**Vimentin-mediated signalling is required for IbeA+ *E. coli* K1 invasion of human brain microvascular endothelial cells**

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**INTRODUCTION**

NSM (neonatal bacterial sepsis and meningitis) continues to be the most common serious infection in newborns, with high morbidity and mortality rates [1]. The high morbidity and mortality rates are due to insufficient knowledge of the pathogenesis of NSM. *Escherichia coli* is the most common Gram-negative bacterial pathogen that causes NSM [1]. Premature infants, immunocompromised hosts and children with underlying severe gastrointestinal diseases are especially prone to *E. coli* sepsis and meningitis [1]. Several *E. coli* virulence factors, including Ibe proteins (IbeA, IbeB, IbeC, IbeR and IbeT), AslA, FimH, TraJ and OmpA, have been identified and characterized. Most of these invasion genes are present in the genomes of *E. coli* K1 and K12 strains [2–4]. Among these virulence factors, the GimA genetic island, which encodes IbeA, IbeR and IbeT, is unique to *E. coli* pathogens, but is not present in non-pathogenic *E. coli* K12 strains. We and others have shown recently that IbeA as an outer membrane protein is important for *E. coli* K1 invasion of HBMECs [human BMECs (human brain microvascular endothelial cells)] in vitro as well as in the murine models of NSM [4,5]. Recent studies have shown that the *ibeA* gene was significantly more prevalent in the *E. coli* strains causing early infections of human NSM and avian colibacillosis [6,7].

Abbreviations used: ASFV, African swine fever virus; BBB, blood–brain barrier; BMEC, brain microvascular endothelial cell; CaMKII, Ca2+/calmodulin-dependent protein kinase II; CAV1-KD, caveolin-1 small interfering RNA; CtxB, cholera toxin B; Cy5, indodicarbocyanine; DIC, differential interference contrast; EMT, epithelial–mesenchymal transition; ERK, extracellular-signal-regulated kinase; GFP, green fluorescent protein; GST, glutathione transferase; HBMEC, human BMEC; HMP, HBMEC membrane protein; IF, intermediate filament; Ni-NTA, Ni 2+ nitrilotriacetate; NSM, neonatal bacterial sepsis and meningitis; PSF, PTB (polypyrimidine-tract-binding protein)-associated splicing factor; RFP, red fluorescent protein; siRNA, small interfering RNA; VDM, vimentin head domain deletion mutant; VHD, vimentin head domain; VIM, full-length vimentin; WFA, withaferin A; YGF, yellow/green fluorescent.

Key words: caveola, caveolin-1, IbeA, lipid raft, vimentin.

A fundamental issue in the pathogenesis of NSM is how specific surface structures on both microbial pathogens and host cells contribute to the tissue tropism of this disease and pathogen crossing the BBB (blood–brain barrier) that is sealed together by the tight junctions of BMECs. The specific IbeA–BMEC surface protein interaction and subsequently induced signal transduction were shown to be essential for *E. coli* K1 invasion [8,9]. Two IbeA-binding proteins have been identified: vimentin, which is constitutively present in the surface of HBMECs, and PSF [PTB (polypyrimidine-tract-binding protein)-associated splicing factor], which is inducibly expressed in both mesenchymal (endothelium) and non-mesenchymal (epithelium) cells [10,11]. The binding sites of the IbeA–vimentin interaction are located in the middle region of IbeA (271–370 residues) and VHD (vimentin head domain) [10]. However, it is still unclear how vimentin contributes to IbeA-mediated *E. coli* K1 invasion of HBMECs.

Vimentin is the major IF (intermediate filament) protein of mesenchymal cells such as endothelium [10]. Various novel functions of vimentin in cell adhesion, migration and signalling have received considerable attention [12]. The presence or absence of vimentin markedly affects the organization and expression of surface molecules critical for adhesion and migration [12]. Several studies have shown that vimentin is involved with bacterial and viral pathogenesis. *Salmonella* virulence protein

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SptP directly interacts with vimentin, which is recruited to the membrane ruffles [13]. The toxin protein from Pasteurella multocida is able to bind VHD [14]. Cowpea mosaic virus, a plant comovirus, is able to target vascular endothelial cells via surface vimentin [15]. ASFV (African swine fever virus) infection leads to rearrangement of vimentin into a cage surrounding viral factories. The cage structure is involved with phosphorylation of the N-terminal domain of vimentin at Ser^{12} by CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II) to facilitate disassembly and transport of vimentin on microtubules [16]. It has been shown that an accumulation of phosphorylated vimentin is associated with the cytoskeleton, which may be a consequence of the ERK (extracellular-signal-regulated kinase) activation [17]. Binding of vimentin to phospho-ERK can protect the activated kinase from dephosphorylation [18]. A number of microbial pathogens, including Mycobacterium leprae, Helicobacter pylori and Epstein–Barr virus, are able to hijack the ERK signalling pathway during infection of their hosts [19–21].

