

# Molecular interaction between caveolin-1 and ABCA1 on high-density lipoprotein-mediated cholesterol efflux in aortic endothelial cells

Yu-Chun Lin, Chang Ma, Wei-Ching Hsu, Hsiao-Fan Lo, Vivian C. Yang\*

*Department of Life Science, Tunghai University, Taichung, Taiwan, ROC*

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## Abstract

**Objective:** Caveolin-1 and ATP-binding cassette transporter A1 (ABCA1) are proteins that are involved in cellular cholesterol efflux. In this study, we analyzed the relationships between caveolin-1 and ABCA1 on high-density lipoprotein (HDL)-mediated cholesterol efflux in rat aortic endothelial cells.

**Methods and results:** Overexpression of caveolin-1 by transfection with caveolin-1 cDNA in aortic endothelial cells up-regulated ABCA1 expression and enhanced cholesterol efflux. Suppression of caveolin-1 by siRNA decreased ABCA1 expression and reduced cholesterol efflux. The number of caveolae increased after transfection with caveolin-1 into cells. Immunoprecipitation assays revealed a molecular interaction between caveolin-1 and ABCA1 in the plasma membrane and in the cytoplasm after HDL incubation. Immunoelectron microscopy demonstrated that caveolin-1 colocalized with ABCA1 in the caveolae and in the cytoplasmic vesicles; it was also found that caveolin-1 and ABCA1 colocalized with cellular cholesterol by immunofluorescence microscopy. Blocking of intracellular lipid transport by inhibitors disrupted the interaction between caveolin-1 and ABCA1 and reduced cholesterol to methyl- $\beta$ -cyclodextrin and HDL.

**Conclusions:** The molecular interaction between caveolin-1 and ABCA1 is associated with the HDL-mediated cholesterol efflux pathway in aortic endothelial cells.

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*Keywords:* Caveolin-1; ABCA1; HDL; Cholesterol efflux; Aortic endothelial cells

## 1. Introduction

Caveolae are free cholesterol-rich, invaginated microdomains (50–100 nm in diameter) at the surface of most peripheral cells. They have been implicated in many cellular activities [1]. Caveolin-1, the main structural protein of caveolae, is involved in the regulation of cellular cholesterol metabolism and lipid uptake, as well as efflux [2]. However, the effect of caveolin-1 expression on cholesterol efflux is still controversial [3–8]. For example, cholesterol efflux increased in L1210-JF cells and human fibroblasts after transfection with caveolin-1 [3,4]. On the contrary, the inhibition of caveolin-1

expression was shown to stimulate high-density lipoprotein (HDL)-mediated cholesterol efflux in NIH-3T3 fibroblasts [5]. Several other studies have indicated that there was no alteration of cholesterol efflux after transfection with caveolin-1 [6–8]. Aortic endothelial cells (ECs) represent one of the major cell types involved in atherogenesis, however, the formation of caveolae after overexpression of caveolin-1 and its effect on cholesterol efflux have not yet been investigated.

In addition to caveolin-1, it is well known that ATP-binding cassette transporter A1 (ABCA1) at the plasma membrane also functions in cellular lipid efflux in the presence of apolipoproteins [9]. Tangier disease, a rare genetic condition caused by loss-of-function mutations in the ABCA1 transporter that eliminate apoAI-stimulated cholesterol efflux [10]. Tangier disease patients have near-absence of circulating HDL [11]. Loss of ABCA1 activity specifically in macrophages may predispose them to foam cell formation [12]. The aortic

*Abbreviations:* ABCA1, ATP-binding cassette transporter A1; HDL, high-density lipoprotein; siRNA, small interfering RNA; ECs, endothelial cells.

\* Corresponding author. Tel.: +886 4 23590121x2481; fax: +886 4 23590296.

E-mail address: vcyang@thu.edu.tw (V.C. Yang).

endothelium interacts continuously with plasma proteins and is the most resistant to cholesterol accumulation among the cells that compose the atherosclerotic plaque. They do not undergo the foam cell phenotype changes seen in macrophages and smooth muscle cells in the plaque. We, therefore, speculate that ABCA1 might play a role in aortic ECs during the cellular cholesterol efflux.

The caveolin-1 and ABCA1 are expressed coordinately in differentiated THP-1 cells [13]. Our previous study provided evidence for an interaction between ABCA1 and caveolin-1 in the cell lysate of aortic ECs after HDL incubation [14]. It is appealing to assume that the interaction of caveolin-1 and ABCA1 may be important in the cholesterol efflux in aortic endothelium. In the present study, we aimed to further clarify the relationships between caveolin-1 and ABCA1 on HDL-mediated cholesterol efflux in aortic ECs.

## 2. Methods

### 2.1. Cell culture and HDL isolation

Sprague–Dawley rats 4 weeks old were sacrificed. Rings 1 mm thick were cut from the thoracic aorta, cultured at 37 °C in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum and penicillin–streptomycin (50 U/ml) under 5% CO<sub>2</sub>/95% air atmosphere. In order to establish pure endothelial cell cultures, ring explants were removed after 3–4 days of culture. Cultures exhibiting pure ECs, maintained for 2–7 passages, were used in the experiment. Prior to the experiment, subconfluent monolayers of endothelial cells were washed twice with PBS containing fatty acid-free albumin (FAFA, 2 mg/ml) and incubated with DMEM containing FAFA (2 mg/ml) and cholesterol (50 µg/ml) in ethanol (10 mg/ml) for 24–48 h at 37 °C. HDL isolation was performed according to the method by Chao et al. [15]. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

### 2.2. Transient transfection of plasmids and small interfering RNA (siRNA)

A full length rat caveolin-1 cDNA was subcloned into pcDNA 3.1 vector using *EcoRI* sites; the resulting plasmid was called pCav-cDNA. siRNA targeting to caveolin-1 (Dharmacon), siRNA targeting to ABCA1 (Qiagen) and rat scrambled-siRNA (Dharmacon) was used to modulate caveolin-1 and ABCA1 expression. Twenty-four hours before transfection,  $3 \times 10^4$  cells were seeded per 24-well plate. On the day of transfection, 1 µg of plasmid DNA or 50 nM siRNA was diluted in 25 µl of serum-free DMEM. In a separate tube, 1 µl of lipofectamine (Invitrogen) was diluted in 25 µl of serum-free DMEM. The diluted DNA and the lipofectamine were then gently mixed and incubated at 25 °C for 20 min. After the incubation, 250 µl of serum-free DMEM was added to the DNA/lipofectamine mixture. The

final mixture was added into cultured cells which were grown on 24-well plate. After incubation at 37 °C for 5 h, the cells were added to 250 µl of DMEM containing 20% serum and grown for an additional 24 h.

