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Endothelial Cell Derived MVs and Exosomes: Release and Functional Study

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ENDOTHELIAL CELL DERIVED MICROVESICLES AND EXOSOMES: RELEASE AND FUNCTIONAL STUDY

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Master of Science

By

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WRIGHT STATE UNIVERSITY

GRADUATE SCHOOL

Date:_____

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Langni Liu ENTITLED Endothelial Cell Derived MVs and Exosomes: Release and Functional Study BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Langni Liu, M.S. Department of Pharmacology and Toxicology, Wright State University, 2013. Endothelial Cell Derived MVs and Exosomes: Release and Functional Study.

Extracellular vesicles are membrane derived vesicles exists in all organs. The two major functioning extracellular vesicles are exosomes (EXMs) and microvesicles (MVs). EXMs released from cell membranes via exocytosis with size between 30-150 nm in diameter and MVs directly budded from plasma membrane and size between 100-1000 nm. Ceramide is one of the most important factor in lipid mechanism for EXM release and also associated with MV release. Ceramide generated from breakdown of sphingomyelin by neutral sphingomyelinase (nSMase). In this study, we used an nSMase inhibitor (GW4869) and an analogue of ceramide (C6-ceramide) to modulate the release of MVs and EXMs in human brain microvascular endothelial cells (ECs), and to test the effects of these modulations on EC proliferation, microRNA126 (miR-126) expression and MV uptake ability and the interaction between EC derived MVs/EXMs (EC-MVs/EXMs) with target cell, human brain smooth muscle cells (SMCs). Endothelial cells were treated with GW4869 in 10 μM and C6-ceramide in 10 μM for 24 h. MVs were isolated from by centrifuge and EXMs were isolated by centrifuge at 20,000 x g for 2 h and EXM were isolated by ultracentrifuge at 120,000 for 2 h with 100 nm filtration. MVs and EXMs size and concentration

were analyzed by Nanoparticle Tracking Analysis (NTA) technique. Cell proliferation was detected by MTT. The levels of miRNA126 expression in ECs and EC-MVS/EXMs were detected by qRT-PCR. MV uptake measured by fluorescent microscope. Migration measured by wound healing method. GW4869 significantly decreased EXM release and C6-ceramide significantly increased both MV and EXM release in ECs. ECs treated with C6-ceramide and GW4869 had different miR-126 expression and MV uptake ability. Modulated EC-MVs/EXMs had no effect on normal SMC proliferation and migration, but EC-EXMs under both release regulation decreased angiotensin II (Ang II) induced SMC migration independent of miR-126 level in EXMs. Our data demonstrate that modulation of MV/EXM via C6-ceramide and GW4869 could affects EC function and it`s derived MVs/EXMs function, which may imply in vascular physiology and pathology.

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INTRODUCTION

Endothelial cells

Endothelial cells (ECs), the main cell components of blood vessels, form a thin monolayer lining in the interior vessel surface which acts as an anticoagulant barrier between the vessel wall and blood. This thin layer of ECs, also defined as endothelium, plays an important role in maintaining normal vascular structure and function.

As early as the 1850s, the structure and function of the endothelium were first being explored by Ludwig. However, scientists in that period more likely believed that the endothelium works as an inert semipermeable barrier that only controls the exchange of substrates between blood vessels and tissues. At the beginning of 1953, a series of studies of ultrastructure and physiology finally led to the idea that the endothelium is a dynamic, heterogeneous, disseminated organ that possesses vital secretory, synthetic, metabolic, and immunologic functions [1].

Endothelial function

ECs regulate various physiological functions, such as blood fluidity, vascular tone, platelet adherence, cell proliferation, vascular inflammation and the balance between coagulation and fibrinolysis (Table 1).

Under normal conditions, the endothelium releases a variety of relaxing and contracting factors to regulate vascular tone. Stimuli such as thrombin, bradykinin or increasing shear stress will release EC nitric oxide (NO). NO is produced from L-arginine by a functional endothelial NO synthase (eNOS) [2]. NO is also known as endothelium-derived relaxing factor (EDRF) [3]. NO activates the enzyme guanylate cyclase in vascular smooth muscle cells, thus resulting in cyclic GMP increase and causing vasorelaxation [3]. ECs can also mediate hyperpolarization of vascular smooth muscle cells via endothelium-derived hyperpolarizing factors (EDHF), an NO-independent pathway, which increases potassium conductance and subsequent propagation of depolarization of vascular smooth muscle cells [4]. Of note, the basal tone is also modulated by vasoconstrictors such as endothelin-1 (ET-1), reactive oxygen species (ROS), endothelin, angiotensin II (Ang II) and prostacyclin [5, 6].

ECs also play important roles in immune and inflammatory reactions via regulating lymphocytes and leukocytes. Lymphocytes can interact with ECs under normal homeostasis condition by the L-selectin receptor. Lymphocyte activation up-regulates the expression of leukocyte function-associated antigen-1 (LFA-1) and very late antigen-4 (VLA-4), which promote lymphocyte-EC adhesion via interacting with pro-inflammatory factors such as EC adhesion molecules, Intercellular adhesion molecules 1 and 2 (ICAM-1 and ICAM-2), and vascular cell adhesion molecule (VCAM). Under normal conditions, there is little to no expression of pro-inflammatory factors. During inflammation processes, leukocyte adhesion is increased and ECs are activated [7]. NO has been shown

to inhibit leukocyte adhesion [8]. The activation of ECs promotes the generation of a variety of cytokines including interleukins, tumor necrosis factor and cell adhesion molecules. The inflammatory cytokines produced by ECs activate nuclear factor-kappa beta (NF-κβ) and up-regulate EC adhesion molecules, which direct the process of inflammation [9]. As the barrier that is in direct contact with blood, the endothelium controls blood fluidity and coagulation through the factors that regulate the clotting cascade, platelet activity, and the fibrinolytic system [9].

Endothelial function	Associated regulator
Maintenance of vascular tone	NO, PGI2, TxA2, EDHF, E-1, Ang II
Balancing blood fluidity and thrombosis	NO, t-PA, PAI-1, TF, PGI2, TxA2, vWF, fibrinogen, thrombomodulin, heparin
Control of the vascular inflammatory process	VCAMs and ICAMs, NF-κβ, MCP-1, P and E selectin, IL-1, IL-6, IL-18, TNF
Vasculogenesis/angiogenesis	VEGF, PDGF, TGF-β

Table 1. Endothelial functions and function associated regulators. Ang II: Angiotensin II; EDHF: Endothelium-derived hyperpolarizing factor; ET-1: Endothelin-1; ICAM: Intercellular adhesion molecule; IL: Interleukin; MCP-1: Monocyte chemotactic factor-1; NF-κβ: Nuclear factor kappa beta; NO: Nitric oxide; PAI-1: Plasminogen activator inhibitor-1; PDGF: Platelet-derived growth

factor; PGI2: Prostacyclin; TF: Tissue factor; TGF-β: Transforming growth factor-beta; TNF: Tumor necrosis factor; t-PA: Tissue plasminogen activator; TxA2: Thromboxane A2; VCAM: Vascular cellular adhesion molecule; VEGF: Vascular endothelial growth factor; vWF: von Willebrand's factor.

Endothelial Dysfunction

Endothelial dysfunction refers to abnormalities in the phenotype and functions of endothelial cells. It causes reduced anti-inflammatory properties and prothrombic properties of the endothelium, impaired modulation of vascular growth, dysregulation of vascular remodeling and an imbalance in the endothelium-derived relaxing and contracting factors (Fig 1).

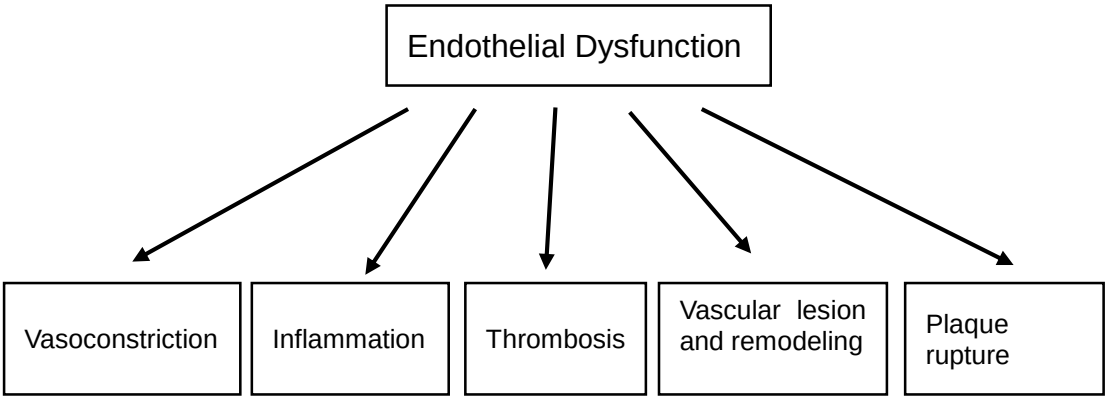


Fig 1. Consequences of endothelial dysfunction.

