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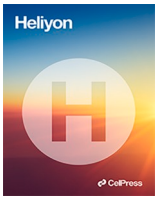

Research article

Plasma-derived exosomes contributes to endothelial-to-mesenchymal transition in Moyamoya disease

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ABSTRACT

Background: Moyamoya disease (MMD) is a cerebrovascular disease with a high disability rate; however, its pathogenesis remains unknown. Endothelial-mesenchymal transition (EndMT) is the pathological basis of many vascular diseases; however, the key role of EndMT in MMD has not yet been reported.
Method: We collected vascular tissues from three control samples and six patients with MMD to detect the expression of EndMT-related genes. To elucidate the mechanism of EndMT in MMD, we performed in vitro cell experiments. Plasma-derived exosomes (PDEs) can transmit information between cells and tissues and are of considerable importance in several disease studies. PDEs were used to stimulate EndMT phenotype in cerebrovascular endothelial cells.
Results: Multiplex fluorescent immunohistochemistry staining confirmed that CD31, VE-cadherin and E-cadherin down-regulated, whereas α-SMA and vimentin were significantly up-regulated in moyamoya vascular endothelial cells than in control samples. PDEs from MMD patients significantly promoted cell proliferation and migration, resulting in slender cells. PDEs induce EndMT-related phenotype changes in cerebral vascular endothelial cells, including decreased endothelial cell marker expression and increased mesenchymal cell marker expression. We demonstrated that EndMT phenotypic alterations are mediated, in part, by microRNA(miRNAs).
Conclusion: This study was the first to propose that EndMT may exist in the vessels of patients with MMD. PDEs induce the EndMT phenotype to promote the development of MMD. This study aimed to provide a new theoretical basis for elucidating the pathogenesis of MMD.

1. Introduction

Moyamoya disease (MMD) is a chronic progressive cerebrovascular disease characterized by partial stenosis or occlusion of the distal end of the internal carotid artery and abnormal vascular network formation at the base of the brain [1]. MMD mostly manifests as ischemic or hemorrhagic stroke, epileptic seizures and cognitive dysfunction, with high rate of disability and even death [2]. The annual incidence of MMD has been increasing in recent years, and a higher incidence is reported in East Asia [3]. Owing to the

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insidious onset of MMD and the lack of specificity and sensitivity indicators for early screening, timely diagnosis and treatment remain challenging in most patients [3]. Because of the difficulty in obtaining clinical samples and the limited availability of relevant studies, conducting basic research on the pathogenesis of MMD is imperative.

The pathogenesis of MMD was not fully understood, and it was currently believed to be related to genetic, inflammatory, environmental, and other factors [1,4]. Our findings indicated that the blood vessels of patients with MMD may exhibit a biological process of endothelium-mesenchymal transition (EndMT). EndMT was first discovered by Markwald et al. during atrioventricular valve development in the embryonic heart and has been observed in a variety of pathological conditions characterised by abnormal shear stress, vascular damage, and chronic inflammation [5]. EndMT is a complex cellular transdifferentiation process in which the endothelial cells lose their endothelial properties and acquire mesenchymal characteristics, as well as enhanced migratory and proliferative capacities [6]. The molecular markers of EndMT include endothelial markers such as decreased CD31 and mesenchymal markers such as N-cadherin and vimentin, which are increased [7]. EndMT is the common pathological basis of various vascular diseases [8–10], and our findings suggest that EndMT plays a key role in MMD. However, the specific role of EndMT in the occurrence and development of MMD and its upstream regulatory mechanisms have not been reported.

Exosomes are membranous vesicles secreted into the extracellular environment by the fusion of intracellular multivesicular bodies with the cell membrane [11]. PDEs in MMD significantly promote the proliferation of cerebral vascular endothelial cells compared to those in healthy samples; however, has no effect on peripheral vascular endothelial cells, suggesting that PDEs inMMD specifically act on cerebrovascular endothelial cells [12]. Exosomes secreted from highly metastatic cancer cells can promote vascular permeability by downregulating the expression of VE-cadherin and ZO-1 in endothelial cells, thereby promoting tumour recurrence and metastasis [13], indicating that exosomes have the potential to regulate EndMT. Our study showed that the PDEs in MMD significantly promoted the EndMT phenotype in cerebral vascular endothelial cells. In conclusion, we believe that PDEs promote the occurrence and development of MMD by regulating EndMT.

2. Materials and methods

2.1. Patient selection and plasma samples

This study recruited patients with the following criteria who visited the hospital between 16 November 2021 and 4 June 2022: All patients aged $\leq$60 years with a confirmed diagnosis of MMD by cerebral angiography. Patients with pre-existing comorbid diseases such as cerebral arteriosclerosis and severe cardiopulmonary disease were excluded. The discarded vascular tissue from neurosurgery patients with MMD during surgery and the discarded vascular tissue from cardiac surgery and vascular bypass surgery were obtainedfor tissue-level experiments. Plasma samples from patients with MMD and control group were collected for exosome isolation, extraction, and in vitro experiments. Plasma samples of the control group were obtained from healthy individuals at the Physical Examination Centre.