### 2.3. Reverse transcription PCR

Total RNA was isolated from ECs with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The cDNA was prepared with an oligo(dT) primer and Omniscript<sup>®</sup> Reverse Transcription kit (Qiagen) and amplified by polymerase chain reaction (PCR) with Taq DNA polymerase and standard protocol (Promega). Primers used in this experiment were as follows: rat caveolin-1 forward, 5' ATGTCTGGGGGGTAAATAC3' and reverse, 5'CTATA-TCTCTTCCTGCGTG3'; rat ABCA1 forward, 5'GGGTGGAGGACAGAATGACATC3' and reverse, 5'CCCAGTTTTTCGAATTGCC3'; rat  $\beta$ -actin forward, 5'ACACTGTGCCCATCTACGAG3', and reverse, 5'CGGA-ACCCTCATTGCCAAT3'. The amplified products were separated on a 1% agarose gel, stained with ethidium bromide, and photographed under ultraviolet illumination.

### 2.4. Immunoblot analysis

Cell lysate was harvested in lysis buffer (Tris–HCl, 10 mM, pH 7.4, containing 150 mM NaCl, 1 mM benzamidine, 0.5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride dissolved in dimethyl sulfoxide), homogenized, and centrifuged for 10 min at 800 g at 4 °C. The protein concentration of post-nuclear supernatants was measured with the BCA protein assay (Pierce). The protein was separated by 8% SDS-PAGE for ABCA1 and 12% SDS-PAGE for caveolin-1. After electrophoresis, proteins were transferred to a PVDF membrane (Millipore). Blots were probed with mouse anti-human ABCA1 (1:1000, v/v, Abcam), rabbit anti-human caveolin-1 (1:5000, v/v, Santa Cruz), and mouse anti- $\beta$ -actin (1:10,000, v/v, Sigma) primary antibody. The appropriate HRP-conjugated secondary IgG antibodies were visualized using enhanced chemiluminescence reagent (NEN). The intensity of reaction bands was analyzed by an Image Gauge system (Fuji).

### 2.5. Immunoprecipitation analysis

The cholesterol-loaded ECs were grown on a T25 flask and incubated with HDL medium (50 µg/ml) at 37 °C for 1 h. Cells were then placed on ice for 15 min and washed 3 times with PBS. Dithiobis (DSP, Sigma) was dissolved in dimethyl sulfoxide (DMSO) immediately before use, diluted to 250 µM with PBS, and added in the flask. Cells were then incubated with DSP solution at room temperature for 1 h. After incubation, the cells were washed twice with PBS and lysed with lysis buffer (1% NP-40, 0.25% deoxycholic acid and 15 mM imidazole) overnight at 4 °C. The immunoprecipitation was performed with a reversible immunoprecipitation system kit (Upstate). In brief, a 500 µl aliquot of diluted cell lysate

(500  $\mu\text{g}$ ) was incubated with 10  $\mu\text{l}$  (4  $\mu\text{g}$ ) rabbit anti-human caveolin-1 antibody (Santa Cruz), 10  $\mu\text{l}$  (4  $\mu\text{g}$ ) mouse anti-human ABCA1, 10  $\mu\text{l}$  (4  $\mu\text{g}$ ) non-immune rabbit IgG and 10  $\mu\text{l}$  (1  $\mu\text{g}$ ) antibody capture affinity ligand on a rocking platform at 4 °C overnight. The lysate and antibody mixture were transferred to an affinity spin column and centrifuged for 5–10 min at 1500  $g$ . After centrifugation, the final “flow through” samples were tested by western blot analysis as the controls. The spin column was washed with release lysis/wash buffer and IP elution buffer. The eluted proteins and the control samples were determined by immunoblot analysis.

## 2.6. Subcellular membrane fractionation

Subcellular membrane fractionation was performed as described by Wu et al. [16] with a minor modification. In

brief, the cholesterol-loaded cells grown on a T25 flask were harvested and lysed with cold extract solution (0.02 M boric acid, 0.3 mM EDTA, 1 mM PMSF, 1 mM benzamidine, pH 10) for 15 min on ice with vortexing every 5 min. The cell debris and nuclei were discarded after centrifugation at 650  $g$  for 10 min at 4 °C, and supernatant was centrifuged at 12,000  $g$  for 1 h at 4 °C. Subsequently, the harvested pellet was plasma membrane fraction, and the supernatant was concentrated by Nanosep® Centrifugal Devices (Pall) as cytoplasm fraction.

## 2.7. Immunofluorescence staining

Endothelial cells grown on a cover slip in a 24-well plate were incubated with cholesterol (50  $\mu\text{g}/\text{ml}$ ) for 24 h and treated with HDL (50  $\mu\text{g}/\text{ml}$ ) for 5 min at 37 °C. The cells were then chilled on ice, washed 3 times with cold PBS–albumin, and fixed in 2% paraformaldehyde for 25 min at room temperature. After fixation, the cells were blocked with PBS–albumin either in the presence or absence of filipin (0.05%). After washing with PBS, cover slip cultures were incubated with rabbit anti-human caveolin-1 antibody (1:200; Santa Cruz), mouse anti-human ABCA1 (1:50; Abcam) and Golgi marker, mouse anti-rat 58 k Golgi protein (1:50; Abcam), for 30 min at room temperature. Cover slips were then washed three times with PBS and incubated for 30 min with FITC-conjugated goat anti-rabbit IgG (1:200; Zymed), and TRITC-conjugated goat anti-mouse IgG (1:200; Zymed). Cover slips were washed 3 times with PBS, mounted on slides, and photographed with a Nikon E400 immunofluorescence microscope (Nikon).