The loss of NO bioactivity is defined as the hallmark of endothelial dysfunction. NO maintains the vascular wall in a quiescent state by targeting several regulators such as NF-κβ to silence the cellular inflammatory process. The decline in NO bioavailability causes EC dysfunction. NO reduction may be caused by decreased expression of eNOS or eNOS activity (a lack of substrate or cofactors) or accelerated NO degradation by reactive oxygen species (ROS) [10].

Oxidative stress appears to play a pivotal role in the alteration of endothelial function. Oxidative stress increases vascular endothelial permeability and promotes leukocyte adhesion, which is coupled with alterations in endothelial signal transduction and redox-regulated transcription factors such as NF-κβ [11]. It is also increasingly recognized that cell differentiation and proliferation, cytokine expression, and programmed cell death are determined by oxidation-sensitive regulatory pathways [12].

Cardiovascular disease risk factors initiate a chronic inflammatory process that is accompanied by a loss of vasodilators and anti-thrombotic factors and an increase in vasoconstrictors and pro-thrombotic products. Mostly, risk factors as diverse as smoking, aging, hypercholesterolemia, hypertension, hyperglycemia, and a family history of premature atherosclerotic disease, obesity, elevated C-reactive protein, and chronic systemic infection are all associated with endothelial dysfunction [9].

Extracellular vesicles

Extracellular vesicles, also called microparticles, are membrane-derived vesicles that can be released from almost all cells. They carry various DNAs, proteins, mRNAs, and microRNAs (miRNAs) that have potential diagnostic and therapeutic purposes. Extracellular vesicles can transfer molecular contents to recipient cells. This ability to carry and transfer biological information makes extracellular vesicles an important way of intercellular communications and could service as biomarkers.

Extracellular vesicles are mainly characterized into 3 categories: 1) exosomes (EXMs); 2) microvesicles (MVs); and 3) apoptotic bodies. EXMs and MVs are the functional extracellular vesicles. Even though both EXMs and MVs are released from cellular membranes, the differences in their biogenesis and parental cells provide them with function differences.

Key features of EXMs and MVs

Exosomes are vesicles surrounded by a phospholipid bilayer and their size is approximately 30–150 nm in diameter. Endocytic vesicles bud into early endosomes and multi-vesicular body (MVB) as form of intraluminal vesicles (ILVs). MVBs either fuse with lysosomes or fuse with the plasma membrane. Fusion with the plasma membrane allows them to release their content, which are exosomes [13]. EXMs feature phosphatidylserine (PS) on the outer membrane leaflet and exosomal markers such as CD63 and CD9 [14]. The widely accepted protocol for EXM isolation includes ultracentrifugation, sometimes in combination with sucrose density gradients or sucrose cushions

to float the relatively low-density exosomes [15]. Flow cytometry is a popular tool to detect extracellular vesicles. It is more commonly used in detection of MVs because of its limitation in detected particle size. Nanoparticle tracking analysis (NTA) technique is a new technique to track the size and distribution of small particles. The ability of NTA for tracking small particles makes it the more sensitive and accurate method for tracking EXMs, compared to flow cytometry.

MVs are structures surrounded by a phospholipid bilayer and their size is approximately 100–1000 nm in diameter. MVs are released from cells by directly budding from membranes. MVs have bound PS on the outer membrane leaflet. Several studies also consider that MVs lack PS externalization due to the inability of PS to bind to annexin V [16]. One of the main isolation methods of MVs is by centrifugation. The methods of detection for MVs are the same as EXMs.

Functions of MVs and EXMs

MVs and EXMs can directly or indirectly control a variety of biological processes by transferring membrane proteins, signaling molecules, mRNAs, and miRNAs and activate receptors in recipient cells. I Interaction of extracellular vesicles with other cells occurs in various physiological and pathological processes, including inflammation, cancer, infectious diseases and neurodegenerative disorders.

MVs and EXMs derived from different parental cells have their respective

functions. Several studies have indicated that extracellular vesicles participate in the development and progression of atherosclerosis. Atherosclerotic plaques contain large amounts of extracellular particles. The variety of factors expressed in plaques vesicles stimulate T lymphocytes that might causes B lymphocytes activation and production of specific immunoglobulins which have the ability to against atherosclerotic plaque antigens or apoptotic cell debris [17, 18].

MVs and EXMs also have pro- or antiangiogenic activities, depending on their cellular origin. Several studies indicated that angiogenesis is enhanced by MVs or EXMs released by endothelial cells and platelets [19, 20]. EXMs derived from CD34+ hematopoietic stem cells contribute to hypoxia-induced angiogenesis [21]. Cancer cell derived MVs and EXMs favor angiogenesis by promote endothelial cell proliferation, migration, tube formation and vascular leakiness [22].

MV and EXM releasing mechanism

The releasing mechanism of EXMs can be mainly described as two parts: The endosomal sorting complex required for transport (ESCRT) mechanism and ESCRT-independent mechanism.

The ESCRT consists of four complexes: ESCRT-0 is responsible for cargo clustering in an ubiquitin-dependent manner, ESCRT-I and ESCRT-II responsible for promoting bud formation, ESCRT-III drives vesicle scission [13]. Members of the ESCRT family had been proved shown in a variety of cells and

control EXM release. Inhibition of HRS, an ESCRT-0 member, decreased exosome secretion [23]. Depletion of TSG 101, an ESCORT-1 member, decreased exosome in tumor cells [13, 24]. ALIX-syntenin, which belongs to the ESCRT-III complex induced intraluminal budding of vesicles in endosomes, which regulates exosome formation and secretion [25].

Besides ESCRT mechanism, several studies suggested that ESCRT-independent mechanisms are also involved in ILV formation and EXM biogenesis. These mechanisms involve lipids (including ceramide), tetraspanins, or heat shock proteins [13]. Ceramide, one of the most important factors of the lipid ESCRT-independent mechanism has been shown to regulate exosome formation and release in several cells [25, 26]. Ceramide is a central second messenger that has been shown to cause cell growth arrest and apoptosis. It is generated from the breakdown of sphingomyelin (SM) by neutral sphingomyelinase (nSMase) [27]. Inhibition of nSMase reduces ceramide generation and causes decrease in EXM release. As the analog of ceramide, the exogenous C6-ceramide increases the EXM release via this lipid mechanism (Fig 2).

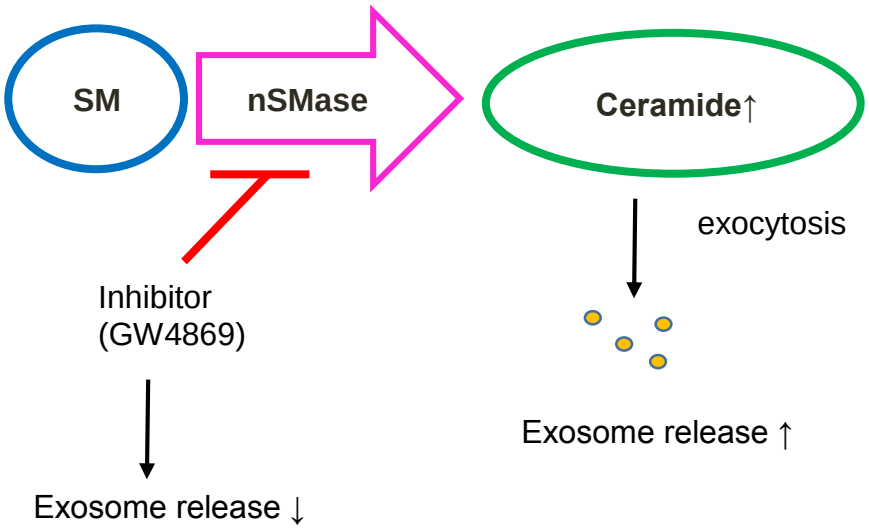


Fig 2. Simple diagram of the nSMase-ceramide mechanism regulating exosome release. SM: sphingomyelin; nSMase: neutral sphingomyelinase;

Endothelial MVs and EXMs

EC derived MVs (EC-MVs) and EC derived EXMs (EC-EXMs) carry endothelial proteins such as platelet endothelial cell adhesion molecule-1, intercellular cell adhesion molecule (ICAM)-1, or E-selectin. Several studies showed that EC-MVs or EC-EXMs provide protective effects to recipient cells. Endothelial progenitor cells derived MVs contribute to hypoxia/reoxygenation induced endothelial dysfunction and apoptosis via their carried micorRNA126 (miR-126) associated with ROS production and the PI3K/eNOS/NO pathway [28]. miR-126 is highly expressed in endothelial cells. It had been reported to be specific for vascular system in zebrafish [29]. In many studies, miR-126 had been reported can regulate vascular integrity and angiogenesis [30, 31]. The expression of miR-126 in MVs and EXMs proved that MVs and EXMs can transfer messages molecules from endothelial cells and affect recipient cells functions.