Vimentin has been identified as a raft protein present in lipid raft proteomes of endothelial cells [22]. Lipid rafts are highly ordered membrane subdomains enriched in not only cholesterol and sphingolipids, but also ganglosides (G_{M1} and G_{M0}) and glycosylphosphatidylinositol-anchored proteins [23,24]. Caveolin-1, a 22-kDa caveolar scaffolding protein, may concentrate signalling molecules in caveolae, allowing for their rapid activation by post-translational protein modification, such as by phosphorylation [25]. Caveolae/lipid rafts are being increasingly recognized as significant entry portals for the endocytosis of a wide variety of microbial pathogens and toxins [26–28]. For example, lipid rafts have been shown to contribute to the entry and survival of bacterial pathogens, including Shigella flexneri, Chlamydia trachomatis, uropathogenic E. coli, Mycobacterium kansasi, Salmonella and Listeria monocytogenes [29,30]. Recently, we have shown that the use of caveolae/lipid rafts as the entry portal is essential for meningitic Cryptococcus neoformans penetration across HBMECs [28]. E. coli K1 entry into HBMECs was shown to be clathrin-independent, but caveolae-dependent [31]. The E. coli K1-specific virulence factors, however, were not identified in those studies. Our previous studies have shown that vimentin is the primary receptor of IbeA, which is present in HBMECs, but not in epithelial cells [11]. In the present study, we have demonstrated that the contribution of CaMKII and ERK in vimentin-mediated signalling is required for IbeA+ E. coli K1 invasion of HBMECs via caveolin-1-enriched lipid rafts/caveolae.

**MATERIALS AND METHODS**

**Brain endothelial cells, E. coli strain and invasion**

HBMECs were isolated and cultured as described previously [9]. E44 is a rifampicin-resistant derivative of E. coli K1 strain RS218 (serotype 018:K1: H7) [1,8]. ZD1 is an ibeA in-frame-deletion mutant of E44 [8]. GFP (green fluorescent protein)-expressing E44 was made by transformation with pHC60 encoding the gfp gene that is constitutively expressed [32]. E. coli invasion was performed as described previously [1,9]. For blocking invasion assays, the proteins and rabbit anti-vimentin (H84) antibody (Santa Cruz Biotechnology) were dialysed overnight at 4°C, diluted to different concentrations and then incubated with the HBMEC monolayers for 60 min at 37°C before addition of bacteria. The inhibitors WFA (withaferin A) (ChromeDex), acrylamide (Sigma), KN93 (Alexis Biochemicals), P098059 (Calbiochem), filipin (Sigma), nystatin (Sigma) and the ERK peptides (synthesized by GenScript) were pre-incubated with the HBMEC monolayers for 60 min at 37°C before addition of bacteria. The two ERK peptides are ERK89 (vimentin-binding domain), RAPTIQMVDYIVQ, and ERK312 (control peptide), EQYYPSDEPIAAEP. All of the inhibitors, proteins, antibodies and peptides were present throughout the invasion experiment until the medium was replaced with experimental medium containing gentamicin.

**Protein expression and purification**

pET17A and pETFH for expression of His_{6}–IbeA and FimH proteins were transformed into E. coli BL21(DE3) cells. Purification of His_{6}–IbeA and FimH was performed under the denaturing condition (8 M urea) using Ni-NTA (Ni^{2+}-nitrilotriacetate)–agarose columns. The eluted protein was dialysed against decreasing concentrations of urea in the same buffer as described previously [8]. The construction of the plasmid for protein expression of GST (glutathione transferase)–VHD in pGEX-KG was described by Zou et al. [10]. The expression and purification of recombinant proteins in E. coli BL21(DE3) cells were carried out according to the manufacturer’s instructions (EMD Biosience). The soluble proteins GST–VHD and GST were purified, dialysed and used for invasion assays.