## 2.8. Immunoelectron microscopy

The pcDNA and pCav-cDNA transfected cells were treated with cholesterol for 24 h. After washing in PBS–albumin three times, cells were incubated with HDL medium (80  $\mu\text{g}/\text{ml}$ ) at 37 °C for 5 min. After incubation, the cells were chilled on ice, washed 3 times with ice-cold PBS–albumin, and then prefixed with 4% paraformaldehyde in PBS for 30 min at 4 °C. After washing, the prefixed cells were incubated with rabbit anti-caveolin-1 primary antibody (1:40 in PBS–albumin; Santa Cruz) and mouse anti-ABCA1 primary antibody (1:5 in PBS–albumin; Abcam) for 1 h at room temperature. The cells were then washed 3 times with PBS–albumin and incubated with either goat anti-rabbit IgG-15 nm colloidal gold (1:40 in PBS–albumin; EMS), or goat anti-mouse IgG-10 nm colloidal gold (1:40 in PBS–albumin; EMS) for 1 h. After washing 3 times with PBS–albumin, the cells were post-fixed with 3 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, at 4 °C for 2 h. The cells were then washed 3 times with 0.1 M cacodylate buffer, incubated with 1% OsO<sub>4</sub> for 30 min at 4 °C, dehydrated in a graded ethanol series, and embedded in epon 812 (EMS). Thin sections (80 nm) were stained with uranyl acetate and lead citrate and viewed under a Hitachi H-600 transmission electron microscope (Hitachi). Quantitative data were obtained

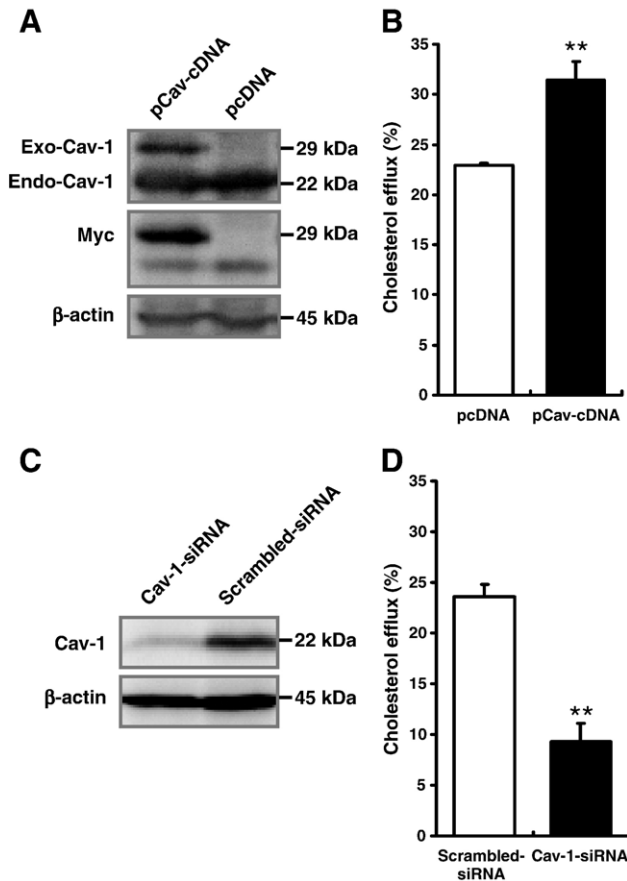


Fig. 1. Effect of caveolin-1 on cholesterol efflux in aortic ECs. ECs were transfected with pCav-cDNA, pcDNA, scrambled-siRNA, and caveolin-1-siRNA, respectively, in the presence of [<sup>3</sup>H]cholesterol (0.5  $\mu\text{Ci}/\text{ml}$ ) for 24 h. Cholesterol efflux was initiated by adding HDL (50  $\mu\text{g}/\text{ml}$ ) and allowed to proceed for 24 h. Immunoblots of protein expression (A) and cholesterol efflux (B) from pCav-cDNA transfected cells and pcDNA transfected cells. Immunoblots of protein expression (C) and cholesterol efflux (D) from scrambled-siRNA transfected cells and caveolin-1-siRNA transfected cells. The data of cholesterol efflux are from 3 independent experiments performed in triplicate. Values are expressed as mean  $\pm$  SD.  $n=3$ ; \*\* $p<0.01$  for test versus control (pCav-cDNA versus pcDNA in [B], caveolin-1-siRNA versus scrambled-siRNA in [D]).

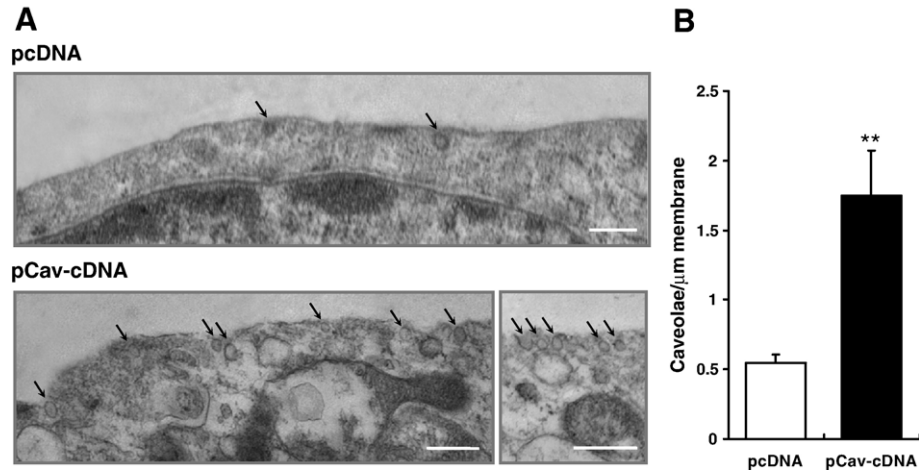


Fig. 2. Caveolin-1 expression induces caveolae formation. (A) Transmission electron micrographs of pcDNA cells and pCav-cDNA transfected cells. Plasma membrane invaginations with the typical bulb-shape and characteristic size are recognized as caveolae (indicated with arrows). Scale bar, 300 nm. Quantitative data indicated that the incidence of caveolae/ $\mu\text{m}$  significantly increased (\*\* $p < 0.01$ ) in the pCav-cDNA group as compared with pcDNA group.

from three of the six tissue blocks from each group. Fifteen to 25 sections were cut from each block, electron micrographs were taken from each of the five cells in a randomly selected section. The numbers of caveolae per  $\mu\text{m}$  were counted over the plasma membrane. Statistical analysis was performed where appropriate using Student's *t*-test.

