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The Endoplasmic Reticulum-Stressed Head and Neck Squamous Cell Carcinoma Cells Induced Exosomal miR-424-5p Inhibits Angiogenesis and Migration of Humanumbilical Vein Endothelial Cells Through LAMC1-Mediated Wnt/β-Catenin Signaling Pathway

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Abstract

Under endoplasmic reticulum (ER) stress, tumor plays multifaceted roles in endothelial cell dysfunction through secreting exosomal miRNAs. However, for the head and neck squamous cell carcinoma (HNSCC), it is still unclear about the impact of ER-stressed HNSCC cell derived exosomes on vascular endothelial cells. To address this gap, herein, systemic research was conducted including isolation and characterization of ER-stressed HNSCC cell (HN4 cell line as an *in vitro* model) derived exosomes, identification of regulatory exosomal miRNAs, target exploration and downstream signaling pathway investigation of exosomal miRNAs in human umbilical vein endothelial cell (HUVEC). ER-stressed HN4 cell-derived exosomes inhibited angiogenesis and migration of HUVEC cells *in vitro*. Furthermore, RNA-seq analysis demonstrated that miR-424-5p was highly upregulated in ER-stressed HN4 cell-derived exosomes. Through matrigel tube formation and transwell assays of HUVEC cells, miR-424-5p displayed great capabilities on inhibiting angiogenesis and migration. Finally, based on western blot and luciferase reporter, it was demonstrated that LAMC1 is the target of miR-424-5p which could inhibit the angiogenesis and migration of HUVEC cells by repressing the LAMC1-mediated Wnt/β-catenin signaling pathway. ER-stressed HNSCC cell-induced exosomal miR-424-5p inhibits angiogenesis and migration of HUVEC cells through LAMC1-mediated Wnt/β-catenin signaling pathway. This study offers a new insight for understanding the complicated mechanism behind ER-stress induced anti-angiogenesis of HNSCC.

Keywords

head and neck squamous cell carcinoma, endoplasmic reticulum stress, exosomal miRNAs, Wnt/β-catenin, anti-angiogenesis

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Introduction

Head and neck tumors are the sixth largest malignancy worldwide[1]. More than 90% of them belong to head and neck squamous cell carcinoma (HNSCC), with more than 890,000 new cases and 450,000 deaths every year[2]. Despite the thriving advancement of diagnostic technology and treatment schemes, over 65% of head and neck cancer patients are profoundly jeopardized by recurrence or metastasis[3]. The 5-year survival rate has remained under 50% for the past 30 years, while 65% of head and neck cancer patients without metastasis can survive for more than 5 years[4].

Like most solid tumors, such as breast cancer and hepatocellular carcinoma, there are two main ways accounting for distal metastasis of HNSCC: one is to directly enter the blood circulation system, and the other is to enter the lymph nodes near the primary focus[5]. However, as to HNSCC metastasis, present study has mainly focused on lymph nodes. Angiogenesis is involved with the migration, proliferation and differentiation of vascular endothelial cells[6]. Additionally, angiogenesis plays a critical role in facilitating the metastasis of tumor cells to distant organs as blood vessels support the tumor growth by supplying nutrients and oxygen. But it remains a misty question for HNSCC blood metastasis. Hence, it is urgent to explore HNSCC cell impacts on intratumoral vessels.

It is well established that endoplasmic reticulum (ER) stress is closely related to angiogenesis[7]. The ER in cells is extremely sensitive to the external environments under abnormal conditions such as nutrient deficiency, hypoxia and oxidative stress, and proteins are not properly folded or conformed in the endoplasmic reticulum[8,9]. ER stress is controlled by several ER stress-related proteins, including protein kinase R-like ER kinase (PERK), activating transcription factor 6 (ATF6), glucose-regulated protein 78 (GRP78) and inositol-requiring enzyme 1 (IRE1)[10]. It has been reported ER stress is involved in the occurrence, development and metastasis of various tumors[11]. However, recent studies demonstrated the inhibitory influence of tumor ER on angiogenesis and metastasis. This discrepancy makes it necessary to investigate whether ER stress could play some role in blood metastasis in HNSCC.

When referring to blood metastasis, the relationship between tumor cells and vascular endothelial cells is inevitably under consideration. It has been universally known exosomes are the key bridge of cell-cell interaction. Exosomes refer to nano-sized lipid bilayer membrane vesicles (30–200 nm), acting as a significant role in the local and systemic intercellular communication by transferring their cargo (e.g., mRNAs, proteins, DNA, and microRNAs [miRNAs]) to adjacent or distant cells[12,13]. Specific miRNAs perform as key regulators in the inflammation, tumorigenesis, and cancer development when transported by exosomes[14–16]. Recent studies have suggested that tumor cells can have various effects on the surrounding HUVEC cells through exosomal miRNAs. For example, nasopharyngeal carcinoma cell induced exosomal miR-9 inhibits angiogenesis by targeting MDK and regulating PDK/AKT pathway[17]. However, under ER stress, it has not been reported about the relationship between HNSCC cell-derived exosomes and vascular endothelial cells.

In our study, it is the first time to demonstrate that, under ER stress, HNSCC cell-derived exosomal miR-424-5p contributes to inhibiting the angiogenesis and migration of HUVEC cells via targeting LAMC1-mediated Wnt/β-catenin signaling pathway. These findings offer new understanding into the effect of exosomal miRNAs secreted by HNSCC cells in carcinogenesis.

Materials and Methods

Cell Lines and Cell Culture

The HNSCC cell line was purchased from Cell Bank of the Chinese Academy of Sciences, Shanghai. HN4 cells were cultivated in DEME/F12 Medium (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). HUVEC cells was acquired from China Pharmaceutical University (Nanjing, China). HUVEC cells were cultivated in RPMI 1640 medium with 10% FBS (Gibco) and water-saturated air. The two cell lines were incubated in a humid environment with 5% CO_2 at 37°C.

