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Exosomes Released from Multiple Myeloma Cells Influence the Angiogenic Function of Endothelial Cells by Regulating MicroRNA-29b

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EXOSOMES RELEASED FROM MULTIPLE MYELOMA CELLS INFLUENCE THE ANGIOGENIC FUNCTION OF ENDOTHELIAL CELLS BY REGULATING MICRORNA-29B

A Thesis submitted in partial fulfillment of the
requirements for the degree of
Master of Science

By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Qinmao Ye ENTITLED Exosomes Released from Multiple Myeloma Cells Influence the Angiogenic Function of Endothelial Cells by Regulating MicroRNA-29b BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Ye, Qinmao, M.S. Department of Pharmacology and Toxicology, Wright State University, 2018. Exosomes Released from Multiple Myeloma Cells Influence the Angiogenic Function of Endothelial cells By Regulating MicroRNA-29b

Multiple myeloma is a hematological malignancy characterized by clonal proliferation of plasma cells generally caused by chromosomal abnormalities. It occurs in the bone marrow, which is the microenvironment of multiple myeloma. Exosomes (EXs) are 30-100 nm membrane-derived micro-vesicles containing various of bioactive molecules, such as microRNAs, to mediate the cell-cell interaction. Numerous studies reported that exosomes play a significant role in tumor microenvironment. Angiogenesis has the important implication in tumor exacerbation to supply nutrients to promote the progression of cancer cells through endothelial cells (ECs). Some studies demonstrated that microRNA-29b (miR-29b) can suppress tumor development and inhibit angiogenesis. Therefore, in this study, we designed experiments to research the relationship between exosomes released from multiple myeloma, miR-29b and angiogenesis in ECs. Two types of multiple myeloma cells, OPM2 and RPMI-8226 cell lines, were treated with C6-Ceramide. Their released exosomes (MM-EX$^{C6\text{-}Cer}$) were collected, which enriched in miR-29b. MM-EX$^{C6\text{-}Cer}$ were cocultured with human umbilical vein endothelial cells (HUVECs) to test the effect on angiogenic function of

HUVEC. The results showed that the EC proliferation, the tube formation, migration and vascular endothelial growth factor A (VEGFA) expression were decreased in ECs. In addition, miR-29b inhibitor was used in ECs, and could decrease the level of the miR-29b in ECs. Exosomes released from multiple myeloma cells (MM-EX) were cocultured with ECs, which were treated with miR-29b inhibitor, to examine the effects on EC angiogenic function. We found that EC proliferation, VEGFA expression, migration and tube formation were promoted. This data demonstrated that miR-29b can negatively modulate the angiogenic function of ECs through exosomes secreted by multiple myeloma cells.

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I. INTRODUCTION

Multiple Myeloma

Multiple Myeloma (MM) is a deadly hematological malignancy [1]. It is characterized by the clonal proliferation of plasma cells in the bone marrow (BM), and a monoclonal protein existing in the serum or urine [2].

MM accounts for approximately 13% of hematologic cancers [2], but it has nearly 20% deaths of hematological malignancy [3]. MM was first time to be documented in 1844. In the next period, it is gradually descripted detailly, and the new treatment are continuously discovered. MM diagnosed in Africa and America is 10-12/100,000, and it is in Asia is 0.5-1/100,000 [4]. Most of patients are approximately 70 years old, and 37% of patients are younger than 65 years old [2].

Chromosomal Abnormalities in MM

There are some factors to arouse MM, such as obesity, family history, genetic factors and even environmental factors. In general, MM is most thought to be preceded by a monoclonal gammopathy of undetermined clinical significance (MGUS), and process to malignant MM [5]. DNA damage occur in malignant plasma cells during the process of MM pathogenesis [2]. Aneuploidy is a common finding in MM.

The primary chromosomal abnormalities are typically involved the

immunoglobulin heavy chain (IgH) that switch region on chromosome 11 and 14. The secondary translocations are usually discovered during tumor progression, and there are four abnormalities often reported [1] (Table 1). Chromosomal abnormalities can activate proto-oncogenes or inactive tumor suppressor gene inactivation [6], and they also correspond to stimulation in MM microenvironment [2].

Table 1. Chromosomal Translocations in MM

Table 1. Chromosomal Translocations in MM		
	Location	**Development of MM**
Primary Translocations	**t (11:14) (q13; q32)** [7-9]	Most Common Chromosomal Abnormality; Involves bcl-1 Oncogene; Found in 15% Patients; Associated with a Favorable Outcome
	t (4:14) (p16; q32) [10-13]	Involves *Wolf-Hirschhorn syndrome candidate 1 gene* (WHSC1) & *fibroblast growth factor receptor 3* (FGFR3); Poor Survival
	t (14:16) (q32; q23) [9,10]	Less Common, but significant importance in clinical process; Juxtaposes IgH locus and c-MAF locus; Poor Outcome
	MYC Oncogene & Ig Locus (8q24) (Bergsagel and Kuehl, 2001; Gabrea et al., 2008)	Involve in 45% of the patients; Late-Stage events in tumor progression

Secondary Translocations	**Deletion of Chromosome 13** [9] [13,14]	Found in 50% of patients; Close Associated with the translocation t (4：14) (p16; q32)
	Deletion of 17p13 [15,16]	Rare in Late-Stage events; Reported in 10% of patients; Correlation with a Poorer Outcome
	Chromosome 1 [8,9] [17,18]	Deletion generally to 1p; Amplifications correspond to 1q

The Microenvironment of MM

The BM as the microenvironment of MM exerts a significantly important effect on cell proliferation, growth and survival of MM cells [19]. Bone cells, myeloma cells, endothelial cells and extracellular matrix take close interactions in this microenvironment. MM cells adhere to bone marrow stromal cells (BMSCs) to maintain cell proliferation and invasion [20].

Certain proteins in extracellular matrix can mediate via cell receptors to induce tumor cells growth, such as vascular endothelial growth factor (VEGF), interleukin-6 (IL-6) [21], insulin-like growth factor 1, tumor necrosis factor and transforming growth factor β1. The involvement of IL-6 enhances the adhesion of multiple myeloma cells to promote cell survival in BM [1]. These proteins are produced and secreted by cells, including myeloma cells, to the BM to influence the microenvironment of MM.

Exosomes are novel sight in tumor microenvironment that are thought to mediate the cell-cell interactions in MM microenvironment. BMSCs derived Exosomes directly facilitate MM progression [22,23]. Exosomes released from MM cells regulate the BM

microenvironment via enhancing the angiogenesis and immunosuppression [19]. Nucleic acids also play an important role in the microenvironment of MM through Exosomes. Exosomes carry nucleic acids, such as microRNAs, mRNAs, long non-coding RNAs, DNA fragments triggering significant phenotypic changes in the tumor microenvironment [24].

Angiogenesis in MM

Angiogenesis is a process that new vessels are generated from existed vasculature [25]. Some studies reported that angiogenesis is regulated by molecules and micro-vesicles. At meanwhile, angiogenesis plays a significantly role in tumor progression, which can supply nutrients and growth factor to promote the development of tumor cells [25]. In the microenvironment of MM, cell-cell interaction promotes the production of growth factors, such as VEGF, which up-regulates the angiogenesis in MM [2].

Exosomes (EXs)

EXs are 30-100 nm membrane-derived vesicles that contain a wide range of functional proteins, lipids, mRNAs, and microRNAs to mediate the cell-cell communications. They usually present in blood, urine, breast milk and semen. Numerous studies identified that various cell types can release EXs including immune cells, tumor cells, endothelial cells, stem cells and among others [25]. CD9, CD81, and CD63 are classic exosomal markers [26] carried by EXs.

It is reported that EXs fused with the recipient cells membrane are more likely to occur in the acidic extracellular environment [27]. Depend on the parental cells of EXs, they can play a variety of specific role with carried different nucleic acids, proteins or lipids. For instance, when EXs released from tumor cells, the content of EXs promote angiogenesis and cell proliferation [28]. If EXs secreted from immune cells, they can assist in antigen presentation [29]. Otherwise, EXs can travel to distant cells to have effects via body fluids [30]. Recently, tumor-derived EXs are thought as the potential vaccines for the tumor [31], and they have potential diagnostic abilities in cancer disease [32].

The Biogenesis of EXs

The biogenesis of EXs starts with endocytosis that are from the inward budding of plasma membrane to form the early endosomes [33]. Early endosomes mature to late endosome, which is also known as multivesicular bodies (MVBs). In this process, intraluminal vesicles (ILVs) are accumulated inside of MVBs through the endosomal sorting complex required for transport (ESCRT) machinery [34] and ESCRT-independent pathways, such as ceramide-dependent mechanism [35] (Table 2). EXs secreted by MVBs fusing with the plasma membrane to release ILVs to extracellular space, which are referred to EXs. MVBs can fuse with lysosomes to degrade content [33] (Figure 1).

