ISSN 1672-9145 **Acta Biochimica et Biophysica Sinica** 2007, **39**(3): 224–233 **CN** 31-1940/Q

Small Interfering RNA-mediated Caveolin-1 Knockout on Plasminogen Activator Inhibitor-1 Expression in Insulin-stimulated Human Vascular Endothelial Cells

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Abstract Using human vascular endothelial cells (ECV304) as the target, we studied the effect of caveolin (CAV)-1 in the course of insulin-stimulated expression of plasminogen activator inhibitor (PAI)-1. The appropriate single-stranded oligonucleotides representing the RNAi CAV-1 gene were analyzed by Ambion software. After annealing to generate double-stranded oligonucleotides (ds oligo), it was cloned into the pENTR/U6 entry vector containing RNA polymerase III expression element by T4 DNA ligase. The short hairpin (shRNA) sequences transferred from the pENTR/U6 entry were cloned into the pLenti6/BLOCK-iT-DEST vector with an LR recombination reaction. After identification by sequencing, we successfully constructed the CAV-1 RNAi lentiviral expression system using Gateway technology. Silencing efficiency was assayed by real-time reverse transcription-polymerase chain reaction, immunofluorescence staining and Western blotting. ECV304 cells were cultured in the medium containing different concentrations of insulin (1×10^{-9} to 1×10^{-7} M) with the CAV-1 gene silenced or not. The expression level and subcellular localization of PAI-1 and CAV-1 were compared using reverse transcription-polymerase chain reaction, immunofluorescence staining and Western blot assay. The results showed that the potent inhibition of CAV-1 expression could reach 85%, and it was specific to the CAV-1-derived shRNA, not the S100A13-derived shRNA. There was no dramatic difference in PAI-1 expression between the $RNAi^+$ and $RNAi^-$ ECV304 cells incubated with physiological insulin, but PAI-1 protein did accumulate under the cell membrane. As the concentration of insulin increased, the expression of PAI-1 was up-regulated, whereas the expression of CAV-1 attenuated. Furthermore, PAI-1 clearly augmented after CAV-1 knockdown. These results indicated that hyperinsulinism could promote PAI-1 expression by inhibiting CAV-1, and stabilizing or up-regulating CAV-1 expression in endothelial cells might reduce complications of the great vessels and capillary vessels in diabetes.

Key words caveolin-1; plasminogen activator inhibitor-1; gene silencing; insulin

The decrease of insulin sensitivity is also called insulin resistance (IR). IR is a major cause of diabetes, heart disease, hypertension, hyperlipemia and metabolism syndrome [1]. Insulin receptor (InsR) is a kind of transmembrane tyrosine kinase (TK) complex molecule on the cell membrane that is an $\alpha2\beta2$ tetramer connected by a disulfide bond between two α and β subunits. When it does not combine with the ligand, the conformation of α subunits inhibits β subunit kinase activity. When insulin combines with insulin receptor α subunits, it causes receptor allosterism, relieves its inhibition on β subunits, and induces autophosphorylation of the TK region on β subunits. It also activates TK phosphorylation insulin receptor substrates (IRS-1 and IRS-2), which combines with the SH2 structural domain proteins (PI-3K), initiates phosphorylation or

Received: December 4, 2006 Accepted: January 7, 2007
This work was supported by the grants from the National Natural Science Foundation of China (30400265 and 30671047), the Youth Foundation of Hunan Province Education Department (No. 06B079) and Traditional Chinese Medicine of Hunan (No. 204061)
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DOI: 10.1111/j.1745-7270.2007.00270.x

dephosphorylation cascade reactions of a serials of protein substrate, and accommodates intracellular biological processes such as glucose transport and glucogen synthesis [2].

The direct cause of IR is the decreased activity of InsR and induced abnormality of the post-receptor signal transduction pathway thereafter [1,2]. High free fatty acids, hyperglycemia, and increased oxidative stress might inhibit phosphorylation in β subunits of tyrosine, and block the activity of TK [3,4]. Hyperinsulinemia not only features in type 2 diabetes patients, it also exists in patients with IR but without diabetes. It has been proved that hyperinsulinemia can cause vascular endothelial dysfunction, and is an important risk factor for angiopathy, especially coronary heart disease. In hyperinsulinism angiopathy, it is important to pay attention to the effects of the impairment of fibrinolysis caused by increased plasminogen activator inhibitor (PAI)-1 [5]. PAI-1 is the physiological inhibitor of tissue-type plasminogen activator and urokinase plasminogen activator. In some metabolic conditions the activity and concentration of PAI-1 paralleled the level of hyperinsulinemia [6].

Caveolae are specific plasma membrane rafts mainly formed by cholesterol, glycophosphosphingolipid and caveolae protein. They belong to the signaling molecule pond, which presents in most cells, and were first characterized morphologically as small flask-shaped plasma membrane invaginations. Caveolin (CAV)-1 is a principal component of caveolae where insulin receptor enriches *in vivo*. The typical CAV-1 protein might combine with TK, threonine protein kinase, serine protein kinase such as Src-family TK, PKCα, H2Ras and others, and inhibit their activities [7–9,15–18]. A diverse array of functions have been proposed for caveolae and CAV-1, including modulation of signal transduction, endocytosis, potocytosis, and cholesterol trafficking. The roles played by caveolae and CAV-1 in IR and hyperinsulinemia remain controversial [5,6]. Plasma PAI-1 originates mainly from endothelial cell (EC), and the expression of CAV-1 on its membrane is also abundant. We cultivated human umbilical vein epithelial cells (ECV304), added high insulin (100 nM) in medium to simulate PAI-1 expression on ECV304 *in vitro*, and used a CAV-1-small interfering RNA technique to study the function of CAV-1 in insulin-stimulated PAI-1 expression.