Exosome Isolation and Identification

Exosomes were isolated from the supernatant of HN4 by using Total Exosome Isolation Reagent (Invitrogen). Shortly, in order to remove cell debris, acclimatized supernatant was centrifuged 2000 × g for 30 min. Then the supernatant was added with Total Exosome Isolation reagent, and then incubated at 4°C overnight. On the next day, the mixture was centrifuged at 10,000 g for 60 min at room temperature. Afterwards, exosomes were resuspended in PBS. Isolation of exosomes from the supernatants was confirmed by nanoparticle tracking analysis (NTA). The exosomes diluted in PBS (25 μg/mL) was determined by an ELSZ-DN2 zeta-potential analyzer (Otsuka Electronics) to measure zeta potential values, according to the manufacturer's instructions.

Constructing Library, Sequencing, and Analysis

Exosomal miRNA sequencing was performed by Capital Bio Technology., LTD (Shanghai, China). Shortly, an estimated 0.1 μg of RNA was utilized to make a library by use of NEBNext Multiplex Small RNA Library Prep Set for Illumina (Illumina, San Diego, CA) as per the protocol. Libraries constructed before were at this point amplified and aligned through HiSeq Rapid SBS Kit V2 (50 cycles) together with HiSeq Rapid SR Cluster Kit V2 in the HiSeq 2500 system

(Illumina). In order to confirm the identified miRNAs, the sequences we acquired were used to compared with the sequences in the database (miRBase). The relative amount of miRNAs in this study was further standardized through the following formula: RPM = (number of reads mapped to miRNA/number of reads in clean data) × 1,000,000. Association constants were calculated by Pearson formulae ($R^2 > 0.8$). Cut-off thresholds of$|\log_2$ (fold change)$| \geq 1$ and FDR < 0.05 were applied to identify miRNAs which were considered to be significantly differentially expressed.

Total RNA Purification and Quantitative Real-Time PCR

In this study, total RNA extraction of maintained cells or frozen tissues was implemented by TRIZOL reagent (Invitrogen). After that, the PrimerScript™ reagent kit (TaKaRa, Shiga, Japan) was usedto obtain cDNA. Then, we implement SYBR Premix Ex Taq II (TaKaRa) to exert the qRT-PCR following the manufacturer's instructions. GAPDH and U6 were used as the internal reference. All Primer sequences were designed by RiboBio (Guangzhou, China). Primer sequences of related genes for RT-qPCR:

ATF6: forward 5'-CCAGCAGAAAACCCGCATTC-3' and reverse

5'-AACTTCCAGGCGAAGCGTAA-3';

GRP78: forward 5'-AACCCAGATGAGGCTGTAGCA-3' and reverse

5'-ACATCAAGCAGAACCAGGTCAC-3';

β-catenin: forward 5'- TGACAAAACTGCTAAATGACGAGG -3' and reverse

5'-CGCATGATAGCGTGTCTGGA-3';

c-myc: forward 5'- CCACGAAACTTTGCCCATAG -3' and reverse

5'- TGCAAGGAGAGCCTTTCAGAG-3';

Cyclin D1: forward 5'- TGTCCCACTCCTACGATACGC -3' and reverse

5'- CAGCATCTCATA AACAGGTCACTA C-3';

LAMC1: forward 5′-ATTTCAATCAACCGCTCT-3' and reverse

5'-GTTATGGACCTCCTTCGT -3';

GAPDH: forward 5'-GACGTAGGGAGTGAAGGTC-3' and reverse

5'-GAGAGTTCAGATGTTGATGG-3.'

Cell Transfection

In this study, HUVEC cells were performed with cell transfection. Specific targeting LAMC1 (si-LAMC1),non-target siRNA control (si-NC), miR-424-5p mimic, miR-424-5p inhibitor and corresponding negative control used for this experiment were all synthesized by the Gene Pharma (Gene Pharma). Lipofectamin™2000 (Invitrogen) was applied in the cell transfection experiment according to the manufacturer's instructions.

In Vitro *Matrigel Tube Formation Assay*

HUVEC cells (2×10^4 cells per well) were seeded onto per well of 96-well plate while the bottom of each well was coated with 50 μL matrigel (Growth factor-enriched; BD Biosciences, USA) and cultured for 12 h at 37°C in 5% CO_2. Capillary-like structures were evident and counted using a phase-contrast microscope and the networks formed by HUVEC cells were quantified with ImageJ software (NIH, USA). Data are summarized as means ± SD.

Transwell Assays

The HUVEC cells migration assay were exerted through applying the transwell chambers (24 well, 8 μm, Corning, Corning, NY, USA). In migration assay, each chamber was loaded with approximately 2.0×10^4 of HUVEC cells. After incubation for 24 h, the bottom of the chambers were put in 4% paraformaldehyde for 30 min to fix and stained by 0.1% crystal violet for 15 min (Beyotime, Shanghai, China). The cells were observed, counted and photographed in five randomly chosen fields under a microscope.

Protein Extraction and Western Blot

Western blot was performed as common methods. The protein of HUVEC cells was isolated in radioimmunoprecipitation (RIPA) buffer supplemented with PMSF (Beyotime). The protein were separated by using 10% SDS-PAGE gels and immediately moved onto polyvinylidenedifluoride membranes (PVDF; Millipore, Boston, MA, USA) via a wet transfer method. Subsequently, these membranes were incubated in specific antibodies at 4°C overnight. On the next day, protein band visualization was implemented by exposing to enhanced chemiluminescent (ECL) reagent after incubated with secondary antibodies. GRP 78 antibodies (Abcam, Cat No: ab21685), ATF 6 antibodies (Abcam, Cat No: ab203119) and primary antibody: CD63, TSG-101, LAMC1, β-catenin, c-myc, cyclin D1 and GAPDH (Cell Signaling Technology, Danvers, MA, USA).