### 2.9. Cholesterol efflux

ECs were grown in a 24-well plate for 24 h, followed by transfection with either plasmids or siRNA using lipofectamine for 5 h. The cells were then labeled with 0.5  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ] cholesterol for 24 h. In the inhibition experiment, ECs labeled with [ $^3\text{H}$ ]cholesterol (0.5  $\mu\text{Ci/ml}$ ) for 24 h, followed by incubation with progesterone (10 mg/ml), monensin (50  $\mu\text{M}$ ), and brefeldin A (50  $\mu\text{M}$ ), respectively, for 24 h. Before each efflux experiment, cells were washed with DMEM and then incubated with either HDL medium (50  $\mu\text{g/ml}$ ) for 24 h or methyl- $\beta$ -cyclodextrin (2 mM) for 30 min. After incubation, the medium was collected and the cells were solubilized in 0.5 N NaOH. The radioactivity of the medium and cell extract was measured by TOPcount machinery (PerkinElmer). The results represent radioactivity in the medium as a percentage of the total radioactivity (medium plus cell lysate).

### 2.10. Statistics

Data are shown as mean  $\pm$  SD. The 2 groups were compared by a 2-tailed *t*-test using SAS software.

## 3. Results

### 3.1. Caveolin-1 induces cholesterol efflux and caveolae formation in aortic endothelial cells

To evaluate the effect of caveolin-1 on cholesterol efflux in aortic ECs, the level of cholesterol efflux was measured in

up-regulated and down-regulated caveolin-1 cells. Both exogenous c-myc tagged caveolin-1 (29 kDa) and endogenous caveolin-1 (22 kDa) existed in pCav-cDNA transfected cells. The total level of caveolin-1 expression in the pCav-cDNA transfected cells increased by 44% compared with control cells (Fig. 1A). The cholesterol efflux from the pCav-cDNA transfected cells was 37% higher than that in control cells ( $p < 0.01$ , Fig. 1B). However, the expression of caveolin-1 protein decreased 90% in the siRNA targeting caveolin-1 transfected cells (Fig. 1C), and the cholesterol efflux was reduced by 61% ( $p < 0.01$ , Fig. 1D). These results indicated that caveolin-1 was a positive regulator of HDL-mediated cholesterol efflux in aortic ECs.

Several studies have demonstrated that caveolae are the major portal for HDL-mediated cholesterol efflux [1,17]. A possible mechanism by which caveolin-1 enhances cholesterol efflux may result from the formation of more caveolae. To address this, we observed an abundance of caveolae in the pCav-cDNA transfected cells and the pcDNA transfected cells by electron microscopy. Numerous caveolae were found in the pCav-cDNA transfected cells, but only a few caveolae were revealed in the control cells (Fig. 2A). Quantitative data indicated that the numbers of caveolae per unit membrane significantly increased in the pCav-cDNA group ( $1.74 \pm 0.32/\mu\text{m}$ ) compared with the pcDNA group ( $0.54 \pm 0.06/\mu\text{m}$ ) (Fig. 2B). Thus, overexpression of caveolin-1 seems to contribute to the formation of caveolae in cells.

### 3.2. Caveolin-1 positively regulates the ABCA1 expression

In view of the possible link between expression of caveolin-1 and ABCA1, we analyzed the expression of the ABCA1 gene in up-regulated and down-regulated caveolin-1 cells. Semi-quantitative RT-PCR analysis showed that the level of ABCA1 mRNA increased in the up-regulated caveolin-1 cells and decreased in the down-regulated caveolin-1 cells in a dose-