Materials and Methods

Materials

ECV304 cells were obtained from the Clinic Research Institute at the First Affiliated Hospital of Nanhua University (Hengyang, China). BLOCK lentiviral RNA interference (RNAi) expression system, SuperScript III cDNA reverse transcription kit and DMEM were purchased from Invitrogen (Carlsbad, USA). Plasmid extraction kit (Promega, Madison, USA), and BCA protein quantitative kit (Pierce, Rockford, USA) were purchased. BlueRanger pre-dye protein molecule standard and protein fluorescence detection kit were from HyClone (South Logan, USA). Rabbit anti-human CAV-1 and PAI-1 polyclonal antibodies, mouse anti-human β-actin monoclonal antibody, sheep anti-rabbit secondary antibody labeled with fluorescein-isothiocyanate or Cy3, sheep anti-mouse and sheep anti-rabbit secondary antibody labeled with horseradish peroxidase were all from Santa Cruz Biotechnology (Santa Cruz, USA). SYBR green I fluorchrome was from Shangene (Shanghai, China). Primers of PAI-1 (forward, 5′-CCTGGCCGACTTCACAAGTC-3′ and reverse, 5′-TTGCAGTGCCTGTGCTACAGA-3′; amplification fragment 570 bp), β-actin (forward, 5′-TTGCAGTGCCTGTG-3′ and reverse, 5′-CAAACATGATCTGGGTCATCTTCTC-3′; 350 bp) and CAV-1 (forward, 5′-AGAC2GAGCTGAGCGAGAAGC-3′ and reverse, 5′-CCAGATGTGCAGGAAAGA-3′; amplification fragment 540 bp) were designed and synthesized by Sangon (Shanghai, China).

Short hairpin RNA (shRNA)-pENTR/U6 entry vector of CAV-1

Induced silent sites were selected from the CAV-1 gene where the G/C ratio was 30%–50% with Ambion shRNA analysis software (http://www.invitrogen.com/rnai), and BLAST analysis (http://www.pubmed.gov) suggested that it had no homology with other genes. Two complementary oligonucleotide strands were designed based on the following sites: top strand, 5′-CACCGGGACATCTCTAC-ACCGTTCCCGAAGGAACGGTGTAGAGATGTCCC-3′ (underline shows haripin loop sequence), and bottom strand, 5′-AAAAGGGACATCTCTACACC-GTTCCTTCGGGAACGGTGTAGAGATGTCCC-3′. Five microliters of each complementary oligonucleotide strand (200 μM) and 2 μl of 10×denaturation buffer solution was mixed, and sterilized deionizated H_2O was added to a final volume of 20 μl. The reaction mixture was denaturated at 95 °C for 4 min, then annealed at room temperature for 10 min until it formed double-stranded oligonucleotides (ds-oligo). T4 DNA ligase was used to clone the ds-oligo into the linear vector pENTR/U6, and the reaction system was built following instructions. TOPO 10 competent cells of *Escherichia coli* were transformed

at room temperature for 5 min, on ice for 30 min, and spread by Luria Broth (LB) solid medium with kanamycin. Monoclonal colonies were selected 1 d later, and sequenced by TaKaRa (Dalian, China).

Construction of RNAi lentiviral expression vector targeting CAV-1

CAV-1 pENTR/U6 entry plasmid and pLenti6/BLOCK-iT lentiviral expression plasmid were used to carry out the LR recombined reaction. Four microliters of entry plasmid, 1 μl pLenti6/BLOCK-iT DEST vector, 3 μl TE, and 2 μl LR recombinase compound Clonase II enzyme mix were incubated at 25 ºC for 1 h, then 1 μl protease K was added and the mixture was incubated at 37 ºC for 10 min. Stbl3-competent cells of *E. coli* were transformed by heat-shock techniques using 3 μl LR reaction compound, and spread with selective LB medium cultivate board containing 100 μg/ml ampicillin and 50 μg/ml blasticidin. Monoclonal colonies that could not live on the screening chloromycin medium were chosen 1 d later. After plasmids were extracted, U6 upstream primer and V5 downstream primer were used to carry out polymerase chain reaction (PCR) for identification.

CAV-1 RNAi-induced gene silencing

CAV-1 shRNA pENTR/U6 entry vector plasmid, pLenti6/BLOCK-iT lentiviral expression plasmid and S100A13 RNAi lentiviral expression plasmid (negative shRNA control, and there is no homology between S100A13 and the CAV-1 gene) were extracted and transfected into human ECV304 cells using a liposome-mediated method. Fluorescence quantitative reverse transcription (RT)-PCR and Western blotting were used to validate gene silencing efficiency induced by CAV-1 RNAi after 48 h or 72 h.