Dual-Luciferase Reporter Detection

The wild type (Wt) sequences of LAMC1 (LAMC1-Wt) including the speculative miR-424-5p binding site and the matched mutant type (Mut) ones of LAMC1 (LAMC1-Mut) containing the mutated seed regions of the miR-424-5p–binding site were designed and synthesized by Gene Pharma (Gene Pharma). Subsequently, the pEX-3 vector inserted by LAMC1-Wt and LAMC1-Mut, respectively, were named as LAMC1-WT, LAMC1-MUT. Then, HUVEC cells were co-transfected by LAMC1-WT, LAMC1-MUT with miR-424-5p

mimic or negative control (NC) by lipofectamine™2000 (Invitrogen) respectively. The transected cells were harvested and lysed to be used in luciferase assay after incubated for 48 h. The Dual-Luciferase Reporter Assay System (Promega) was implemented to test luciferase activities of cells.

Statistical Analysis

ALL experimental data analyses were performed using GraphPad Prism 7.0 and SPSS software (version 22.0, SPSS Inc, Chicago, IL, USA). The significant differences among groups were compared through using analysis of variance (ANOVA, with Sidak post hoc test) or the Student's *t*-test. In this study, *P* value less than 0.05 was considered to be statistically significant.

Results

ER-Stressed HN4 Cell-Derived Exosome Inhibits Angiogenesis and Migration of HUVEC Cells in Vitro

To determine if ER stress is upregulated in HNSCC patients, we compared the difference between the expression of ER stress-related proteins in surgically resected HNSCC tissues and paracarcinoma tissues by Western Blot and qPCR. These assays verified that a much higher expression of ER stress-related indicators (GRP78 and ATF6) in tumor tissues than in para-carcinoma tissues (Fig. 1A–C). It has been reported that IFN-γ could induce endoplasmic reticulum stress in cells[18,19]. To investigate how ER stress modulates the angiogenesis and migration of HUVEC cells *in vitro*, HN4 cells were cultured with IFN-γ (500 U/ml for 48 h) to induce ER stress and then the expression of ER stressed related marker were determined by Western Blot. The results showed that IFN-γ inducedHN4 cell displayed significantly elevated GRP78 and ATF relative to HN4 cell without IFN-γ treatment (Fig. 1D, E). Since exosomes are important communicators between different cell types, to systemically investigate HNSCC derived exosomes on angiogenesis, we derived exosomes from HN4 cells (Exo-ER) treated with or without IFN-γ (defined as Exo-NOR). Western blot analysis demonstrated the expression of two exosomal-marker proteins CD63 and tumor susceptibility gene (TSG)-101 indicating successful extraction of exosomes (Fig. 1F). The purity of these exosomes was confirmed by nanoparticle tracking analysis (NTA). The NTA image of Exo-NOR or Exo-ER confirmed the expected size range of 30–200 nm in diameter (Fig. 1G), and the zeta potential confirmed the stability of the exosome particles in suspension (Fig. 1H). Then, we collected the exosomes of NH4 cells to verify whether it has an impact on angiogenesis and migration of HUVEC cells. The angiogenesis and migration ability of HUVEC cells were suppressed by Exo-ER compared to Exo-NOR (Fig. 1I, J). Thus, we found ER-stressed HN4 cell-derived exosome inhibited the angiogenesis and migration of HUVEC cells *in vitro*.

ER-Stressed HN4 Cell-Derived Exosomal miR-424-5p Inhibit Angiogenesis and Migration of HUVEC Cells in Vitro

To detect factors concerning HN4 cell-derived exosomes inhibited angiogenesis and migration of HUVEC cells, we subsequently performed comprehensive miRNA sequencing using RNA from Exo-ER (n = 3) and Exo-NOR (n = 3). The data indicated a strong dysregulation in the expression of miRNAs in Exo-ER. Remarkably a number of miRNAs, including miR-99a-5p, miR-99b-5p, miR-424-5p, miR-96-5p and miR-26a-5p, were highly upregulated in Exo-ER compared to Exo-NOR (Fig. 2A–C). TaqMan probe-based qRT-PCR assays additionally indicated miR-424-5p was identified as the mostly upregulated miRNAs in Exo-ER (Fig. 2D). Next, we assessed the amount of miR-424-5p in HUVEC cells cocultured with Exo-ER or Exo-NOR. The results showed that Exo-ER up-regulated the expression of miR-424-5p in HUVEC cells compared to control cells (Fig. 2E). To further evaluate the role of miR-424-5p in the inhibition of angiogenesis and migration in HUVEC cells, we transfected miR-424-5p mimics or inhibitor into HUVEC cells to over-express or knockdown the levels of miR-424-5p (Fig. 2F). The ability of angiogenesis and migration was significantly enhanced by miR-424-5p inhibitor in HUVEC cells (Fig. 2G, I). Adversely, the over-expression of miR-424-5p inhibited the angiogenesis and migration in HUVEC cells by matrigel tube formation assay and transwell assays (Fig. 2H, J). Overall, these data revealed exosomes secreted from ER-stressed HN4 cell could increase the expression of miR-424-5p to prevent the angiogenesis and migration of HUVEC cells *in vitro*.