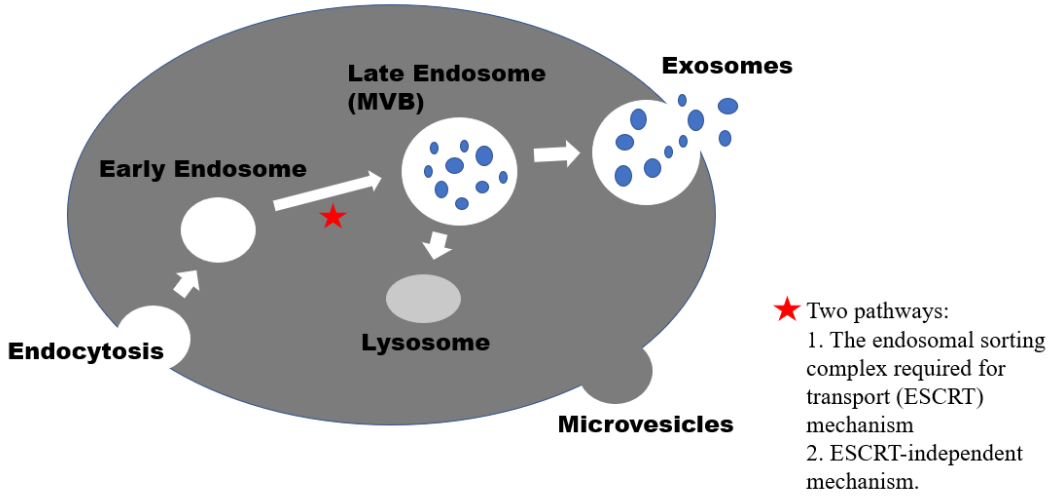


Figure 1. The Biogenesis of EXs and MVBs

Table 2. The Mechanism of EXs Generation

Table 2. The Mechanism of EXs Generation [36,37]		
	Type	Functions
ESCRT Pathway	ESCRT-0	Cluster cargo in a ubiquitin-dependent way
	ESCRT-I	Involve in budding
	ESCRT-II	
	ESCRT-III	Vesicle scission
ESCRT Independent Pathway		Involve lipids (ceramide, cholesterol, phospholipase D2), or tetraspanins

MM Derived EXs

EXs released from tumor cells have been identified that MHC class I molecules is

loaded in EXs [38]. MM derived EXs (MM-EX) also have the same characterization. MHC class I, CD44 and bone marrow stromal antigen 2 (BST-2) are rich in the membrane of MM-EX. Moreover, antigen presenting molecules, adhesion molecules, membrane transport, cytoskeletal proteins and other bioactive proteins play important roles in development of MM from MM-EX [39].

It has been reported that EXs released from MM cells can promote angiogenesis in BM to enhance MM growth [40]. MM cells produced EXs with the hypoxia conditions can increase the endothelial tube formation through HIF-FIH signaling pathway [41]. MM-EX also play a role in enhancement the osteoclastic activity in the BM [42], and it induces osteoclast precursors to differentiate to osteoclasts [43]. Of note, EXs secreted from MM can directly increase MM cell proliferation [44].

EXs in Angiogenesis

EXs have been indicated that exert salutary or deleterious effects on angiogenesis due to their different content or origins [25].

The function of EXs secreted from tumor cells are also high-profile. The support of numerous blood vessels is necessary to cancer exacerbation, which can supply nutrients and growth factors to tumor. EXs from chronic myeloid leukemia cells affect vascular remodeling via IL-8 activated VCAM-1 [45]. There are other studies that identify that EXs released from tumor cells influence enhancement in angiogenesis. Moreover, EXs are thought as a new therapeutic target for the treatment of certain

tumor.

Endothelial cells (EC) derived EXs play potential roles in regulation the autocrine or paracrine factors via proteins and RNAs/microRNAs. MicroRNA-146a-laded exosomes released from ECs cocultured with cardiomyocytes leading to a decrease in metabolic activity [46]. However, it is also reported that endothelial exosomes promote capillary-like structure formation for neighboring ECs through the Delta like ligand/Notch pathway [47].

In addition, EXs generated from platelets, stem cells, and cardiomyocyte cells are reported that they have pro- or anti- effects in angiogenesis [25].

MicroRNAs

MicroRNA (miR) is approximately 22-nt-long non-coding RNA molecules, which regulates the activity of specific mRNA targets. It exerts functions on cell proliferation, inflammation, angiogenesis, apoptosis and oncogenesis [48]. MiRs are widely found in animal, plants and unicellular eukaryotes [49].

MiRNAs play function through the RNA-induce silencing complex (RISC), which is mature miR loaded into the ribonucleoprotein complex. RISC specifically bind in the specific 3'-untranslated regions (3'-UTR) of the target genes to inhibit translation or degrade mRNA [48].

MiRs are potentially involved in MM as oncogenes or tumor suppressors through signaling pathways [50].

The Biogenesis of Human MiRs

Human miRNA biogenesis is briefly formed through two-step cleavage events by two ribonuclease III endonucleases, Drosha and Dicer, respectively performed in nuclear and cytoplasm [51].

MiRs are transcribed by RNA polymerase II or III to produce primary miRNA (pri-miRNA) molecule. Following Drosha-DGCR8 complex process the pri-miRNA into approximately 70-nucleotide precursor miRNA (pre-miRNA). The pre-miRNA is then exported to the cytoplasm via RAN-GTP and Exportin-5, and it is next cleaved by Dicer, assisted by TRBP, to the ~22 nt miRNA duplex containing mature miRNA [52] (Figure 2).

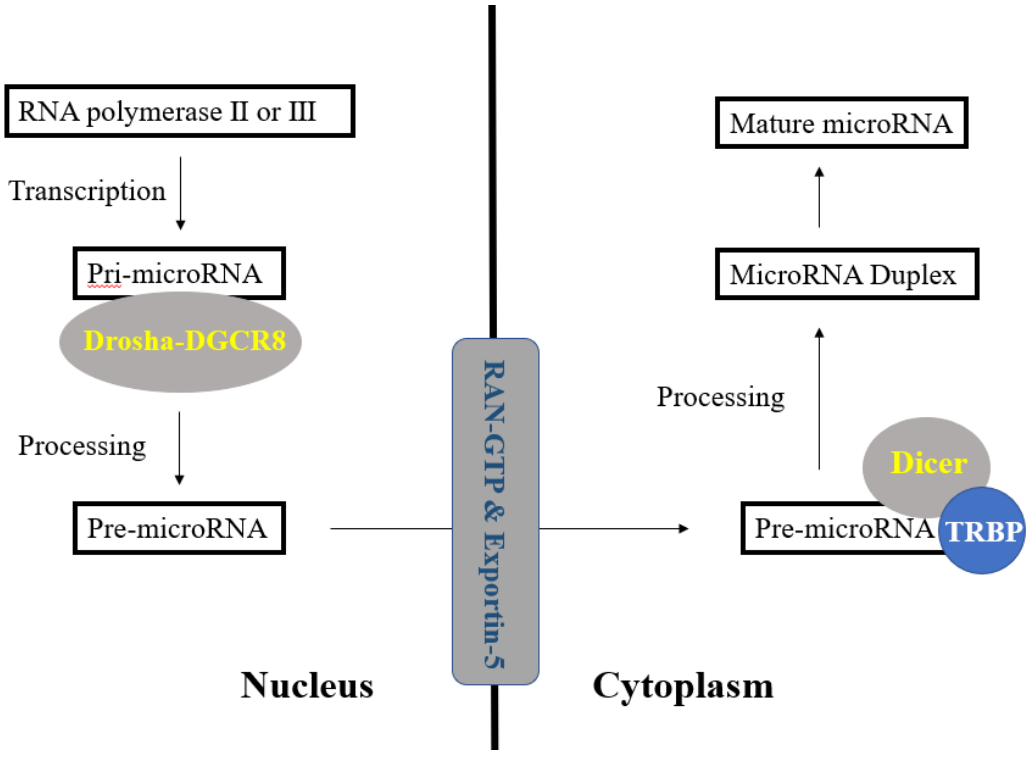


Figure 2. The Biogenesis of Human MiRs

EXs-derived MiRs

EXs, as the delivery tool in vivo, can contain functional miRs to exert effects. According to the biogenesis of EXs, the components of EXs form in the MVBs before they secreted rom parental cells. Therefore, the mature miRs are loaded into EXs in this process. Some studies reported that miR sorts into EXs through a ceramide-dependent secretory mechanism [53]. Also, the miR loaded into EXs is probably in a miR-induced silencing complex independent manner [54]. Interestingly, EXs-derived miRs do not completely copy from parent cells, suggesting that miRs are selectively packed into EXs [55,56].

It has been indicated EXs-derived miRs regulate the recipient cell migration, inflammation, immune responses, angiogenesis and metastasis [48]. In addition, miRs in EXs released from tumor cells play dual roles in tumor progression. Numerous studies demonstrated that exosomal miRs can be used as diagnostic biomarker. Luo et al [57] reported that EXs-derived miRs can serve as biomarkers in pregnancy disorders.

MiR-29b

MiR-29b has two sub-family members – miR-29b1 and miR-29b2. These two types of miR-29b come from two different per-miRNA, but the mature miR-29bs are identical [58]. Many studies have indicated that miR-29b can serve as a tumor suppressor [59]. In MM, miR-29b negatively modulates the migration of MM and ECs

[60]. In breast cancer, miR-29b is involved in angiogenesis, and targets VEGFA in this microenvironment [61].

Cheng et al measured the miR-29b levels of exosomes released from MM cells treated with C6-ceramide (MM-EX$^{C6\text{-}Cer}$) and found that miR-29b expression were significantly increased (Figure 3). Also, Cheng et al examined the proliferation of MM cells after cocultured with MM-EX$^{C6\text{-}Cer}$ and found that the MM cell proliferation was remarkably inhibited [62].

