



Non-negligible factors in studying the ApoM-S1P axis using EA.hy926 cells

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Background: The apolipoprotein M (ApoM)-sphingosine-1-phosphate (S1P) axis was recently identified, and research into its function has received increasing attention. However, there are some factors which might influence the results of studies into the function of the ApoM-S1P axis using the EA.hy926 cells. This study investigated related factors, including coagulation factor VIII (FVIII), ApoM, S1P receptor subtypes (S1PRs), C-myc-tagged, and His-tagged proteins in EA.hy926 cells, as well as the effects of ApoM overexpression on S1PRs.

Methods: The expression of FVIII, ApoM, S1PRs, C-myc, and His-tagged proteins in EA.hy926 cells was investigated through cellular immunofluorescence. EA.hy926 cells were infected with lentiviruses carrying (OE group) or lacking (NC group) the ApoM gene sequence. A stable cell line expressing ApoM was obtained, and the expression of ApoM mRNA was detected through single tube duplex fluorescence reverse transcription quantitative polymerase chain reaction (RT-qPCR). S1PRs expression was detected by RT-qPCR and Western blotting.

Results: The results showed that EA.hy926 cells expressed FVIII, ApoM, C-myc-tagged, and His-tagged proteins. Moreover, they highly expressed S1PR1, slightly expressed S1PR3, weakly expressed S1PR2, and did not express S1PR4 and S1PR5. ApoM overexpression significantly increased S1PR1 mRNA and protein expression but did not affect the expression of S1PR3. EA.hy926 cells expressed FVIII, suggesting the cell line possesses endothelial cell characteristics and could be used for *in vitro* studies of the ApoM-S1P axis.

Conclusions: EA.hy926 cell line is suitable for investigation of the ApoM-S1P axis *in vitro*. However, since EA.hy926 cells expressed endogenous ApoM, C-myc and His tagged proteins, the exogenous recombinant ApoM should not be labeled with C-myc and His tags for distinguishing from endogenous ApoM. In addition, overexpression of ApoM should be considered to significantly increase the expression of S1PR1 when studying the APOM-S1P axis.

Keywords: Apolipoprotein M (ApoM); ApoM-S1P axis; S1PRs; EA.hy926 cells; coagulation factor VIII; tagged proteins

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Introduction

In 2013, Arkensteijn *et al.* (1) published a review exploring the relationship between the ApoM-S1P axis and lipid metabolism. The ApoM-S1P axis consists of apolipoprotein M (ApoM), sphingosine-1-phosphate (S1P), and S1P receptor subtypes (S1PRs). First identified by Xu and Dahlbäck in 1999 (2), ApoM, an important component of high-density lipoprotein (HDL), is a member of the lipocalin protein family and a physical carrier of plasma S1P (1,3). ApoM is required for the formation and maturation of pre β -HDL particles. Since the main function of pre β -HDL is to promote the outflow of intracellular cholesterol, lack of ApoM will significantly affect the reverse transport of cholesterol, and lead to the occurrence of atherosclerosis (4,5).

A lipid regulator with biological activity, S1P and its corresponding receptors have five subtypes (S1PR1 to S1PR5), which belong to the G protein-coupled receptor family and are expressed in different cells (6). ApoM delivers S1P to S1PR1 on the cell membrane, where it plays a significant role in the barrier protective effects of endothelial cells, as demonstrated *in vivo* and *in vitro* (6). Despite the importance of the ApoM-S1P axis, the role ApoM carries out in the delivery of S1P to other S1PRs remains unclear. Further research is needed to elucidate the underlying mechanism.

The human cell line EA.hy926 and human umbilical vein endothelial cells (HUVECs) are frequently used in the exploration the pathophysiological functions of endothelial cells *in vitro*. HUVECs were demonstrated to express S1PR1 and S1PR3 at the nucleic acid level by Dunlap *et al.* (7); however, the basic properties of EA.hy926 cells in relation to functional studies of the ApoM-S1P axis remain unclear.

In the present study, the expressions of coagulation factor VIII (FVIII), ApoM, C-myc-tagged and His-tagged proteins, and S1PRs was examined, and the effect of ApoM on S1PRs in EA.hy926 cells was investigated, thus providing a basis for further study into the function of the ApoM-S1P axis.

Methods

Materials

EA.hy926 cells (American Type Culture Collection, ATCC) were grown in 60-mm Petri dishes in Dulbecco's Modified Eagle Medium (DMEM, with 4.5 g/L glucose, L-glutamine,

and sodium pyruvate) with 10% FBS. Cells were cultured at 37 °C in 5% CO₂ and passaged with trypsin-EDTA. Then, the cell culture medium was changed to DMEM with 1% fatty acid-free Bovine Serum Albumin (BSA) and cultured for 24 h before the experiment. ApoM antibody was purchased from GeneTex (CA, USA), and FVIII, S1PR2, S1PR4, S1PR5, and C-myc tag antibodies were obtained from Santa Cruz (CA, USA). S1PR1, S1PR3, and His tag antibodies and FITC-conjugated secondary antibodies were purchased from Abcam (Cambridge, UK). Cy3-conjugated secondary antibody was purchased from Bethyl (TX, USA). Deep-sea fish gelatin was obtained from Sigma (CA, USA). 4'-diamidino-2-phenylindole (DAPI) dye and anti-fade solution were sourced from Boster Biological Engineering Company (Wuhan, China). Cell culture plates (6-well and 24-well) were purchased from Costar (NY, USA).

