using a commercial kit (BestBio, Shanghai, China), and the exosome samples were mixed with sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) loading buffer (P0015, Beyotime) and heated for 5 min at 95 °C. The protein samples were separated on 10% SDS-PAGE and transferred into a 0.22 μm polyvinylidene difluoride (PVDF) membrane (Millipore, MA, USA). Then, the PVDF membranes were blocked in 5% nonfat milk (BD, USA) and dissolved in Tris-buffered saline with Tween 20 (0.15 M NaCl, 0.05% Tween-20, 10 mM Tris–HCl [pH 8.0]) for 90 min at room temperature. Rabbit polyclonal anti-CD63 antibodies (1:100, 25682-1-AP, Proteintech Group) and TSG101 antibodies (1:100, ab125011, Abcam) were used for immunoblotting. After incubating in the primary antibody overnight at 4 °C, the PVDF membranes were incubated with horseradish peroxidase-labeled secondary antibodies for 60 min at room temperature. The signals were detected using Image Studia Digits Ver 4.0.

Immunofluorescent staining

Primary ESCs, HUVECs, and DRG neurons were identified via immunofluorescent staining. The cells were fixed in ice-cold methanol for 5 min, and the samples were washed twice with ice-cold PBS. The samples were incubated for 10 min in PBS containing 0.25% Triton X-100 and washed twice with PBS for 5 min, followed by 1%

bovine serum albumin (BSA) in phosphate-buffered saline with Tween-20 (PBST) for 30 min to block unspecific binding of the antibodies. The sample were incubated with anti-vimentin antibody (1:400, ab92547, Abcam), HUVECs with anti-CD31 antibody (1:500, ab28364, Abcam), and DRG neurons with MAP2 antibody (1:400, ab18207, Abcam) and diluted in 1% BSA in PBST in a humidified chamber overnight at 4 °C. After cells were washed with PBS twice for 5 min, they were incubated with the secondary antibody (1:500, ab150065, ab150068, Abcam) in 1% BSA for 1 hour at room temperature in the dark. The secondary antibody solution was decanted and washed with PBS twice for 5 min in the dark. Cells were incubated with 4′,6-diamidino-2-phenylindole (DAPI, ab104139, Abcam) for 1 min and finally maintained in the dark. To detect the direct transfer of exosomes into HUVECs and DRG neurons, ESC-derived exosomes were labeled using PKH67 Fluorescent Cell Linker Kit (Sigma-Aldrich, Saint Louis, MO). Purified exosomes resuspended in PBS were labelled with PKH67; and PBS without exosomes was used as control. The internalization was performed at 37 °C by incubating HUVECs and DRG neurons with exosomes. Then, the fluorescence signals were detected after 24 h.

Flow cytometry

The validation of ESCs, HUVECs, and DRG neurons and the internalization of exosomes by HUVECs and DRG neurons were detected via the flow cytometry analysis. The cells were incubated with anti-human vimentin (1:200, ab92547; Abcam), anti-human CD31 (1:100, ab28364, Abcam), and anti-mouse MAP2 (1:400,

ab18207, Abcam) for 30 min on ice. After centrifugation (300 ×g), the cells were incubated with the second antibody (1:500, ab150065, ab150068, Abcam) for 1 hour at room temperature in the dark. After washing with PBS, the cells were resuspended in PBS for FCM analysis. The internalization of exosomes was also detected via FCM, the purified exosomes resuspended in PBS were labelled with PKH67 and added to the HUVECs or DRG neurons culture supernatant. After incubating with exosomes for different time periods, HUVECs and DRG neurons were collected for FCM analysis.

In vitro tube formation assay

Endothelial cell tube formation is an in vitro method to measure angiogenic effects. To analyze the effect of ESC-derived exosomes on HUVEC tube formation, we stimulated HUVECs with EmESC-exosomes and CoESC-exosomes (10 μg/ml or 20 μg/ml) for 24 h. The doses of exosomes that we used were according to previous studies with minor modification [20, 21]. To further investigate the role of exosomes in pro-angiogenesis, the neutral sphingomyelinase (N-SMase) inhibitor GW4869 [22] to block ESCs that were secreting exosomes to measure the ability of releasing the exosomes to produce the angiogenic effect of ESCs. We prepared the conditioned medium from ~10^5 ESCs with or without GW4869 (10 μM). 96-Well plates and 1 ml tips were pre-chilled at -20 °C for 6 h. The growth factor-reduced Matrigel (Corning, USA) was thawed in a refrigerator for 1 day before use; 40 μl of growth-reduced Matrigel were aliquoted to each well in ice. Approximately 10^4 HUVECs were plated in one well resuspended in

the conditioned medium as described previously. Dimethyl sulfoxide (DMSO, 1‰, v/v) was used as the control group. After 12 h, HUVECs branching was examined under light contrast microscopy, and images were captured. Tube formation was assessed using Image J v1.8.0 (National Institutes of Health, USA).