LAMC1 Is the Target of miR-424-5p

miRNA is through specific complementary pairing with the base of the target mRNA, causing the target mRNA degradation and translational repression[20]. To deeply reveal the potential downstream molecule target of miR-424-5p, bioinformatics software was used to predict that LACM1 is the target gene of miR-424-5p. And its combination with miR-424-5p is shown in TargetScan database and the related score is very high. In the 3' UTR of LAMC1, the binding sites for miR-424-5p were highly conserved among seven species. The results of the dual-luciferase reporter analysis indicated that miR-424-5p could interact with the 3' UTR sequence of wild-type LAMC1 to suppress luciferase activity. However, no decrease was observed in the mutant LAMC1 3'UTR reporter (Fig. 3A, B). Furthermore, to investigate the relation between miR-424-5p and LACM1, the LACM1 expression levels were measured in the HUVEC cells with the

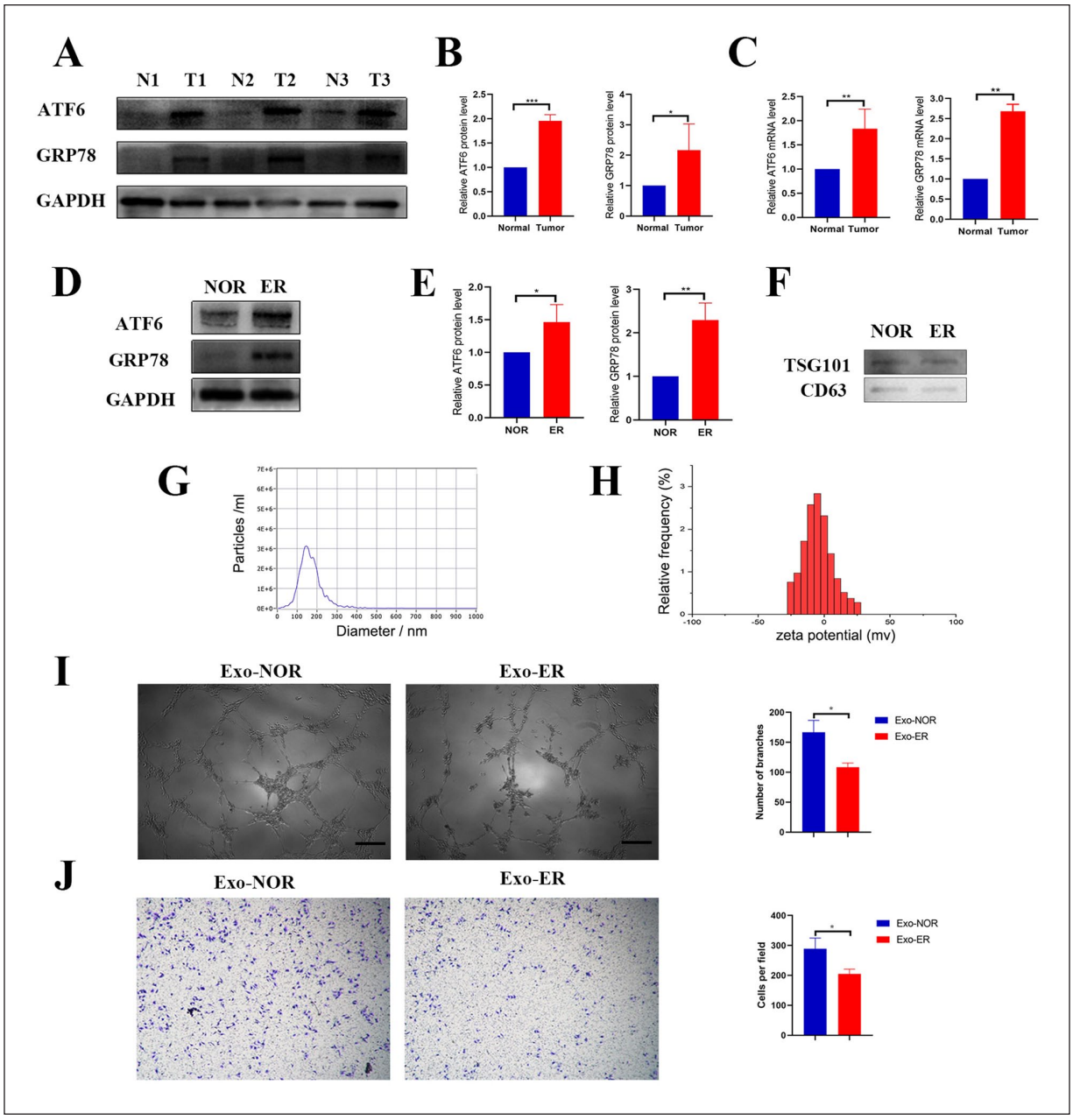

Figure 1. ER-stressed HN4 cell-derived exosome inhibits angiogenesis and migration of HUVEC in vitro. (A, C) Relative expression of GRP78 and ATF6 in three pair samples of HNSCC and matched adjacent normal tissues by Western blotting and qRT-PCR. (B) Quantification of protein levels of ATF6 and GRP78 in (A). (D) Protein expression of GRP78 and ATF6 in HN4 cultured with or without IFN-γ. (E) Quantification of protein levels of ATF6 and GRP78 in (D). (F) Analysis of the presence of the exosomal markers TSG101 and CD63 by Western blotting. (G) Nanoparticle tracking analysis of Exo-NOR (exosomes from HN4 cells treated without IFN-γ) or Exo-ER (exosomes from HN4 cells treated with IFN-γ) confirming the expected size range of 30–200 nm in diameter. (H) Zeta potential measurements for the Exo-NOR or Exo-ER. (I) HUVEC cultures were treated with Exo-NOR or Exo-ER for 24 h and then subjected to in vitro tube formation assay, scale bar: 100 μm. (J) The cell migration abilities of HUVEC cells treated with Exo-NOR or Exo-ER were determined by transwell assays. The data are presented as the mean ± SD, analyzed using Student's t-test or analysis of variance. ER: endoplasmic reticulum; HUVEC: human umbilical vein endothelial cell; qRT-PCR: quantitative real-time polymerase chain reaction; IFN-γ: interferon-γ; NOR: normal; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; HNSCC: head and neck squamous cell carcinoma. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

overexpression or knockdown of miR-424-5p expression respectively. The results showed that the expression of LACM1 was higher by the knockdown of miR-424-5p and its expression was lower by the overexpression of miR-424-5p (Fig. 3C, D). Collectively, these data confirmed that LAMC1 was indeed the target of miR-424-5p.