**Immunofluorescence and confocal microscopy studies**

HBMECs were grown in eight-well chamber slides coated with collagen and incubated for 2–3 days before infection with E. coli K1 or E. coli K1 carrying pH60 [32]. After bacterial infection for 1.5 h, the HBMEC monolayers were then washed with PBS and fixed with 4% (w/v) paraformaldehyde for 30 min at room temperature (25°C). After blocking with 5% (w/v) BSA in PBS, the cells were incubated with primary antibodies for each protein and then with fluorescently labelled secondary antibodies. The lipid raft marker G_{M1} was stained with CtxB (cholera toxin B)–FITC (Sigma). The other lipid raft marker G_{M0} was stained with a mouse monoclonal anti-G_{M0} IgM (Wako Chemicals) and rhodamine-conjugated anti-mouse IgM (Millipore). Samples were examined under a fluorescence microscope at the Congressan Dixon Cellular Imaging Core Facility, Children’s Hospital Los Angeles. The same parameters were used for all of the images to ensure the fluorescence strength of each treatment in a comparable manner. For confocal microscopy, samples were examined with a Leica TCS SP microscope. Images were acquired with Leica confocal software. Three lasers with wavelengths of 488, 561 and 633 nm were used. Images were acquired with three fluorescence photomultiplier tube detectors, and the DIC (differential interference contrast) image was taken with a transmitted light photomultiplier tube detector. Images were acquired with the same optimized parameters in each set of experiments. The microscope was equipped with a Marzhauser motorized XY stage with joystick interface and internal ‘Wide Z’ motorized focus drive. Z-stacks of images were taken for some cells in order to confirm whether the label was on the membrane or in an intracellular location. Orthogonal section function was used to show a side view of the Z-stack to demonstrate the subcellular location of labelling in cells.

**His_{6}–IbeA-coated bead association assay**

The bead-coating procedure was carried out as described previously [33]. To test the IbeA-mediated entry, IbeA- or BSA-coated latex beads were added to the cells at a ratio of 100 beads/cell, and incubated for 1.5 h. The cells were washed four times with PBS and fixed with 4% (w/v) paraformaldehyde for 30 min at room temperature. After washing with PBS and
blocking with 5% BSA at room temperature for 30 min, cells were then washed twice with PBS containing 0.1 M glycine. The cells were stained with anti-vimentin antibody (V9) conjugated to Cy5 (indodicarbocyanine) and for actin with rhodamine-phalloidin. The total number of beads associated with HBMECs (extracellular binding plus intracellular beads) was quantified by randomly selecting at least 10 fields under fluorescence microscopy. Results are expressed as the percentage of particles associated with HBMECs among the total number of particles.

Preparation of lipid rafts from HBMECs

The Caveolae/Rafts Isolation kit was purchased from Sigma–Aldrich. HBMECs were pre-incubated with or without filipin (1 μM) and triggered by medium (control), E44, ZD1 (5 × 10^5/plate) or His6–IbeA-coated latex beads (5 × 10^5/plate) for 2 h. After incubation, lipid rafts were prepared in a cold room as described previously [34]. The cells were lysed by passage through a 22 gauge needle 20 times. Lysates were centrifuged at 1000 g for 10 min. The resulting post-nuclear supernatant was transferred to a separate tube and adjusted to 35% of OptiPrep with 60% original stock, and transferred into SW40 centrifuge tubes and overlaid with 2 ml each of 30, 25 and 20% OptiPrep in lysis buffer and 1 ml of lysis buffer on the top. Gradients were fractionated into nine fractions (1 ml for each fraction) by centrifugation at 21 000 rev./min for 3 h using an SW-41 rotor. The fractionated proteins were detected by Western blotting with the following antibodies: a rabbit anti-caveolin-1 polyclonal antibody (BD Bioscience), and mouse monoclonal antibodies against vimentin (V9) (Santa Cruz Biotechnology) and PSF (Sigma).

Ligand overlay assay

The ligand overlay assay was carried out as described previously [10]. The purified His6–IbeA protein was resolved by SDS/PAGE (12.5% gels) and transferred on to PVDF membranes, and the membranes were blocked with 5% (w/v) non-fat dried skimmed milk powder in PBS. After washing four times with PBST (PBS containing 0.1% Tween 20) for 10 min, the membranes were incubated with total HBMEC cell lysate or lipid rafts (Fractions 2 and 3 from lipid raft preparation) in PBS overnight at 4°C, and the protein-binding bands were detected with the anti-vimentin (V9) and anti-caveolin-1 antibodies as described above.

IbeA pull-down assay

The His6–IbeA pull-down assay was carried out as described previously [10]. The pull-down complex was immunoblotted with mouse anti-His6 antibody, mouse anti-vimentin (V9) antibody and rabbit anti-caveolin-1 antibody. His6–FimH protein was used as a negative control as this protein binds to glycosylphatidylinositol that is linked to CD48 protein, but not to vimentin or caveolin-1 [35]. A control experiment was to incubate HMP (HBMEC membrane protein) with the Ni-NTA resin. The proteins eluted from the resin and crude HMP were used as negative and positive controls respectively.