II. HYPOTHESIS AND SPECIFIC AIMES

Hypothesis:

It is hypothesized that modulation of microvesicle (MV)/exosome (EXM) release, without affecting endothelial cell (EC) proliferation, alters the microRNA 126 (miR-126) content and function of ECs, and affects the miR-126 content and function of EC derived MVs/EXMs on vascular smooth muscle cell (SMC) proliferation and migration.

Specific Aims:

Aim 1: To evaluate the effects of C6-Ceramide and GW4869 on EC MV and EXM release.

Aim 2: To determine the impacts of C6-Ceramide and GW4869 on EC proliferation, miR-126 expression and MV uptake ability.

Aim 3: To evaluate the effects of EC-MVs/EXMs released under C6-Ceramide or GW4869 modulation on SMC proliferation and migration.

III. EXPERIMENTAL DESIGN

Design for Aim 1:

ECs were cultured and treated with C6-Ceramide at 0, 10, 15 μM or with GW4869 at 0, 5, 10 μM for 24 hours. Culture medium samples were collected for MV and EXM isolation. Particle size and concentration were analyzed by NTA to determine the effective concentration for further experiments. The levels of miR-126 in MVs and EXMs were measured by Quantitative real-time PCR (qRT-PCR). ECs at passages 8-16 were used in this study.

Design for Aim 2:

ECs were cultured and treated with C6-Ceramide at 0, 10, 15 μM or with GW4869 at 0, 5, 10 μM for 24 hours. Cell proliferation was tested using the MTT method. The NTA and cell proliferation results, provided the effective concentration for further experiments. ECs were divided into 3 groups: Vehicle; C6-Ceramide treatment; GW4869 treatment. Expression of miR-126 in ECs was detected by qRT-PCR. MV uptake rates of ECs with different treatment groups were analyzed by fluorescence microscopy and image J software. ECs at passage 8-16 were used in this study.

Design for Aim 3:

SMCs were cultured and treated with EC derived MVs or EXMs (10 μg/ml) for

24 hours. Cell proliferation was measured by the MTT method and cell migration was measured by the wound healing method. SMCs at passage 5-8 were used in this study.

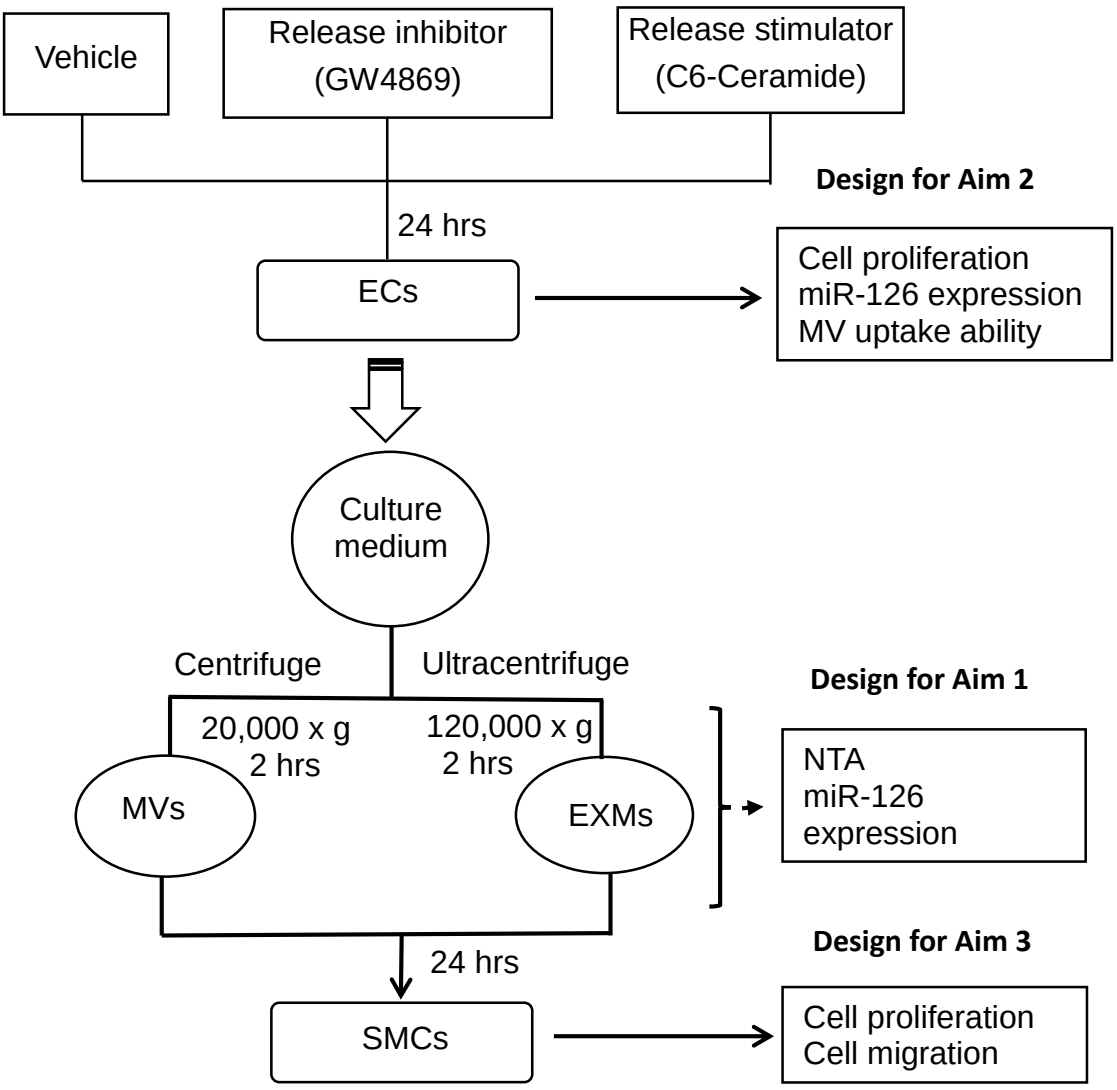


Fig 3. Flow chart of the experimental design in this study. EXMs: exosomes; ECs: endothelial cells; SMCs: smooth muscle cells; miR-126: microRNA 126; MVs: microvesicles; NTA: nanoparticle tracking analysis.

IV. MATERIALS AND METHODS

Materials

Human brain microvascular endothelial cells, CSC complete medium, human recombinant growth factors (CSC CultureBoost-R), attachment factor, CSC serum free medium and RocketFuel supplements were purchased from Cell Systems Corp. (Kirkland, WA, USA). DMEM high glucose medium, L-glutamine, Fetal Bovine Serum (FBS), antibiotic-antimycotic solution, trypsin, methyl thiazolyl tetrazolium (MTT), TRIzol reagent were purchased from Invitrogen (Carlsbad, CA). N-Hexanoyl-D-sphingosine (C6-Ceramide), GW4869, PKH26 were purchased from Sigma-Aldrich Co. LLC (Saint Louis, MO, USA).

Cell culture and treatment

Human brain microvascular endothelial cells and human brain microvascular smooth muscle cells were used in this study. ECs were cultured in CSC complete medium with 10 % FBS, 2 % human recombinant growth factors, 1 % L- glutamine and 1 % antibiotic-antimycotic solution in a cell incubator with a standard cell culture atmosphere (37 °C, 5 % CO_2). Culture medium was replaced every 2-3 days. ECs at passage 8-16 were used in this study. For passaging, with 85-90 % cell confluence, culture medium was discarded and replaced With 0.025 % trypsin/0.01% EDTA at 37 °C for 5-10 min, then added DMEM/F12 medium with 10 % FBS to stop the reaction. After centrifuge at 300

x g for 5 min, pellet was resuspended with CSC complete medium and passage in a 1:3 ratio for further culture.