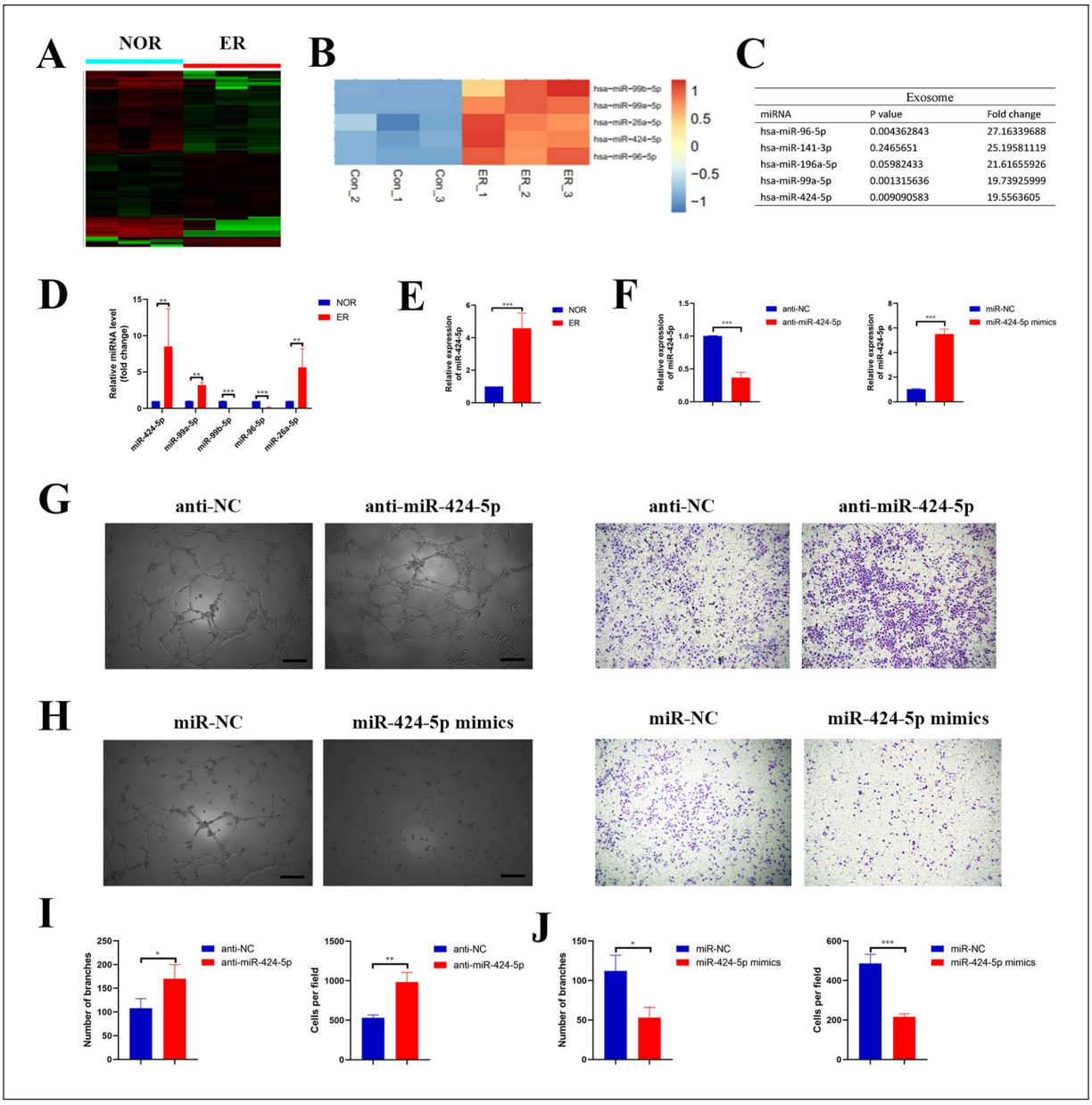

Figure 2. ER-stressed HN4 cell-derived exosomal miR-424-5p inhibits angiogenesis and migration of HUVEC in vitro. (A) Heat-maps showing the relative expression of miRNAs in exosomes isolated from Exo-NOR or Exo-ER. (B) Heat-maps analysis of differential expression of the top five miRNAs with high expression. (C) Selected top five high expression of miRNAs in Exo-ER group by FC. (D) The expression of top five miRNAs in exosomes derived from Exo-NOR and Exo-ER. (E) The expression of miR-424-5p in HUVEC cells treated with Exo-NOR or Exo-ER. (F) The relative expression of miR-424-5p in HUVEC cells transfected with miR- 424-5p mimics or inhibitor was determined by qRT-PCR. (G–J) Tube formation tests and cell migration abilities of HUVEC cells transfected with miR-424-5p mimics or inhibitor, scale bar: 100μm. The data are presented as the mean ± SD, analyzed using Student's t-test or analysis of variance. ER: endoplasmic reticulum; HUVEC: human umbilical vein endothelial cell; NOR: normal; FC: fold change; qRT-PCR: quantitative real-time polymerase chain reaction; NC: negative control. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

ER-Stressed HN4 Cell-Derived Exosomal miR-424-5p Inhibits the Angiogenesis and Migration of HUVEC Cells Depending on LAMC1 in Vitro

To explore whether LAMC1, an already confirmed target of miR-424-5p, also participates in miR-424-5p-mediated inhibition of angiogenesis and migration in HUVEC cells, we knocked down LAMC1 in HUVEC cells by the transfection of si-LAMC1 (Fig. 3E). The knockdown of LAMC1 expression led to the significant inhibition of angiogenesis and migration of HUVEC cells compared to the negative control group (Fig. 3F, G). Studies have shown that Wnt/β-catenin pathway plays