Co-immunoprecipitation and Western blotting

HBMECs grown in 100-mm-diameter dishes were harvested, washed and lysed in 400 μl of lysis buffer containing 20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM 2-glycerophosphate, 1 μg/ml leupeptin, 1 mM PMSF, 100 mM okadaic acid and 1 mM Na3VO4. The lysate (200 μl) was mixed with primary antibodies and incubated with gentle rocking overnight at 4°C. Then, the mixture was added with Protein G– or Protein A–agarose beads and incubated with gentle rocking for 1–3 h at 4°C. After centrifugation five times at 8000 g for 30s and washing, the pellet was resuspended with 30 μl of 3 × SDS sample buffer. The samples were analysed by SDS/PAGE (12.5% gels) and immunoblotted with rabbit antibodies against caveolin-1 (BD Biosciences) and vimentin (Biomeda). For vimentin phosphorylation, the total lysate samples were immunoblotted with a mouse anti-phospho-vimentin (Ser82) antibody (MBL International) and a rabbit anti-vimentin (H84) antibody (Santa Cruz Biotechnology). For ERK activation, the total lysate samples were immunoblotted with rabbit antibodies against phosphorylated ERK1/2 (Thr202/Tyr204) and ERK1/2 (Cell Signaling Technology).

Construction and overexpression of VIM (full-length vimentin) and VDM (VHD deletion mutant) in the HBMECs transduced with lentivirus

The DNA for VIM and VDM were amplified from human vimentin cDNA (OriGene) and cloned into pEGFP-C1 (BD Biosciences) at the BglII and EcoRI sites in the C-terminus of GFP to make GFP–VIM and GFP–VDM fusions respectively. VIM and VDM cDNAs were amplified with two primer pairs: VIM5 (5′-TCCGGATCCATGTCCACCAGGTCCGTGCT-3′) and VIM3 (5′-AGTAATTCCTGACTGAGTGTGTCG-3′), and VDM5 (5′-AACCGATCTTCAAGAAACCCGACCT-3′) and VIM3. The fragment GFP–VIM, GFP–VDM and GFP (vector control), were double-cut with the NheI/Sall sites and cloned into pRLsinhCMV (lentiviral vector) at the XbaI/Sall sites respectively. These constructs were used to co-transfect HEK (human embryonic kidney)-293T cells together with two packaging vectors, pCMVDR8.91 and pCMV-VSVG, according to the calcium phosphate transfection protocol as described by Chen et al. [36]. After 48 h, conditioned medium was collected and filtered, and the lentivirus stock was stored at −70°C. The 60–80% confluent HBMECs were infected with the lentivirus, the GFP expression was examined under a fluorescence microscope at 48 h incubation after lentiviral infection. The transfected HBMECs with more than 90% expression of GFP were subjected to Western blotting, fluorescence microscopy examination and invasion assays.

Co-transduction/transfection

The GFP–VIM-transduced HBMECs with lentivirus were grown to 40% confluence in eight-well chamber slides and transfected with plasmid containing caveolin-1–RFP (red fluorescent protein) (Addgene) using FuGENE™ HD transfection regent (Roche). The cells were incubated for 48 h and examined by fluorescence microscopy.

Caveolin-1 knockdown experiments

Knockdown experiments were performed using the human caveolin-1 siRNA (small interfering RNA) kit from Santa Cruz Biotechnology according to the manufacturer’s instructions. siRNA duplex (60 pmol) was diluted with transfection medium and incubated with HBMECs. The cells were incubated for 18–24 h. After replacing with fresh medium and incubating for an additional 24–48 h, cells were assayed as described above.

Statistical analysis

All results given are means ± S.D. of triplicate determinations. The statistical analysis of the data from our in vitro studies involved ANOVA. Raw data were entered into Microsoft Excel files and were analysed using the statistical package. ANOVA and co-variates were followed by a multiple comparison test.
such as the Newmann–Keuls test, to determine the statistical significance between the control and treatment groups. $P < 0.05$ was considered to be significant.

RESULTS

Blockage of vimentin resulted in decreased IbeA+E. coli K1 invasion

In order to test the role of vimentin in E. coli K1 invasion of HBMECs, we first tested whether IbeA-mediated bacterial entry was inhibited by vimentin inhibitors WFA and acrylamide. The effect of WFA on vimentin cleavage was examined by Western blotting as described previously [37]. WFA was able to induce cleavage of vimentin (Figure 1A) and to block E. coli K1 invasion (Figure 1B) in a dose-dependent manner, suggesting that vimentin is required for IbeA+E. coli K1 invasion. Acrylamide, a known disrupter of vimentin IF [38], was used to examine whether the integrity of the vimentin network was important for IbeA+E. coli K1 invasion. The result showed that acrylamide was also able to dose-dependently inhibit E44 (IbeA+) invasion of HBMECs, indicating that the integrity of the vimentin IF network was required for IbeA+E. coli K1 entry (Figure 1C).