Lentivirus infection

EA.hy926 cells were infected with lentiviruses (Genechem, China), in line with the manufacturer's instructions. Cells (2×10^5 /well) were seeded in six-well plates and cultured for 24 h. Then, cells were infected with recombinant lentiviruses carrying the ApoM gene sequence (NM_019101, overexpression group, OE group) and lentiviruses carrying no ApoM gene sequence (negative control group, NC group), and exposed to polybrene. The transduced cells were positive for green fluorescent protein (GFP), and green fluorescence was observed by fluorescence microscopy 48 h later.

Immunofluorescence staining

Endogenous FVIII, ApoM, S1PR1-5, C-myc, and His-tagged proteins in EA.hy926 cells were detected by immunofluorescence staining. EA.hy926 cells were plated into 24-well plates at a density of 5×10^4 /mL in 500 μ L/well. When cells reached 60–70% confluence, immunofluorescence detection was performed. Samples were fixed in a 4% paraformaldehyde solution for 15 min and washed 3 times with PBS. They were then exposed to 0.3% Triton X-100 solution for 10 min and washed 3 times before the addition of 1% fish gelatin for 1 h to block nonspecific binding sites. Incubation with specific antibodies (1:200 dilution) took place at 4 °C overnight. An equal volume of 1% BSA was added to the control group instead of primary antibody, before the samples were

washed 3 times, incubated with FITC-conjugated (1:1,000 dilution) or Cy3-conjugated secondary antibody (1:200 dilution) at 37 °C for 1 h in the dark, and washed with PBS 3 more times. The nucleus was stained with DAPI for 6 min, and samples were washed 3 times prior to the addition of anti-fade solution. An immunofluorescence microscope (Olympus IX73) was used to capture fluorescence images, which were then processed with CellSens software.

RNA extraction and Duplex fluorescence RT-qPCR.

EA.hy926 cells were seeded into 6-well plates at a density of 1×10^5 /mL in 2 mL/well. Total RNA was extracted with an RNA purification kit (Shenergy Biocolor Bioscience and Technology, China) once cells reached 80–90% confluence, according to the manufacturer's instructions. Each group had six wells and the process was repeated twice. A spectrophotometer (at 260/280 nm) assisted with the detection of the RNA quality. Aliquots of 2 µg RNA were reverse transcribed to cDNA with a First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). The PCR primers and probe sets are listed in *Table 1* (synthesized by Sangon Biotech Co. Ltd., China) and were designed using Primer Premier 6.00 (Premier Biosoft International, USA). The levels of target gene mRNA were quantified relative to the internal reference gene mRNA (8). RT-PCR was performed in 25 µL volumes containing 2 µL cDNA, 0.04 µL of 100 µM S1PR or ApoM primer and probe, 0.04 µL of 100 µM GAPDH primer and probe, 0.25 µL Taq DNA polymerase, 2.5 µL 10 × PCR buffer, 2.5 µL MgCl₂ (25 mM), 0.5 µL 4 × dNTPs (10 mM), and 17.01 µL water. The PCR conditions were as follows: 95 °C for 3 min followed by 40 cycles of 95 °C for 5 s, and 58 °C for 12 s (S1PR2 amplification curves were analyzed at 40 and 50 PCR cycles). The relative mRNA expression was calculated by the $2^{-\Delta\Delta C_t}$ method.

Western blotting

After 48 h of transfection, total cellular protein was harvested, and bicinchoninic acid assays were carried out to measure protein concentration. Each group had three wells and the process was repeated twice. Protein lysates (60, 30, or 15 µg) were separated by 10% SDS-PAGE, before transferral to polyvinylidene fluoride membranes, which were blocked with 3% BSA buffer and incubated with

S1PRs and GAPDH primary antibodies. After extensive washing, membranes were incubated along with goat anti-mouse or goat anti-rabbit secondary antibodies at 37 °C for 2 h. Following incubation in ECL luminescence solution (Pierce, USA), the light signal was visualized by exposing blots to radiographic film. The radiographic film was scanned with Microtek ScanMaker i800. Quantification was performed with Quantity One software (BioRad, Germany), and GAPDH was used as a loading control to normalize target protein expressions.

Statistics

All data are expressed as mean ± SD. Statistical analysis was performed in GraphPad Prism version 6.0 (GraphPad Software, USA). Student's *t*-test (two-tailed) facilitated comparisons between two groups to be performed. A value of $P < 0.05$ was considered to be statistically significant.