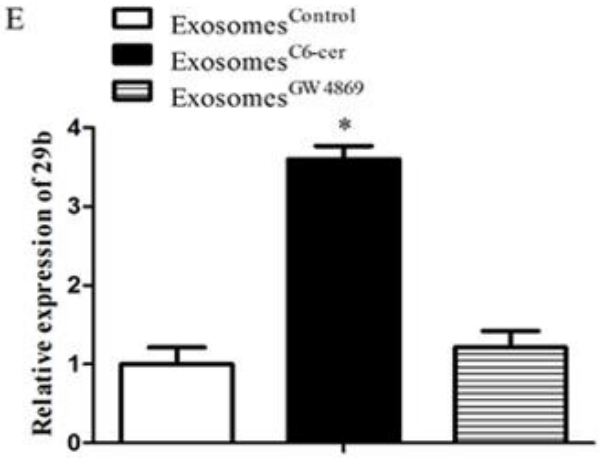


Figure 3. The MiR-29b Expression in MM-EXs with Different Condition.

The miR-29b level in MM-EX$^{C6\text{-}Cer}$ was significantly increased compared to MM-EX^{Control} and MM-EX^{GW4869}.

II. HYPOTHESIS AND SPEICIFIC AIMS

Hypothesis:

The angiogenic function of ECs is partially regulated by miR-29b with the MM-EX.

Specific Aims:

Aim 1: to determine the effects of exosomes derived from C6-Ceramide stimulated multiple myeloma cells (MM-EX$^{C6\text{-}Cer}$) on ECs angiogenic function (migration, tube formation ability and VEGFA expression), and the levels of miR-29b in ECs.

Aim 2: to determine the role of miR-29b in mediating the angiogenic function of MM-EX on ECs.

III. EXPERIMENTAL DESIGN

Design for Specific Aim 1:

Two types of MM cell lines, OPM2 and RPMI-8226, were used in this study. Each cell lines were respectively cultured in serum-free medium (Vehicle group) and in 10 μM/μl C6-ceramide serum-free medium (C6-Cer group) for 48 hours. Culture media samples were collected for EXs isolation. MM-EX^{veh} and MM-EX$^{C6\text{-}cer}$ were respectively suspended in HUVEC completed media, and then cocultured with ECs in 48 hours to examine the EC proliferation, tube formation, migration and VEGFA expression (Figure 4).

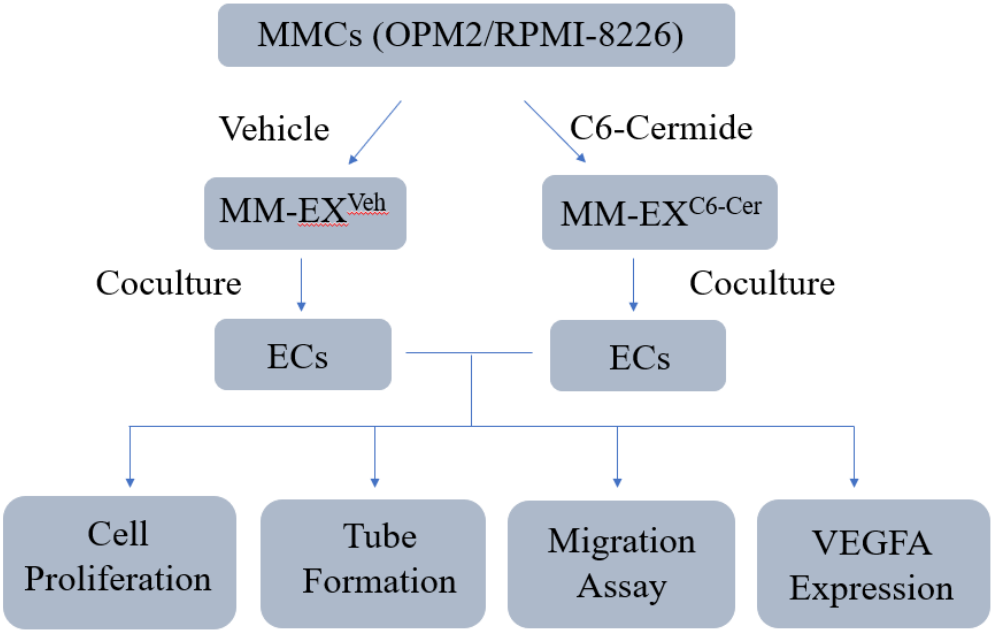


Figure 4. The Flow Chart of Experimental Design 1

Design for Specific Aim 2:

MiR-29b inhibitor and miRs inhibitor control were respectively transfected into ECs for 6 hours in advance. All MM cells were cultured in serum-free media for 48 hours; the cell supernatant was then used for EXs isolation. Then EXs were suspended with HUVEC completed media to be cocultured with ECs for 48 hours to test the cell proliferation, tube formation, cell migration and VEGFA expression of ECs (Figure 5).

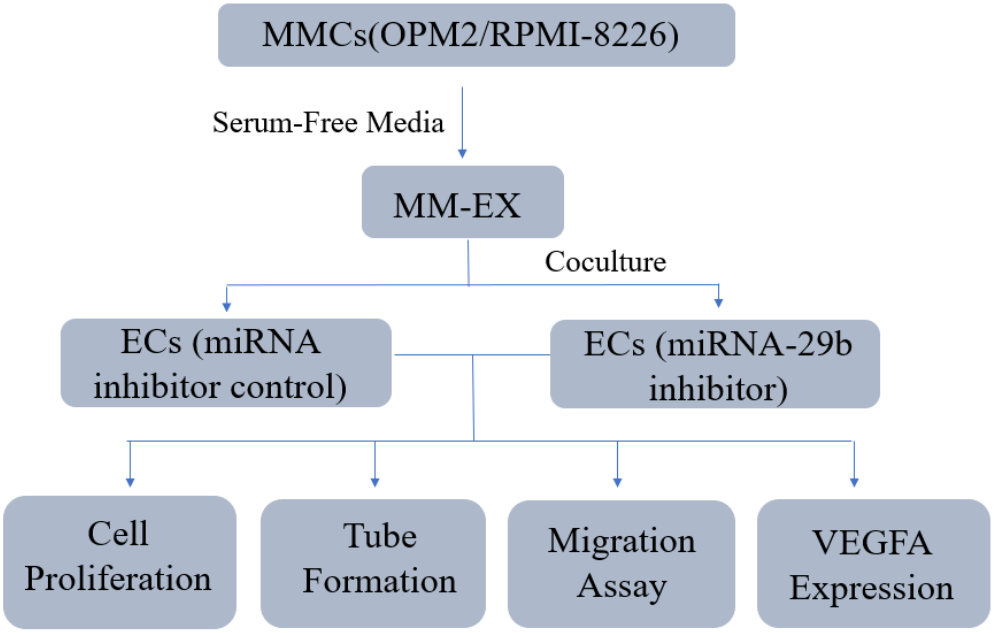


Figure 5. The Flow Chart of Experimental Design 2

IV. MATERIALS AND METHODS

Materials

OPM2 MM cell lines was a gift by Dr. Zhan (University of Iowa). RPMI-8226 cell lines, HUVEC lines and F-12K medium for HUVEC were purchased from ATCC company. RPMI 1640-medium for MM cells and fetal bovine serum (FBS) were purchase from Hyclone Corp. Endothelial cell supplement factor, heparin for cell culture, antibiotic-antimycotic solution, trypsin, methylthiazolyl tetrazolium (MTT), antibodies for western blotting, N-Hexanoyl-D-sphingosine (C6-Ceramide) and PKH26 were purchased from Sigma-Aldrich company. TRIzol reagent was from Invitrogen, and Matrigel matrix was purchased from Corning company. CDNA synthesis kit was purchased from Takara company, and q-PCR kit was from GeneCopoeia. MiR-29b inhibitor (SiRNA-29b), miR inhibitor control, and transfection reagent was purchased from Dharmacon company.

Methods

1. Cell Culture

HUVECs were culture in F-12K medium with 10%fetal bovine serum (FBS), 0.05 mg/ml endothelial cell growth supplement, 0.1mg/ml heparin sodium salt from porcine intestinal mucosa, and penicillin/streptomycin. Human MM cells line OPM2, RPMI-8226 were culture in RPMI 1640 medium, supplemented with 10% fetal

bovine serum and antibiotics. All of cells in this experiment were maintained at 37°C with 5% CO2 and 95% humidity.

2. EXs Extraction and Immunofluorescence Staining

EXs were collected from the culture supernatants of MM cells by multiple centrifugations. Firstly, cell medium was harvested when MM cells cultured in serum-free medium in 48 hours. Then, the medium was centrifuged for 20 min at 2000g to remove cells and cell debris. Next, the cell-free culture medium was centrifuged again at 20,000g for 70min, and ultra-centrifuged at 170,000g for 1.5h. The pelleted EXs were resuspended with filtered PBS or medium to be used for following experiments.

3. Nano Tracking System Analysis (NTA)

Nano Tracking System Analysis (NTA) 300 was used to analyze the size and concentration of these exosomes. EXs suspended in PBS were loaded into the sample chamber. Light scatter mode of the tracking system used the camera level 10 and the camera filter 1. 30 seconds videos were taken three times with a frame rate of 30 frames per second. NTA 3.0 software was used to analyze the data.