2.2. Exosomes isolation and identification

PDEs extraction was performed using the kit (UR52136) from Shanghai Umibio Biotechnology Co., Ltd. Exosome morphology was observed using electron microscopy, and the particle size was measured using a Nanosight LM10 system. Exosome markers were confirmed by western blotting.

2.3. Cell proliferation assays

A Cell Counting Kit-8 (Dojindo, Japan) was used to detect cell proliferation, and the cells were seeded in 96-well culture plates at 5000 cells per well and stimulated bEnd.3 for 24h and HUVEC for 72h with PDEs. After stimulation, the cells were incubated with the WST-8 reagent for 2h, and the OD value was detected using a microplate reader. Since bEnd.3 cells grew faster, they were only stimulated for 24 h for the proliferation experiments.

2.4. Cell migration assays

Transwell plates (Corning 3422) were used for cell migration experiments. A total of 10,000 cells per well were seeded in the upper chamber of the Transwell. The volume of the culture medium in the upper chamber was 200 μL without serum, and the volume in the lower chamber was 600 μL with 10% serum. PDEs were added to each chamber, cultured for 24 h, and observed under a microscope.

2.5. Tube formation assay

Tube formation assays were performed using the bEnd.3 cells with PDEs for 6h, and 100 μL Matrigel per well was added to a 96-well plate and incubated at 37 °C for 30 min. After the Matrigel solidified, 10,000 cells were seeded into each well.

2.6. Immunofluorescence assays

A total of 50,000 cellsper well were seeded onto cell slides and stimulated with PDEs for 48 h for immunofluorescence staining. The

5-color multiplex fluorescence kit (abs50013) was used for five-colour multiplex fluorescence, and the primary antibody dilution ratio of each molecular indicator was 1:100.

2.7. Western blot analysis

After PDEs stimulation, the cell pellets were collected for proteolysis, and SDS-PAGE gel electrophoresis was performed to detect changes in the protein expression of various molecular indicators. The primary and secondary antibody dilution ratios were 1:1000 and 1:5000, respectively.

2.8. qRT-PCR

After PDEs stimulation, the cells were harvested for RNA lysis and subsequently assayed for gene expression using qRT-PCR. The 2-ΔΔCq method was used for data analysis. The mRNA relative expression was normalized to β-actin and the miRNA relative expression was normalized to U6. The primers sequences used for the experiment were as follow: β-actin, Forward primer: GGCTGTATTCCCCTCCATCG; Reverse primer: CCAGTTGGTAACAATGCCATGT. E-cadherin, Forward primer: CTCCAGTCATAGGGAGCTGTC; Reverse primer: TCTTCTGAGACCTGGGTACAC. Snail, Forward primer: CACACGCTGCCTTGTGTCT; Reverse primer: GGTCAGCAAAAGCACGGTT.

ERG, Forward primer: CGTGCCAGCAGATCCTACG; Reverse primer: GGTGAGCCTCTGGAAGTCG. VE-cadherin, Forward primer: GTTCACCTTCTGCGAGGATATG; Reverse primer: GATGGTGAGGATGCAGAGTAAG. N-cadherin, Forward primer: AGCCAACCTTAACTGAGGAGT; Reverse primer: GGCAAGTTGATTGGAGGGATG. Snail2, Forward primer: CGAACTGGACACACATACAGTG; Reverse primer: CTGAGGATCTCTGGTTGTGGT. U6, Forward primer: GGAACGATACAGAGAAGATTAGC; Reverse primer: TGGAACGCTTCACGAATTTGCG. miR-151a-3p, Forward primer: GGGCAACCTAGACTGAAGCTC; Reverse primer: GTGCGTGTCGTGGAGTCG. miR-128–3p, Forward primer:GGGGAATCACAGTGAACCG; Reverse primer: CAGTGCGTGTCGTGGAGT. miR-200c-3p, Forward primer:GGGGTAATACTGCCGGGTAA; Reverse primer:CAGTGCGTGTCGTGGAGT. miR-125b-5p, Forward primer: GCTCCCTGAGACCCTAAC; Reverse primer: GTGCGTGTCGTGGAGTCG.

2.9. Statistical analysis and date availability

All results are presented as the mean Values ± SD. Normality tests and homogeneity of variance were performed before the significance of differences was evaluated using SPSS software. The statistical significance of the results was analyzed by a 2-tailed unpaired Student *t*-test using GraphPad Prism. The statistical significance of all the data is indicated by *P < 0.05, **P < 0.01, ***P < 0.001.

3. Results

3.1. Patients information

This study recruited 53 adults (comprising 28 MMD patients and 25 controls) between November 2021 and April 2022. Six blood vessels were reserved for tissue-level experiments. Control vascular samples were obtained from the internal mammary artery resected viavascular bypass surgery during cardiac surgery. MMD vascular samples were obtained from the middle cerebral artery that was resected via vascular reconstruction surgery during neurosurgery. The detailed patientinformation is presented in Table 1.

3.2. Expression of EndMT markers in human MMD

Multiplex fluorescent immunohistochemistry was used to detect the expression of EndMT-related molecules in MMD and control vessels. The results showed a decreased expression of VE-cadherin and CD31 (Fig. 1A-C, F) and increased expression of vimentin, Snail, and α-SMA (Fig. 1A, B, D, E, G, and H), suggesting that EndMT plays an important role in MMD. Compared with the control group, the intimal layer of blood vessels was significantly thickened (Fig. S1), indicating abnormal proliferation of vascular endothelial cells in MMD. The ETS (E−26 transformation-specific) transcription factor ERG (ETS-related gene) is essential for maintaining endothelial homeostasis. We detected that ERG was absent in the vascular endothelial cells of patients with MMD, suggesting that the vascular endothelial cells were dysfunctional (Fig. S2A).