Real-time quantitative PCR validated RNAi efficiency

Total RNA from normal ECV304 cells, and those cells transfected by CAV-1-shRNA pENTR/U6 entry plasmid, CAV-1-pLenti6/BLOCK-iT lentiviral expression plasmid and S100A13 RNAi lentiviral plasmid was extracted using Trizol, and reversely transcribed into cDNA following the instructions of the SuperScript III cDNA reverse transcription kit. The solution containing 10^6 copies of pcDNA3.1 CAV-1 expression plasmid was diluted at five multiple proportionally and used as a template to draw a standard curve [10,11]. SYBR green I (0.5 μl) was added into 25 μl PCR reaction, denaturated at 94 ºC for 45 s, renaturated at 55 ºC for 30 s, and extended at 72 ºC for 40s, 30 cycles. Gel electrophoresis (1.5%) was used to eliminate the interference of the primer dimer and to ascertain the specificity of the end products of PCR.

Cell culture and experimental groups

ECV304 cells were cultured in DMEM containing 10% calf serum, 2 mmol glutamine, 0.1 mM Minimum Essential Medium non-essential amino acid and 1% penicillin and streptomycin, then were incubated into monolayer attached cells and passaged. Cells were cultured in serum-free DMEM solution for 24 h before adding insulin, and kept growing at equal pace in the G_0 period. DMEM solution containing 10% calf serum was divided into: (1) physiological dose insulin group (1×10^{-9} M); (2) medium dosage insulin group (1×10^{-8} M); (3) high insulin group (1×10^{-7} M); (4) physiological dose insulin group after CAV-1-RNAi (1×10^{-9} M); (5) medium dosage insulin group after CAV-1-RNAi (1×10^{-8} M); (6) high insulin group after CAV-1-RNAi (1×10^{-7} M); (7) blank ECV304 cells group. Changes in expression of CAV and PAI-1 were detected after cells were cultured for 24 h.

Indirect immunofluorescence

The efficiency of CAV-1 shRNA-mediated CAV-1 knockout and the expression of PAI-1 were detected by indirect immunofluorescence (IIF) after insulin induction. Cells in each group were fixed with methanol:glacial acetic acid (3:1) for 15 min, penetrated with permeabilizing solution (0.25% Triton+5% dimethylsulfoxide), incubated at 37 ºC for 20 min, and blocked with Tris-buffered saline-Tween 20 (TBST) containing 5% dried skim milk for 2 h. Rabbit anti-human CAV-1 first antibody (diluted 1:200) or rabbit anti-human PAI-1 polyclonal first antibody was added, blocked at 4 ºC overnight, and washed with TBST three times. Then 1:3000 diluted fluorescein-isothiocyanate and Cy3 fluorescence-labeled sheep anti-rabbit secondary antibody was added and incubated at room temperature for 45 min, and washed three times with TBST. Images were acquired and observed by fluorescence microscope.

Western blotting

Efficiency of CAV-1 shRNA and expression of PAI-1 were detected by Western blotting. Proteins in normal cells, interfered CAV-1, and S100A13 cells were collected. BCA protein quantitative kit was used to quantify each sample, SDS-PAGE was used, then the samples were transfered to polyvinylidene difluoride membrane. TBST containing 5% dried skim milk (20 mM Tris, pH 7.6, 50 mM NaCl, 0.1% Tween) was used to block at 4 ºC overnight, incubated with 1:200 CAV-1 sheep anti-rabbit first antibody,

1:250 PAI-1 sheep anti-rabbit first antibody for 4 h, washed with TBST three times, incubated with 1:3000 horseradish peroxidase-labeling sheep anti-rabbit secondary antibody at room temperature for 45 min and washed again three times with TBST. The protein fluorescence detection kit (HyClone) showed the results on X-ray film. Images were acquired by an Epson 1650 photo scanner (Epson, Taiwan, China).

Statistical analysis

These experiments were repeated three times. Data are presented as the mean±SD. Differences between groups were analyzed by Student's *t*-test. $P<0.05$ was considered statistically significant.

Results

shRNA-pENTR/U6 entry vector of CAV-1 gene

The CAV-1 gene shRNA-pENTR/U6 entry vector was sequenced. Results are shown in **Fig. 1**. The size and open reading frame of the shRNA *CAV-1* gene pENTR/U6 vector were correct, and were the same as the sequence designed by the Ambion shRNA analysis software. The figure shows the beginning and end regions of the vector with shRNA inserted.

Construction of CAV-1 RNAi lentiviral expression vector

Long-term stable expression of the shRNA-pENTR/U6 entry vector can not be obtained in target cells because of the lack of a related antibiotic screening marker. It is easy to recombine the shRNA sequence in the shRNA-pENTR/U6 entry vector into the CAV-1 RNAi lentiviral expression vector using LR recombinase. The resistance of the target vector to ampicillin, chloromycin, and blasticidin was obtained before LR recombination. After LR recombination, chloromycin deactivation and the shRNA-pENTR/U6 entry vector's chloromycin deactivation led to the death of the vectors on the ampicillin LB medium, so the CAV-1-RNAi lentiviral expression vector could be screened and constructed conveniently. PCR was used to amplify the DNA fragment (280 bp) containing shRNA transcription element in the expression vectors (**Fig. 2**, lanes 4, 5 and 6). The single-stranded oligonucleotides and ds-oligo after annealing are also shown in **Fig. 2** (lanes 1 and 2, and lane 3, respectively).