C6-Ceramide and GW4869 were dissolved in DMSO to 90 mM as stock solution and stored in -20 °C. ECs were mainly divided into 3 groups: Vehicle group, cells treated with DMSO as control; +C6-Ceramide group， cells treated with C6-ceamide for 24 hours, +GW4869 group, Cells treated with GW4869 for 24 hours. Before treating with drugs, ECs were cultured with serum free CSC medium (supplemented with 1.4 % RocketFuel, 1 % L-glutamine and 1 % antibiotic-antimycotic solution) overnight, then replaced with fresh serum free CSC medium and treated with C6-Ceramide at 0, 10, 15 µM or GW4869 at 0, 5, 10 µM for 24 hours. NTA and EC proliferation were analyzed to determine the effective concentration for further experiments. From the NTA and cell proliferation results, a concentration of 10 µM for C6-Ceramide and GW4869 was identified as an effective concentration. After treatment with the effective concentration for 24 hours, ECs were collected for further experiments, culture medium samples were collected for MV and EXM isolation.

SMCs were cultured in DMEM high glucose medium with 10 % FBS, 1 % L-glutamine and 1 % antibiotic-antimycotic solution in a cell incubator with a standard cell culture atmosphere (37 °C, 5 % CO2). Culture medium was replaced every 2-3 days. SMCs at passage 5-8 were used in this study.

Cell proliferation assay

EC proliferation was tested by MTT assay, an easy and rapid colorimetric assay

for assessing cell metabolic activity. In cells, NAD(P)H-dependent cellular oxidoreductase enzymes which reflect the number of viable cells present are capable of reducing the tetrazolium dye MTT to its insoluble formazan, which has a purple color. The differences in the color level showed the different ability in cell proliferation [32, 33]. ECs were cultured in 96-well plate. When cells reached 85-90 % confluence, the culture medium was replaced with serum free CSC medium overnight, then cells were treated with C6-Ceramide at 0, 10, 15 μM or GW4869 at 0, 5, 10 μM for 24 hours. After that, 20 μL of MTT solution (12 mM) were added to each well and ECs were incubated at 37 °C for 4 hours in the dark. Afterwards, the cultured medium was discarded, and 150 μL of DMSO was added to each well and cells were incubated for 15 min at room temperature in the dark. The dye optical absorbance was read at 572 nm. The percent of cell proliferation was expressed as the relative optical absorbance of treated groups versus the vehicle group [28].

Isolation of EC-MVs and EC-EXMs.

ECs were cultured with serum-free CSC medium overnight and treated with C6-Ceramide or GW4869 at 10 μM in serum-free medium for 24 hours. After treatment, culture medium samples were collected and spun at 300 x g for 10 min followed by centrifugation at 2,000 x g for 20 min to remove cells and cell debris. The supernatant were then spun at 20,000 x g for 2 hours to pellet EC-MVs. The MV pellet was resuspended with 1 ml sterile-filtered phosphate buffered saline (PBS) (filtered through 0.2 μm filter). The supernatant of the MV pellet was collected and spun in an ultracentrifuge at 120,000 x g for 2 hours and filtered through a 0.1 μm filter. After ultracentrifugation, the supernatant

was removed, and EXM pellet was resuspended with 100 µl sterile-filtered PBS. All MV and EXM samples were stored in -80 °C for further use.

Nanoparticle tracking Analysis (NTA)

The size and concentration of EC-MVs and EC-EXMs were detected by NTA using an NS300 instrument (NanoSight, Amesbury, UK). The NTA technique can measure the size distribution of vesicles ranging from 50 nm – 1 µm in diameter. The threshold of particle concentration detection is between 10^7 - 10^9 particles/ml. For more accurate detection, MV and EXM samples are diluted with PBS to a particle concentration of about 10^8 particles/ml. After dilution, 700 µl of diluted sample was loaded to the machine for recording particle movement videos in a rate of about 30 frames/s. Particle movement videos were recorded 3 times per test at different positions and particle movements were analyzed by the NTA software (version 2.2, NanoSight). The results of NTA were presented as the mean of the 3 tests. The final particle concentration was calculated after considering the dilution factor of the NTA results.

Co-incubation of EVs with ECs and SMCs

To track the MVs and EXMs uptake of ECs and SMCs, isolated MVs and EXMs were added with 2 µl of the PKH26, a lipid-bound red fluorescent dye, for 5 min at room temperature in the dark. All the samples were then spun in an ultracentrifuge at 120,000 x g for 2 hours and washed with sterile-filtered PBS once. The pellets were resuspended with sterile-filtered PBS as PKH26 labeled MVs and EXMs. The PKH26 labeled MVs or EXMs were added to ECs or SMCs

at 10 µg/ml and incubated for 24 hours. Fluorescence images were taken by a fluorescence microscope to determine the communication of MVs and EXMs with ECs and SMCs.

MV uptake measurement

ECs were incubated in 12-well plate with CSC complete medium. Cells at 85-90 % confluence were cultured with CSC serum-free medium overnight then cultured with PKH26 labeled EC derived MVs at 10 µg/ml for 24 hours. Both cell images and fluorescence images were taken by the Essen IncuCyte ZOOM instrument (Essen BioScience) every 3 hours within 24 hours. The intensity of red fluorescent particles were analyzed by the NTA machine.

Gene expression analysis for miR-126

miR-126 from treated ECs, EC-MVs, and EC-EXMs was extracted by using the TRIzol reagent. ECs were cultured in T-75 flasks. After treatment, the culture medium was removed and the cells were dissolved in 1 ml TRIzol reagent and collected into 1.5 ml microcentrifuge tubes. Afetrwards, 200 µl chloroform was added to each tube and shaken for 15 sec. The tubes were left for 10 min at room temperature and then spun at 12,000 x g for 15 min at 4 °C. After centrifugation, the samples were separated in 3 layers with the upper clear layer containing the RNA extracted from the samples. The middle white cloudy interphase contains DNA. The upper layer was transferred to a new tube avoiding contamination with the second layer. Pre-cooled isopropanol (500 µl) was added to the RNA-containing sample, mixed by pipetting up and down.

After 5-10 min at room temperature, samples were spun at 1,200 x g for 10 min at 4 °C. The supernatant was discarded and the pellet suspended with 1 ml 75 % ethanol. Samples were spun once more at 7,500 x g for 5 min at 4 °C and the supernatant discarded. Afterwards, all the tubes were left at room temperature until the ethanol had evaporated, the RNA was dissolved in RNase-free water for later processing. cDNA was synthesized using the Prime Script reverse transcription Reagent kit (QIAGEN) following the manufacturer's instructions. qRT-PCR was conducted with miR-126 specific primers and miScript SYBR Green PCR Kit (QIAGEN) on a real-time PCR system (Bio-Rad). Small nuclear RNA U6 (U6) was used as an internal control. Relative expression of miR-126 was calculated using the $2^{-\Delta\Delta}$ CT method [28].

Cell migration assay

SMC migration was determined in 12-well plates using the wound healing methods described previously with small modifications [34]. Briefly, 80 to 90% confluent SMCs were used in this study. Cells cultured in plates were scraped with a sterile pipette tip across the diameter of each well to produce wounds about 1 to 1.5 mm wide. Then cells were rinsed once to remove cellular debris and replaced with fresh serum-free DMEM high glucose medium. Images were taken at the initial time of wounding (0 h) using an inverted fluorescence microscope. Cells were then treated with EC-MVs and EC-EXMs in the presence and absence of Angiotensin II (Ang II, 100 nM). Images were taken every 3 hours at 3 different positions per well. Images were analyzed by Image J software. Data are presented as the fold changes of the differences in the wounded area at 0 and 24 hours.

Statistical analysis

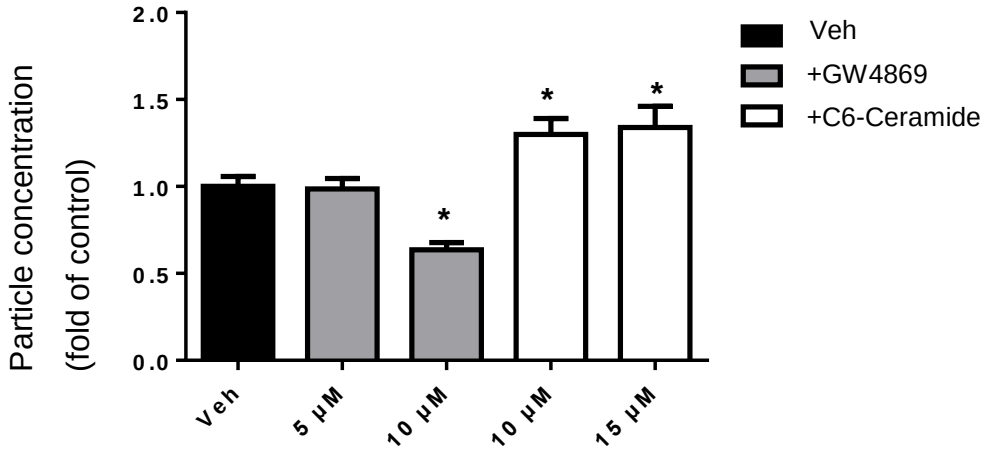
All results were presented as means ± SEM of at least three experiments. Student t-test was used to compare significant differences between two groups. Multiple comparisons were analyzed by one or two-way ANOVA. A value of $P<0.5$ was considered statistically significantly different. All statistical analyses were performed using GraphPad Prism 6.01 software.