Results

Identification of FVIII, ApoM, S1PR1-5, C-myc, and His-tagged proteins in EA.hy926 cells

Immunofluorescence staining demonstrated that EA.hy926 cells expressed FVIII and ApoM, with FVIII evenly distributed in EA.hy926 in contrast to ApoM, which was strongly expressed and widely distributed in the cell (*Figure 1*). S1PR1–3 expression was detected in EA.hy926 cells, whereas S1PR4 and S1PR5 were barely detected (*Figure 2*). S1PR1 was highly expressed and showed a dense, patchy distribution, whereas S1PR2 and S1PR3 were expressed at low levels and showed a low-density punctate distribution. The immunofluorescence staining results also showed that C-myc and His-tagged proteins were uniformly and densely distributed in the EA.hy926 cells (*Figure 3*). No signals were detected when immunostaining was performed in the absence of primary antibodies.

S1PRs mRNA and protein expression in EA.hy926 cells

The generation of smooth amplification curves of S1PRs genes took place in the FAM channel (465–510 nm, *Figure 4A,B*). The GAPDH gene amplification curve was produced from the same cDNA amplification that appeared in the CY5 channel (618–660 nm, *Figure 4C,D*). The expression of S1PR1 and S1PR3 mRNA was detected in EA.hy926 cells in 40 cycles of PCR, whereas S1PR2,

Table 1 Human primers and fluorescent probes for RT-qPCR

Name	Sequence (5'-3')
ApoM	
Forward primer	CTGACAACCTCTGGGCGTGGAT
Reverse primer	TGTCCACAGGGTCAAAAGTTGC
Probe	FAM-AGTTCCCAGAGGTCCACTTGGGCCA-BHQ1
S1PR1	
Forward primer	GGGCTCTCCGAACGCAAC
Reverse primer	GGACCCCGACTCGAGCTG
Probe	FAM-TCCGAGGCCCTCTCCAGCCAA-BHQ1
S1PR2	
Forward primer	GGGACGCAGACGCCAAG
Reverse primer	TGTTCTGGACCTTGTGGG
Probe	FAM-CCTAGCCAGTTCTGAAAGCCCCAT-BHQ1
S1PR3	
Forward primer	CACCCGCTAGGATGCCG
Reverse primer	CTCCAGCGAGGGCGTTG
Probe	FAM-TCAGCCGACGGAGGAGCCCTT-BHQ1
S1PR4	
Forward primer	CCCGCACCTTCCGTCTG
Reverse primer	CAGGCTGAAGGTGGAGGC
Probe	FAM-CGCCCCGCCAGTGGTTCCTAC-BHQ1
S1PR5	
Forward primer	CGCACGACCAGGGCG
Reverse primer	AGTTGTAATGCAGGACGATGACC
Probe	FAM-CCCATGGAGTCGGGGCTGCT-BHQ1
GAPDH	
Forward primer	GGAAGGTGAAGGTCGGAGTC
Reverse primer	CGTTCTCAGCCTTGACGGT
Probe	CY5-TTTGGTTCGTATTGGGCGCCTG-BHQ2

ApoM, apolipoprotein M.

S1PR4, and S1PR5 were not detected. When the number of PCR cycles increased to 50, S1PR2 was detected, and the CT value was 40.3. Western blotting detection of S1PR1–5 in the EA.hy926 cells indicated that S1PR1, S1PR2, and S1PR3 were expressed in cells, whereas S1PR4 and S1PR5 were not (Figure 4E).

ApoM overexpression in EA.hy926 cells

Cells infected with GFP-tagged overexpression lentiviruses and mock-control lentiviruses were labeled as the OE and NC groups, respectively. In the OE group, ApoM mRNA levels were significantly increased ($P < 0.0001$, Figure 5; ~2,000-fold). The vector map used to construct recombinant lentiviruses is shown in Figure 5D. The results demonstrated the successful establishment of a high-expression ApoM cell model.

The effects of ApoM on S1PR1–3 mRNA and protein expression

The assessment of S1PR1–3 mRNA levels in the OE and NC cells showed that the expression of S1PR1 mRNA was 1.44-fold higher ($P = 0.0015$) in the OE group than in the NC group (Figure 6A). There was no significant difference between the S1PR3 mRNA levels of the two groups (Figure 6B). S1PR2 mRNA was not detected in the two groups, even when the number of PCR cycles was increased to 50. To determine whether ApoM affected S1PR1–3 protein expression, the gray scale value of the protein bands from the Western blotting was analyzed (Figure 6C). The overexpression of ApoM increased S1PR1 protein expression 1.65-fold ($P = 0.0025$, Figure 6D), whereas there were no significant differences observed between the S1PR2 and S1PR3 protein levels of the two groups (Figure 6E,F).