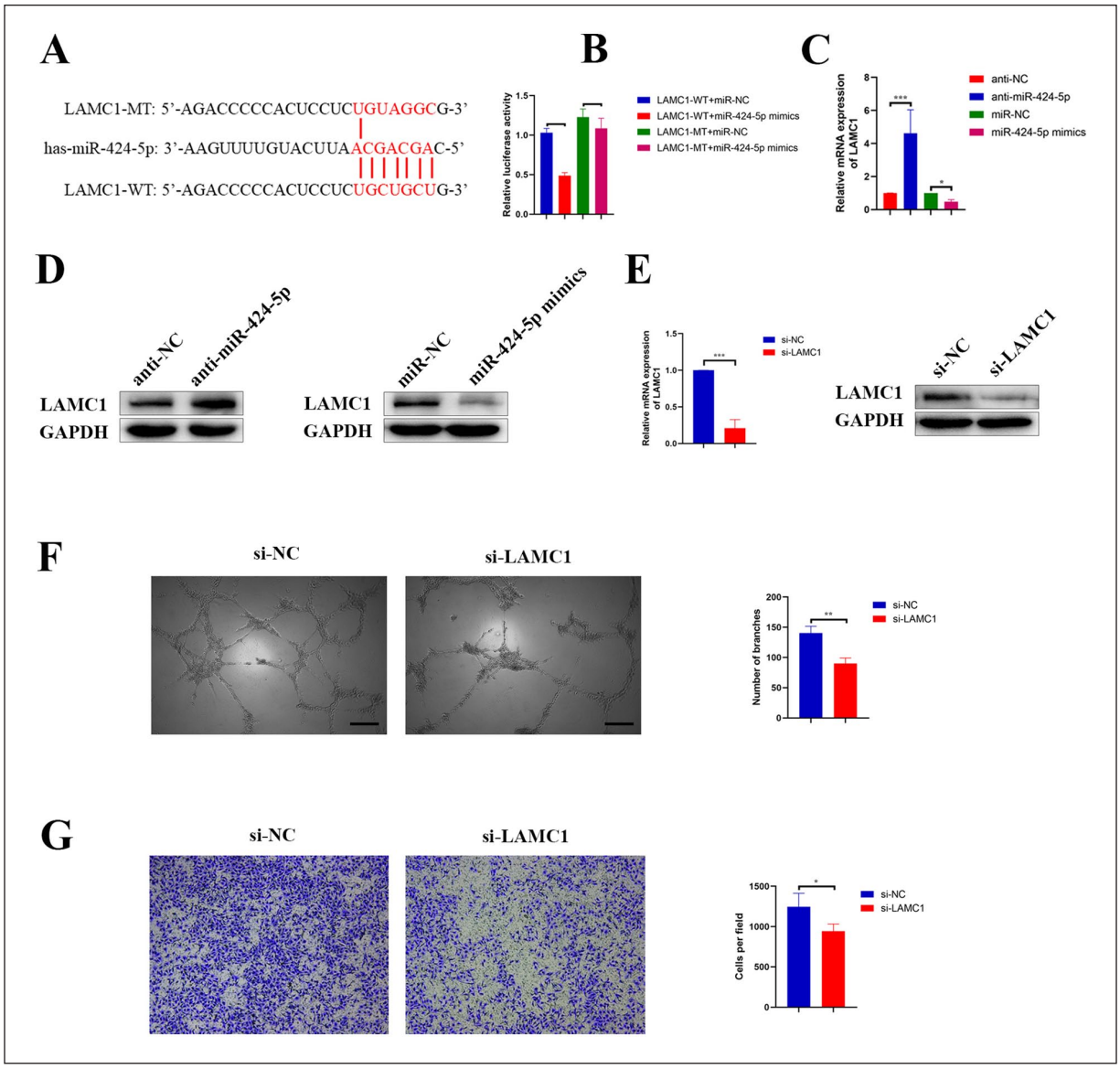

Figure 3. LAMC1 is the target of miR-424-5p. (A) The predicted binding sites of LAMC1 to the miR-424-5p sequence. (B) Luciferase activity was detected after HUVEC cells were co-transfected by pEX-3-LAMC1-WT, pEX-3-LAMC1-MUT with miR-424-5p mimics or inhibitor. (C, D) Relative LAMC1 expression in HUVEC cells after transfection with miR-424-5p mimics or inhibitor by qRT-PCR and Western blotting. (E) Relative LAMC1 expression in HUVEC cells after transfection with si-LAMC1. (F, G) Tube formation tests and cell migration abilities of HUVEC cells transfected with si-LAMC1, scale bar: 100 μm. The data are presented as the mean ± SD, analyzed using Student's t-test or analysis of variance. HUVEC: human umbilical vein endothelial cell; WT: wild type; MUT: mutant type; qRT-PCR: quantitative real-time polymerase chain reaction; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; NC: negative control. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

an important role in angiogenesis. To explore whether Wnt/β-catenin pathway is involved in the process of inhibiting the angiogenesis and migration of HUVEC cells induced by miR-424-5p/LAMC1, we co-transfected miR-424-5p inhibitor and si-LAMC1 into the HUVEC cells. As shown in Fig. 4A, B, silencing LAMC1 inhibited Wnt/β-catenin pathway. Western Blot demonstrated non-active-β-catenin decreased and the expression of related proteins (c-myc and cyclin D1) of Wnt/β-catenin pathway also decreased after LAMC1 knockdown. Moreover, downregulated LAMC1, non-active-β-catenin, c-myc and cyclin D1 expression after si-LAMC1 treatment could be reversed by the treatment with miR-424-5p inhibitor in HUVEC cells (Fig. 4A, B). In the rescue experiment, the enhancement of angiogenesis and migration of HUVEC cells induced by miR-424-5p inhibitor was reversed by si-LAMC1 after co-transfection of miR-424-5p inhibitor and si-LAMC1 in HUVEC cells (Fig. 4C–F). Overall, the above experiments showed that ER-stressed HN4 cell-derived exosomal