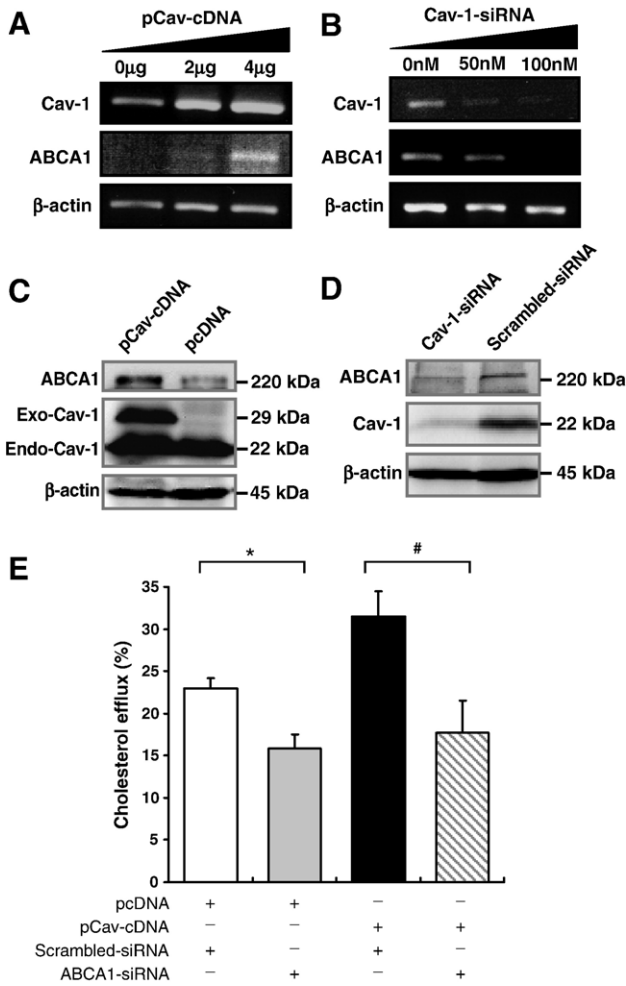


Fig. 3. Caveolin-1 positively regulates ABCA1 expression. ABCA1 is involved in caveolin-1-mediated cholesterol efflux. ECs were transfected with different concentrations of either pCav-cDNA or caveolin-1-siRNA in the presence of cholesterol (50  $\mu$ g/ml) for 24 h. Reverse transcription PCR (A and B) and immunoblotting (C and D) for caveolin-1, ABCA1, and  $\beta$ -actin in pCav-cDNA transfected cells and caveolin-1-siRNA transfected cells. (E) ECs were co-transfected with pcDNA and scrambled-siRNA (open bar), pcDNA and ABCA1-siRNA (gray bar), pCav-cDNA and scrambled-siRNA (solid bar), pCav-cDNA and ABCA1-siRNA (hatch bar), respectively, in the presence of [ $^3$ H]cholesterol (0.5  $\mu$ Ci/ml) for 24 h. Cholesterol efflux was initiated by adding HDL (50  $\mu$ g/ml) and allowed to proceed for 24 h. The data of cholesterol efflux are from 3 independent experiments performed in triplicate. Values are expressed as mean  $\pm$  SD. \* $p$ <0.05 for pcDNA+scrambled-siRNA versus pcDNA+ABCA1-siRNA, # $p$ <0.05 for pCav-cDNA+scrambled-siRNA versus pCav-cDNA+ABCA1-siRNA.

dependent manner (Fig. 3A and B). Immunoblotting analysis also showed that the protein level of ABCA1 increased in the up-regulated caveolin-1 cells and decreased in the down-regulated caveolin-1 cells (Fig. 3C and D). These data indicate that caveolin-1 positively regulates ABCA1 expression both at the RNA and protein levels.

We have demonstrated that caveolin-1 positively regulated the cholesterol efflux (Fig. 1A–D). This raises the possibility that caveolin-1 facilitates the cholesterol efflux by inducing ABCA1 expression. To address this, we further examined the effect of ABCA1 expression on the caveolin-1-mediated

cholesterol efflux pathway. The percentage of cholesterol efflux in pCav-cDNA transfected cells was increased by 40% compared with that in pcDNA transfected cells. This increased cholesterol efflux was also markedly reduced by ABCA1-siRNA treatment ( $p$ <0.05, Fig. 3E). Both in pCav-cDNA transfected cells and pcDNA transfected cells, the cholesterol efflux reduced to similar levels after ABCA1-siRNA treatment ( $p$ <0.05, Fig. 3E), indicating that suppression of ABCA1 expression inhibits the caveolin-1-mediated cholesterol efflux. We conclude that ABCA1 is involved in the caveolin-1-mediated cholesterol efflux pathway.

### 3.3. Caveolin-1 interacts with ABCA1 in plasma membrane and in cytoplasm

Immunoprecipitation analysis indicated that caveolin-1 have protein–protein interaction with ABCA1 in ECs (Fig. 4A). The plasma membrane and cytoplasm were fractionated as described in Methods. Each fraction was analyzed by immunoblotting with the plasma membrane marker, integrin  $\beta$ 1, and the cytoplasmic markers, GAPDH and tubulin. ABCA1 and caveolin-1 were found in both fractions. The fraction-specific immunoprecipitation analysis showed that ABCA1 was co-immunoprecipitated with

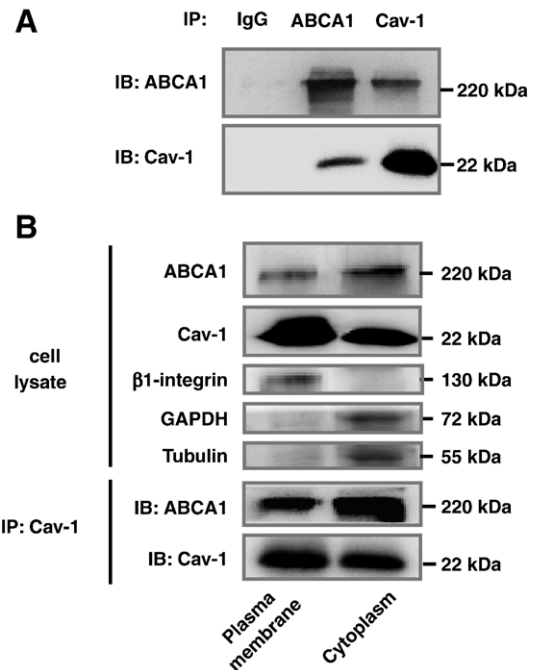


Fig. 4. The molecular interaction between caveolin-1 and ABCA1 occurs in plasma membrane and cytoplasm. ECs were incubated with cholesterol (50  $\mu$ g/ml) for 48 h followed by treatment with HDL (50  $\mu$ g/ml) for 1 h. Cells were washed 3 times with PBS and exposed to DSP cross-linker for 1 h. (A) Total cell lysates were immunoprecipitated with ABCA1 antibody, caveolin-1 antibody and non-immune rabbit IgG, respectively, and were subjected to immunoblot analysis. (B) ECs were fractionated as described in Methods. Fractionated cellular protein was examined by immunoblot analysis. Fractionated cellular protein was also immunoprecipitated with caveolin-1 antibody and was subjected to immunoblot analysis.