Discussion

HUVECs, which are derived from the maternal umbilical cord, are commonly used in the study of vascular endothelial

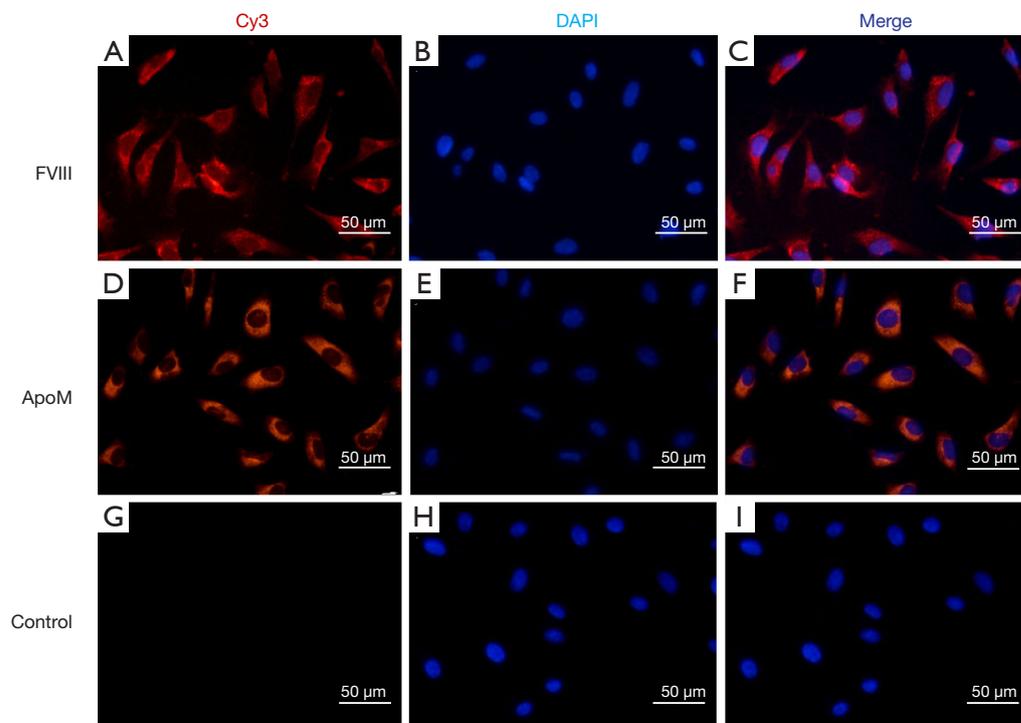


Figure 1 Immunofluorescence staining of FVIII and ApoM in EA.hy926 cells. FVIII and ApoM antibodies were incubated at 4 °C overnight. The corresponding cy3-conjugated secondary antibodies were incubated at 37 °C for 1 h in the dark. Reddish orange indicates positive staining. EA.hy926 cells expressed FVIII and ApoM. FVIII was evenly distributed in EA.hy926, whereas ApoM was strongly expressed and widely distributed in the cells. Magnification, $\times 200$, scale bar = 50 μm . FVIII, factor VIII; ApoM, apolipoprotein M.

cell function in *in vitro* experiments. However, nutrient-rich conditions are needed for their growth. Even so, they grow slowly after 6–8 passages. EA.hy926 cells have been fused with HUVECs and thioguanine-resistant A549 lung cancer cells, which are induced by polyethylene glycol. The resulting cell line shares the characteristics of endothelial cells and is able to grow and proliferate indefinitely (9). Because the culturing of EA.hy926 cells can be carried out *in vitro* quickly and easily, EA.hy926 cells served as a model in this study. The results indicated that FVIII and endogenous ApoM were expressed by EA.hy926 cells. FVIII expression and secretion are characteristics of endothelial cells which point towards the source of EA.hy926 cells. As a secretory protein, ApoM primarily localizes to liver and kidney tissues, and it is secreted by hepatocytes and renal tubular epithelial cells (10). The results of this study showed EA.hy926 cells to express ApoM, and this could be applied to future studies of the ApoM-S1P axis. However, only S1PR1 has thus far been reported to be affected

by ApoM in EA.hy926 cells (11), and it is still not clear whether expression of other S1PRs is affected by ApoM; we therefore examined the basal expression of S1PRs and the impact of ApoM on S1PRs in EA.hy926 cells.

In the present study, cell immunofluorescence staining, Western blotting, and RT-qPCR were used to demonstrate that EA.hy926 cells highly expressed S1PR1, slightly expressed S1PR3, weakly expressed S1PR2, and did not express S1PR4 and S1PR5. We also showed that the levels of S1PR1 were significantly higher in EA.hy926 cells infected with lentivirus carrying the ApoM gene than in control cells, which is consistent with the findings of our previous research (11). In contrast, S1PR3 expression was not affected, indicating that ApoM upregulates S1PR1 mRNA and protein expression. Our results indicated that ApoM carried S1P and played a physiological role in endothelial cells, mainly through S1PR1. However, S1PR1 expression was affected by ApoM itself and needed to be considered. Since S1PR4 and S1PR5 are not expressed