As our previous study has shown that IbeA could bind to VHD [10], we next tested whether VHD was able to block IbeA+E. coli K1 invasion. A recombinant GST–VHD fusion protein was constructed, expressed and purified. Two bands were detected in the purified GST–VHD sample owing to the easy cleavage of the GST-fusion protein (Figure 1D). As shown in Figure 1(E), GST–VHD was able to block IbeA+E. coli K1 invasion of HBMECs in a dose-dependent manner when compared with that of GST (control), suggesting that VHD is required for IbeA+E. coli K1 invasion. Next, a rabbit polyclonal antibody (H84) recognizing VHD was used for blockage of IbeA+E. coli K1 invasion. As shown in Figure 1(F), the H84 antibody was able to block E44 invasion in a dose-dependent manner, but was unable to alter the residual activity of theibeA mutant ZD1. This result confirms further the important role of VHD in IbeA-mediated E. coli K1 invasion of HBMECs.

IbeA+E. coli K1 invasion activity was enhanced and reduced respectively by overexpression of VIM and VDM in HBMECs

To study further the role of vimentin in E. coli K1 invasion, we generated two lentivirus constructs, GFP–VIM and GFP–VDM, with fusion of GFP to the N-terminus of the targeted protein VIM or VDM (Figure 2A). The transduced HBMECs with lentiviral constructs of GFP–VIM, GFP–VDM and GFP were subjected to GFP fluorescence analysis (Figure 2B), Western blotting (Figure 2C) and invasion assays (Figure 2D). As shown in Figure 2(B), the network structure in control cells. IbeA+E. coli K1 (E44) was able to induce reorganization and clustering of GFP–VIM. However, in the GFP–VDM-transduced cells without E44, the structure of GFP–VDM was disrupted by the head domain deletion, resulting in GFP–VDM distribution homogeneously in the cytoplasm without any network structure. E44 infection could not induce GFP–VDM reorganization or clustering owing to loss of the IbeA-binding domain. Compared with the GFP-transduced cells in which GFP was homogeneously distributed throughout the whole cell including the nucleus, GFP–VDM only existed outside

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Figure 2 Genetic effects of lentivirus-mediated overexpression of VIM and VDM on IbeA+E. coli K1 invasion of HBMECs

(A) Schematic diagram of GFP–VIM and GFP–VDM constructs. a.a., amino acids. (B) Immunofluorescence images of the transduced HBMECs with GFP–VIM, GFP–VDM or GFP were infected with or without E44 for 1.5 h. Scale bar, 25 μm. (C) Western blot of the transduced HBMECs using a mouse anti-vimentin (V9) antibody and a rabbit anti-GFP antibody. Lanes 1, GFP–VIM; lanes 2, GFP–VDM; lanes 3, GFP. Band a, GFP–VIM (82 kDa); band b, GFP–VDM (72 kDa); band c, VIM (55 kDa); band d, GFP (27 kDa). (D) The transduced HBMECs were tested for their ability to affect bacterial entry into cells by the invasion assay with E44 (grey bars) and ZD1 (white bars). Invasion assays were carried out as described in the Materials and methods section. **P < 0.01.

Vimentin phosphorylation (Ser^{82}) is involved with IbeA+E. coli K1 invasion

The IF organization is dynamically regulated by the phosphorylation state [16]. It has been reported that vimentin rearrangement during ASFV infection was regulated by the activation of CaMKII and phosphorylation of the N-terminal domain of vimentin on Ser^{82} [16]. Since IbeA+E. coli was able to induce the vimentin reorganization and clustering during the entry process, we next examined whether vimentin (Ser^{82}) phosphorylation was induced during the E44 invasion process. The results of the time course (0–90 min) study on phosphorylation of vimentin (Ser^{82}) showed a maximum level at 30 min after being challenged with E44, whereas no changes in phospho-vimentin (Ser^{82}) were detected when cells were challenged with ZD1 (results not shown). The level of phospho-vimentin (Ser^{82}) after 30 min of E. coli treatment indicated a significant increase in E44 treatment compared with ZD1 treatment or non-treatment control (Figure 3A), suggesting that IbeA was able to induce vimentin (Ser^{82}) phosphorylation during the E. coli K1 invasion. To ensure further whether the vimentin (Ser^{82}) phosphorylation was regulated by CaMKII in this process, we tested whether KN93, an inhibitor of CaMKII, was able to block vimentin phosphorylation and IbeA+E. coli K1 invasion. As shown in Figures 3(B) and 3(C), KN93 inhibited the vimentin (Ser^{82}) phosphorylation and invasion in a dose-dependent manner upon E44 infection, whereas slight changes occurred in ZD1 treatment, suggesting that IbeA-induced
vimentin (Ser82) phosphorylation was regulated by CaMKII in the process of E44 invasion.