CAV-1 RNAi-induced gene silencing

Real-time fluorescence quantitative RT-PCR [**Fig. 3(A,**

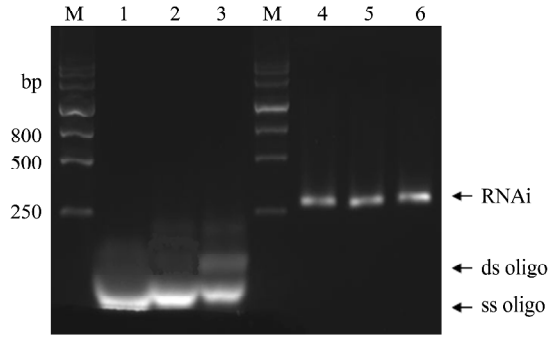

Fig. 2 Construction of the pLenti6/BLOCK-T-iT-DEST vector against caveolin (CAV)-1

1, CAV-1 top strand oligonucleotide; 2, CAV-1 bottom strand oligonucleotide; 3, CAV-1 double-stranded oligonucleotide (ds oligo) annealing reaction; 4–6, DNA sequences transfered from the pENTR/U6 entry clone consist of a U6 RNA interference (RNAi) cassette containing the human U6 promoter and CAV-1 ds oligo encoding the short hairpin RNA and Pol III terminator. M, DNA marker III; ss, single-stranded.

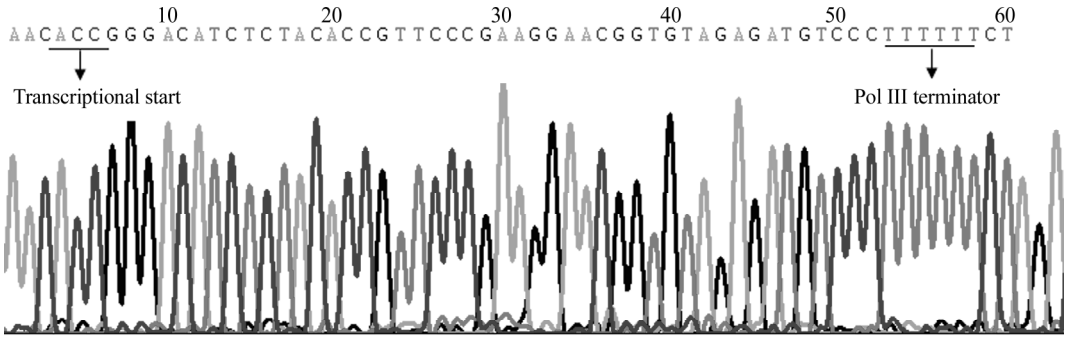

Fig. 1 Sequence of pENTR/U6 RNA interference against caveolin-1

B)], IIF (**Fig. 4**), and Western blotting (**Fig. 5**) were used to compare the level of CAV-1 gene and protein among four groups of ECV304 cells: (1) untransfected ECV304 cells; (2) S100A13 RNAi plasmid transfected ECV304 cells (negative shRNA control); (3) CAV-1-shRNA pENTR/U6 entry plasmid transfected ECV304 cells; (4) CAV-1-pLenti6/BLOCK-iT lentiviral plasmid transfected ECV304 cells. The results showed that levels of the CAV-1 gene and protein in groups 1 and 2 were higher, but noticeably decreased in groups 3 and 4. However, no significant difference was detected between the groups 1&2 and groups 3&4. Expression of the CAV-1 gene and protein after transfection with CAV-1-shRNA decreased by 85% compared with groups 1 and 2 [**Figs. 3(B1), 4** and **5**, lanes 3 and 4]. The expression of the CAV-1 gene was not affected by S100A13 shRNAi [**Fig. 3(B), 4** and **5**, lane 2]. This suggested that the transfection of constructed CAV-1 shRNA pENTR/U6 entry vector and CAV-1 pLenti6/BLOCK-iT lentiviral expression vector could efficiently and specifically inhibit the expression of CAV-1 gene and protein.

Changes in the insulin-induced expression of PAI-1 after shRNA induced CAV-1 gene silencing

ECV304 cells were incubated in medium with different doses of insulin, and changes in the insulin-induced expression of PAI-1 after shRNA-mediated CAV-1 knockout were studied with IIF. There were no obvious changes in total PAI-1 expression between blank and physiological