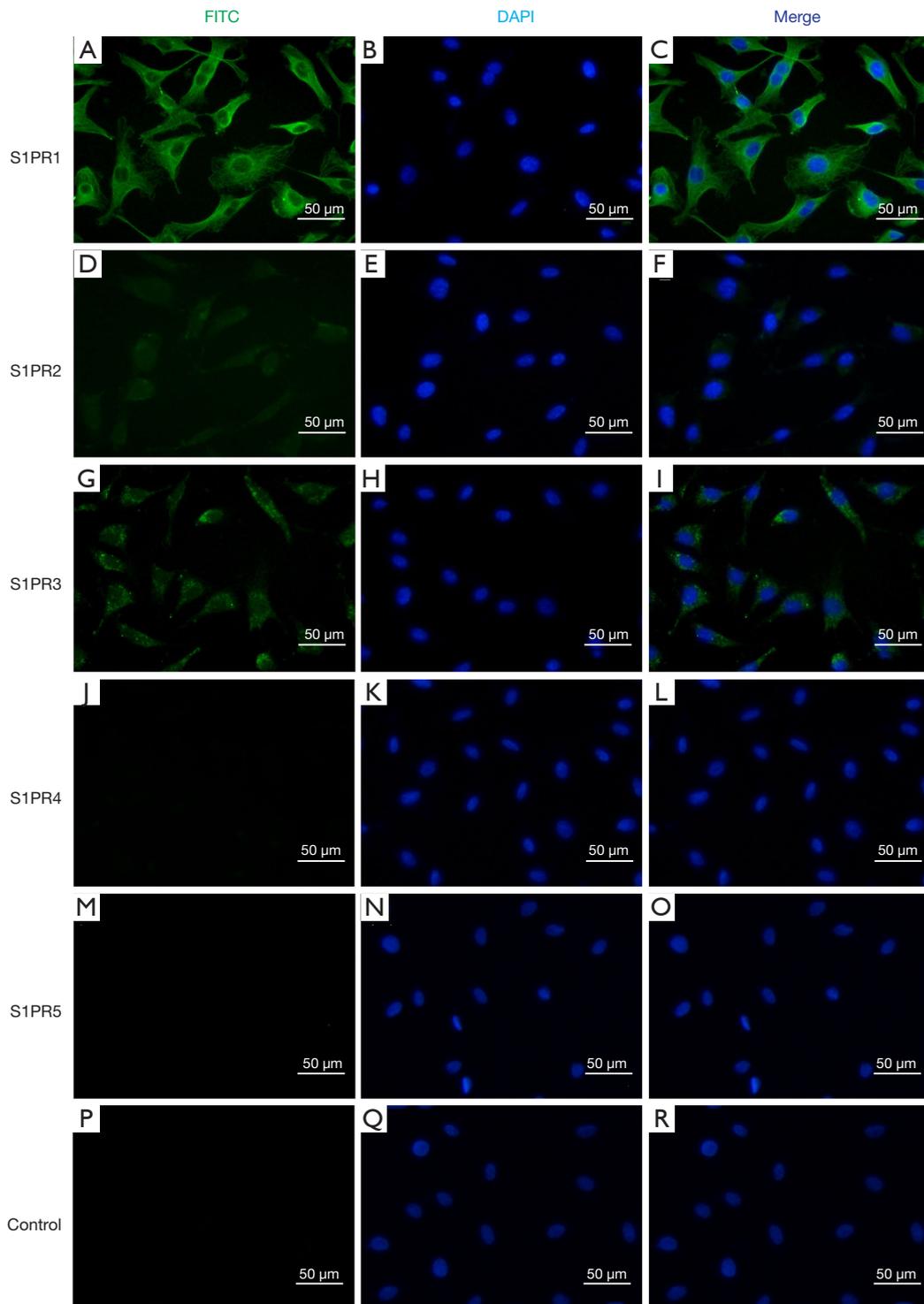


Figure 2 Immunofluorescence staining of S1PR1, S1PR2, S1PR3, S1PR4, and S1PR5 in EA.hy926 cells. S1PR1–5 antibodies were incubated at 4 °C overnight. FITC-conjugated secondary antibody was incubated at 37 °C for 1 h in the dark. Green indicates positive staining. S1PR1–3 expression was detected in EA.hy926 cells, whereas S1PR4 and S1PR5 were barely detected. S1PR1 was highly expressed and showed a dense patchy distribution, whereas S1PR2 and S1PR3 were expressed at low levels and showed a low-density punctate distribution. Magnification, $\times 200$, scale bar = 50 μm .