4. EXs Uptaking

To examine the uptake of EXs, PKH67 was used to label EXs. 4 µl of PKH26

with 1 ml PBS to staining every 30 μg of EXs for 4 min protected from light. Then added an equal volume of 1% BSA to stop staining reaction, and centrifuged at 170,000g in 90 min at 4°C. Next, EXs pellet was washed with PBS and centrifuged at 170,000g for 90 min at 4°C. The EXs were resuspended with completed F-12K medium and cultured with ECs for 24 hours. After removed the old medium, HUVECs were washed with PBS and stained with DAPI for 5-10 minutes in room temperature pretend from light. Then, HUVECs were washed with PBS again and fixed with 4% formaldehyde. LSM710 laser scanning microscope was used to take the pictures of the EXs uptake in ECs.

5. Co-culture System

For aim 1: MM cell were respectively cultured in serum-free medium and in 10 μM/μl C6-ceramide serum-free medium for 48 hours. Culture media samples were collected for EXs isolation. MM-EX^{veh} and MM-$EX^{C6\text{-}cer}$ were respectively suspended in HUVEC completed media, and then cocultured with ECs in 48 hours.

For Aim 2, miR-29b inhibitor and miRs inhibitor control were respectively transfected into ECs for 6 hours in advance. All MM cells were cultured in serum-free media for 48 hours; the cell supernatant was then used for EXs isolation. Then EXs were suspended with HUVEC completed media to be cocultured with ECs for 48 hours.

6. Cell Proliferation Assay

HUVEC cell proliferation was examined by MTT assay. ECs were seeded in a 96-well plate at 1×10^3 cells in each well, and cocultured with OPM2 and RPMI-8226 released exosomes for 48 hours. Then, the cells were incubated with 20μl of 5 mg/ml MTT solution for 4 h at 37°C. After removed the medium containing MTT, 150 μl dimethyl sulfoxide (DMSO) was added to each well. The microplate reader was used to measure the optical density at 490 nm.

7. Migration Assay

HUVECs were seeded in the 6-well plate and cultured until 90% confluent. A 1ml-pipette tip was used to make a scratch on cells and the pictures were captured by using LSM710 laser scanning microscope. After cocultured with EXs in 37°C for 12 hours, ECs were taken pictures again at the same condition. The migration distance of ECs wase analyzed with Image J (NIH).

8. Tube Formation Assay

Matrigel matrix was dissolved at 4°C overnight in advance. 200 μl Matrigel was coated each well on the 48-well plate without air bubbles and gelled in incubator for 30 min. Then, 1×10^5 HUVECs were seeded in the plate and cultured for 4 hours in 37°C. Then, LSM710 laser scanning microscope was used to take the picture of tubes

formed by the cells. Next, the length of tubes was measured by using Image J.

9. RNA Extraction and Quantitative Real-Time PCR Analysis (qRT-PCR)

RNA was extracted with TRIzol. Transcript cDNA synthesis kit was used to transcribed RNA samples to cDNA. QRT-PCR was performed cDNA samples using a Bio-Rad 96 System with GeneCopoeia qRT-PCR kit. The primers U6, miR-29b, GAPDH, VEGFA were used in this experiment. U6 and GAPDH were used as the control gene to calculate the expression of miR-29b and VEGFA.

10. Protein Extraction and Western Blot Analysis

Exosomal proteins and whole-cell lysates were prepared by using lysis buffer. The concentration of protein samples is measured by protein assay, and equal amounts of proteins samples were separated in 6%-10% sodium dodecyl sulfate-polyacrylamide gels. Proteins in gel were transferred to immobilon polyvinyldifluoride (PVDF) membranes. Western blot analyses were performed with mouse antibodies, anti-β-actin and VEGFA (1:500), and goat antibodies, anti-Annexin V, anti-CD63 (1:500). The secondary antibodies were used anti-mouse and anti-goat peroxidase-linked (1:10000). The results were visualized by ECL Prime Western Blotting Detection Reagent.

11. MiR-29b Inhibition

HUVECs were seeded in 6-well plates or 12-well plates with 80%-90% confluency. Then miR-29b inhibitor and miR control (miRCtrl) respectively transfected to ECs by using DharmaFECT 1 Transfection Reagent for 6 hours. Transfection efficacy was examined by qRT-PCR.

12. Statistical Analysis

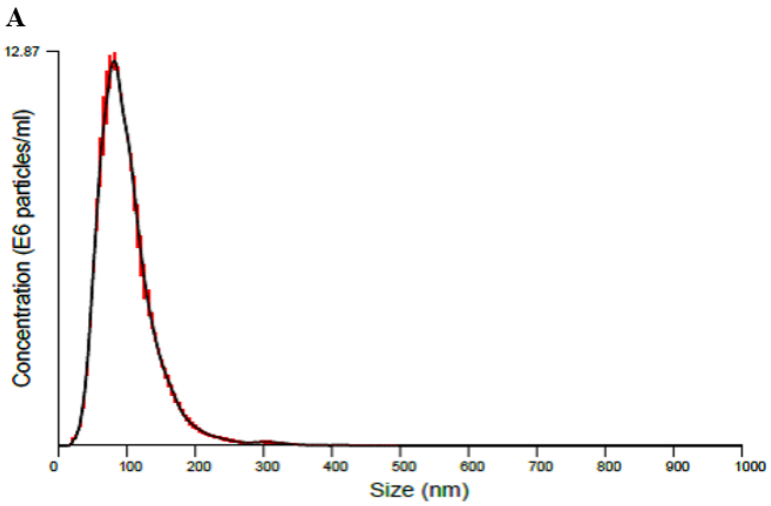
All experimental results were replicated at least three times. Statistical analysis was performed by using t-test or one-way analysis of variance (ANOVA) to compare between two or three groups. In all case, $p < 0.05$ was considered statistical significant. GraphPad Prism 6.0 software was used in statistical analyses.

V. RESULTS

Results for Aim 1

1. The Characterization of MM-EXs

As NTA results shown in Fig 6 (A&B), the diameter of the isolated OPM2-exo and RPMI-8226-exo were both around 100nm. The concentration was respectively 12.47 x 10^6 and 13.51 x 10^6 particles each milliliter. To further identify the micro-vesicles secreted from MM cells were EXs, we measured the protein levels of Annexin V and CD63 (Fig 6C). The data showed that the expression of Annexin V and CD63 was much more in these micro-vesicles than in OPM2 and RMPI-8226 cell themselves.



OPM2

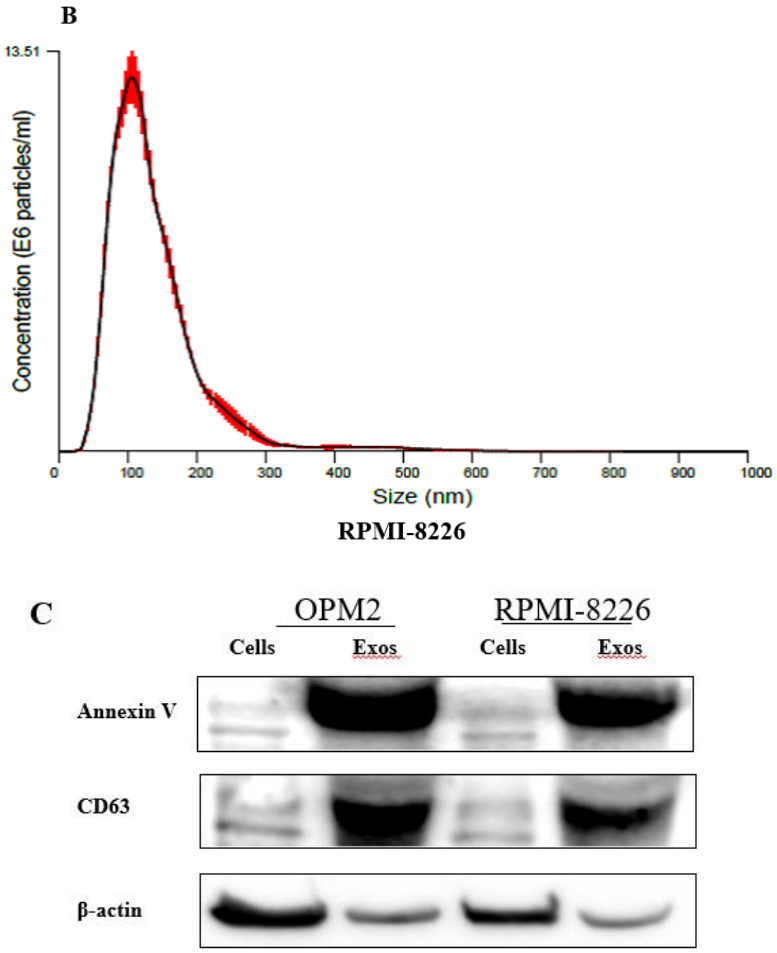


Figure 6. The characterization of EXs released from MM cells.

A), B) Exosomes respectively isolated the culture medium from OPM2 cell lines and RPMI-8226 cell lines was analyzed by NTA. The NTA results of OPM2 and RPMI-8226 derived exosomes indicated the diameter of these particles was both ~ 100 nm.