Table 1
Samples information used in experiments.

Assay	group	Sample	n	sex		Age
				Male	Female	
Tissue level	MMD	artery	6	2	4	45.00 ± 7.14
	Control	artery	3	3	0	58.67 ± 11.11
Cell level	MMD	Plasma	22	13	9	46.50 ± 9.04
	Control	Plasma	22	10	12	44.14 ± 6.22

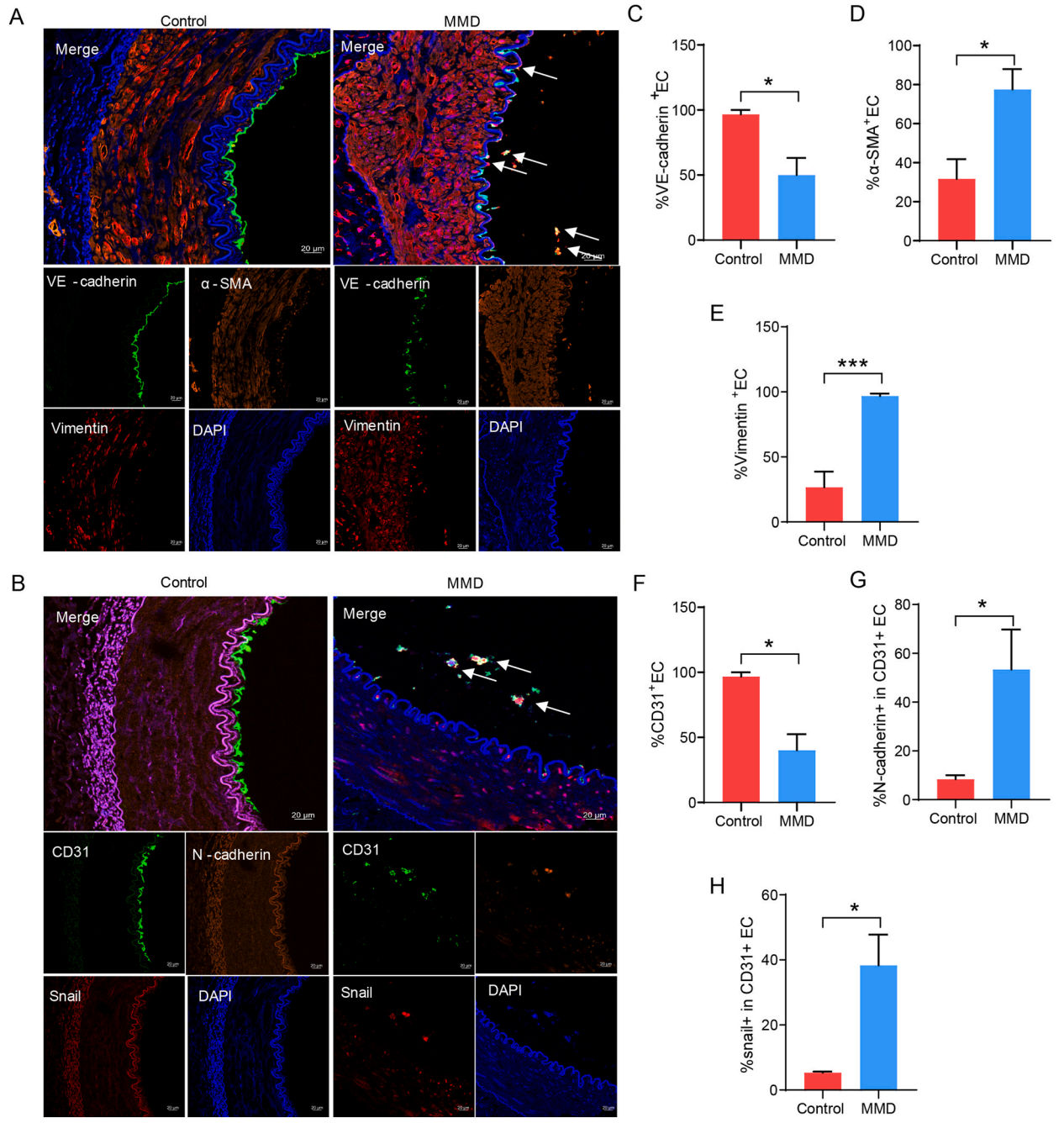

Fig. 1. Expression of EndMT-related molecular markers in arterial tissue (A)VE-cadherin (green), Vimentin (red) and α-SMA (orange) expressionin control and MMD tissues. (B) CD31, Snail(red) and N-cadherin(orange) expressionin control and MMD tissues.(C) Percentage of VE-cadherin + ECs in arterial intima (*$p < 0.05$). (D)Percentage of α-SMA + VE-cadherin + ECs in arterial intima (*$p < 0.05$). (E)Percentage of Vimentin + VE-cadherin + ECs in arterial intima (***$p < 0.001$). (F) Percentage of $CD31^+$ ECs in arterial intima (*$p < 0.05$). (G)Percentage of N-cadherin + $CD31^+$ ECs in arterial intima. (H)Percentage of snail + $CD31^+$ ECs in arterial intima(*$p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. Exosome identification