**Activation of ERK1/2 is required for IbeA-induced signalling and E44 invasion**

In our previous study, IbeA has been shown to be an outer membrane protein, but not a secreted protein [4]. Since IbeA+*E. coli* was able to induce extensive vimentin reorganization (Figure 2B), we assumed that IbeA-induced vimentin clustering was involved with the ERK pathway. In the present study, we tested whether ERK activation was required for IbeA–vimentin-mediated *E. coli* K1 invasion. PD908059, an ERK inhibitor, was used to examine the inhibition of E44 invasion of HBMECs. Figure 4(A) shows that PD908059 could block E44 invasion in a dose-dependent manner as compared with ZD1 invasion. The level of phospho-ERK (activated ERK) was also detected by Western blotting upon E44 and ZD1 treatment. The result showed that phosphorylation of ERK increased in 5 min and reached a maximum level at 30 min during the time course from 0 to 90 min with E44 treatment, whereas ZD1 did not induce any changes of phospho-ERK during the time course (results not shown). Figure 4(B) shows that E44 significantly increased the level of phospho-ERK after 30 min of treatment as compared with ZD1 treatment or the control without treatment. PD098059 was able to almost completely eliminate ERK phosphorylation in all treatments at the concentration of 50 μM, demonstrating that ERK phosphorylation was involved with IbeA-mediated signalling. We next used synthesized ERK peptides ERK89 (vimentin-binding domain) and ERK312 (non-binding peptide) to examine whether E44 invasion and the activation of ERK could be blocked. As shown in Figure 4(C), ERK89 was able to block the E44 invasion, but not the residual invasion activity of ZD1, whereas the control peptide ERK312 had no effect on both strains. Phosphorylation of ERK was inhibited upon ERK89 treatment in all three settings as compared with ERK312 or the negative control (no peptide) (Figure 4D). These results suggest that the activation of ERK is critical for the IbeA–vimentin-induced signalling.

**IbeA is a major virulence factor for inducing vimentin clustering in HBMECs**

As vimentin has emerged as an organizer of a number of important signalling molecules [12], and our studies showed that E44 was able to induce molecular signalling leading to phosphorylation of vimentin and ERK activation, we next examined how IbeA was able to induce vimentin reorganization and clustering. Using GFP-tagged E44 and ZD1, we demonstrated that E44 (IbeA+) and IbeA protein were able to significantly induce vimentin clustering and reorganization in HBMECs compared with ZD1 (IbeA−) and the untreated control (Figures 5A and 5B). This finding suggested that IbeA played a major role in inducing vimentin reorganization during *E. coli* K1 entry into HBMECs. Vimentin clustering induced by E44 was next examined by confocal microscopy sectional scanning. When comparing the control cell without any treatment, vimentin strongly aggregated towards the perinuclear region and clustered in the apical side of the cell upon binding with E44 (Figure 5C). However, the vimentin clustering did not form a cage-like structure to wrap *E. coli*. It was very interesting that E44 and IbeA were able to induce extensive vimentin reorganization, including, but not limited to, the co-localized region with E44. Vimentin clustering was also common to neighbouring non-infected cells, suggesting a ‘bystander effect’, which can be induced by certain viruses [39]. The enhanced fluorescence intensity is due to the change in the structure of vimentin IF from monomer to polymerized IF which may be caused by clustering [40]. The cell membrane in the horizontal direction at the basal side was stained much more strongly than the control with the V9 antibody (indicated by arrowheads), indicating that more vimentin shifted to the cell membrane of E44-infected HBMECs, which was correlated with IbeA+*E. coli* K1 entry.

**Association of IbeA-coated beads and GFP-tagged IbeA+*E. coli* K1 with HBMECs was correlated with clustering of vimentin**

To determine whether IbeA alone was sufficient for bacterial entry that was correlated with clustering of vimentin, we tested whether it could stimulate adhesion and internalization of inert beads. YGF (yellow/green fluorescent) beads were covalently coupled to purified His6–IbeA, which was treated with polymyxin B–agarose to remove contaminated LPS (lipopolysaccharide). IbeA-coated beads were tested for efficiency of entry, using as control beads coupled to similar amounts of BSA. HBMEC-associated (internalized plus extracellular) beads were determined by quantification of immunofluorescence as described in the...
Figure 4  Blockage of ERK1/2 activation results in reduced IbeA+ E. coli K1 invasion

(A) E44 invasion of HBMECs was blocked by the ERK inhibitor PD098059 in a dose-dependent manner. (B) ERK1/2 phosphorylation (p-ERK1/2) after 30 min of stimulation with E44 or ZD1 (2 × 10⁷/ml) with or without the ERK1/2 inhibitor PD098059 (50 μM). (C) Inhibition of E44 invasion of HBMECs by ERK peptides. HBMECs were incubated with or without ERK89 (vimentin-binding domain) and ERK312 (control peptide, 25 μg/ml) for 1 h before adding E44 or ZD1. (D) ERK1/2 phosphorylation was inhibited by the ERK89 peptide. The two ERK peptides are ERK89 and ERK312. HBMECs were incubated with or without the ERK peptides for 1 h before infection with E44 or ZD1 (2 × 10⁷/ml) for 30 min. In (A) and (C), invasion assays were carried out as described in the Materials and methods section. **P < 0.01. In (B) and (D), ERK1/2 phosphorylation (p-ERK1/2) was detected using a rabbit anti-phospho-ERK1/2 (Thr 202/Tyr204) antibody, and the total ERK1/2 (T-ERK1/2) protein was detected with a rabbit anti-ERK antibody as the loading control. CON, control; WB, Western blot.