Neurite outgrowth assay and flow cytometry for apoptosis of DRG neurons

To determine the effect of ESC-derived exosomes on neurite outgrowth, DRG neurons were placed into 24-well plates and stimulated by CoESC-exosomes and EmESC-exosomes (10 and 20 μg/ml) for 24 h. Exosome production was blocked using GW4869 at the concentration of 10 μM. Briefly, the upper chambers of cell culture inserts (0.3 μm in pore size and 24-well plate, Corning, USA) were added in 200 μl 10% FBS culture medium-containing ~10^5 ESCs with or without GW4869 (10 μM). The DMSO at same concentration with the treatment groups was used as the control group. The co-culture system was maintained at 37 °C at an atmosphere of 5% CO_2 for 24 h. To minimize individual variation, the DRG neurons derived from six mice were mixed. After co-culturing for 24 h, the neurite outgrowth length was measured via immunofluorescent staining and evaluated using Image J v1.8.0 (National Institutes of Health, USA). A series of three images was randomly selected from each sample and taken for each immunostaining parameter to obtain the mean. To explore whether ESC-derived exosomes can affect the DRG neuron apoptosis, DRG neurons were subjected to EmESC-exosomes. The percentage of apoptotic cells was assessed via FCM analysis of fluorescein isothiocyanate-annexin V/propidium iodide staining. The

staining procedure was performed according to the manual instruction.

Statistical analysis

GraphPad Prism 7 (GraphPad Software, Inc., USA) was used for statistical analysis, and the data were presented as mean ± standard deviation. All the experiments were repeated three times. Multiple group comparisons were performed using one-way ANOVA with post-hoc test or Kruskal–Wallis tests. $P<0.05$ indicated statistically significant differences.

Results

Identification of ESCs and characterization of ESC-derived exosomes

After collecting clinical specimens, endometrial cells were obtained via digestion. We observed the morphology of ESCs through microscopy and used immunofluorescent staining to identify ESCs. Vimentin was used to label ESCs in green (Fig. 1A). The purified ESCs were identified via flow cytometry (Fig. 1B). Similar morphology, size, and purity of EmESCs and CoESCs were observed. We collected the exosomes of ESC-culture supernatant via differential centrifugation. Their sizes and morphology were assessed through NTA and TEM (Fig. 1C and 1D). The size and morphology of EmESC-exosomes and CoESC-exosomes were found to have no measurable differences. They exhibited a biconcave morphological feature and a typical diameter of ~100 nm. The results of western blot analysis confirmed the proteins expected to be

present in the exosomes such as CD63 and TSG101 (Fig. 1E). We also blocked exosome formation by treating the ESCs with GW4869. GW4869 is a neutral sphingomyelinase inhibitor, which inhibits the ceramide-mediated inward budding of MVBs and the release of mature exosomes from MVBs; it is the most widely used pharmacological agent for blocking exosome generation. Following the drug treatment, the pellet contained significantly reduced exosomes as determined by NTA quantification and immunoblots (Fig 1E and 1F).

EmESCs secrete exosomes interacting with HUVECs and promote angiogenesis

We obtained HUVECs through collagenase digestion (Fig. 2A). The results of FCM and immunofluorescent staining showed that the purity of the cells reached nearly 100% (Fig. 2B and 2C). Immunofluorescent microscopy showed that EmESC-exosomes labeled with PKH67 (green) were internalized by HUVECs stained with CD31 (Fig. 2D). FCM analysis showed that with the passage of time (3, 6, 12, 24, and 30 hours), increasing number of exosomes were internalized by HUVECs and generally showed an S-shaped curve (Fig. 2E). These results suggested the crosstalk in ESCs and HUVECs via the exosome pathway. In the tube formation assay, we observed that the EmESC-exosome exerted a greater potential in pro-angiogenesis than CoESC-exosomes. When we doubled the dose of exosomes to 20 μg/ml, the role of exosomes in promoting angiogenesis was even more pronounced (Fig. 2F). We co-cultured HUVECs with EmESCs with or without blocking exosome secretion, and

the results showed that after blocking the exosome secretion, the pro-angiogenic effect was decreased (Fig. 2G). This in vitro functional assay demonstrated the critical role of EmESC-exosomes-mediated angiogenesis in endometriosis.