V. RESULTS

C6-Ceramide increased both EC-MV and EC-EXM release, whereas, GW4869 only decreased EC-EXM release at 10 µM.

Cultured ECs were treated with the MV/EXM releasing regulator C6-Ceramide at 0, 10, 15 µM or with GW4869 at 0, 5, 10 µM for 24 hours. Total particles including MVs and EXMs were collected from the culture medium. The NTA results showed that GW4869 significantly reduced particle release at 10 µM and C6-Ceramide significantly induced particle release starting at 10 and 15 µM compared to the control (Fig 4A). At 15 µM, C6-Ceramide did not show significant changes in inducing particle release compared to the treatment at 10 µM. Based on these results, the concentration of 10 µM for both C6-Ceramide and GW4869 was considered as an effective concentration for MV/EXM release for further experiments. To confirm the effectiveness of this concentration, MVs and EXMs were isolated separatedly from the culture medium of C6-ceramide/GW4869 treated ECs by ultracentrifugation. As shown in Figure 3, C6-Ceramide significantly induced both MV and EXM release (~1.5 fold). GW4869 significantly reduced EXM release but had no significant effect on MV release.

A)



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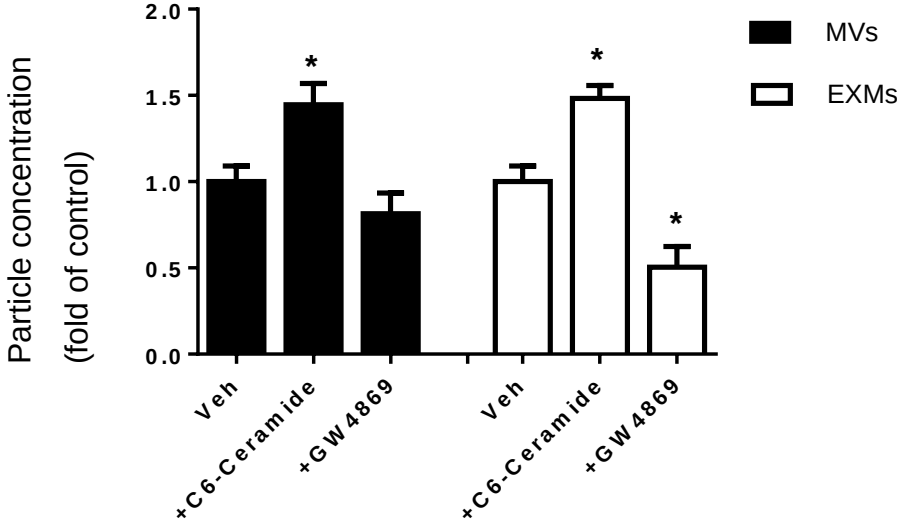


Fig4. C6-Ceramide / GW4869 modulates MV/EXM release in ECs. A) NTA results showing that C6-Ceramide or GW4869 modulates particle concentration in ECs at different concentrations. ECs were treated with C6-ceramide at 0, 10, 15 µM or GW4869 at 0, 5, 10 µM for 24 hours. The total particle concentration in culture medium was analyzed by NTA. B) Data summary of NTA represents MVs/EXMs concentration changes as fold change of control after MV/EXM release regulation. ECs were treated with C6-ceramide or GW4869 at 10 µM

for 24 hours. MVs and EXMs were isolated from cell culture medium by centrifugation and ultracentrifugation methods. Size and concentration of MVs and EXMs were measured by NTA. Data are presented as mean ± SEM, n=4/group, ***** P<0.05 versus Veh. Veh: Vehicle group; MVs: EC derived microvesicles; EXMs: EC derived exosomes; NTA: nanoparticle tracking analysis.

Both C6-Ceramide and GW4869 had no effect on EC cell proliferation at 10 μM.

Similarly to the previous treatment steps, cultured ECs were treated with C6-Ceramide at 0, 10, 15 μM or GW4869 at 0, 5, 10 μM for 24 hours. As shown in figure 5, C6-Ceramide significantly decreased EC proliferation at 15 μM compare to the control or 10 μM. Both C6-ceramide and GW4869 had no significant effects in the parental EC proliferation at 10 μM. This result further confirmed the effective concentration of 10 μM that allowed C6-Ceramide/GW4869 regulates MV/EXM release without significant affects the parental ECs.

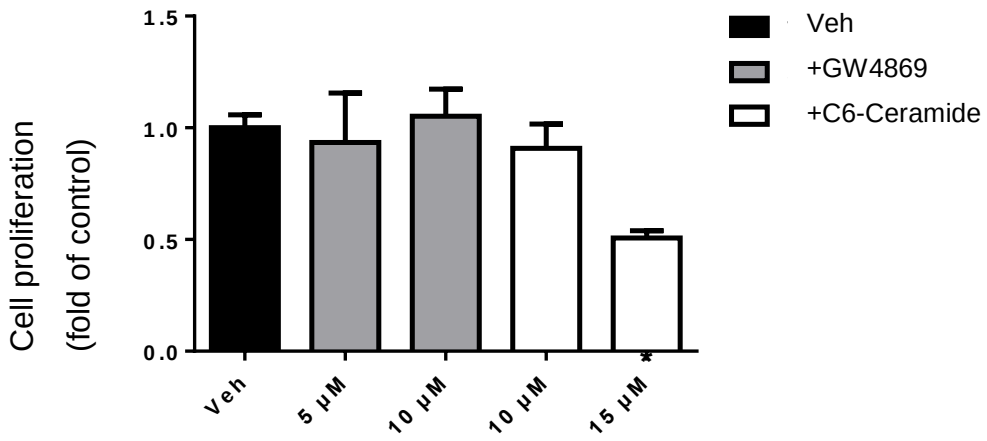


Fig 5. The effect of C6-Ceramide/GW4869 on EC proliferation. ECs were treated with C6-Ceramide at 0, 10, 15 µM or GW4869 at 0, 5, 10 µM for 24 hours. Cell proliferation was tested by the MTT method and was calculated as fold changes in the treated groups versus the vehicle group. Data is presented as mean ± SEM, n=5/group, ***** P<0.01 versus Veh. Veh: Vehicle group; +C6-ceramide: cells treated with C6-Ceramide; +GW4869: cells treated with GW4869.

C6-Ceramide but not GW4869 treatment increased miR-126 expression in ECs.

To evaluate the effects of MV/EXM releasing regulation in EC function, the EC specific microRNA miR-126 expression in ECs was analyzed by qRT-PCR. GW4869 and C6-Ceramide treatment at a concentration of 10 µM had no effect on miR-126 expression in ECs compared to the control (Fig 6). However miR-

126 expression in C6-Ceramide treated ECs was significantly different from GW4869 treated ECs (Fig 6).