C) The Annexin V and CD63 were enriched in EXs compared to the cells.

2. The Uptake of MM-EXs by HUVECs

MM-EXs were stained by PKH26 and suspended in HUVEC completed medium to coculture with HUVEC for 24 hours. After old medium of HUVECs was removed,

ECs were stained with DAPI for 5-10 minutes in room temperature in dark. Then ECs were washed with PBS and fixed with 4% formaldehyde. The images of the EXs uptake were taken by using LSM710 laser scanning microscope. Figure 7 A, B showed that MM-EXs was clustered around the nucleus of ECs.

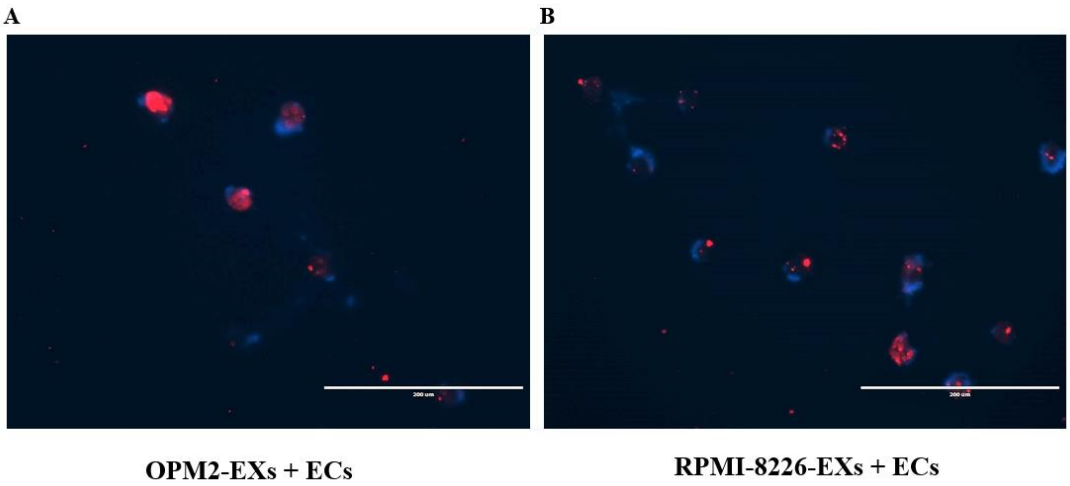


Figure 7. The Uptake of EXs Secreted from OPM2 and RPMI-8226 cells.

A), B) Red: PKH26, EXs; Blue: DAPI, nucleus. Scale bars: 200 μm.

3. MM-EX$^{C6\text{-}Cer}$ Decreased the Cell Proliferation of HUVECs

MM-EX$^{C6\text{-}Cer}$ were collected from OPM2 and RPMI-8226 cells treated with 10 μM/μl C6-ceramide to increase the microRNA-29b level in exosomes, and they were cocultured with endothelial cells for 48 hours. Then, these ECs were incubated with MTT solution for 4 h at 37°C. Next the medium containing MTT was removed and DMSO was added to each well. In Figure 8, the EC proliferation was decreased after ECs cocultured with MM-EX$^{C6\text{-}Cer}$ compared to ECs treated with MM-EX^{Veh} ($p<0.05$).

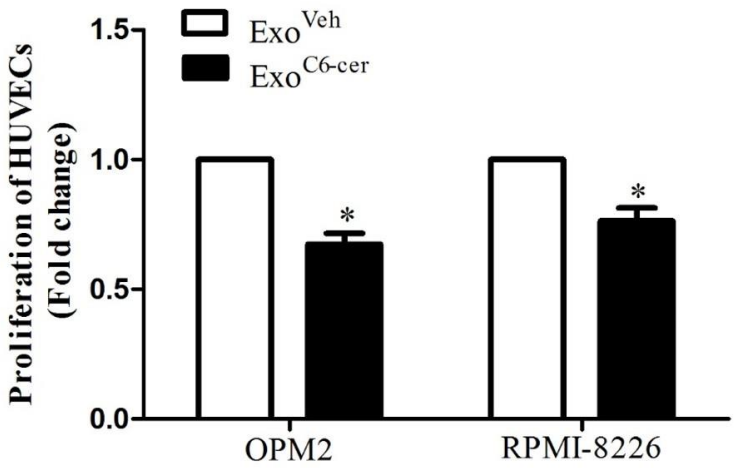


Figure 8. The Effect of MM-EXs on EXs Proliferation.

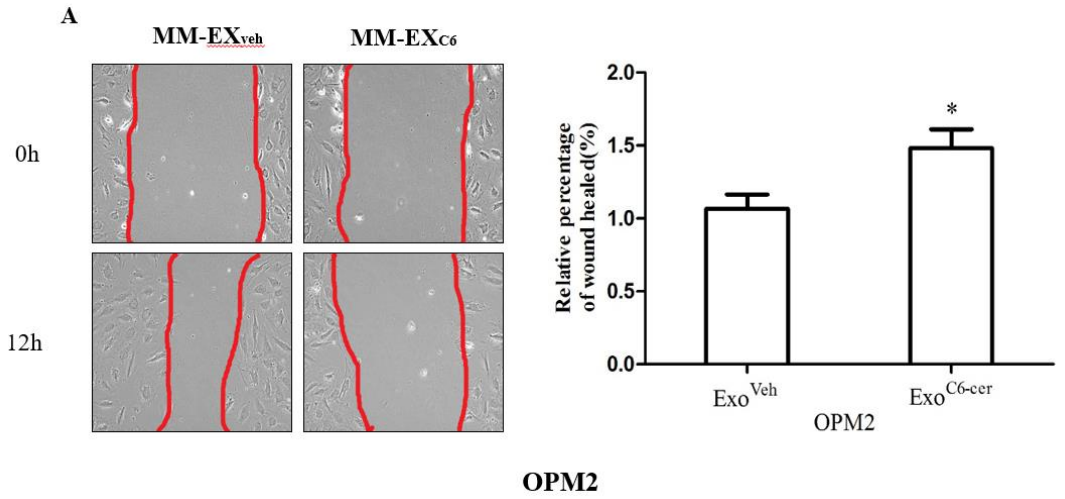
After MM-EX$^{C6\text{-}Cer}$ cocultured with ECs for 48 hours, the cell proliferation was down-regulated compared to the result of MM-EX^{Veh} cocultured with ECs.

4. MM-EX$^{C6\text{-}Cer}$ Inhibited the Cell Migration and Tube Formation of HUVECs

For migration assay, when ECs were cultured until 90% confluent, a 1ml-pipette tip was used to make a scratch on cells. Then pictures were taken by laser scanning microscope (Figure 9 A, B) at 0 hour and 12 hours. The results showed that the migration of HUVEC was reduced after ECs cocultured with MM-EX$^{C6\text{-}cer}$ compared to the HUVEC cocultured with MM-EX^{veh}. It suggests that MM-EX$^{C6\text{-}cer}$ had an inhibitory effect on the migration of HUVECs.

For tube formation, Matrigel was coated each well in plate firstly, then ECs were cultured in this plate. MM-EXs were suspended in medium to be cultured ECs on Matrigel for 4 hours in 37°C. LSM710 laser scanning microscope was used to take

pictures of tubes formed by ECs. In Figure 9 C showed that the tube formation of HUVEC was reduced after treatment with MM-EX$^{C6\text{-}cer}$ compared to the result cocultured with MM-EX^{Veh}. With the analysis of Image J (Figure 9 (D)), the result also demonstrated that the tube length was inhibited in HUVEC cells which were cocultured with MM-EX$^{C6\text{-}cer}$ ($p<0.05$).



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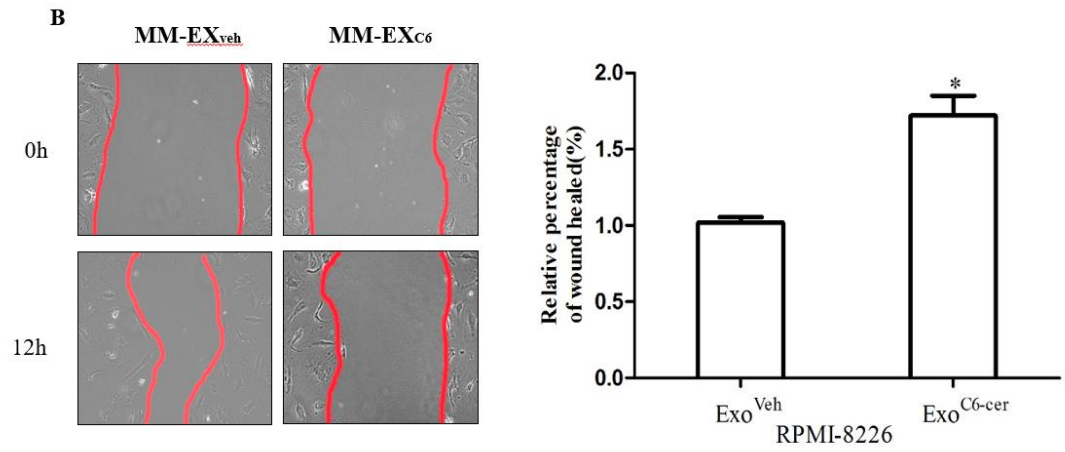