A series of cellular experiments were conducted to confirm whether peripheral blood exosomes regulated EndMT. Peripheral blood samples were collected for exosome extraction and identification. Electron transmission microscopy revealed that the exosomes were shaped like tea saucers (Fig. 2A). The Nanosight LM10 system showed that the exosomes were mainly concentrated at 100 nm

(Fig. 2B). Western blotting analysis showed that the exosome markers TSG, CD63, and CD9 were positively expressed (Fig. 2C). The non-adjusted images of western blotting were shown in the Figs. 2 CS1-3 as supplementary material.

3.4. Exosomes from MMD patients promote cerebrovascular endothelial cell proliferation and migration

In vitro experiments were performed to demonstrate the functional effects of exosomes on the occurrence and development of MMD. The CCK-8 assay showed that, compared with the control group, exosomes in MMD significantly promoted the proliferation of cerebrovascular endothelial cells; however, they had no effect on umbilical vein endothelial cells (Fig. 3A and B). Similarly, compared with the control group, Transwell assays showed that plasma exosomes in MMD significantly promoted the migration of cerebrovascular endothelial cells but had no effect on umbilical vein endothelial cells (Fig. 3C–E). To further confirm the exosomes induce migration of endothelial cells, we use HCMEC/D3 cell line to perform transwell assay to validate the founding, and the results showed that exosomes derived from patients with MMD induced migration ability compared with control group (Fig. S3A). Interestingly, compared with healthy controls, exosomes in patients with MMD had minimal effect on the angiogenic ability of cerebrovascular endothelial cells, which was consistent with the results of Duanet al. (Fig. 3F and G), indicating that exosomes inpatients with MMD specifically act on cerebrovascular endothelial cells and that there may be a dysfunction of endothelial cells [12].

3.5. Exosomes from MMD patients promote cerebrovascular endothelial cell EndMT phenotype

Our results showed that cells stimulated by exosomes from patients with MMD underwent significant morphological changes. Immunofluorescence was performed to detect the cell length via staining β-tubulin, which demonstrated that the cytoskeleton was significantly elongated (Fig. 4A and B). We hypothesised that exosomes regulate cellular morphological changes by regulating EndMT. To further demonstrate the regulatory effects of the exosomes on EndMT, we measured the expression of EndMT-related markers. RNA analysis showed that E-cadherin was down-regulated and Snail expression was down-regulated (Fig. 4C). Immunofluorescence showed that the expressions of CD31 and E-cadherin were down-regulated (Fig. 4D), whereas the expression of Snail was up-regulated (Fig. 4D) after exosome stimulation. Western blotting showed that the expression of VE-cadherin, E-cadherin, and CD31 was down-regulated (Fig. 4E and F). The non-adjusted images of western blotting were shown in the Figs. 4 ES1-4 as supplementary material. The expression of vimentin and Snail was upregulated in MMD patients after exosomes stimulation compared with those in the control group(Fig. 4G and H). The non-adjusted images of western blotting were shown in the Figs. 4 GS1-3 as supplementary material. These results suggested that exosomes promote the EndMT phenotype in patients with MMD. In addition, western blotting and immunofluorescence showed downregulation of ERG expression (Figs. S2B and C), indicating that endothelial homeostasis was impaired after exosome stimulation, which may be the reason for the lack of ability to promote tube formation (Figs. 3F and G). We use HCMEC/D3 cell line to verify the expression of EndMT-related genes, and the results also showed that exosomes from patients with MMD promote the EndMT (Fig. S3B).

3.6. Exosomal miRNAs promote endothelial cells EMT phenotype

Previously, our research group carried outa sequencing analysis of miRNAs in plasma-derived exosomes and screened themfor differentially expressed miRNAs. After exosome stimulation, miRNA detection was performed, which showed that miRNA151a-3p and miRNA125b-5p were up-regulated (Fig. 5A). We speculated that miRNA-151a-3p and miRNA-125b-5pmay play important roles inEndMT phenotypic changes. Overexpression of miRNA-125b-5p enhanced the migratory ability of endothelial cells, whereas miRNA-125b-5p knockdown weakened the migratory ability of endothelial cells (Fig. 5B,D,E). Similarly, miRNA-151a-3p overexpression enhanced the migratory ability of endothelial cells, whereas miRNA-151a-3p knockdown weakened the migratory ability of