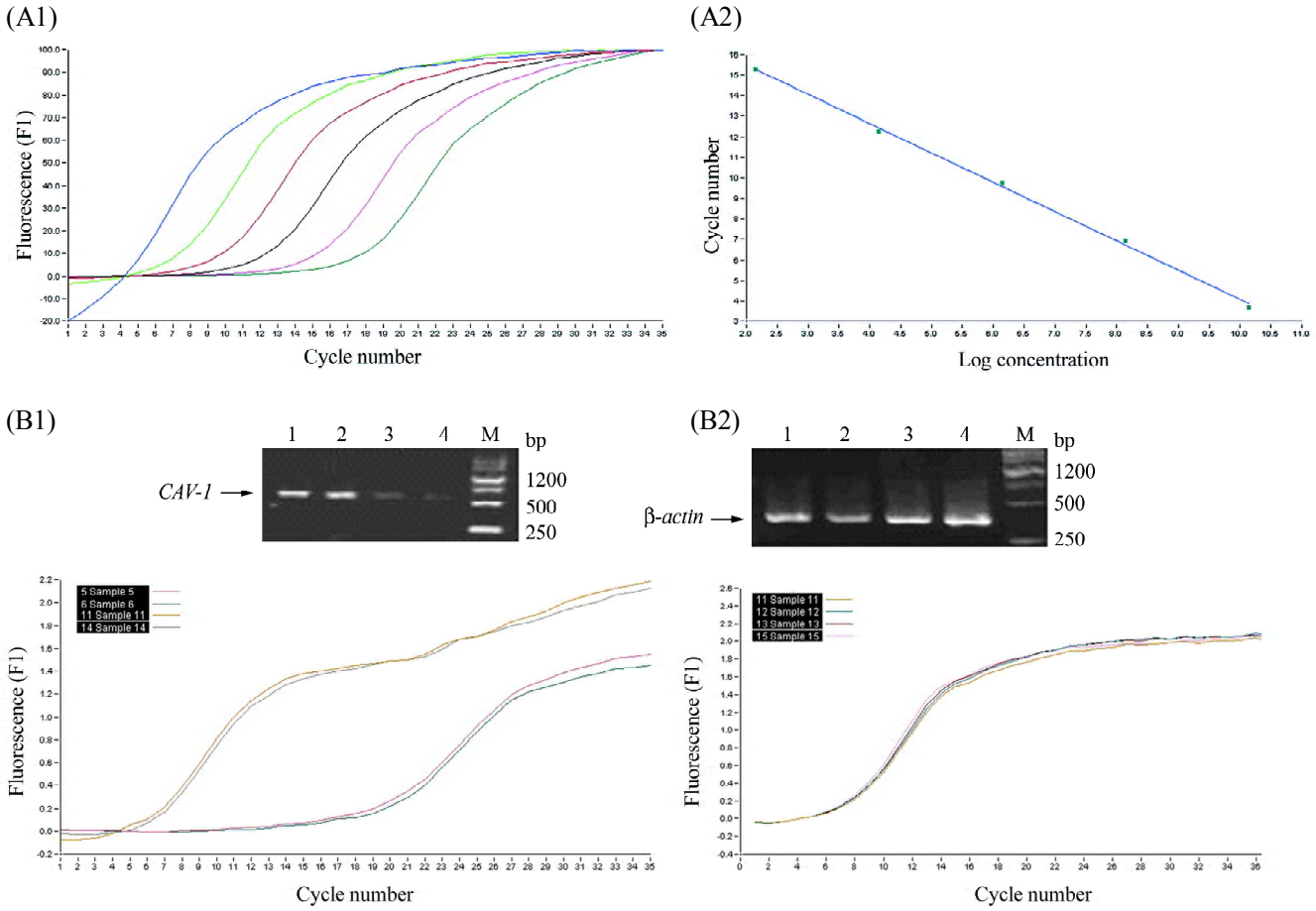

Fig. 3 Assay for caveolin (CAV)-1 gene silencing efficiency by real-time fluorescence quantitative reverse transcription-polymerase chain reaction (RT-PCR)

The results of short hairpin (sh)RNA targeting CAV-1 were assayed by real-time RT-PCR. (A1,A2) Standard curve (fluorescent light of CAV-1 plasmid template (diluted at five multiple proportionally). (B1) CAV-1 expression fluorescent light curve was compared by real-time RT-PCR using four group cells. (B2) β-actin expression fluorescent light curve was compared by real -time RT-PCR using the same four group cells. Only the CAV-1-specific shRNA (entry vector and expression vector, lanes 3 and 4, respectively) inhibited expression of the CAV-1 gene. No CAV-1 knockdown was observed with the S100A13 shRNA (negative shRNA control, lane 2). 1, untransfected human vascular endothelial cell (ECV304); 2, S100A13RNAi cell (negative shRNA control); 3, pENTR/U6-CAV-1 shRNA; 4, pLenti6/BLOCK-iT-DEST-CAV-1 shRNA.

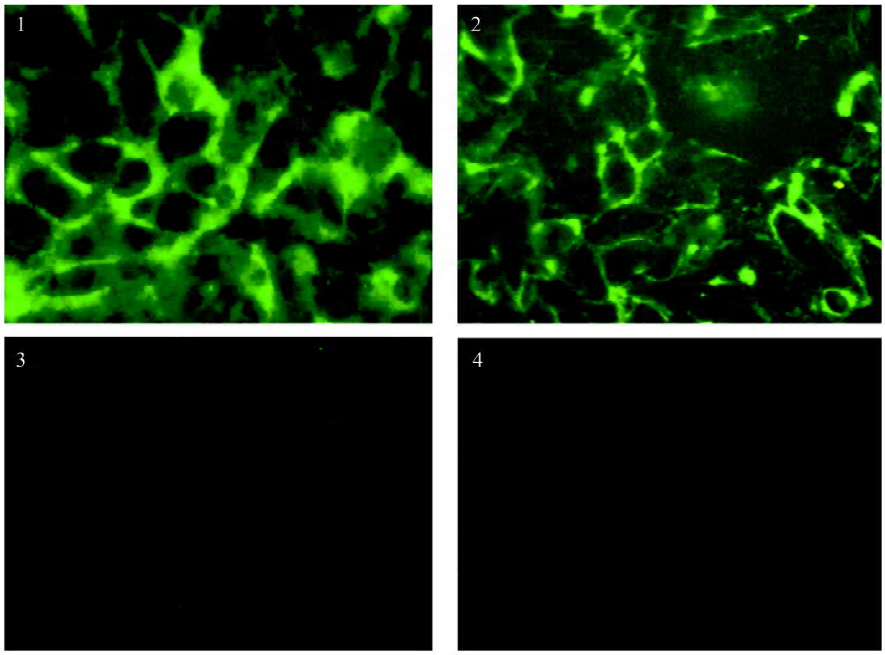

Fig. 4 Assay for caveolin (CAV)-1 gene silencing efficiency by immunofluorescence staining

1, untransfected human vascular endothelial cell (ECV304); 2, S100A13RNAi cell (negative shRNA control); 3, pENTR/U6-CAV-1 shRNA; 4, pLenti6/BLOCK-iT-DEST-CAV-1 shRNA.