RPMI-8226

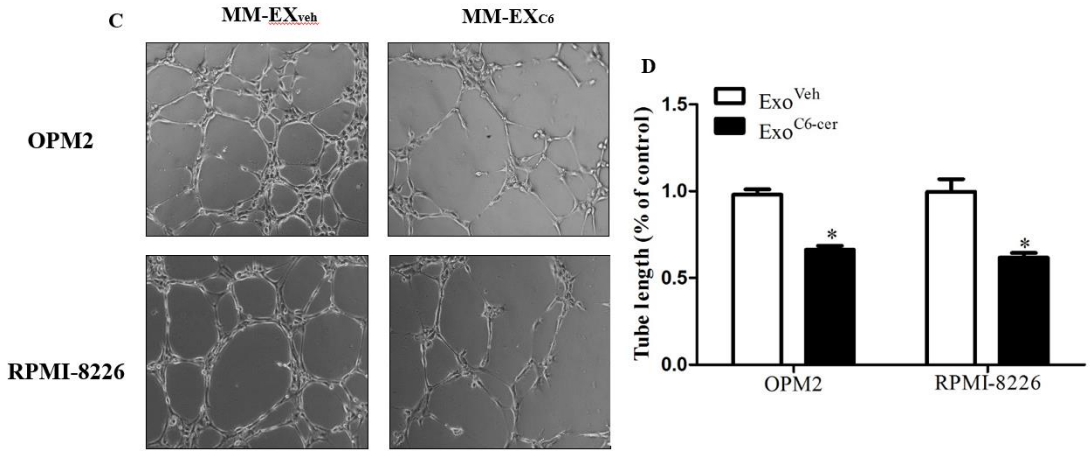


Figure 9. The Effect of MM-EXs on ECs Migration and Tube Formation.

A), B) ECs cocultured with MM-EXs for 12 hours, and the wound healing was reduced with the treatment of MM-EX$^{C6\text{-}Cer}$ compared with the ECs cocultured with MM-EX^{Veh}. **C)** MM-EXs cocultured with ECs on Matrigel for 4 hours, the tube lengths of ECs in MM-EX$^{C6\text{-}Cer}$ group were less than the lengths of ECs treated with MM-EX^{Veh}. **D)** The result of tube formation on ECs was analyzed by Image J, and tube lengths were decreased in ECs cocultured with MM-EX$^{C6\text{-}Cer}$.

5. MM-EX$^{C6\text{-}Cer}$ Down-regulated the VEGFA Expression Level and Up-regulated the miR-29b level in HUVECs

Endothelial cells were cocultured with MM-EXs for 48 hours, and the RNA and protein in HUVEC were extracted. RNA samples were reverse transcription to cDNAs. Then cDNAs was analyzed by qRT-PCR to measure the GAPDH, VEGFA mRNA expression and microRNA U6, miR-29b level (Figure 10 A, D). U6 and GAPDH were a normalization in PCR. The result (10 A) shows that VEGFA mRNA levels in HUVEC was significantly down-regulated after MM-EX$^{C6\text{-}Cer}$ cocultured

with ECs. In figure 10 D, the level of microRNA-29b was obviously up-regulated in ECs which coculture with MM-EX$^{C6\text{-}Cer}$ compared to the vehicle group.

Western blotting was used to analyze the VEGFA and β-actin protein expression in ECs. β-actin was as a standard control. In Figure 10 B, C, VEGFA expression was inhibited in HUVEC when ECs treated with MM-EX$^{C6\text{-}Cer}$ compared to the expression levels in ECs cocultured with MM-EX^{Veh} ($p<0.05$).

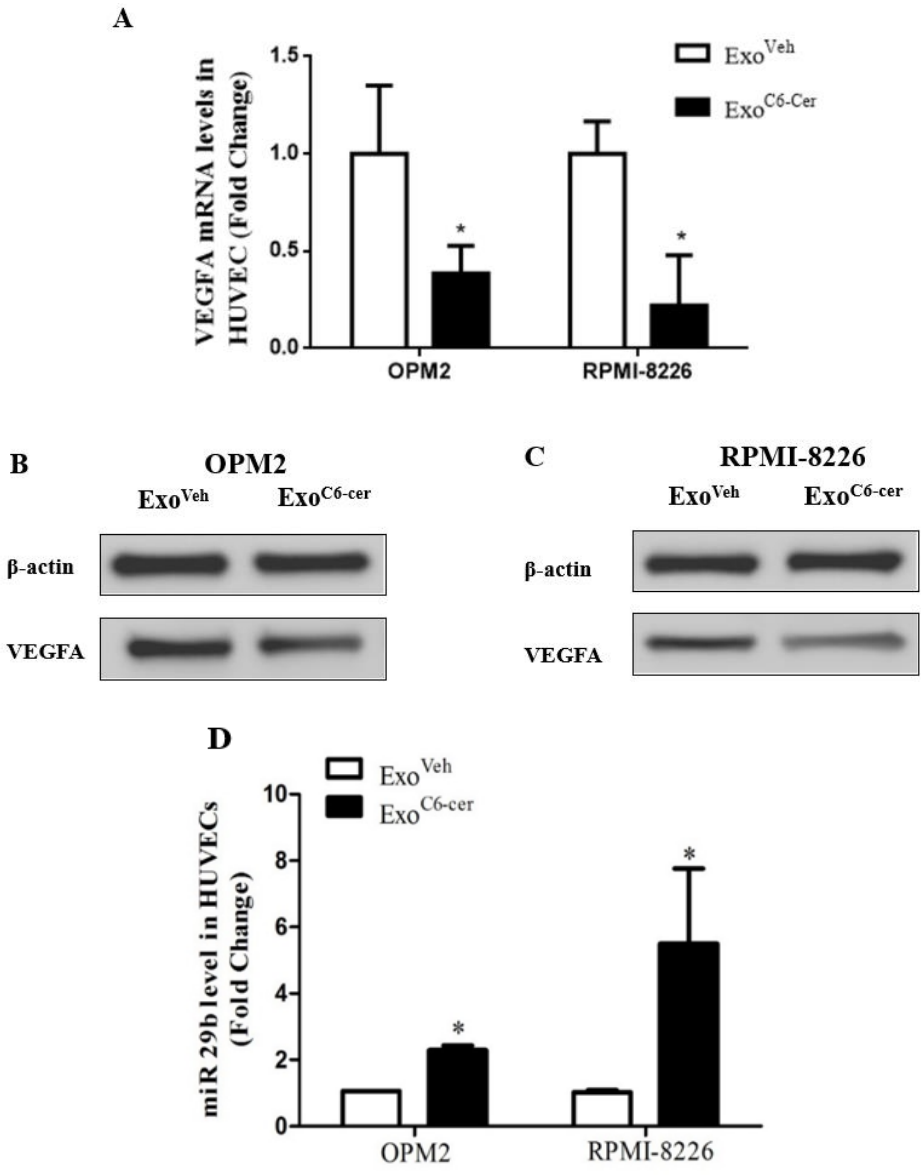


Figure 10. The Effect of MM-EXs on VEGF Expression of ECs.

A) The mRNA expression of VEGFA in ECs was obviously decreased after HUVEC

treated with MM-EX$^{C6\text{-}Cer}$. **B), C)** The protein of VEGF expression in ECs was also down-regulated in ECs cocultured with MM-EX$^{C6\text{-}Cer}$ compared to the protein level in ECs cocultured with MM-EX^{Veh}. **D)** The microRNA-29b expression in HUVEC was significantly increased after ECs cocultured with MM-EX$^{C6\text{-}Cer}$ compared to the miR-29b levels in ECs treated with MM-EX^{Veh}.

Results for Aim 2

1. MiR-29b Inhibitor Down-regulated the Expression of MiR-29b in HUVECs

ECs were grown in plates to 80%-90% confluency. Then miR-29b inhibitor and miR control (miRCtrl) were respectively transfected into ECs for 6 hours. RNA samples were extracted from ECs. Also, RNA sample was collected from normal HUVEC to serve as vehicle group. RNA samples were reverse transcription to cDNAs, and cDNAs was analyzed by qRT-PCR to measure the miR-29b expression. In figure 11, the miR-29b level in ECs of miR-29b inhibitor group was significantly down-regulated compared to the miR control and vehicle group ($p<0.05$).

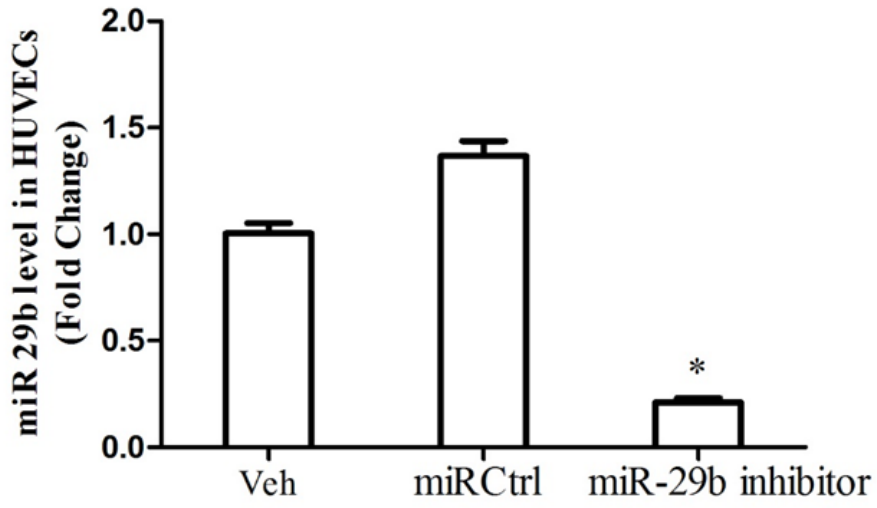


Figure 11. MiR-29b Expression in HUVEC after using miR-29b inhibitor compared to vehicle and miRCtrl group.