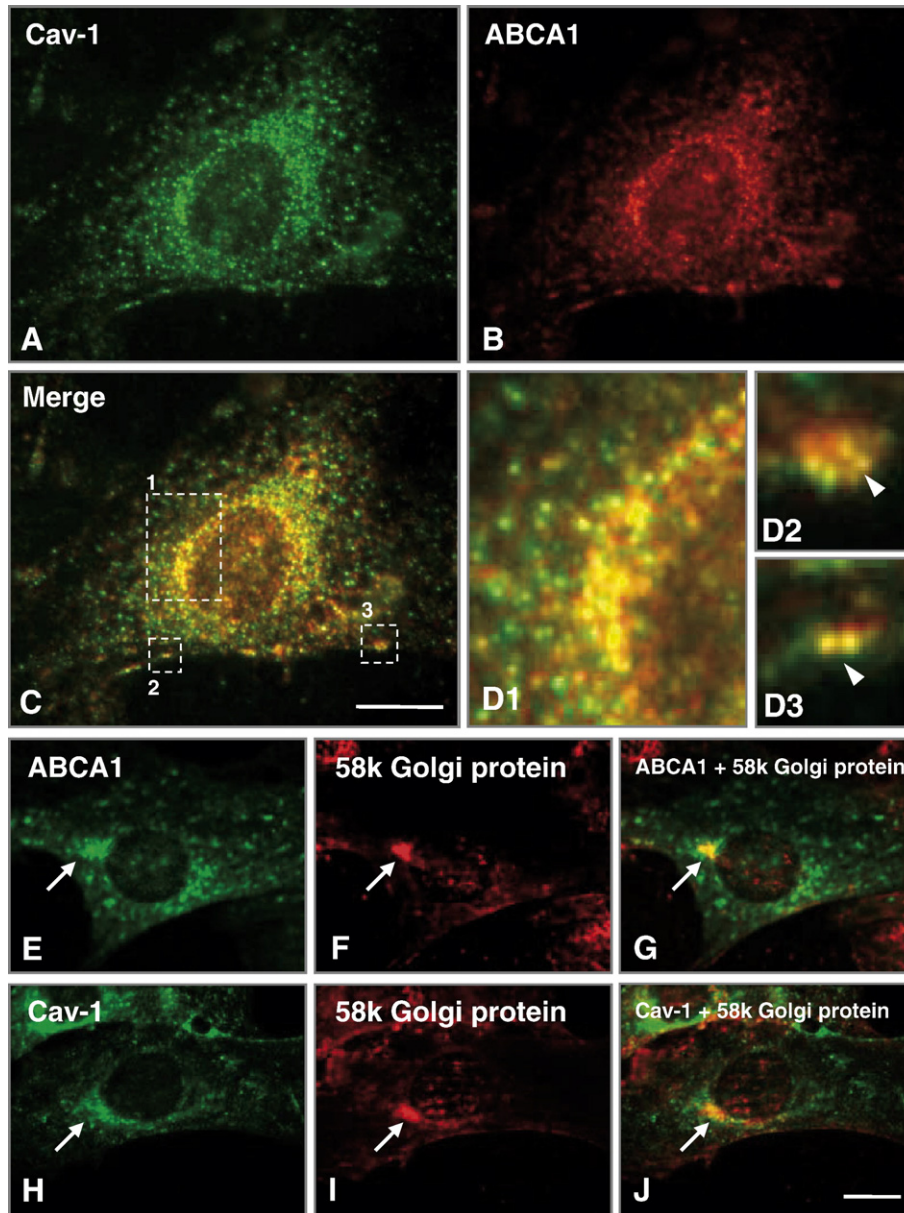


Fig. 5. Immunofluorescence micrographs show the distribution of caveolin-1 (green fluorescence) (A) and ABCA1 (red fluorescence) (B). The caveolin-1 colocalized with ABCA1 (C). Enlarged images of (C) showed the colocalization of caveolin-1 and ABCA1 in the perinuclear region (D1) as well as on the cell surface (D2 and D3). Caveolin-1 (green fluorescence) and ABCA1 (green fluorescence) colocalized with Golgi marker (red fluorescence) in the perinuclear region (E)–(J). Scale bar, 10  $\mu\text{m}$ .

caveolin-1 in the plasma membrane and in the cytoplasm (Fig. 4B). Immunofluorescence microscopy showed that the caveolin-1 intimately colocalized with ABCA1 in the perinuclear region as well as on the cell surface (Fig. 5A–D). The caveolin-1 and ABCA1 colocalized with Golgi marker, 58 k Golgi protein, in the perinuclear region (Fig. 5E–J).

#### 3.4. Colocalization of caveolin-1, ABCA1, and cholesterol in caveolae and cytoplasmic vesicles

ABCA1 was cloned in 1994 [18], and since then the cellular localization of ABCA1 has not been identified at the

ultrastructural level. In the current study, we found that the ABCA1 colloidal gold particles were located predominately in the caveolae structure (Fig. 6A and B) and were occasionally located in the non-caveolae region and in the cytoplasm (data not shown) after HDL incubation. Caveolin-1 colloidal gold particles (15 nm in diameter) colocalized with ABCA1 colloidal gold particles (10 nm in diameter) in the caveolae structure (Fig. 6C and D) and in cytoplasmic vesicles (Fig. 6E and F). We also analyzed the distribution of cellular cholesterol, caveolin-1 and ABCA1 in cholesterol-loaded cells after HDL incubation by immunofluorescence microscopy. We found that caveolin-1 colocalized with ABCA1 in

the perinuclear region and on the cell surface (Fig. 7A–C). The cellular cholesterol, revealed by cholesterol-specific cytochemical staining with filipin [19] (Fig. 7D), was intimately colocalized with caveolin-1 and ABCA1 (Fig. 7E and F).