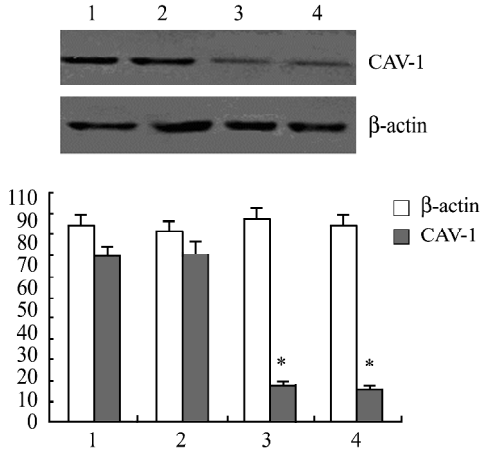

Fig. 5 Assay for caveolin (CAV)-1 gene silencing efficiency by Western blotting

1, untransfected human vascular endothelial cell (ECV304); 2, S100A13RNAi cell (negative shRNA control); 3, pENTR/U6-CAV-1 shRNA; 4, pLenti6/BLOCK-iT-DEST-CAV-1 shRNA.

dose insulin groups [**Fig. 6(A1,A2)**], however, after shRNA induced CAV-1 silencing, PAI-1 mainly located in the membrane and ECM [**Fig. 6(A2)**]. The expression of PAI-1 was enhanced mainly in the cytoplasm under the stimulation of medium dosage insulin [**Fig. 6(B1)**], and a higher level of expression was detected after shRNA induced CAV-1 silencing [**Fig. 6(B2)**]. PAI-1 expressed highly in the high dose insulin group and transported to the nuclear envelope and cell intranuclear, and this change could be enhanced by CAV-1 silencing [**Fig. 6(C2)**].

Changes in PAI-1 and CAV-1 expression after insulin stimulation

The changes in PAI-1 [**Fig. 7(A1,A2,A3)**]and CAV-1 [**Fig. 7(B1,B2,B3)**] expression under different doses of insulin stimulation were compared using RT-PCR and Western blotting. The results suggested that the expression of PAI-1 was less after ECV304 cells were incubated in physiological dose insulin, and did not change significantly after shRNA induced CAV-1 silencing [**Fig. 7(A)**, lanes 1 and 2]. The expression of PAI-1 was obviously high after incubation in high dose insulin [**Fig. 7(A)**, lane 3] and could be enhanced by shRNA-induced CAV-1 silencing [**Fig. 7(A)**, lane 4]. The same results were shown with immunofluorescence. The expression of CAV-1 in blank and physiological dose insulin groups was higher [**Fig. 7(B)**, lanes 1 and 2], with no significant difference between the two groups. This expression could be decreased significantly under high insulin incubation [**Fig. 7**