The miR-29b level in ECs was obviously reduced with the effect of miR-29b inhibitor.

2. The Cell Proliferation of HUVECs Was Augmented in MiR-29b Inhibited HUVECs

MM-EXs were collected from OPM2 and RPMI-8226 cells treated with serum-free media to be cocultured with endothelial cells, which were prior respectively treated with miRCtrl reagent and miR-29b inhibitor. Then, these ECs were incubated with MTT solution for 4 h at 37°C. Next removed the medium containing MTT and added DMSO to each well to read the optical density. In Figure 12, after the miR-29b was inhibited in ECs, the EC proliferation was increased compared to ECs treated with microRNA control. This result showed that miR-29b inhibitor promoted the proliferation of ECs with MM-EXs ($p<0.05$).

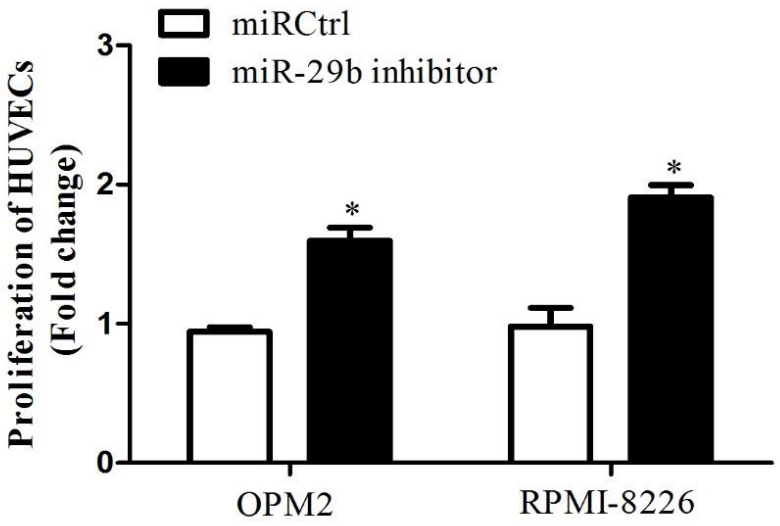


Figure 12. Cell Proliferation of ECs after MiR-29b Inhibition and MM-EXs Cocultured.

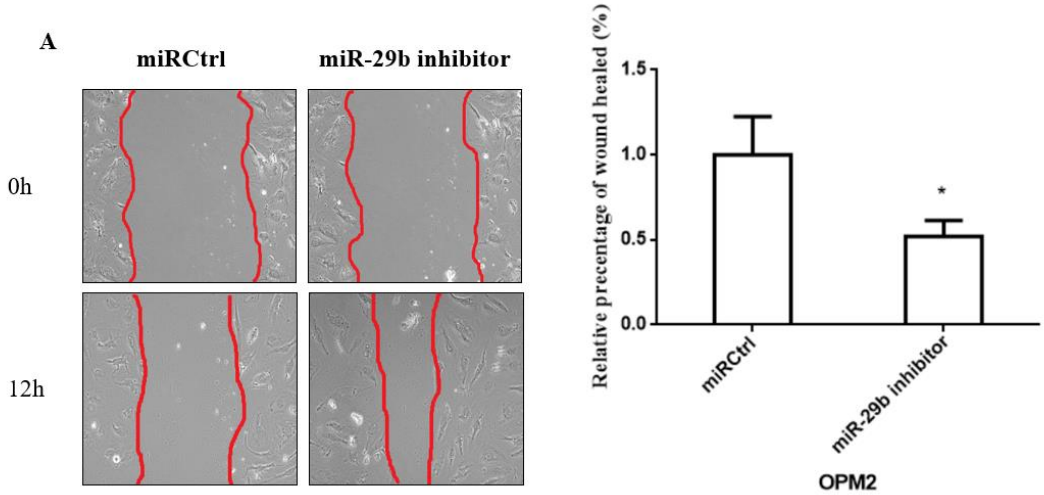
The HUVEC proliferation was upregulated when the miR-29b was inhibited in ECs with MM-EX cocultured.

3. MiR-29b Inhibitor Promoted ECs Migration and Tube Formation in HUVECs

ECs were seeded in 6-well-plate to 90% confluent and were respectively treated with miRNA control and miR-29b inhibitor. After 6 hours treatment, the old medium was removed, and a 1ml-pipette tip was used to make a scratch on cells. Next ECs were cocultured with MM-EXs. Then the picture were taken by using laser scanning microscope (Figure 13 A, B) at 0 hour and 12 hours. The results describe that the migration of HUVEC was augmented after ECs inhibited miR-29b with the influence of MM-EXs compared to the results of HUVEC treated with miRCtrl. It means that microRNA-29b inhibitor had a positive effect on the migration function of HUVEC

with MM-EXs cocultured.

For tube formation, ECs were respectively treated with miRNA control and miR-29b inhibitor for 6 hours. Matrigel was coated each well in plates. Then ECs, which had been treated, were cultured in the plate. MM-EXs were suspended in medium to be cultured with ECs on Matrigel for 4 hours in 37°C. LSM710 laser scanning microscope was used to take picture of tubes of cells. Figure 13 C showed that the tube formation of ECs was promoted after in miR-29b inhibited ECs with coculture of MM-EXs compared to the ECs treated with microRNA control. With the analysis of Image J, the summarized data (Figure 13 D) also indicated that the tube length was increased in HUVEC cells which were treated with miR-29b inhibitor ($p<0.05$).



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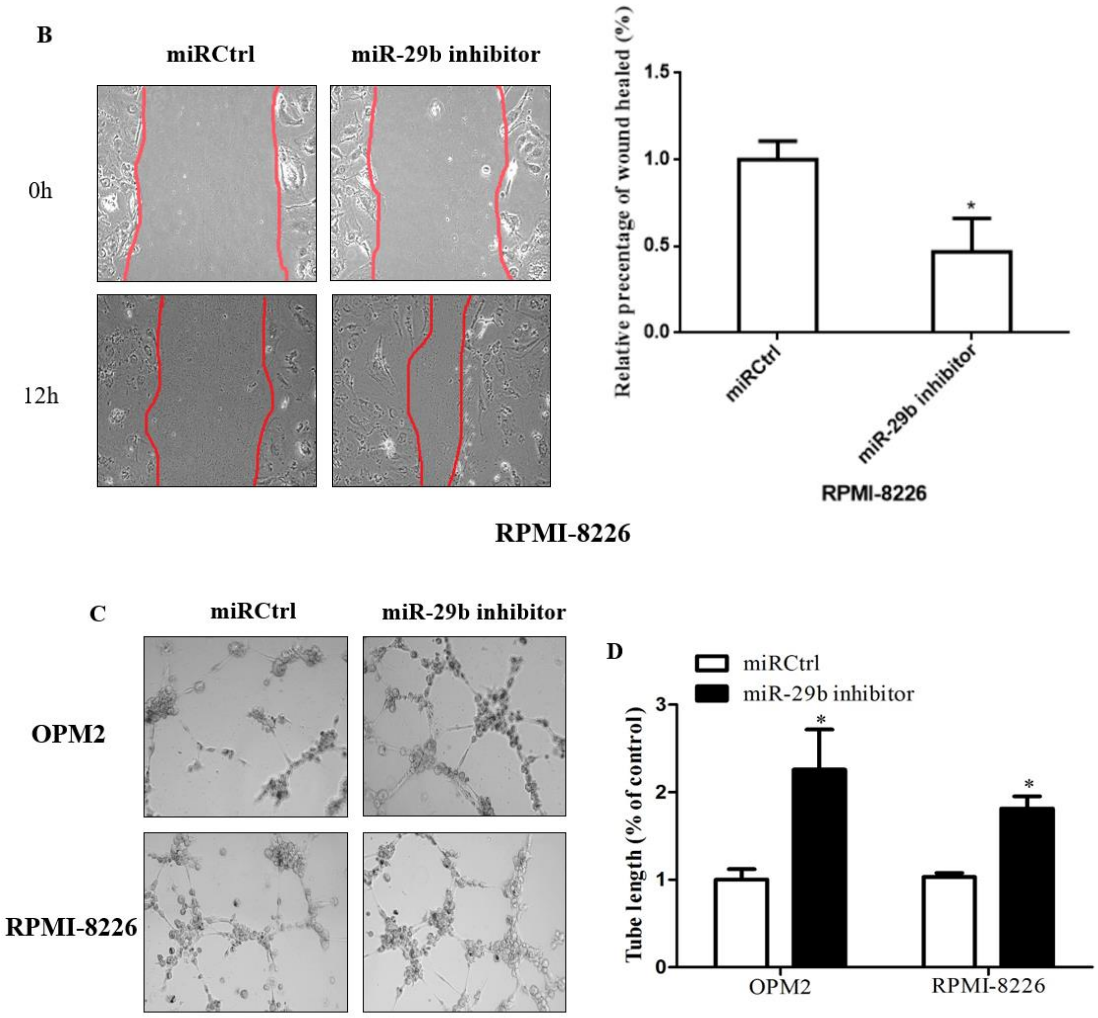


Figure 13. The Effect of MiR-29b Inhibitor on ECs Migration and Tube Formation with MM-EXs Coculture.