EmESCs secrete exosomes interacting with DRG neurons, induce neurite outgrowth, and inhibit neuron apoptosis

We obtained high-purity DRG neurons by improving the previous nerve cell culture methods (Fig. 3A). The DRG neurons were labeled with MAP2, analyzed by flow cytometry, and immunofluorescent staining. The results showed that their purity was found to be higher than 90% (Fig. 3B and 3C). The immunofluorescent staining showed that ESC-derived exosomes with green fluorescence were internalized by DRG neurons stained with MAP2 in red (Fig. 3D). DRG neurons were co-cultured with ESC-derived exosomes at different times and analyzed by flow cytometry (6, 12, 24, and 48 hours). As time progressed, the nerve cells swallowed more exosomes (Fig. 3E). Then, we first used the co-culture system to evaluate the ability of EmESC-exosomes to induce sensory neurite outgrowth. We found that EmESC-exosomes had greater potential in inducing neurite outgrowth than CoESC-exosomes. Likewise, the effect was more obvious when EmESC-exosomes were increased, we observed that 20 μg EmESC-exosomes increased the neurite outgrowth by approximately 2-fold compared with 10 μg EmESC-exosomes (Fig. 4A). Second, we used GW4869 to block exosome secretion, and we found a decrease in total neurite length (Fig. 4B). The flow cytometry

for apoptosis of DRG neurons also revealed that EmESC-exosomes inhibited DRG-derived primary sensory neuron apoptosis. (Fig. 4C). These findings indicated that EmESC likely promoted neurogenesis, and exosomes might be responsible for this phenomenon.

Discussion

In this study, we found that eutopic stromal cells from patients with endometriosis could secrete exosomes as confirmed by their morphological features, NTA analysis, and western blot for the biomarkers of exosomes. Exosomes could be taken up by HUVECs and DRG neurons. The co-culture system suggested that EmESC-derived exosomes promote tube formation, neurite outgrowth and inhibit neuron apoptosis. We also found that after blocking exosome secretion, the effects of pro-neuroangiogenesis were decreased. Our research suggests that eutopic stromal cells may participate in the formation of endometriotic lesions by promoting neuroangiogenesis via the exosome pathway.

The growth of blood vessels and nerve fibers are closely integrated processes linked by common pathways and molecules [23, 24]. Mukouyama, et al. [25] found that nerve fibers produce signals, such as vascular endothelial growth factor (VEGF), to guide blood vessels. Conversely, endothelial cells emit signals, such as artemin and neurotrophin, which can attract axons to the pioneer vessel [26, 27]. In endometriosis, both neurogenesis and angiogenesis can be activated together by the same cytokine.

NGF and VEGF are the most accepted key mediators of both angiogenesis and neurogenesis [28, 29]. Many studies have demonstrated that the peritoneal fluid contains elevated concentrations of different angiogenesis-promoting factors besides VEGF and NGF, including angiopoietin, IL-4, activin A, and IL-17A [30-33]. These efforts contribute to therapies for endometriosis to develop anti-angiogenic treatment strategies [34-37]. As lesions must be vascularized to survive, the blood vessels are presumably innervated by sensory and sympathetic fibers, as many studies demonstrated that nerve fibers are thought to sprout from perivascular and paravascular blood vessels as they vascularize growths [38, 39]. Our study found the pro-angiogenesis role of EmESC-exosomes, which is consistent to the previous study [15], and we also found the pro-neurogenesis role of EmESC-exosomes.