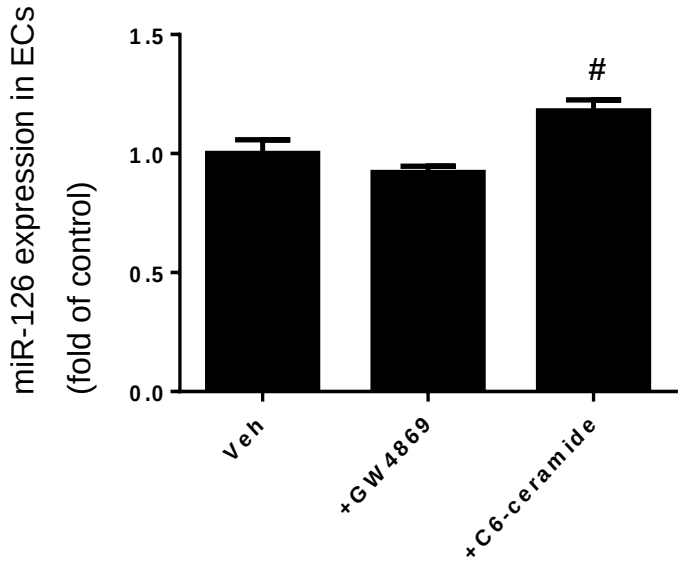
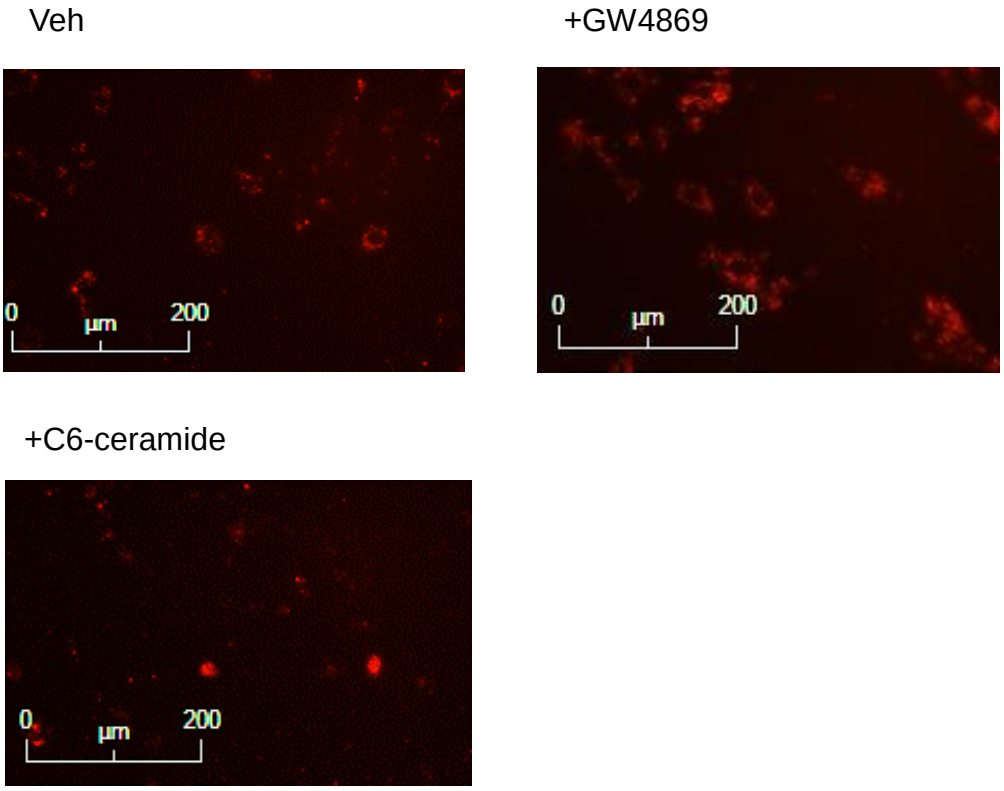


Fig 6. Effect of modulating MV/EXM release via C6-ceramide and GW4869 on miR-126 expression in ECs. ECs were treated with C6-ceramide or GW4869 at 10 μM for 24 hours. miR-126 expression of ECs in different treatment groups was analyzed by qRT-PCR. Data is presented as Mean ± SEM, n=3/group, # $P<0.05$ versus +GW4869. miR-126: microRNA-126; Veh: Vehicle group; +C6-ceramide: cells treated with C6-Ceramide; +GW4869: cells treated with GW4869.

GW4869 increased the EC ability in EC-MV up-taking (merging).

ECs release and uptake MVs and EXMs to transfer biological messages and communicate with other cells. The ability of particle uptake can also be considered as a factor that reflects ECs health and function. The particle uptake ability was measured by fluorescence microscopy. C6-Ceramide-/GW4869-treated ECs were co-incubated with PKH26 labeled normal EC-MVs for 24 hours. Fluorescence images were taken every 3 hours for the measurement of red fluorescence. The images showed that particles merged into cells. After 24 hours, the fluorescence images indicated that the labeled MVs had been uptaken by ECs (Fig 7A). The intensity changes of the red fluorescence images were considered to reflect the MV uptake ability of ECs. Figure 7 showed that under GW4869 treatment at 10 μM, the MV uptake rate of ECs largely increased compared to the control. C6-Ceramide had no effect on the EC MV uptake ability.

A）



B）

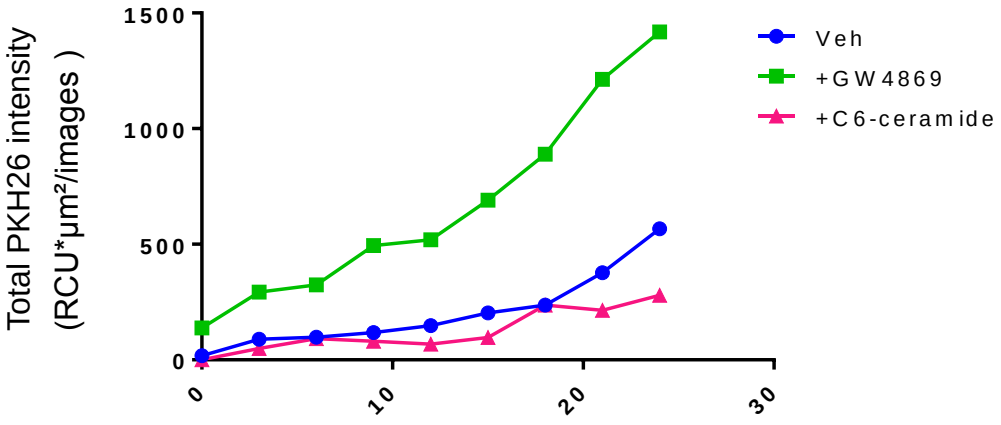


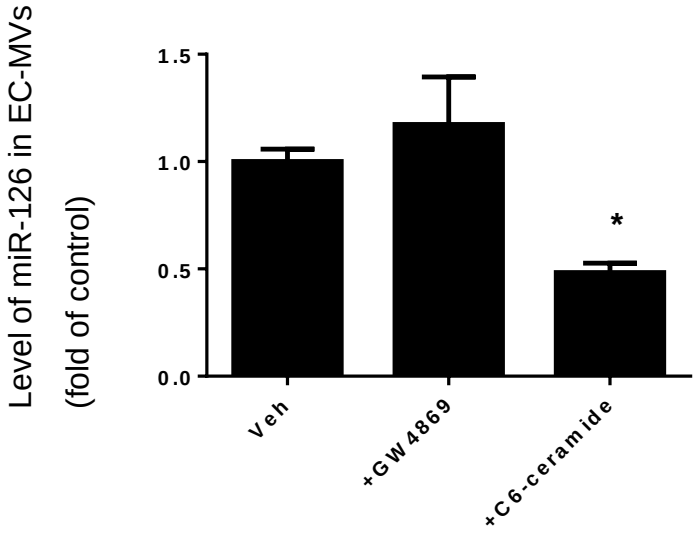
Fig 7. Modulating efect of MV/EXM release on the MV uptake ability of ECs. A) Representative PKH26 staining images of EC-MVs merged into ECs observed under microscope after treatments for 24 hours. Cells were treated with C6-

Ceramide or GW4869 at 10 μM and PKH26 stained normal EC-MVs for 24 hours. Fluorescence images were taken every 3 hours. B) Data summary for the intensity of red fluorescence from PKH26 labeled normal EC-MVs that merged into treated ECs. The intensity changes of the red fluorescence refer to the MV uptake ability of ECs under different treatments. n=3/group. Veh：Vehicle group; +C6-ceramide: cells treated with C6-Ceramide ; +GW4869: cells treated with GW4869 .

C6-Ceramide down-regulates miR-126 level in both EC-MVs and EC-EXMs, whereas GW4869 down-regulates miR-126 level in EC-EXMs, with no effect on EC-MVs.

ECs were treated with C6-Ceramide or GW4869 at 10 μM for 24 hours. Released MVs and EXMs were collected from the culture medium. The level of miR-126 in MVs and EXMs was analyzed by qRT-PCR. The level of miR-126 in both MVs and EXMs was decreased by C6-Ceramide treatment. GW4869 treatment had also decreased miR-126 level in EXMs, but had no effect on MVs (Fig 8).