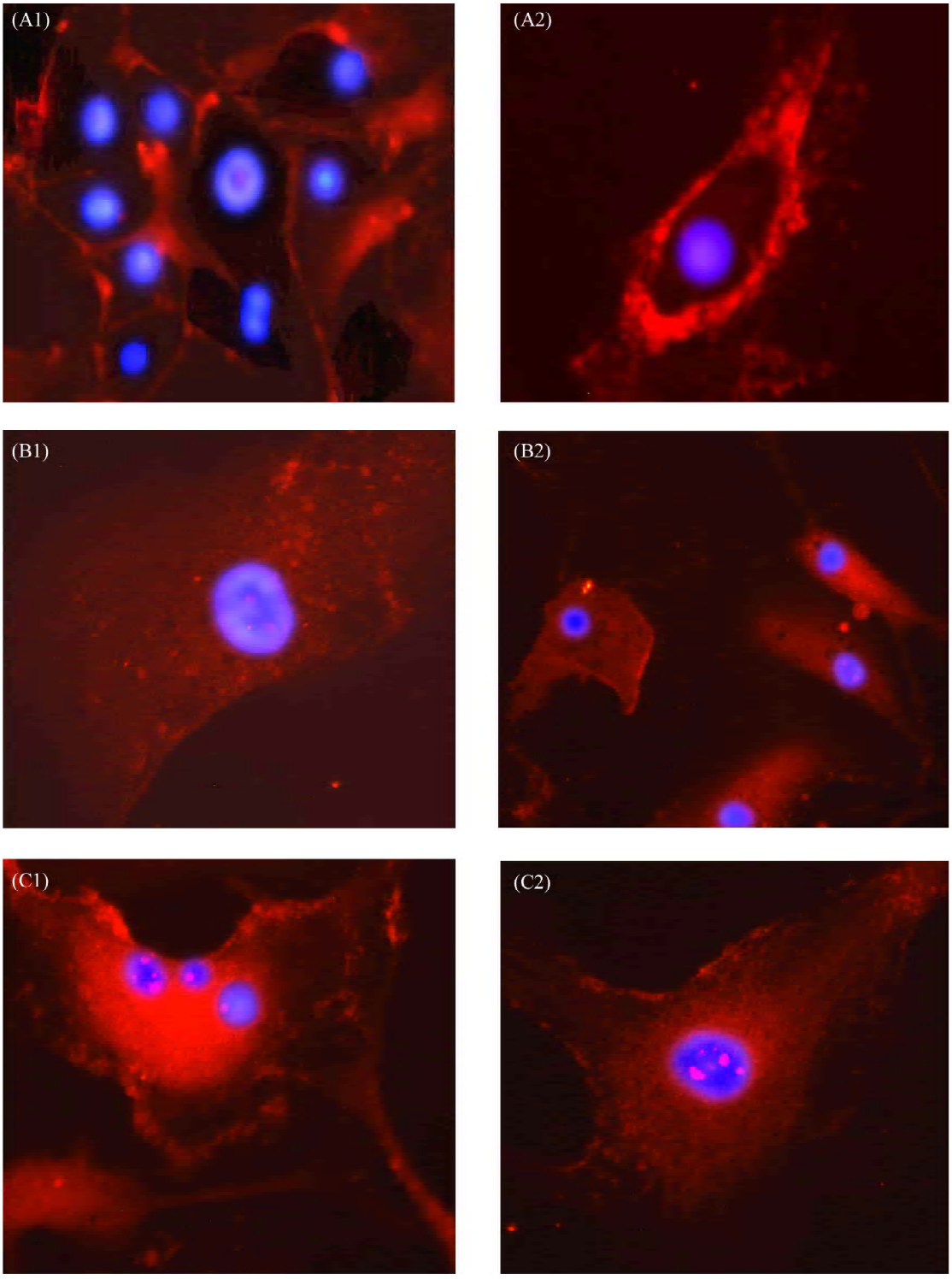

Fig. 6 Subcellular localization changes of plasminogen activator inhibitor (PAI)-1 protein stimulated by insulin in human vascular endothelial cells (ECV304)

The ECV304 cells were cultured with 1×10^{-9}, 1×10^{-8} and 1×10^{-7} M insulin for 48 h. Changes in the insulin-induced expression of PAI-1 after short hairpin RNA-mediated caveolin (CAV)-1 knockout were detected with indirect immunofluorescence, and the nuclei stained by 4′*t*,6′-diamidino-2-phenylindole dihydrochloride as control. Magnification, 200×. (A1) ECV304 cultured by 1×10^{-9} M insulin. (A2) CAV-1 RNA interference (RNAi) ECV304 cultured by 1×10^{-9} M insulin. (B1) ECV304 cultured by 1×10^{-8} M insulin. (B2) CAV-1 RNAi ECV304 cultured by 1×10^{-8} M insulin. (C1) ECV304 cultured by 1×10^{-7} M insulin. (C2) CAV-1 RNAi ECV304 cultured by 1×10^{-7} M insulin.

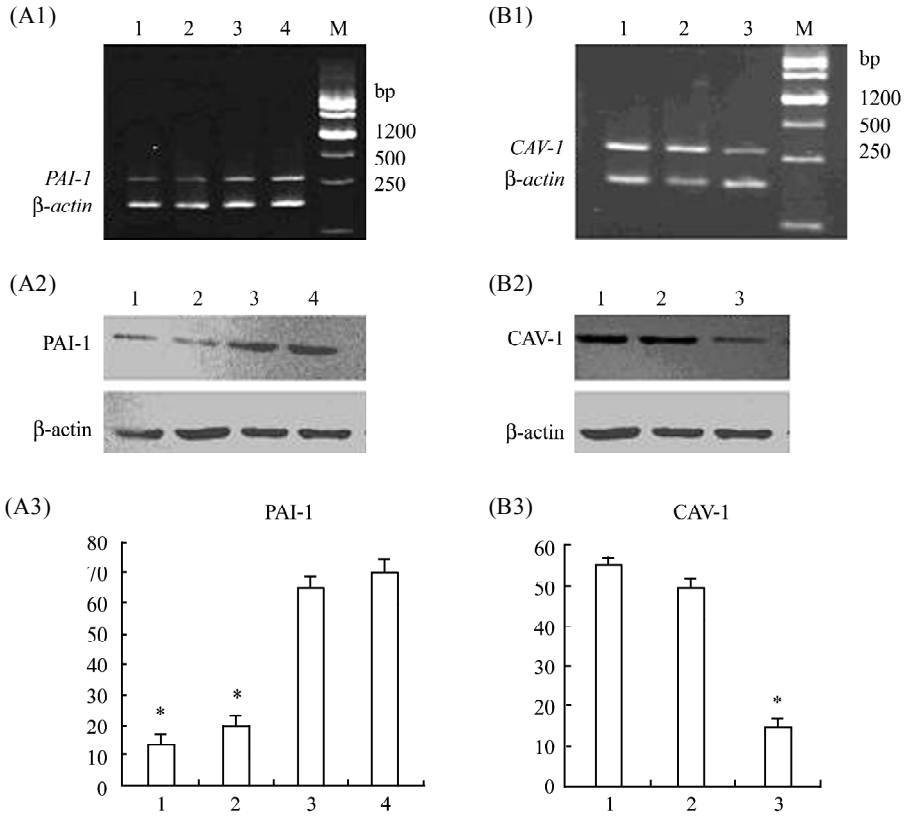

Fig. 7 Plasminogen activator inhibitor (PAI)-1 and caveolin (CAV)-1 expression stimulated by insulin