The exosomes were initially described as unnecessary compounds in the cell [40] . However, we now know that they are not wasted, but a communicator for component exchange between cells and act as signaling vehicles in normal homeostatic processes or as a consequence of pathological development. Exosomes can transfer proteins, lipids, and RNAs to regulate recipient cell functions and serve as mediators of intercellular communication [41]. Exosomes can be obtained noninvasively from all biological fluids, such as blood, saliva, urine, nasal secretions, breast milk, and cerebrospinal fluid [42]; thus, the increasing interest in exosomes has opened up the possibility to identify exosomes as biomarkers for various pathological states [43-46]. Exosomes induce a pro-tumoral microenvironment that promote tumor progression and

survival by promoting angiogenesis, thrombosis, and remodeling of the extracellular matrix. The MET oncoprotein and mutant KRAS have been reported in exosomes, and their uptake was induced by the hypoxic tumor microenvironment [47, 48]. As a benign gynecological disease, endometriosis is characterized by malignant tumors, including hypoxic conditions around the lesion; MET and KRAS also occur in endometriosis. An emerging application of exosomes is using them as vector for loading a particular cargo, which will be delivered to the target cells for modulation of cell functions in an in vivo context. Thus, exosomes hold a great potential for clinical applications. Several studies found that exosomes can promote neuroangiogenesis [13, 49-51]. Different cargoes carried by exosomes that might explain their effects on neuroangiogenesis, especially the miRNAs that contained in the exosomes which were demonstrated in other systems, such as miR-23a, miR-21-3p, miR-939, miR-17-92 and miR124 [12, 52-55]. However, the importance of exosomes in endometriosis progression is less established.

Some of the findings in our research support the previous research, as we showed that exosomes derived from eutopic stromal cells have pro-angiogenesis effects, which is consistent with the previous study by Harp et al. [15]. Harp and his colleagues isolated ESC-exosomes by using a commercial kit and confirmed the presence of exosomes via NTA and TEM. In our study, we used the standard ultracentrifugation method, which is the most commonly used conventional approach for exosome isolation [17]. The characterization of exosomes were confirmed by their morphological features and NTA analysis, as well as with immunoblots for the biomarkers of exosomes. As neurogenesis

and angiogenesis are closely integrated processes [23, 24], we also demonstrated the pro-neurogenesis effect of exosomes derived from endometriosis by using the neurite outgrowth and DRG neuron apoptosis analysis. Importantly, we found that when we blocked exosome secretion by GW4869, the pro-neuroangiogenesis was decreased. The verification of exosome secretion inhibitor further demonstrate the role of their pro-neuroangiogenesis effects.

We acknowledge several limitations in the present work. As exosomes are a heterogeneous group of cell-derived membranous structures, the biogenesis, including the generation, sorting, releasing, internalization, and fate of exosomes in the recipient cells are very complex [56], the regulatory pathways involved in biogenesis and the secretion of exosomes are not well defined [10]. We used only in vitro methods to show the pro-neuroangiogenesis effects of exosomes. Thus, future studies should determine the mechanisms involved in this process. Here we used only two doses of exosomes, guided from previous studies [20, 21, 57]. Future studies should determine dose responses, investigate functional potency assays to standardize exosome dosing, and investigate different routes/timings of administration. Several exosome isolation methodologies [17] exist, including ultracentrifugation, gradient ultracentrifugation, commercial kits, size-exclusion chromatography, field flow fractionation, and also some new enrichment methods (microfluidic filtering, contact-free sorting, and immunoaffinity enrichment). As every isolation method have its own limitations, we choose the most widely used gold standard approach to isolate exosomes according to

an online questionnaire in October 2015 drafted by ISEV [16]. However, much efforts for isolation methods were required for the research of exosomes. In our study, we did not find any significant differences between exosomes from proliferative phase and secretory phase, however the different cargoes contained in the exosomes from different menstrual phase should be studied in the future. Moreover, emerging technologies, such as microarray-based gene expression analysis and proteomics analysis of exosomes should be utilized to improve the efficiency and quality of exosome research in endometriosis, especially the different cargoes potentially carried by exosomes.

Our study show that exosomes may play a key role in the endometriosis progression by promoting neuroangiogenesis. As exosomes are actively secreted by ESCs, they may more easily enter the abdominal cavity through the fallopian tubes than what debris from the menstrual cycle does. Thus, these exosomes with endometriosis status reach the abdominal cavity and have an impact on the microenvironment of the abdominal cavity, affecting the occurrence and development of the disease. Therefore, the results of this study provide a novel insight into the mechanisms of endometriosis. Future studies are needed to identify the specific molecular mechanism of how exosomes affect endometriosis progression.