Figure 5  IbeA protein and IbeA+ E. coli K1 (E44) are capable of inducing vimentin clustering and reorganization in HBMECs

Cells were treated with GFP–E44, GFP–ZD1 or IbeA protein (5 μg/ml) or without any treatment (CON) for 1.5 h. (A) Vimentin was stained with the anti-vimentin antibody (V9) followed by a rhodamine-labelled secondary antibody and DAPI (4′,6-diamidino-2-phenylindole). Scale bar, 25 μm. (B) Mean levels of vimentin clustering. The percentages shown represent the number of cells with vimentin clustering (stained with FITC-labelled antibody) divided by the total cell number (counted with DAPI staining). The data were acquired from five randomly selected fields in each well for each treatment. **P < 0.01. (C) Series sections acquired by confocal microscopy from the apical side to the basal side of HBMECs showed vimentin distribution stained with anti-vimentin antibody (V9) conjugated to rhodamine with or without GFP–E44 treatment. Arrows indicate the horizontal cell-membrane staining. The thickness of each section was 2.0 μm. Scale bar, 25 μm.

Materials and methods section. Beads coated with BSA were rarely found to be associated with cells after washing several times with PBS, and few of the beads observed were found to be intracellular and to induce vimentin clustering. In marked contrast with the control, most of the IbeA-coated beads were found to be associated with cells and to induce extensive vimentin clustering (Figure 6A). The YGF beads coated with IbeA bind to clustered vimentin directly on the top of the cell. The number of...
beads binding to HBMECs and bead-induced vimentin clustering were counted. As shown in Figure 6(B), the rates of HBMEC association and vimentin clustering induced by IbeA-coated beads were significantly higher than those of BSA-coated beads. As a positive control, HBMECs were infected with GFP-tagged IbeA+ E. coli K1 (green) and stained for vimentin with the Cy5-conjugated anti-vimentin antibody (V9) (blue) and for actin with rhodamine-phalloidin (red). Figure 6(C) shows that the green bacteria were able to strongly induce vimentin reorganization and transcellularly co-localized with vimentin clustering on the apical side of HBMECs, whereas, in the untreated control cell or ZD1-induced cell, non-clustered vimentin with bacteria was present at or near the basal side. These results demonstrate that IbeA is sufficient to cause internalization of inert particles into HBMECs which is correlated with vimentin clustering, suggesting that vimentin is the primary IbeA receptor contributing to the E. coli K1 entry process.

Vimentin-enriched lipid rafts/caveolae in HBMECs are required for IbeA-mediated bacterial entry