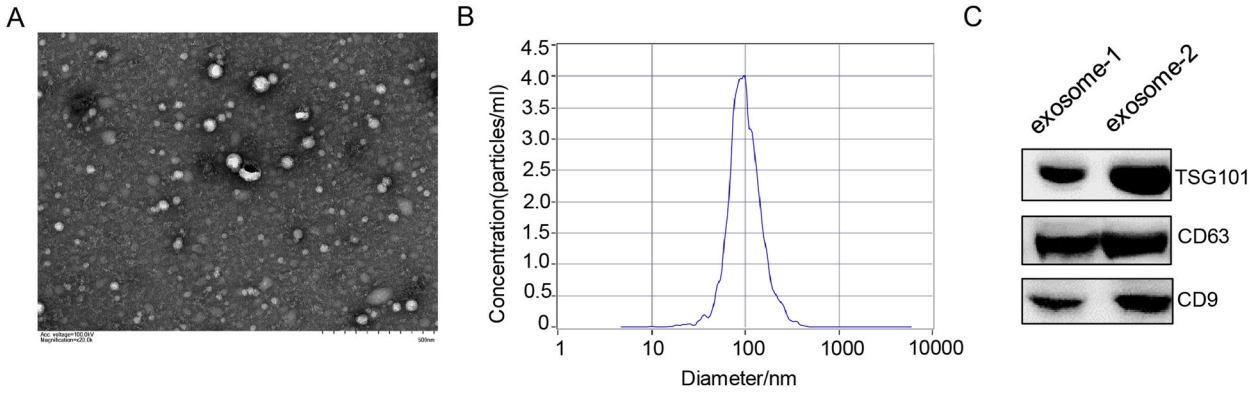

Fig. 2. Identification of plasma-derived exosomes (A) Electron microscope image showing the intact morphology of exosomes isolated.(B) Particle size analysis confirmed that the particle size of exosomes was about 94.5 nm(C) Western blot analysis of the exosome markers TSG101, CD63 and CD9.

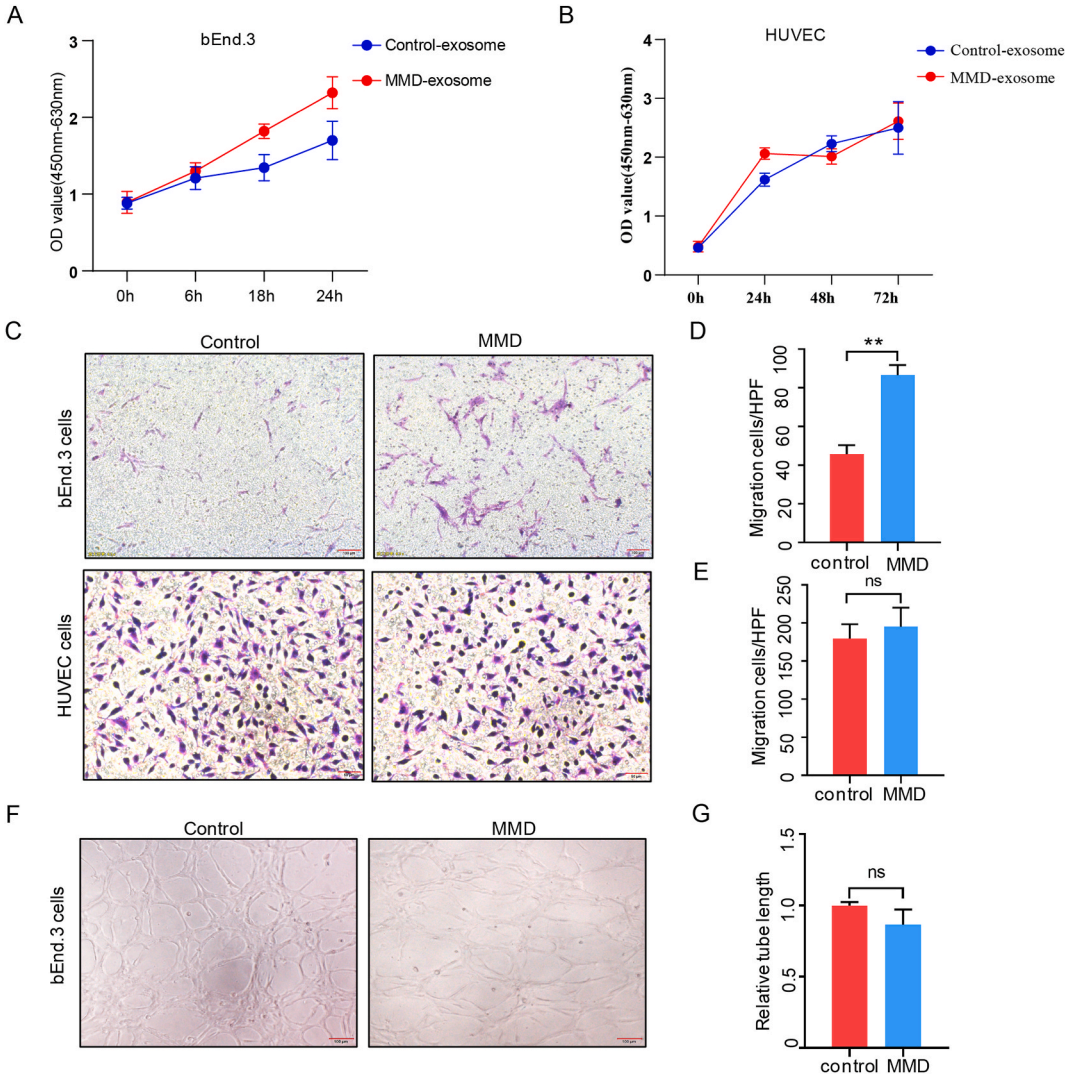

Fig. 3. Exosomes from Moyamoya disease (MMD) patients promote cerebrovascular endothelial cell proliferation, migration but not tube formation (A) (B) CCK-8 assay showed that exosomes (200 μg/mL) from MMD patients promotedmouse brain vascular endothelial cell (bEnd.3)proliferation but had no effect on human umbilical vascular endothelial cells (HUVEC). (C) Transwell assay showed that exosomes (200 μg/mL) from MMD patients promotedbEnd.3migration but had no effect on HUVEC. (D, E)high power field (HPF)showed migration cells of two groups (**$p < 0.01$).(F, G) Exosomes from MMD patients did not promote tube formation by bEnd.3 cells.