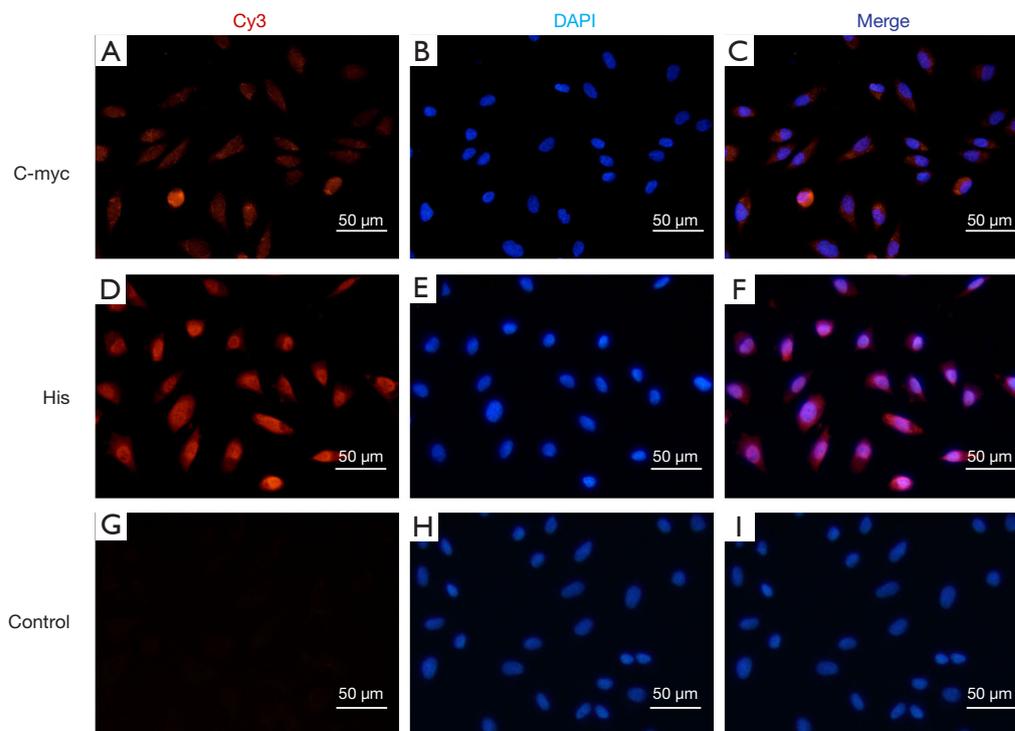


Figure 3 Immunofluorescence staining of C-myc and His-tagged proteins in EA.hy926 cells. C-myc and His-tagged protein antibodies were incubated at 4 °C overnight. Cy3-conjugated secondary antibody was incubated at 37 °C for 1 h in the dark. C-myc and His-tagged proteins were uniformly and densely distributed in the EA.hy926 cells. No signals were detected when immunostaining was performed in the absence of primary antibodies. Reddish orange indicates positive staining. Magnification, $\times 200$, scale bar = 50 μm .

by EA.hy926 cells, it is suggested that it is not necessary for researchers to detect them in future experiments. S1P is transported by ApoM or albumin to the membrane in effector cells, where it binds different receptor subtypes and activates multiple intracellular signaling pathways in target cells. S1PR1 is widely distributed and highly expressed in endothelial cells, lymphocytes, and astrocytes (12). Its main physiological functions include neuronal migration, immune regulation, cardiovascular and nervous system embryonic development, angiogenesis, and endothelial barrier function (13-17). ApoM may promote S1PR1 expression and thus play a part in vascular protection, in turn providing a basis for further research on the physiological role of S1P in S1PR1 activation in the cell membrane.

The results of this study showed S1PR2 to be weakly expressed in EA.hy926-untreated cells; however, the lentiviral-infected cells, including those in the OE and NC groups, showed undetectable levels of S1PR2 mRNA. There are three possible explanations for these findings: (I)

the expression of S1PR2 mRNA may be extremely low and could not be detected, despite an increase in the number of PCR amplification cycles to 50; (II) the S1PR2 protein band may be non-specific or produced by cross-reaction with other receptors; and (III) lentivirus infection may affect growth and S1PR2 expression in EA.hy926 cells. Therefore, other cell lines and detection methods could assist with further verifying our results.

C-myc and His tags are widely used in cell experiments (18-20), such as for the localization of proteins in cellular immunofluorescence assays, and as indicator proteins in Western blots. Exogenous recombinant ApoM with tag proteins could be applied in *in vitro* studies to ascertain whether ApoM acts as a mediator for the delivery of S1P to S1PRs on the cell membrane, as exogenous ApoM can be directly localized by using antibodies against tag proteins, thereby distinguishing it from endogenous ApoM. The present results showed that EA.hy926 cells expressed C-myc and His-tag proteins, which were therefore not suitable as exogenous tag proteins for recombinant ApoM. His-tag