A)



B)

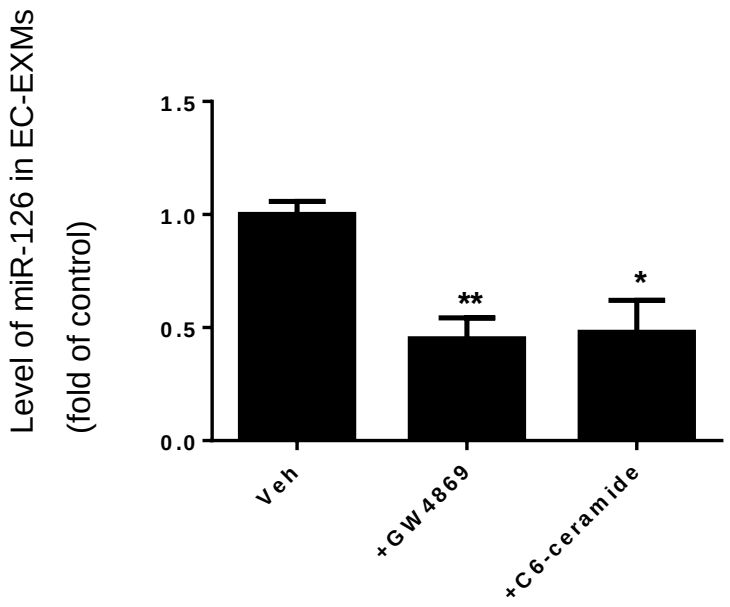


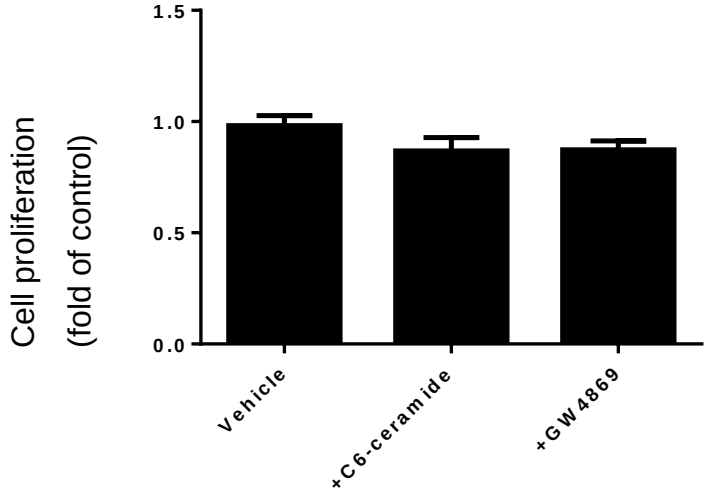
Fig 8. Effect of modulating MV/EXM release via C6-ceramide and GW4869 on miR-126 levels in EC-MVs and EC-EXMs. A) EC-MVs and B) EC-EXMs were isolated and the level of miR-126 in different treatment groups was analyzed by qRT-PCR. Data is presented as Mean ± SEM, n=3/group, *P<0.05, ** P<0.01 versus Veh. miR-126: microRNA-126; Veh: Vehicle group; +C6-ceramide: cells treated with C6-Ceramide; +GW4869: cells treated with GW4869.

EC-EXMs down-regulate Ang II stimulated SMC migration, but have no effect on normal SMC proliferation and migration.

PKH26-labeled EX-MVs and EX-EXMs released from C6-Ceramide or GW4869 treatment were added to SMCs at 10 μg/ml. The fluorescence images showed that PKH26-labeled MVs/EXMs entered and accumulated into SMCs (Fig 9A), indicating that cultured SMCs communicate with co-incubated MVs and EXMs. Cell proliferation and migration of SMCs were analyzed to

determine whether there were function alterations by EC-MVs and EC-EXMs. Cell proliferation was measured by the MTT method, and migration was tested by the wound healing method. EC-MVs and EC-EXMs released by C6-Ceramide or GW4869 at a concentration of 10 μM had no effect on normal SMC proliferation and migration (Fig 10). Under Ang II stimulation (100 nM), EC-EXMs released by both GW4869 and C6-Ceramide reduced SMC migration compared to the control. EC-MVs had no effect on Ang II stimulated SMC migration.

A) EC-EXM treatment



B) **EC-MV** **treatment**

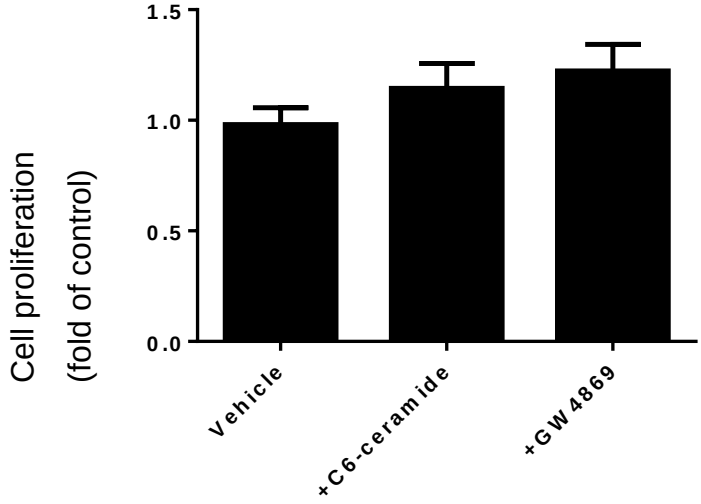
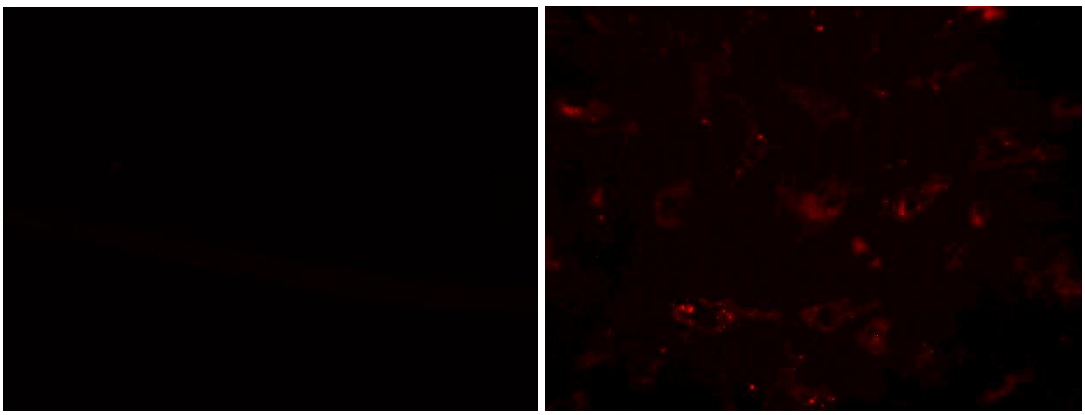


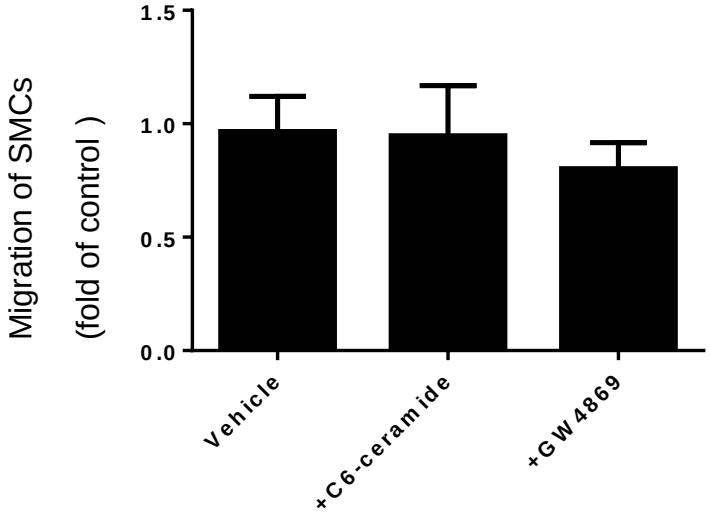
Fig 9. Effects of C6-Ceramide or GW4869 mediated EC-MVs and EC-EXMs in SMC proliferation. A) After incubation with EC-EXMs or B) EC-MVs for 24 hours, cell proliferation of SMCs was tested by the MTT method. Veh: Vehicle group; +C6-ceramide: MVs or EXMs released form cells treated with C6-Ceramide; +GW4869: MVs or EXMs released form cells treated with GW4869.