endothelial cells (Fig. 5C,F,G). To further confirm the role of miRNA-151a-3p and miRNA-125b-5p, the expression of miRNA-151a-3p and miRNA-125b-5p was knocked down during stimulation by plasma-derived exosomes of MMD, and the results showed that the cell migration ability and Snail expression were inhibited (Fig. 5H, I). The non-adjusted images of western blotting were shown in the Figs. 5 IS1, 5IS2 as supplementary material.

4. Discussion

These results demonstrate the key role of EndMT in the occurrence and development of MMD for the first time. PDEs of MMD promote proliferation, migration ability, and EndMT phenotype changes in cerebrovascular endothelial cells. The occurrence of EndMT in MMD is regulated, at least partly, by PDEs.

Currently, MMD is mainly treated by intracranial and extracranial vascular revascularisation [14]. Given the particularity of the disease site, generatingthe discarded vascular tissues during surgery is challenging. Therefore, only intracranial vascular tissue from six patients with MMD was collected, which is a potential limitation of this study. Our findings suggest that EndMT plays a key role in the development of MMD. EndMT is involved in various maladaptive tissue remodelling processes, such as venous graft remodelling and vascular fibrosis caused by stroke [15,16]. Moreover, EndMT causes inflammation, and inflammatory cytokines can destroy