The changes in the PAI-1 (A1–A3) and CAV-1 (B1–B3) expression under different doses of insulin stimulation were determined by reverse transcription-polymerase chain reaction and Western blotting. (A1–A3) PAI-1 expression. 1, 1×10^{-9} M insulin; 2, RNA interference (RNAi) ECV304 cultured by 1×10^{-9} M; 3,1×10^{-7} M insulin; 4, RNAi ECV304 cultured by 1×10^{-9} M. (B1–B3) CAV-1 expression. 1, ECV304 control; 2, 1×10^{-9} M insulin; 3, 1×10^{-7} M insulin.

(B1), lane 3]. There were no statistical differences in the expression of β-actin between each group.

Discussion

RNAi is a technique that uses small interference RNA containing more than 20 nucleotides to induce post-transcriptional gene silencing, taking the place of traditional antisense nucleic acids. shRNA can degrade associated mRNA by homologous complementary sequences, and specifically inhibit the expression of target genes. The pENTR/U6 gateway vector can effectively clone the renaturated double-stranded shRNA into the expression cassette with RNA Pol III polymerase. The lentiviral RNAi expression vector constructed by the Gateway technique was contained in the BLOCK-iT lentiviral RNAi expression system. Not only can a large amount of high titer virus be packaged by host cell 293FT and more than 90% transfection efficiency in the dividing and non-dividing target cells or animals be obtained, but also the liposome-mediated lentiviral shRNA expression vector can be transfected into cells and the long-term stable gene knockout effect can be obtained through blasticidin screening culture [13]. Ambion software was used to analyze the CAV-1 gene coding region, ruling out those homologous to other gene sequences, then 30–50 bp series whose initiated G base matched to U6 shRNA transcription promoter, with a G/C ratio of approximately 50%, were selected. We added a CACC/AAAA, CGAA hairpin loop sequence and an antisense hairpin loop sequence required for cloning, and the RNAi lentiviral expression system to the CAV-1 gene was successfully constructed. mRNA and protein of CAV-1 gene silencing efficiency, detected by real-time fluorescence quantitative RT-PCR, immunofluorescence, and Western blotting, were more than 85%. There was no effect on the expression of other genes such as S100A13 and β-actin, which is essential for further study on CAV-1 function.

High PAI-1 expression is the key indication of EC dysfunction [19]. PAI-1 in blood mainly originates from ECs, adipocytes and hepatic cells, and is an important factor that regulates fibrinolytic activity. The increase in PAI-1 activity inhibits the degradation of fibrin in vascular matrix ,, thickens the basement membrane of vessels, and accelerates artherosclerosis, thrombosis, and the complications of aorta and capillaries in diabetes. It suggested that insulin might stimulate a high expression of PAI-1 with no clear explanation. Banfi *et al*. found that insulin induced more PAI-1 transcription by combining with InsR [20]. Parpal *et al.* found that the energy metabolism in insulin regulation was affected when methyl-β-cyclodextrin broke down the adipocytes caveolae, but had no impact on the insulin-induced mitogen signal molecule EPK 1/2 [21]. It was reported that the PAI-1 expression was clearly increased when they destroyed the IR adipocytes caveolae with methyl-β-cyclodextrin [22], which indicated the possible distinct function of CAV-1 in insulin signal transduction.

Our results (**Fig. 7**) showed that PAI-1 mRNA and protein expression were significantly enhanced by incubating a high concentration of insulin, but the CAV-1 expression down-regulated significantly after 24 h. RNA-mediated CAV-1 knockdown can enhance the PAI-1 expression, suggesting that CAV-1 plays a negative regulation role in the insulin-induced PAI-1 expression process. Although the PAI-1 expression of endothelial cells under physiological dose insulin incubation had no significant difference before or after the CAV-1 shRNA silencing, IIF showed the PAI-1 assembly located around the cell membrane, indicating that CAV-1 might only associate with the transport of PAI-1 protein under physiological conditions and does not stimulate expression of PAI-1. As shown by **Fig. 6(A1,A2)**, inhibition of CAV-1 expression might stimulate PAI-1 transport out of the cell to inhibit fibrinolytic activity. Ma *et al.* found many proteins localized around the nucleus and Golgi body after oxidized and diastatic LDL stimulated EC to express PAI-1, and when the Golgi body was destroyed, release of PAI-1 was suppressed completely [23]. Similarly, we observed that a high concentration of insulin stimulated PAI-1 focused around the nucleus and cytoplasm after CAV-1 RNAi. Whether this collection goes with the Golgi's body increased need further study. Our study indicated that CAV-1 can negatively regulate the expression and transposition of PAI-1 *in vitro*, when the concentration of insulin increased, CAV-1 expression was inhibited, followed by PAI overexpression. PAI-1 expression in high insulin status might be decreased by stabilizing CAV-1 on the membrane or overexpressing it. To stabilize and up-regulate CAV-1 expression could be vital in preventing diabetic complications of the aorta and capillaries.

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