### 3.5. Caveolin-1 and ABCA1 are intimately involved in cholesterol efflux via vesicular transport

It has been reported that caveolin-1-mediated lipid transport from Golgi to the plasma membrane is defective in the patients with Tangier disease and in *Abca1*<sup>-/-</sup> mice [20]. The progesterone, brefeldin A, and monensin were used to examine the effects of these inhibitors on cholesterol efflux [21,22]. Methyl- $\beta$ -cyclodextrin, which strips free cholesterol from the plasma membrane, was used as an indicator of plasma membrane content [23]. The results showed that all three intracellular lipid transport inhibitors

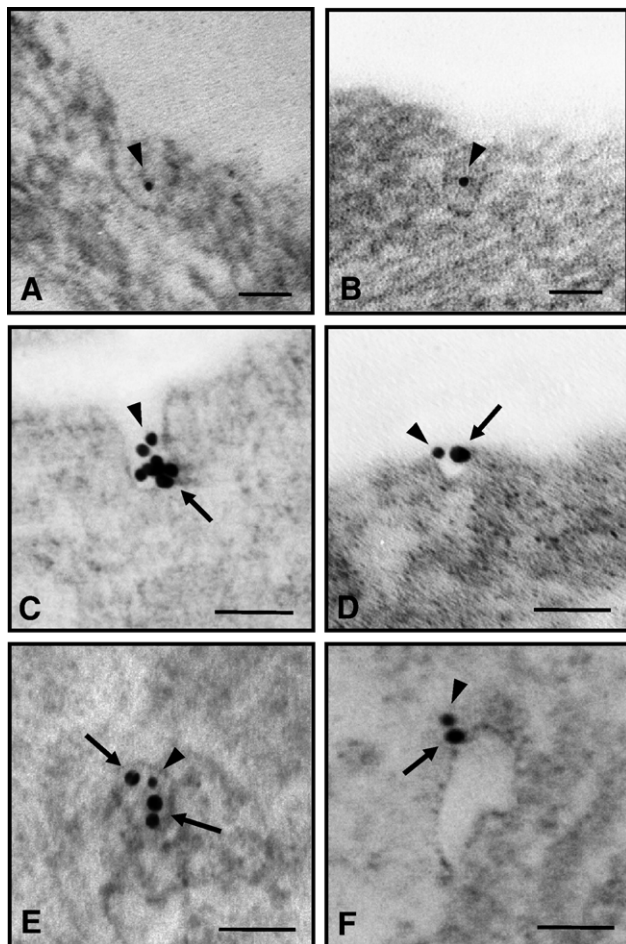


Fig. 6. Caveolin-1 colocalizes with ABCA1 in caveolae and cytoplasmic vesicles. ECs were incubated with cholesterol (50  $\mu$ g/ml) for 24 h, and followed by treatment with HDL (50  $\mu$ g/ml) for 5 min at 37  $^{\circ}$ C. Cells were then processed as described in Methods. (A and B) ABCA1-gold conjugates present in the caveolae (indicated as arrowheads). The ABCA1-gold conjugates (indicated as arrowheads) and caveolin-1-gold conjugates (indicated as arrows) are seen in caveolae (C and D), and in cytoplasmic vesicles (E and F). Scale bars, 100 nm.

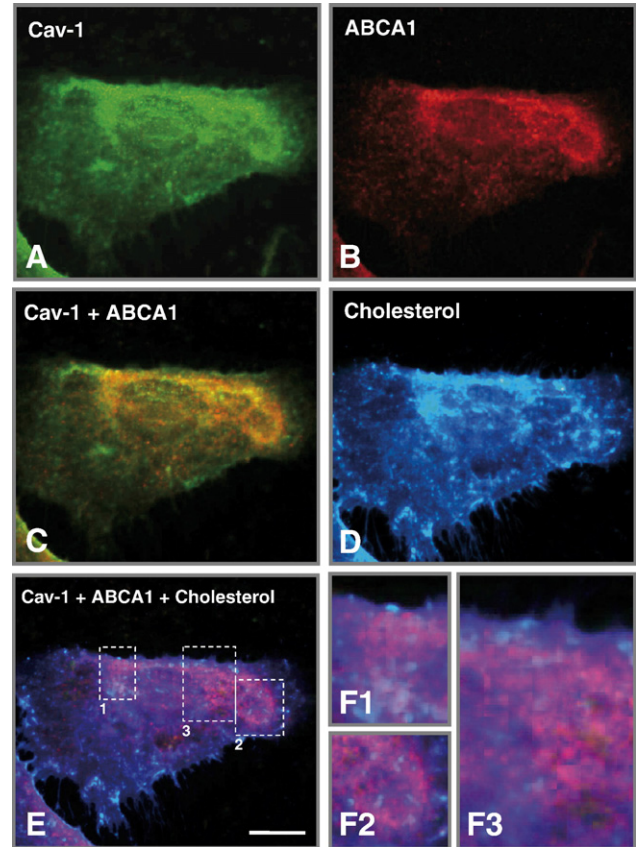


Fig. 7. Caveolin-1 colocalizes with ABCA1 and cholesterol in aortic ECs. ECs were pretreated with cholesterol (50  $\mu$ g/ml) for 48 h, and followed by incubation with HDL (50  $\mu$ g/ml) for 5 min at 37  $^{\circ}$ C. (A)–(E) The fluorescent images of the ECs stained with caveolin-1, ABCA1 and cholesterol. The green fluorescence, red fluorescence, and blue fluorescence indicate the location of caveolin-1, ABCA1, and cholesterol, respectively. (F1)–(F3) Enlarge merged image of (E) showed the colocalization of caveolin-1, ABCA1 and cholesterol. Scale bar, 10  $\mu$ m.

abolished the molecular interaction between caveolin-1 and ABCA1 (Fig. 8A) and reduced the level of cholesterol transport to the plasma membrane (Fig. 8B) as well as efflux of cholesterol from membrane to HDL (Fig. 8C). These results suggested that the interaction between caveolin-1 and ABCA1 modulates cholesterol efflux via vesicular transport.

## 4. Discussion

Many laboratories have shown that the role caveolin-1 plays in the cholesterol efflux pathway in various cell types is equivocal [3–8]. A possible reason for the various interpretations might be related to the varying degree of caveolae formation after transfection with caveolin-1 in different types of cells [21]. In this study, we revealed that the number of caveolae in aortic endothelial cells dramatically increased after overexpression of caveolin-1. Concomitantly, cholesterol efflux also increased. We suggest that caveolin-1 facilitates cholesterol efflux by inducing caveolae formation in aortic endothelial cells.