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Authors' roles

Guoyun Wang conceived and designed the study. Huihui Sun analyzed, interpreted data and drafted the article. Huihui Sun and Ming Yuan performed the experiments. Qiuju Li and Ni li recruited patients and secured tissue samples. All authors participated in the writing and revision of the article.

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Conflict of interest

The authors report no conflict of interest.

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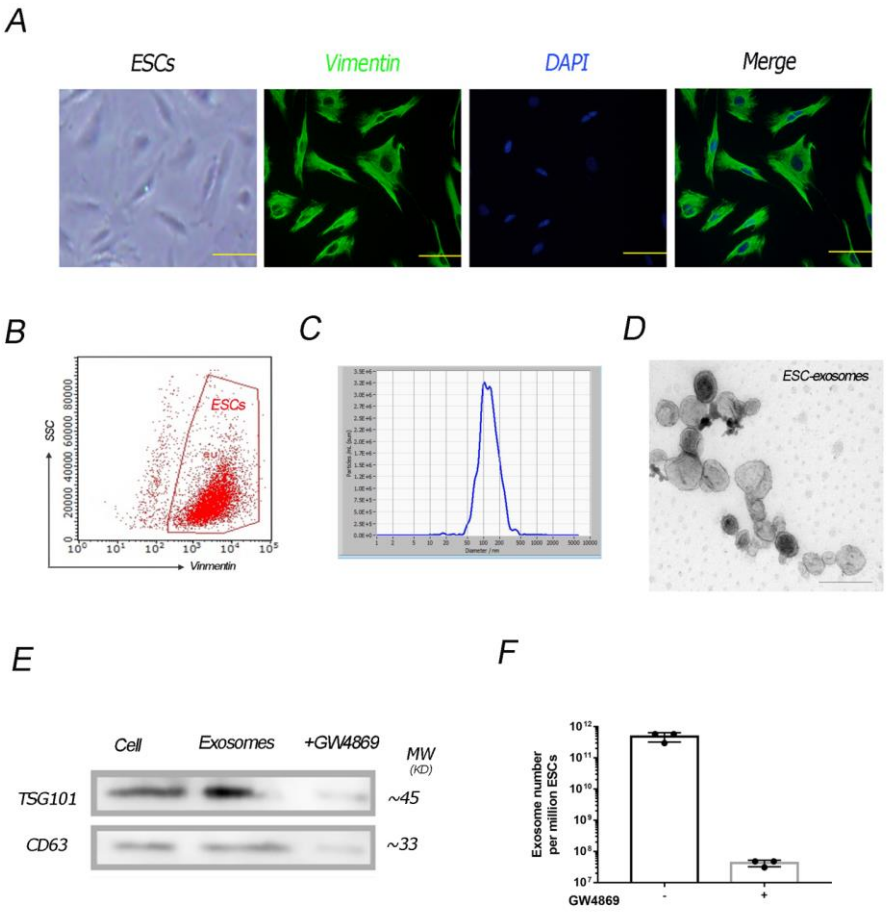

Figure 1. Identification of ESCs and characterization of ESC-derived exosomes. **(A)** ESCs derived from eutopic endometrium with endometriosis were observed under the phase contrast microscope and the immunofluorescent staining of vimentin (green) in ESCs (scale bar=200 μm). **(B)** ESCs were identified via FCM. **(C)** Size distribution of ESC-derived exosomes by using nanoparticle tracking analysis (NTA). **(D)** Morphology of exosomes released by ESCs in 48-h culture supernatants captured via TEM (scale bar=200 nm). **(E)** Western blot of ESC-derived exosomes and immunoblot of protein CD63 and TSG101. **(F)** Exosome quantification of ESCs treated with or without GW4869.