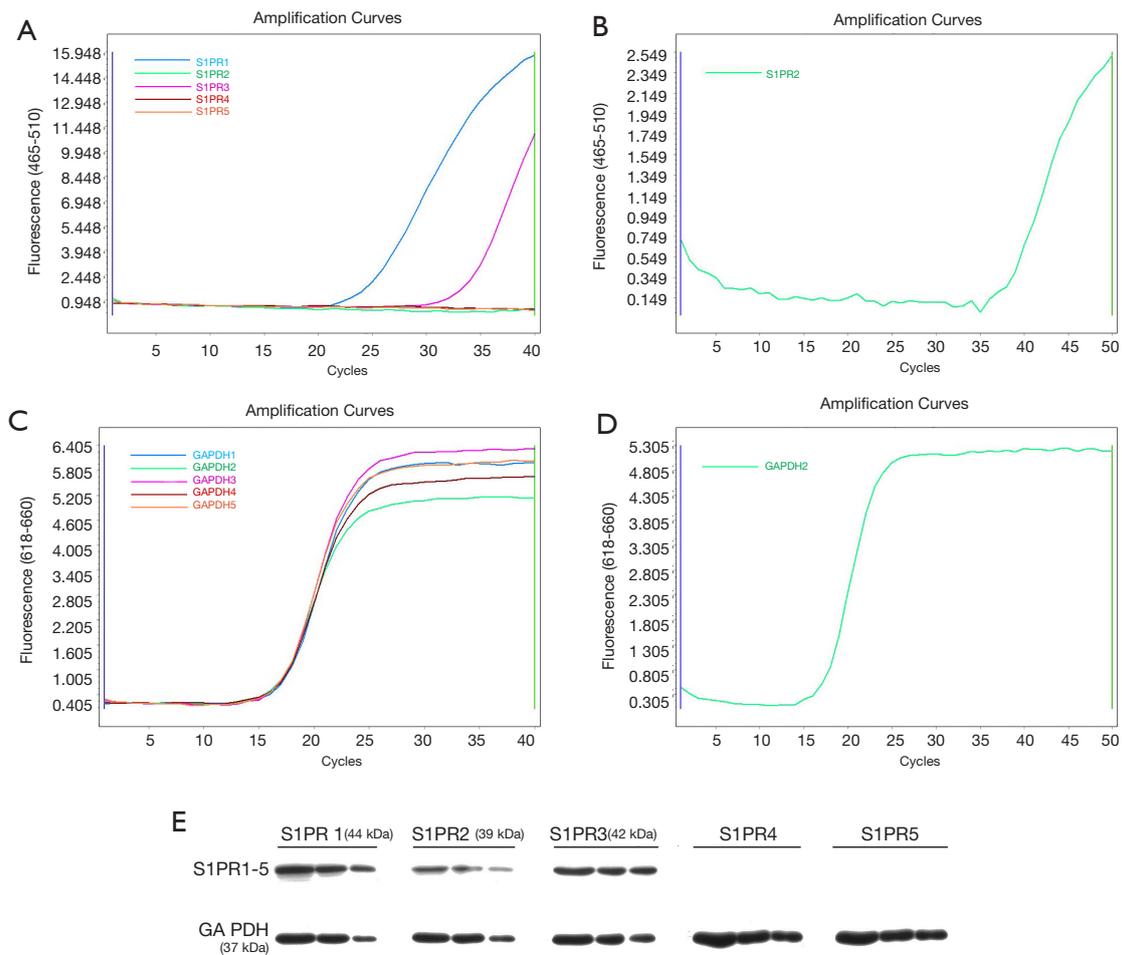


Figure 4 S1PRs mRNA and protein expression in EA.hy926 cells. The amplification curves of human S1PR1–5/GAPDH detected by duplex reverse transcription quantitative polymerase reaction. (A) The amplification curves of S1PR1–5 for 40 cycles (FAM channel, 465–510 nm); (B) the amplification curves of S1PR2 for 50 cycles (FAM channel, 465–510 nm); (C) the amplification curves of GAPDH for 40 cycles (CY5 channel, 618–660 nm); (D) the amplification curves of GAPDH for 50 cycles (CY5 channel, 618–660 nm); (E) the protein mass of S1PR1–5 in EA.hy926 cells. Three wells were loaded for each target protein, and the protein loading for each well was 60, 30, and 15 µg.