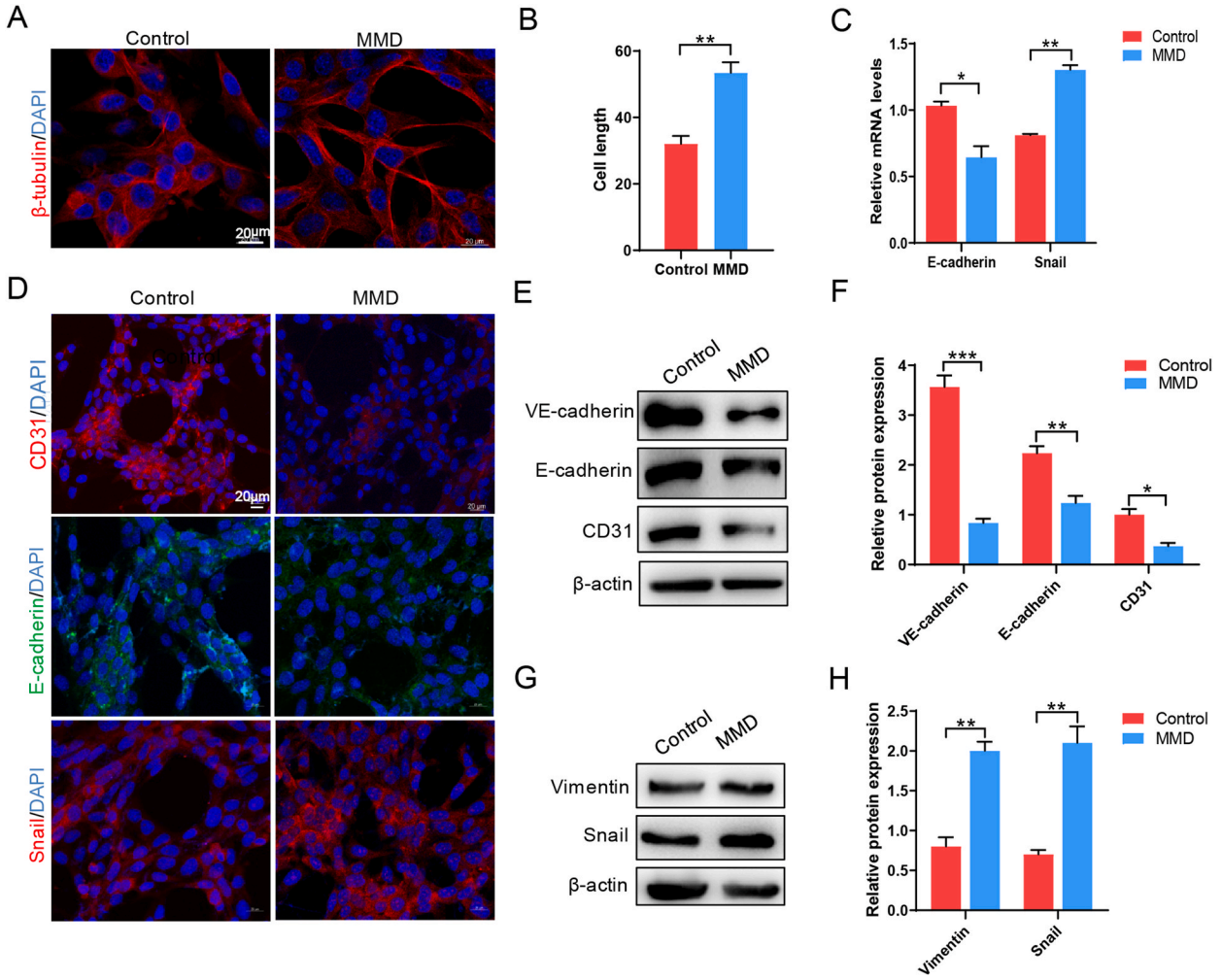

Fig. 4. Exosomes from patients with Moyamoya disease (MMD) promote cerebrovascular endothelial cell EndMT phenotype (A)Immunofluorescence assay showed morphological changes of cells via the localization of β-tubulin. Red represent β-tubulin and blue represent DAPI. (B)The cell length of the control and MMD group were measured by image J tool. (C) qRT-PCR showed the expression of E-cadherin and snail after cells stimulated by exosomes from MMD and control group. (*$p < 0.05$, **$p < 0.01$). (D) immunofluorescenceassay showed the expression of CD31 E-cadherin and Snai after cells stimulated by exosomes from MMD and control group. (E) Western blot showed the expression of VE-cadherin, E-cadherin and CD31 after cells stimulated by exosomes from MMD and control group. (F) The relative protein expression level of VE-cadherin, E-cadherin and CD31 were calculated using β-actin as the loading control (*$P < 0.05$, **$p < 0.01$, ***$p < 0.001$). (G) Western blot showed the expression of vimentin and Snail after cells stimulated by exosomes from control and MMD group. (H) The relative protein expression level of vimentin and Snail were calculated using β-actin as the loading control (**$p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

endothelial cell barrier function, thereby increasing EndMT [5,17]. Our previous study showed that the levels of inflammatory factors IL-1β, TNF-α, and IL-12 in the peripheral blood of MMD patients were significantly higher than those of normal controls, which may be related to vascular EndMT in MMD [18]. The ETS (E−26 transformation-specific) transcription factor, ERG, is a key regulator of endothelial cell homeostasis and contributes to the maintenance of vascular stability [19]. We found that PDEs in MMD promote the proliferation and migration ability of cerebrovascular endothelial cells but do not promote tube formation, which may be closely related to endothelial cell dysfunction caused by ERG deletion. Future studies to verify the regulatory effect of ERG on vascular endothelial cell function in vitro and the ability to treat MMD by inducing ERG expression are warranted.

Existing studies on the functional regulation of PDEs on cerebrovascular endothelial cells are scarce. Exosomes are encapsulation vectors for different molecules, including proteins and coding and non-coding RNAs (such as miRNAs), which can influence protein expression and function in recipient cells, such as vascular endothelial cells [20,21]. In this study, PDEs were isolated and extracted for in vitro experiments, which was the first to demonstrate that PDEs from MMD patients significantly induced EndMT phenotypic changes compared with those in the control group. However, the regulatory effects of exosomes on cerebrovascular endothelial cells require further validation. Our study suggests that exosomal miRNA-151a-3p and miRNA-125b-5p may be involved in inducing the