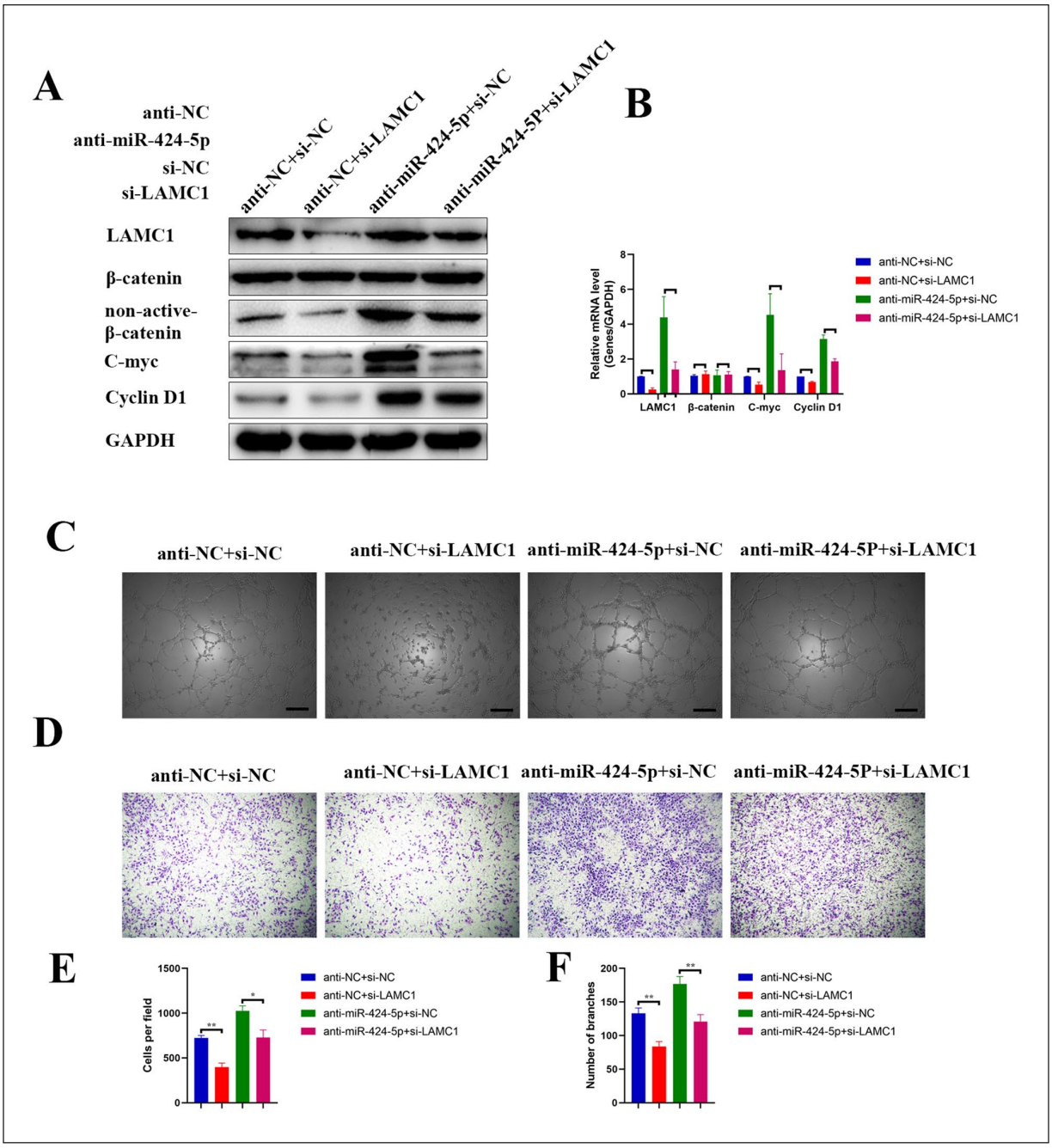

Figure 4. ER-stressed HN4 cell-derived exosomal miR-424-5p inhibits angiogenesis and migration of HUVEC depending on LAMC1 in vitro. (A) Protein expression of LAMC1, β-catenin, non-active-β-catenin, c-myc and cyclin D1 in HUVEC cells transfected with inhibitor negative control (anti-NC), miR-424-5p inhibitor (antimiR- 424-5p), si-LAMC1 negative control (si-NC) or si-LAMC1. (B) Relative expression of LAMC1, β-catenin, c-myc and cyclin D1 in HUVEC cells after treatment as in (A) by qRT-PCR. (C–F) Tube formation tests and cell migration abilities of HUVEC cells after treatment as in (A), scale bar: 100 μm. The data are presented as the mean ± SD, analyzed using Student's *t*-test or analysis of variance. ER: endoplasmic reticulum; HUVEC: human umbilical vein endothelial cell; NC: negative control; qRT-PCR: quantitative real-time polymerase chain reaction; GAPDH: glyceraldehyde-3-phosphate dehydrogenase. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

miR-424-5p inhibited angiogenesis and migration of HUVEC cells by repressing the LAMC1-mediated Wnt/β-catenin signaling pathway.