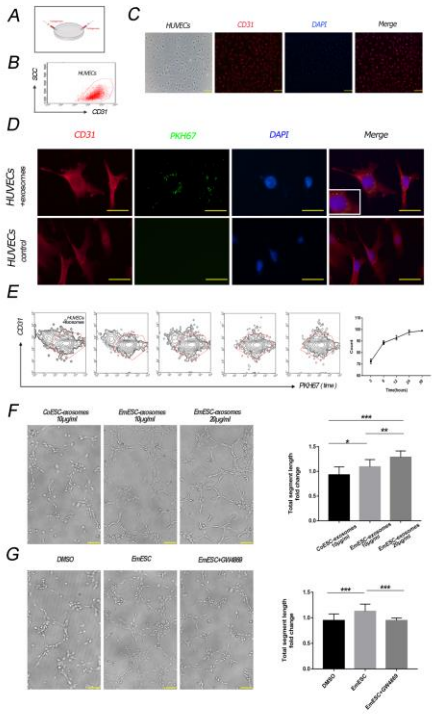

Figure 2. EmESCs secrete exosomes interacting with HUVECs and promote angiogenesis. (A) HUVECs can be obtained via the digestion method. **(B)** HUVECs were identified via FCM. **(C)** Morphology and immunofluorescent staining of CD31(red) in HUVECs (scale bar=200 μm). **(D)** ESC-derived exosomes (green) were interacted by HUVECs stained with CD31 (red) and DAPI (scale bar=100 μm). **(E)** Internalization of exosomes by DRG neurons at different time periods were identified via FCM. **(F)** HUVECs were stimulated by CoESC-exosomes and EmESC-exosomes (10 and 20 μg/ml)**. (G)** HUVECs were incubated with conditioned medium collected from EmESCs treated with or without GW4869. EmESC: primary human endometrial stromal cells isolated from eutopic endometrium with endometriosis. CoESC: primary human endometrial stromal cells isolated from normal endometrium. The results are expressed as mean ±SD. *P<0.05, **P<0.01, and ***P<0.001.