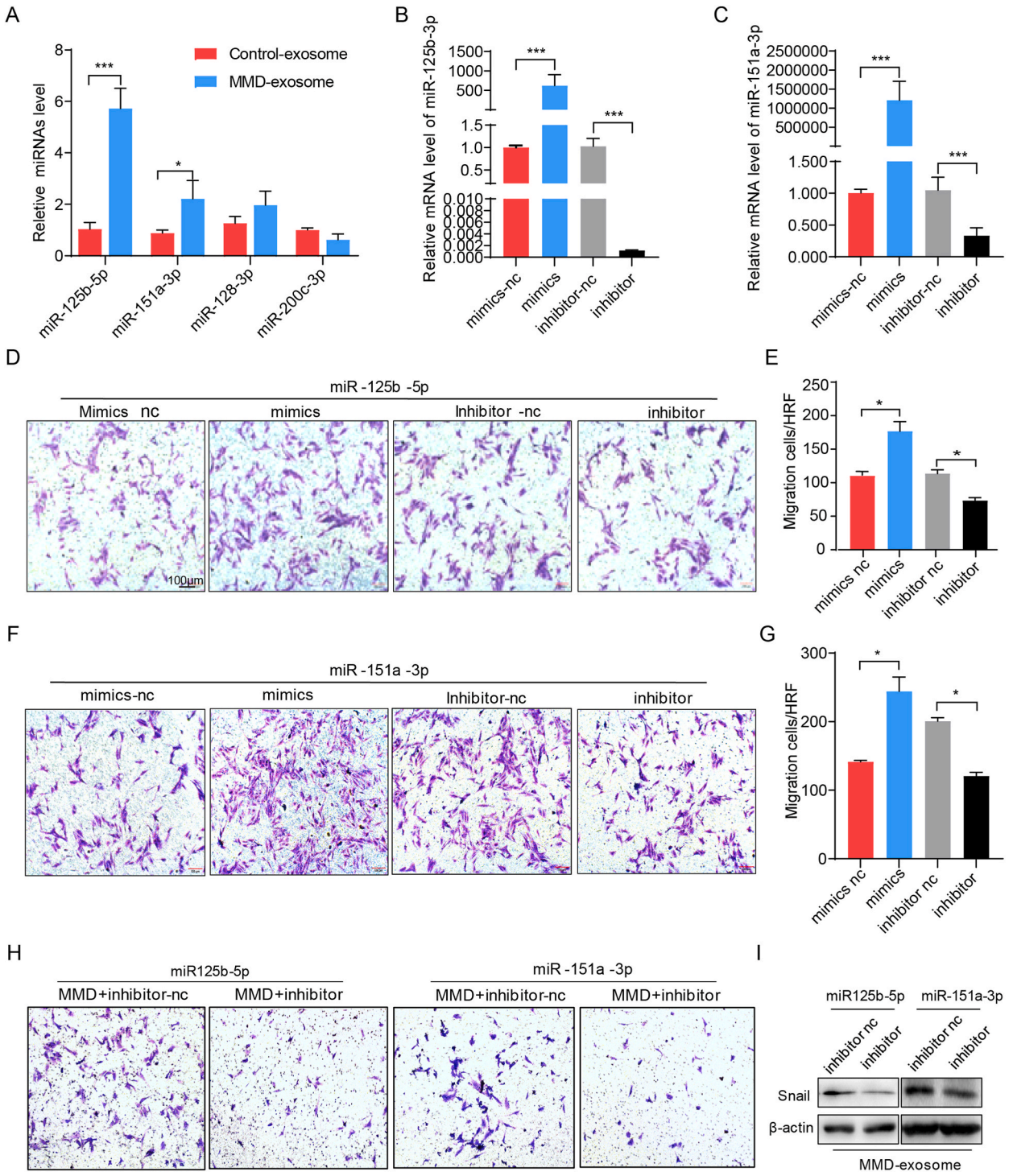

Fig. 5. Exosomal miRNA from Moyamoya disease (MMD) patients promote cerebrovascular endothelial cell migration (A)After bEnd.3 cells stimulated by plama exosome(control and MMD group), the relative mRNA levels of the candidate miRNAs were detected by qRT-PCR. (B)The bEnd.3 cells were transfected with mimics and inhibitor, respectively for the overexpression and knockdown of miR-125b-5p.The mRNA expression of miR-125b-5p was detected by qRT-PCR normalized to U6(***$p < 0.001$). (C)The bEnd.3 cells were transfected with mimics and inhibitor, respectively for the overexpression and knockdown of miR-151a-3p.The mRNA expression of miR-151a-3p was detected by qRT-PCR normalized to U6 (***$p < 0.001$). (D,F) Transwell assay showed the migration ability of each group. Nc, negative control. (E,G)The number of migration cells were counted and showed by bar chart. (H) The bEnd.3 cells were transfected with miRNA inhibitor and inhibitor nc, at the same time, cells were stimulated by exosome from MMD patients. (I)The protein expression of Snail was detected by western blotting.

EndMT phenotype. There are no studies report miRNA-151a-3p and miRNA-125b-5p in MMD. The molecular mechanism, such as downstream target and signal pathway, of miRNA-151a-3p and miRNA-125b-5p need further research. At present, the key signaling pathways that promote EndMT process in endothelial cells including TGF-β signal [22], Notch signal [23], PI3K/Akt/mTOR signal [24], Wnt/β-catenin signal [25] and so on. Bone marrow mesenchymal stem cells and their derived exosomal miRNA-125b-5p offer neuroprotection through modulating Wnt/β-catenin signaling pathways and through reducing inflammatory, apoptotic, and oxidative stress state [26]. Another Study has shown that miR-125b-5p targets STAT3 to inhibit hepatocellular carcinoma cells invasion, migration and growth through Wnt/β-Catenin pathway inactivation. Studies have shown that miR-151a-3p regulate TGF-β signal and Wnt/β-catenin signal [27,28]. We will explore downstream target genes through bioinformatics predictions and verify them through molecular biology experiments such as luciferase in the future. However, this study had certain limitations. First, this study demonstrated only the regulatory effect of exosomes on cerebrovascular endothelial cells. However, the cell types that secreted exosomes into the peripheral blood and whether they were secreted into the plasma by cerebrovascular endothelial cells and could interact with endothelial cells to form a feedback loop are still unclear, which is another limitation of this study.

In the future, we will further collect tissue samples to verify the key role of EndMT in the occurrence and development of Moyamoya disease, and deeply explore the specific regulatory mechanism of plasma exosomes on EndMT, so as to provide new insights into the pathogenesis, diagnosis and treatment of Moyamoya disease.

5. Conclusion

Our study demonstrated that EndMT plays a key role in MMD progression. In addition, PDEs induce EndMT phenotypic changesthat promote the proliferation and migration of vascular endothelial cells. Exosomal miRNAs mediate this EndMT phenotype. Therefore, this study provides new insights into the molecular mechanisms underlying MMD pathogenesis.

CRediT authorship contribution statement

Jilan Liu: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Chao Chen:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. **Xianyun Qin:** Methodology, Formal analysis, Data curation. **Jingtong Wang:** Methodology, Formal analysis, Data curation. **Bin Zhang:** Writing – review & editing, Conceptualization. **Feng Jin:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e26748.

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