Discussion

ER stress is closely correlated with human diseases[21]. Especially in tumors, ER stress exerts an essential role in the

occurrence, development and metastasis of tumor[22]. Besides variously affecting tumor cells themselves, ER stress also has corresponding effects on surrounding cells through intercellular effects[23]. For example, in prostate cancer, ER stress causes the secretion of tumor suppressor (PAWR) into the extracellular space, triggering the apoptosis of nearby cancer cells[24]. According to recent researches, Kanemoto et al[25] reported that ER stress promoted exosome release in IRE1α and PERK-dependent manners. This means that ER stress strengthens the production and release of exosomes, which improves the ability of exosomes to interact between cells. In tumors, ER stress can regulate the crosstalking between cells through exosomes. For example, ER stress-induced exosomal miR-27a-3p promotes immune escape in breast cancer via regulating Programmed Death Ligand 1 (PD-L1) expression in macrophages[26]. Additionally, ER stress causes liver cancer cells to release exosomal miR-23a-3p and upregulate PD-L1 expression in macrophages[27]. Interestingly, we found that ER-stressed HN4 cell-derived exosome could mediate the inhibition of angiogenesis and migration in HUVEC cells *in vitro*. This further shows that transmission through exosomes is indeed one way for ER stress to regulate surrounding cells in tumors.

It is well known that ER stress is closely related to angiogenesis and invasion[28]. Previous studies have revealed that ER stress could induce angiogenesis by PERK, IRE1, and ATF6 hypoxic conditions. For example, Blais et al[29] suggested that PERK-ATF4 arm of the UPR directly upregulates vascular endothelial growth factor (VEGF) while downregulating the inhibitors of angiogenesis. However, Maamoun et al[30] mentioned ER stress could impair the angiogenic capacity of HUVEC cells, and Lin et al[31] found exosomes derived from HeLa cells break down vascular integrity by triggering ER stress in endothelial cells. These suggest ER stress could also inhibit angiogenesis. Evidence suggests that ER stress-generated extracellular vesicles self-perpetuate ER stress and mediate endothelial cell dysfunction independently of cell survival[32]. In addition to directly regulating vascular endothelial cells, ER stress could use a significant approach to mediate them through tumor cell-derived exosomes. In our study, we also found ER-stressed HN4 cell-derived exosomal miR-424-5p inhibits the angiogenesis and migration of HUVEC cells. Moreover, ER stress exerts its effect on HUVEC cells through HN4-derived exosomes and internal miRNAs. We firstly determined exosomes from ER-stressed HN4 cells could inhibit the angiogenesis and migration of HUVEC cells. Then we further determined, miR-424-5p in exosomes plays a regulatory role on HUVEC cells.

During the past decades, more and more evidence accumulated implicate miRNAs in the pathogenesis of many human diseases[33]. Changes in miRNA expression contribute to the cell transformation, tumorigenesis and angiogenesis[34]. MiR-424-5p has been reported in various tumors. Most interestingly, according to previous reports, miR-424-5p could suppress tumor angiogenesis. In hepatocellular carcinoma, miR-424-5p could regulate cell cycle and further inhibit the proliferation of HCC cells by targeting E2F7[35]. In colorectal cancer, FENDRR Sponges miR-424-5p to suppress the cell proliferation, migration and invasion[36]. In addition, MYLK-AS1 facilitates tumor progression and angiogenesis by targeting miR-424-5p/E2F7 axis in hepatocyte[37,38]. Our study showed that miR-424-5p has a negative effect on HUVEC cells, consistent with previous findings. Further investigation is needed to uncover the potential downstream target and mechanism of miR-424-5pin anti-angiogenesis and anti-migration.

Nextly, we further found LAMC1 was the target of miR-424-5p. LAMC1 encodes laminin γ1 chain, a protein essential for assembly of basement membrane. Laminins acted as a signal molecule in the tissue development, tumor cell invasion and metastasis[39]. Recent evidence has shown that LAMC1 could promote the angiogenesis and progression of tumors[40,41]. Similarly, our results revealed that miR-424-5p-induced LAMC1 inhibition could suppress the angiogenesis and migration of HUVEC cells. It has been reported that the Wnt/β-catenin pathway can induce vascular endothelial growth factor to promote angiogenesis[42]. In the classic Wnt/β-catenin pathway, β-catenin accumulates in the cytoplasmic and enters into nucleus, thus regulating the downstream genes expression, which plays an important regulatory role in HUVEC cells[43]. In our study, the miR-424-5p inhibitor could reverse si-LAMC1 induced repression of β-catenin expression. Furthermore, miR-424-5p could inhibit the angiogenesis and migration of HUVEC cells by repressing the LAMC1-mediated Wnt/β-catenin signaling pathway.

These results established that ER-stressed HN4 cell-derived exosome inhibited the angiogenesis and migration of HUVEC cells *in vitro*. miR-424-5p is the main component in exosomes. Moreover, we found that ER-stressed HN4exos-derived miR-424-5p inhibited the angiogenesis and migration of HUVEC cells depending on LAMC1-mediated Wnt/β-catenin signaling pathway *in vitro*. The present study revealed that LAMC1 was a direct target of miR-424-5p and the loss of the LAMC1 repressed Wnt/β-catenin signaling pathway. Taken together, the results of our study showed that exosomes derived from ER-stressed HN4 transferring with miR-424-5p inhibit angiogenesis and migration of HUVEC cells via the downregulation of LAMC1 and inactivation of the Wnt/β-catenin signaling pathway.

Author Contributions

Each author has made an important scientific contribution to the study and has assisted with the drafting or revising of the manuscript.

Ethical Approval

Ethical approval was given by the Affiliated Stomatological Hospital of Nanjing Medical University, Nanjing, China.

Statement of Human and Animal Rights

All procedures in this study were conducted in accordance with the Affiliated Stomatological Hospital of Nanjing Medical University, Nanjing, China.

Statement of Informed Consent

Written informed consent was obtained from the patients for their anonymized information to be published in this article.

Declaration of Conflicting Interests

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Supplemental Material

Supplemental material for this article is available online.

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