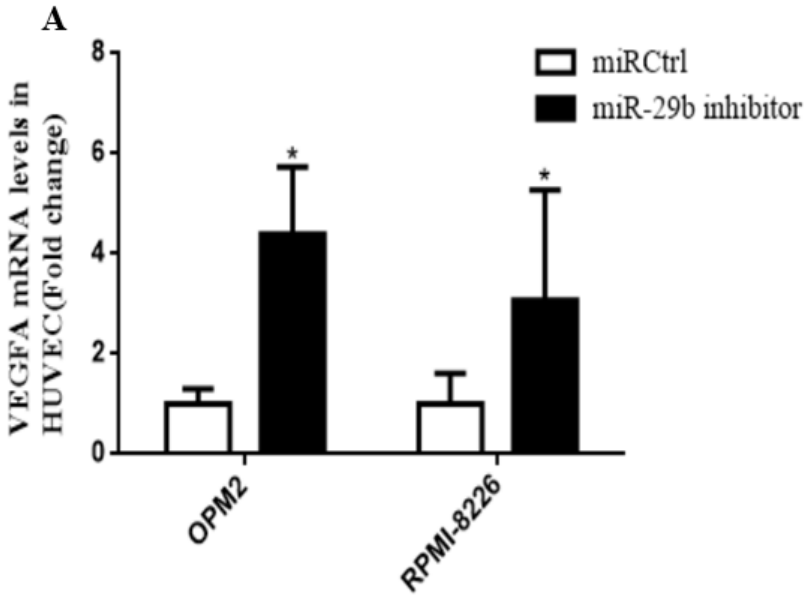
A), B) Inhibited ECs cocultured with MM-EXs for 12 hours, and the wound healing was promoted compared with the miRNA control treated ECs after MM-EXs cocultured. **C)** The tube lengths of ECs were less than the lengths of miR-29b inhibited ECs with MM-EXs. **D)** Summarized data tube formation of ECs in different groups.

4. MiR-29b Inhibitor Up-regulated the VEGF Expression Level in HUVECs

ECs were respectively treated with miRNA control or miR-29b inhibitor for 6 hours. Then the old medium was removed, and ECs were covered with completed medium in which MM-EXs were suspended. Treated ECs and MM-EXs were cocultured for 48 hours, and RNA and protein samples in ECs were extracted.

RNA samples were reverse transcription to cDNAs. Then cDNAs was analyzed by qRT-PCR to measure the GAPDH, VEGFA mRNA expression (Figure 14 A). GAPDH was used to a normalize the mRNA level. The result (14 A) showed that VEGFA mRNA expression in ECs was significantly up-regulated in miR-29b inhibitor group compared to the level in ECs treated with microRNA control.

VEGFA protein levels in ECs were analyzed by the Western blotting. In Figure 14 B, C, VEGFA protein expression was obviously increased in HUVEC when treated with miR-29b inhibitor compare to the level in ECs treated with miR control ($p<0.05$).



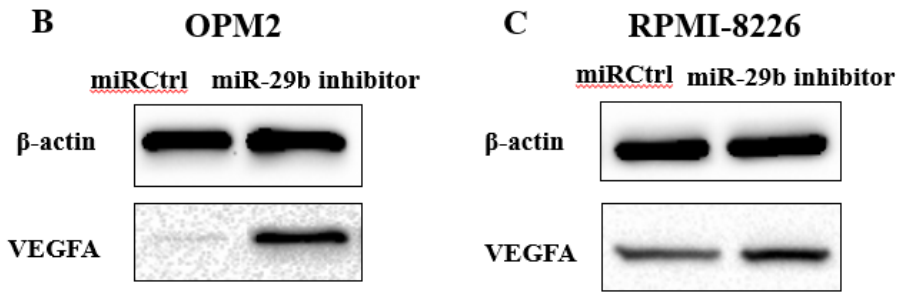


Figure 14. The Effect of MiR-29b Inhibitor on VEGFA Expression in ECs after MM-EXs Coculture.

A) The mRNA expressions of VEGFA in ECs were obviously upregulated after treated with miR-29b inhibitor. **B), C)** The protein levels of VEGFA in ECs were also increased in miR-29b inhibitor treated ECs after cocultured with MM-EX compared to the protein levels in ECs treated with miR control.

VI. DISCUSSION

MM is one of the most common hematological malignancy in the world. Recent research indicates that the microenvironment of MM, the BM, play a crucial role in the development of multiple myeloma.

EXs are membrane-derived micro-vesicles, and they have been demonstrated to play an important role in cell-cell interaction. EXs can serve as a delivery tool *in vivo* to transport functional molecules to neighbor cells and even remoted cells. It has also been found involved into cancer progression or suppression. The miRs are non-coding small RNA molecules, and they consist of a large variety in human. MiRs can bind to the 3'-UTR of target genes to repress translation to exert the modulate function. Of now, numerous studies report that miRs are wrapped in exosomes to participate in some physiological activities, such as immune reaction, tumor progression or suppression. However, the detailed mechanisms between EXs and miRs remain unknown.

In this study, we focused on the roles of EXs and miR-29b in MM cells microenvironment. Some studies indicated that miR-29b can suppress tumor growth via down-regulating angiogenesis. *Amodio et al* demonstrated that miR-29b targets de novo DNA methyltransferase and represses the global DNA methylation in MM cells [63]. Previous study showed that C6-Ceramide stimulated MM cells derived EXs enrich in miR-29b [62]. The miR-29b expression level in ECs after cocultured with MM-$EX^{C6\text{-}Cer}$ was significantly increased (Figure 10 D) when compared to the level in

ECs cocultured with MM-EX^{Veh}. EC proliferation was slightly decreased (Figure 8) after cocultured with MM-EX$^{C6\text{-}Cer}$ when compared to the vehicle group. Also, the angiogenesis functions of ECs (tube formation and migration) were down-regulated (Figure 9). VEGFA level was examined (Figure 10 A, B&C) after ECs cocultured with MM-EX$^{C6\text{-}Cer}$, and the expression was obviously inhibited. This result means the VEGFA might be a target of miR-29b. *Chen et al* reported that miR-29b can target VEGFA to negatively regulate the angiogenesis of ECs via the MAPK/ERK and PI3K/Akt signaling pathways in endometrial carcinoma [64]. This study provides new information for further research. It demonstrated that the angiogenic function of HUVECs is down-regulated by MM cells derived EXs enriched miR-29b.

To deeper research, miR-29b inhibitor was used in in ECs, and the miR-29b expression level was obviously decreased in HUVEC (Figure 11). *Cheng et al* reported that miR-29b level in EXs secreted from serum-free MM cells was appreciably less than the C6-Ceramide treated MM derived EXs [62]. In addition, normal MM cell derived EXs have pro-angiogenesis effects *in vivo* [19]. MiR-29b inhibited ECs and miRCtrl treated ECs both cocultured with normal EX released from MM cells, the EC proliferation was increased in miR-29b inhibited ECs compared to the control group (Figure 12), and the migration and tube formation function of ECs were both augmented (Figure 13). The VEGFA expression in HUVEC was also up-regulated in microRNA-29b inhibited ECs cocultured with MM-EX (Figure 14). These results further confirm the role of miR-29b in mediating the angiogenesis of MM-EX on ECs. MiR-29b inhibitor can promote the angiogenic function of HUVEC

cocultured with MM-EX, suggesting miR-29b plays a negative role in endothelial cells angiogenesis in multiple myeloma.

MM, as a hematological tumor, its microenvironment is more and more being considered in research and clinical treatment. Previous studies indicated the oxygen tension is lower in BM with the exist of MM than the normal BM. Therefore, EXs released from MM in hypoxic condition are also valuable direction. *Umezu et al* reported that exosomal miR-135b regulates the MM angiogenesis in hypoxic condition, and miR-135b from MM derived EXs enhanced the angiogenic function of MM [41].

Tumor microenvironment is increasingly high-profile in recent years. Extracellular vesicles, such as EXs, MVBs and their carried molecules, are thought to mainly involve into cancer cell proliferation, invasion and metastasis, and play a key role in tumor progression [24]. Numerous studies found cancer cells can released much more EXs than the normal cells derived. Tumor derived EXs usually contain special molecules, such as the specific antigen on the surface of EXs, they can be served as a potential biomarker for cancer diagnose and treatment. MiRs, as an important content in EXs, are also focus on recently. Due to the different origins of microRNAs, some microRNAs enrich in certain exosomes, it can be a novel biomarker in diseases diagnosis and treatment.

VII. CONCLUSION

In this study, EXs secreted from C6-Ceramide stimulated MM cells, which have a high level of miR-29b, could inhibit the proliferation, migration, tube formation and VEGFA expression of HUVECs. EXs derived microRNA-29b negatively regulated the angiogenesis of ECs. MiR-29b inhibitor increases proliferation, migration, tube formation and VEGFA expression of ECs with the effect of EXs released from MM cells.

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