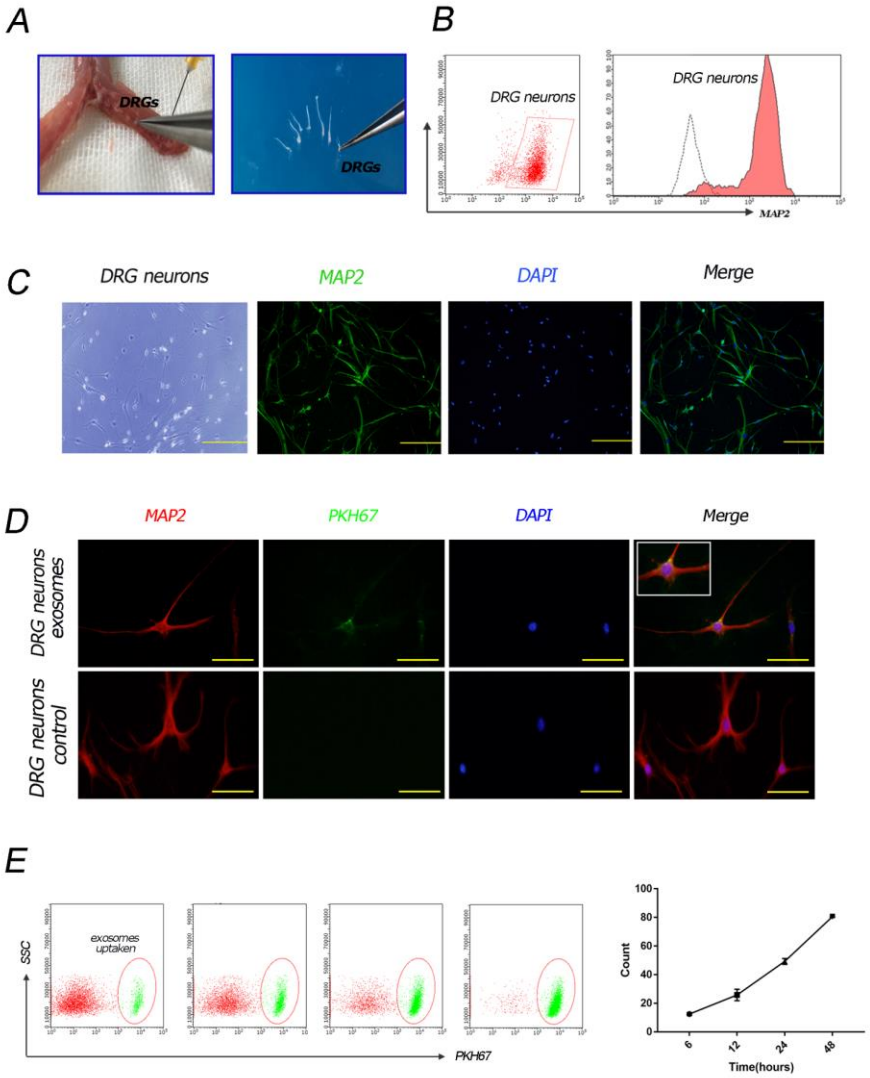

Figure 3. EmESCs secrete exosomes interacting with DRG neurons. (A) DRGs can be found in the intervertebral foramen of the mouse spinal cord. **(B)** DRG neurons were identified via FCM. **(C)** Immunofluorescent staining of MAP2 (green) and DAPI in DRG (scale bar=200 μm). **(D)** ESC-derived exosomes were labeled with PKH67 (green) and co-cultured with DRG neurons. Fluorescent signals were examined by immunofluorescent microscopy. DRG neurons were stained with MAP2 (red) (scale bar=100 μm). **(E)** Internalization of exosomes by DRG neurons at different time periods were identified via FCM.

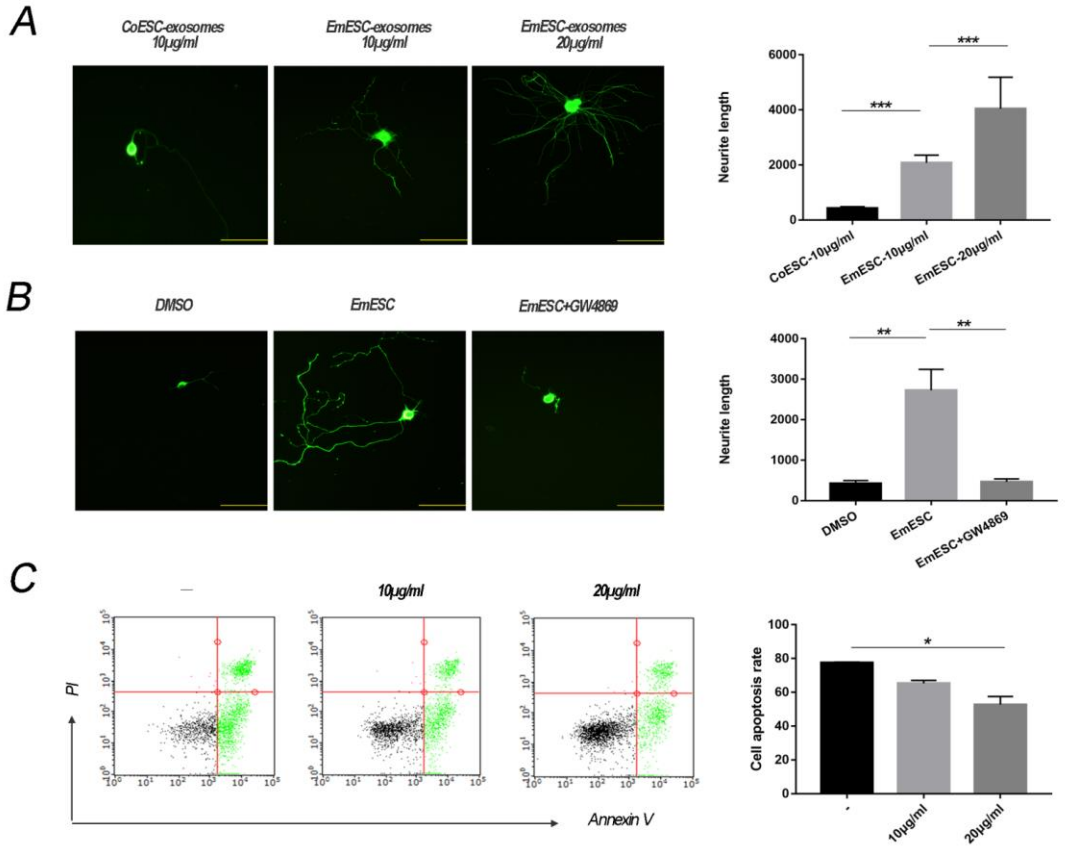

Figure 4. EmESC-exosomes induce neurite outgrowth, and inhibit neurons apoptosis. (A) DRG neurons were stimulated by CoESC-exosomes and EmESC-exosomes (10 and 20 μg/ml)**. (B)** DRG neurons were co-cultured with EmESCs treated with or without GW4869. The total neurite lengths of DRG neurons from (A) and (B) were quantified using Image J. **(C)** FCM detection of the apoptosis of DRG neurons stimulated with EmESC-exosomes (0, 10, and 20 μg/ml). The results are expressed as mean ±SD. *P<0.05, **P<0.01, and ***P<0.001.