A)

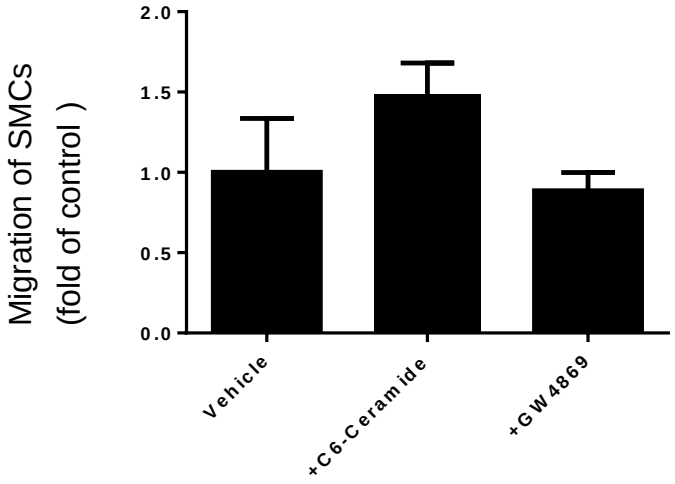


T=0 h **T=24 h**

B）EC-EXMs treatment



C) EC-MVs treatment



D)

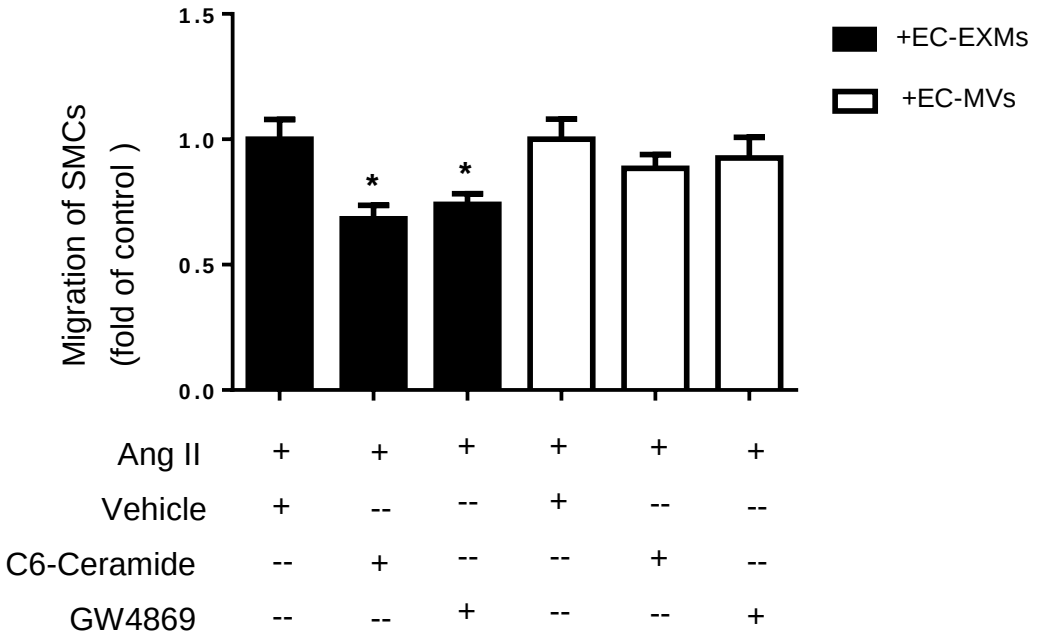


Fig 10. Effect of C6-Ceramide- or GW4869-mediated MVs or EXMs on SMC cell migration. A) Fluorescence images indicate that EC-MVs and EC-EXMs merged into SMCs after 24 hours. SMC were wounded by sterilized pipette tips to cause 1.0 to 1.5 cm wound area. After incubation with B) EC-EXMs or A) EC-MVs C) Ang II with EC-MVs and EC-EXMs for 24 hours, the wound area was measured to evaluate migration of SMCs. Veh: Vehicle group; +C6-ceramide: EC-MVs/EXMs released by the C6-Ceramide group; +GW4869: EC-MVs/EXMs released by the GW4869 group.

VI. DISCUSSION

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MVs and EXMs are extracellular vesicles released from membranes. They carry biological information molecules form parent cells and transfer information to target cells. Given their properties to transfer messages, they could be used as a drug delivery tool for disease treatment, such as cardiovascular disease and cancer. This potential application has generated an increased interest in the budding and releasing mechanisms of MVs and EXMs. In this study, we choose a simple lipid mechanism of MV and EXM release, being the main component ceramide, which is also associated with MV release, to study EC-MVs and EC-EXMs.

The structure of ceramide influence vesicle formation. D-erythro-C6-ceramide (C6-Ceramide) is the most active ceramide analogue tested [35]. C6-ceramide has a dose dependent inhibitory effect on cell growth. Mouse fibroblast cells treated with C6-ceramide at 10 μM produce minor effects on cell growth, modest inhibition of cell growth at 15 μM, and totally block cell growth and significantly reduce cell viability at 20 μM [35]. Similar results were obtained in this study. Treatment with C6-ceramide at 10 μM had no significant effects on EC proliferation (Fig 5). At a concentration of 15 μM, cell proliferation was significantly affected (Fig 5). These results demonstrate the safety effective concentration of the drug. In addition, C6-Ceramide significantly induced EXMs

and MVs release at a concentration of 10 μM without affecting EC proliferation (Fig 4, 5).

MVs and EXMs can transfer molecular messages form parent cells to recipient cells. miR-126 is a short non-coding microRNA which is highly expressed in endothelial cells. In this study, we detected the level of miR-126 in EC-MVs and EC- EXMs (Fig 8). The presence of miR-126 in EC-MVs and EC-EXMs further proved that MVs and EXMs derived from ECs transport their parent proteins and genetic materials. Previous studies showed that miR-126 is the most highly enriched microRNA in ECs [30]. It regulates several aspects of the EC biology, including cell migration, organization of the cytoskeleton, capillary network stability, and cell survival, demonstrating that miR-126 is required for the maintenance of vascular structure. It also plays an important role in angiogenesis. Angiogenic growth factors, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), regulate EC proliferation, migration and adhesion by activating the MAP kinase pathway, which culminates in the nucleus to enhance the expression of genes required for angiogenesis and vascular integrity. miR-126 can directly target the Sprouty-related EVH1 domain-containing protein 1 (SPRED1) and phosphoinositol-3 kinase regulatory subunit 2 (PIK3R2/p85-β), two negative regulators of the VEGF signaling pathway, to regulate angiogenesis [31].

Considering the important vascular protective functions of miR-126, we evaluated the expression of miR-126 in ECs to determine the effects of modulating MV/EXM release by C6-ceramide and GW4869 in their parental EC

functions. However, results showed that both C6-Ceramide and GW4869 had no effect on miR-126 expression in ECs compare to the control （Fig 6）, but the expression in C6-ceramide-treated ECs was statistically significantly increased compared to its expression in GW4869-treated ECs (Fig 6). This finding indicates that the differences in miR-126 expression between C6-ceramide- and GW4869-treated ECs may have altered EC function. To further study the causes of EC function changes upon modulation of particle release, we plan in future studies to perform additional EC functional tests such as measurement of NO production, eNOS expression and ROS production in parental ECs. Moreover, the changes in miR-126 level in EC-MVs and EC-EXMs significantly decreased compared to the control (Fig 8). Considering the cardio-protective properties of miR-126, level changes in EC-MVs and EC-EXMs may lead to a decrease in EC-MV or EC-EXM function. However, Ang II induced SMC migration was decreased by EC-EXMs (Fig 10D). Ang II is the main biologically active agent of the renin-angiotensin system. Several studies found that Ang II induces vascular SMC proliferation and migration, which is associated with various vascular pathological processes in cardiovascular diseases such as hypertension, atherosclerosis, and post-interventional restenosis [34, 36-38]. In this study, EC-EXMs inhibition of ang II-induced cell migration (Fig 10D) appears to uncover the vascular protective properties of EC-EXMs. The miR-126 level of EC-EXMs (Fig 8) may indicate that the protective function in Ang II induced SMC migration is independent of the miR-126 level. Moreover, the effects of decreasing of miR-126 level in EC-MVs and EC-EXMs (Fig 8) and increasing in ECs still remain unclear. To further confirm the changes in C6-ceramide and GW4869 mediated EC-MVs and EC-XMs,

more experiments will be needed to test their functional content at the molecular level.

Another interesting finding in this study is that GW4869 induced MV uptake by ECs (Fig 7). MVs can merged into target cells via phagocytosis or directly fusion into cells. This merging ability may vary due to the EC condition. The fluorescence images of PKH26 labeled EC-MVs co-incubated with ECs, showed that GW4869 induced MV uptake (Fig 7). This ability may refer to EC function. The increasing uptake level also may contribute to the study of MVs and EXMs as drug delivery factors. The mechanisms of MVs and EXMs merging to cells still remain unclear, and this is a new interesting subject for extracellular vesicle studies. The next steps regarding studies on the release mechanism of MVs and EXMs, will combine the release mechanism with the merging mechanism together to study EC-MVs and EC-EXMs more systematically.

VII. CONCLUSIONs

Our data demonstrate that C6-Ceramide induces both MV and EXM release whereas GW4869 only decreases EXM release in ECs without affecting cell proliferation at the concentrations tested. In addition, the C6-Ceramide treatment increased miR-126 expression and GW4869 treatments increased MV uptake ability in ECs. EXMs inhibited Ang II-induced SMC migration with no effect on normal SMCs. In contrast, MVs released from ECs had no effect on Ang II-induced SMC migration. All these effects are independent of miR-126 level in EXMs or MVs. The data suggest that because EXMs released under both stimulatory and inhibitory conditions had no effect on EC proliferation, these particles can be considered as vasoprotective.

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