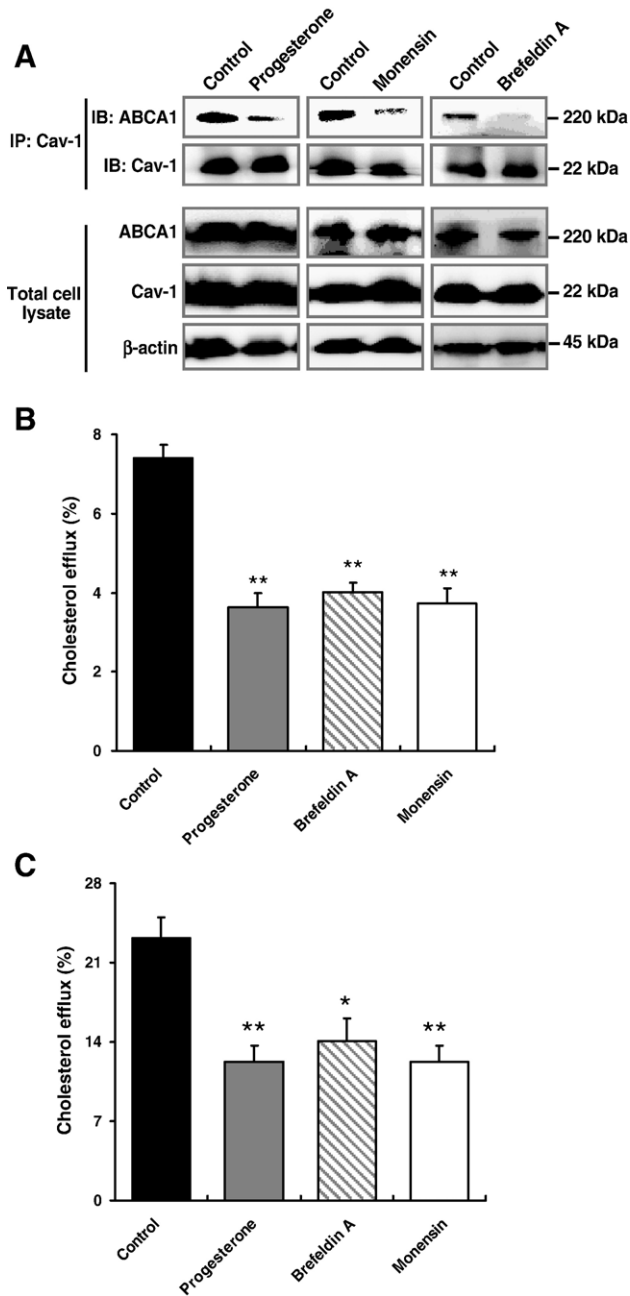


Fig. 8. Vesicular transport inhibitors disrupt the interaction between caveolin-1 and ABCA1 and reduce the cholesterol efflux to methyl- $\beta$ -cyclodextrin and HDL. (A) ECs were pretreated with cholesterol (50  $\mu$ g/ml) and incubated with progesterone (10 mg/ml), monensin (50  $\mu$ M), and brefeldin A (50  $\mu$ M), respectively. The cells were incubated with HDL (50  $\mu$ g/ml) at 37  $^{\circ}$ C for 1 h. The total cell lysates and the protein immunoprecipitated with caveolin-1 antibody were examined by immunoblotting analysis. ECs were labeled with [ $^3$ H]cholesterol (0.5  $\mu$ Ci/ml) and incubated with inhibitors, respectively. Cholesterol efflux was initiated by adding methyl- $\beta$ -cyclodextrin (2 mM) (B) or HDL (50  $\mu$ g/ml) (C) and was allowed to proceed at 37  $^{\circ}$ C for 30 min or 24 h, respectively. The data of cholesterol efflux are from 3 independent experiments performed in triplicate. Values are expressed as mean  $\pm$  SD. \* $p$  < 0.05, \*\* $p$  < 0.01 for test versus control.

Multiple mechanisms for efflux of cellular cholesterol have been reported [24]. Accelerated efflux of cholesterol is mediated by several prominent proteins, such as caveolin-1,

ABCA1, ABCG1, SR-BI, and CYP27A1 [25–28]. In this study, we found a molecular interaction between caveolin-1 and ABCA1 in the plasma membrane and cytoplasm. Caveolin-1 also colocalized with ABCA1 and cholesterol in the plasma caveolae and cytoplasm. We suggested that cholesterol is co-transported with caveolin-1 and ABCA1 in the endothelial cells after HDL incubation.

Several studies indicated that newly synthesized free cholesterol in the ER is transported to caveolae in a single step via the cytosolic complex of caveolin-1, HSP56, cyclophyllins and free cholesterol [3,29]. Another study has indicated that intracellular free cholesterol is transported through the trans-Golgi network (TGN) to caveolae via the vesicular complex of caveolin-1-VIP 21 [30]. We observed that the caveolin-1 and ABCA1 colocalized with cellular cholesterol in the Golgi apparatus, cytoplasmic vesicles, and plasma membrane caveolae. It raised the possibility that the caveolin-1 and ABCA1 transport the free cholesterol from the TGN to plasma membrane caveolae. Monensin, an inhibitor of vesicular transport, has been used as one criterion for verifying passage of molecules through the Golgi apparatus [31]. Brefeldin A disassembles the Golgi apparatus by targeting the cis- and medial Golgi cisternae [32] and blocks anterograde transport of cholesterol to an acceptor particle. Indeed, Mendez has reported that monensin and brefeldin A inhibited HDL-mediated cholesterol efflux [33]. In our study, we demonstrated that monensin and brefeldin A disrupted the interaction between caveolin-1 and ABCA1, and reduced the HDL-mediated cholesterol efflux. Taken together, we assume that the interaction between caveolin-1 and ABCA1 plays an important role in the lipids transport between the Golgi apparatus and the plasma membrane caveolae.

It has been reported that cellular lipid transport from the Golgi to the plasma membrane is defective, and that caveolin-1 is retained in the Golgi complex in the patients with Tangier disease and in *Abca1*<sup>-/-</sup> mice [20]. It raises the possibility that wild-type ABCA1 functions as an accessory protein in caveolin-1-mediated lipid transport from Golgi to plasma membrane. Whether ABCA1 functions as an accessory protein in the aortic ECs during cholesterol transport from Golgi to plasma membrane is an interesting issue and should be investigated in the future.

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