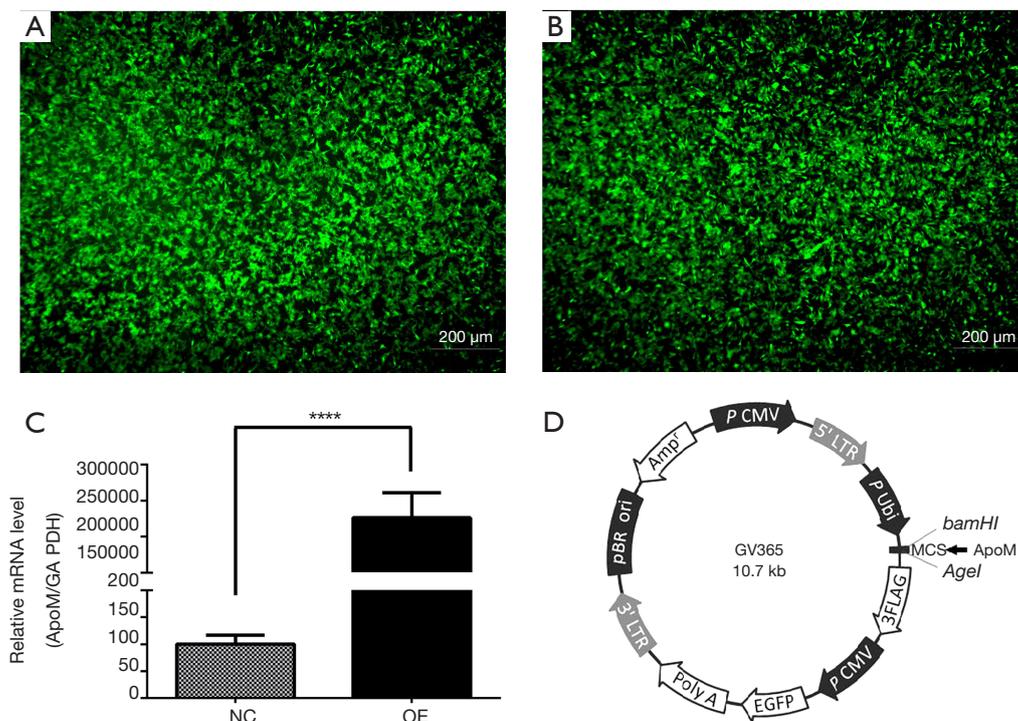


Figure 5 The overexpression of ApoM in EA.hy926 cells. Fluorescence photomicrographs of EA.hy926 cells infected by lentiviruses after 48 h. (A) The NC group; (B) the OE group. Scale bar =200 μ m. Based on the percentage of GFP-positive cells transfected with the GFP expression vector, the transfection efficiency was >90%; (C) the relative expression of ApoM in EA.hy926 cells was analyzed by duplex RT-qPCR. ****, P<0.0001 vs. NC; (D) vector map used to construct recombinant lentiviruses. OE, ApoM overexpression group; NC, control group; ApoM, apolipoprotein M.

is currently the most commonly used tag for prokaryotic protein expression. We detected His-tag expression in EA.hy926 cells by immunofluorescence and analyzed the possible causes. When the EA.hy926 cell line was established, the primary cultured human umbilical vein cells were fused with a thioguanine-resistant A549 clone inserting the His-tag, but this was not specifically mentioned in the article on establishing EA.hy926 cell line (21).

In summary, the EA.hy926 cells with the best macrovascular characteristics expressed ApoM and S1PRs, which points towards their potential value for *in vitro* studies of the ApoM-S1P axis. ApoM overexpression would carry more S1P, promoting the expression of S1PR1, and thereby transferring more S1P to bind to S1PR1, which plays a role in vascular endothelial cell protection. However, the specific underlying mechanism is yet to be determined.

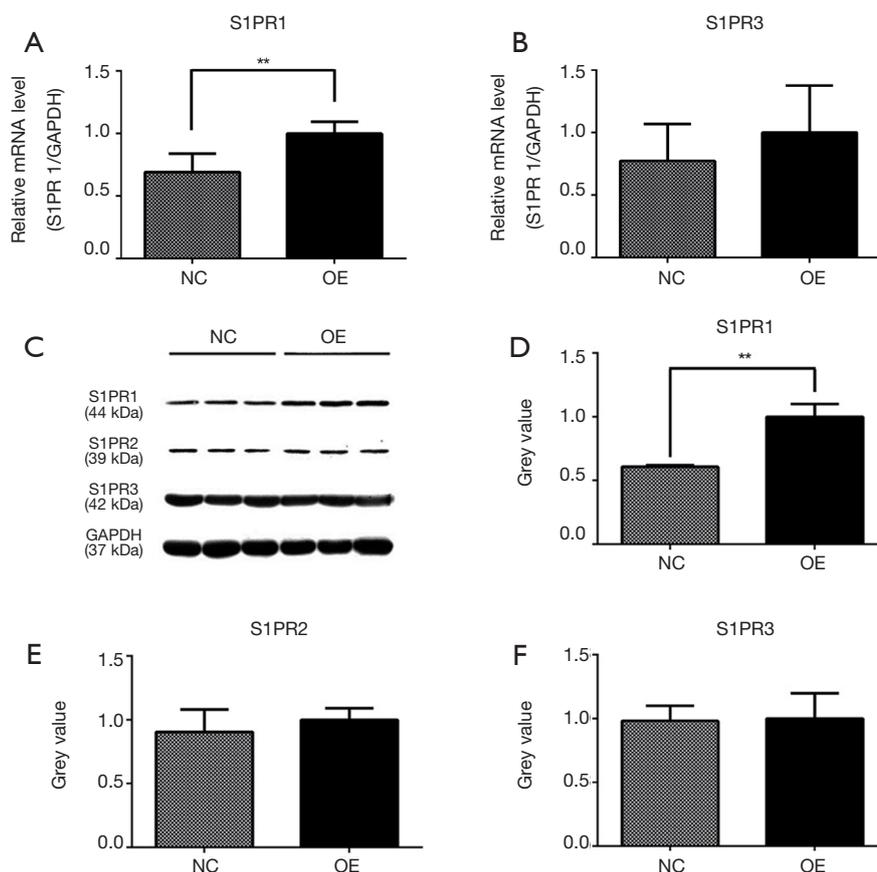


Figure 6 The effects of ApoM overexpression on S1PR1–3 mRNA and protein levels in EA.hy926 cells. (A) S1PR1 mRNA levels in the OE group compared with the NC group. **, $P < 0.01$ vs. NC; (B) S1PR3 mRNA levels in the OE group compared with the NC group; (C) the protein mass of S1PR1–3 in the OE group compared with the NC group; (D) grey value analysis of S1PR1 protein mass in the OE group compared with the NC group. **, $P < 0.01$ vs. NC; (E) grey value analysis of S1PR2 protein mass in the OE group compared with the NC group; (F) grey value analysis of S1PR3 protein mass in the OE group compared with the NC group. The S1PRs/GAPDH relative mRNA levels and bands' grey values in the OE group are set at 1. OE, ApoM overexpression group; NC, control group; ApoM, apolipoprotein M.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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