Previous studies have shown that vimentin is a raft protein present in lipid raft proteomes of endothelial cells [22]. To determine further the role of vimentin in meningitic E. coli infection, we examined whether lipid rafts/caveolae are required for IbeA+ E. coli entry into HBMECs. The raft-disrupting agents filipin and nystatin were used to examine the role of lipid rafts in bacterial invasion. E44 invasion was blocked by these reagents in a dose-dependent manner as compared with ZD1 invasion (Figures 7A and 7B), suggesting that the integrity of the raft microdomains of HBMECs was required for IbeA+ E. coli invasion. We used the density gradient technique to fractionate membrane rafts in order to test whether vimentin and PSF were recruited to the raft microdomains triggered by IbeA or the pathogen, and whether lipid rafts in HBMECs were involved with...
IbeA-mediated signalling. After density gradient centrifugation, glycosphingolipid-rich membrane fractions representing the lipid rafts floated to the interphase between 0 and 20% OptiPrep layers, peaking at Fraction 2 in our studies. As a lipid raft marker protein, caveolin-1 was predominantly present in Fraction 2 (Figure 7C). In HBMECs without treatment (control), only a very small amount of vimentin was constitutively present in Fraction 2, whereas the majority of this protein was present in Fractions 7–9 as a major component of the cytoskeleton (Figure 7D). After triggering with E44 or IbeA-coated beads, more vimentin was recruited to the lipid rafts compared with the control. ZD1-induced distribution of vimentin into Fraction 2 was much less than those induced by E44 and IbeA-coated beads (Figure 7D). However, PSF was not present in the lipid rafts of HBMECs infected with ZD1 and the control without E44 infection. Upon stimulation with E44 or IbeA-coated beads, PSF was recruited to the raft microdomains (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/427/bj4270079add.htm). When the raft-disrupting agent filipin was pre-incubated with HBMECs infected with or without E44, no vimentin and PSF were detected in lipid rafts, whereas caveolin-1 was attenuated in lipid rafts (Figures 7C and 7D and Supplementary Figure S1). These findings suggest that vimentin is the primary receptor for IbeA and that lipid rafts in HBMECs serve as a platform for IbeA-induced signalling. To further demonstrate IbeA–vimentin binding in lipid rafts, E44 and ZD1 bacterial cells were collected and incubated with HBMECs lipid rafts overnight. After washing, the E. coli cells were sampled and subjected to Western blotting analysis with antibodies against vimentin and caveolin-1. Although the binding of vimentin to ZD1 was significantly less than the binding to E44, similar amounts of caveolin-1 were shown to bind to both E44 and ZD1, suggesting that, in contrast with vimentin, caveolin-1 did not show any specific binding of IbeA (Figure 7E). The IbeA-binding protein complex was purified from HBMEC membranes by His$_6$–IbeA pull-down and was then subjected to Western blot analysis with antibodies against His$_{6}$, vimentin and caveolin-1. To exclude the possibility of non-specific binding, His$_{6}$–FimH and Ni-NTA beads as negative controls were also tested following the same protocol. Using an anti-His$_{6}$ antibody, 50-kDa His$_{6}$–IbeA and 28-kDa His$_{6}$–FimH were detected in the pull-down complex. Although both vimentin and caveolin-1 could be detected in HBMEC membrane proteins, vimentin was detected only in His$_{6}$–IbeA complex, and no caveolin-1 was detected in any of the complexes, suggesting that vimentin binds to the IbeA protein specifically and caveolin-1 did not interact with the IbeA protein directly (Figure 7F). The overlay study also demonstrated that
IbeA was able to bind to vimentin in cell lysates and lipid rafts, but that there was no interaction between IbeA and caveolin-1 (Figure 7G). Taken together, these results indicate that IbeA was able to specifically bind to vimentin in lipid rafts/caveolae, but the binding of caveolin-1 to E. coli was IbeA-independent.

Co-localization of GM1 and GM3 with vimentin in E44-infected HBMECs

After our studies showed that caveolae/lipid rafts were required for E. coli K1 invasion of HBMECs, we next examined the co-localization of two lipid raft markers, GM1 and GM3 [24], with vimentin using confocal microscopy. First, the raft microdomains were visualized by GM1 staining using CTxB–FITC. A homogeneous distribution of CTxB–FITC fluorescence was observed in untreated HBMECs (control). No or few co-localizations were found between GM1 and vimentin from the top and side views (Supplementary Figure S2A at http://www.BiochemJ.org/bj/427/bj4270079add.htm). After treatment with E. coli K1 (E44) for 1.5 h, the signals of GM1 and vimentin became much stronger. In addition, a redistribution of GM1 ganglioside towards the cell margin and perinuclear region occurred, resulting in more GM1 being shifted to the apical side of the cell membrane and perinuclear caveolar region (Supplementary Figure S2A). The co-localization of vimentin with the other lipid raft marker GM3 was also examined. Similarly to the pattern of GM1, a homogeneous distribution of GM3 was observed in untreated HBMECs (control) (Supplementary Figure S2B). After treatment with E. coli K1 (E44), a redistribution of GM3 ganglioside towards the cell margins and perinuclear region occurred. A strong co-localization of GM3 with vimentin was observed in HBMECs infected with E44, whereas ZD1-treated HBMECs still shared the same pattern with the control (results not shown). Concurring with the lipid rafts fractionation study (Figure 7), more vimentin was co-localized with GM1 and GM3 upon IbeA+ E. coli treatment.

IbeA+ E44-induced vimentin surface recruitment is caveolin-1-dependent

Caveolin-1 is a scaffolding protein enriched in caveolae/lipid rafts [25]. A previous study showed that caveolin-1 plays an important role in vimentin-mediated signalling [41]. To define the relationship between vimentin and caveolin-1 in IbeA-induced signalling, the co-localization of these two proteins was examined. By co-expression of caveolin-1–RFP and vimentin–GFP in HBMECs, we were able to examine the co-localization of vimentin with caveolin-1 under confocal microscopy (Supplementary Figure S3A at http://www.BiochemJ.org/bj/427/bj4270079add.htm). In untreated cells, as well as HBMECs infected with GFP-tagged ZD1, caveolin-1, which showed a homogeneous distribution, was partially co-localized with non-clustered vimentin. A co-localization of caveolin-1–RFP with vimentin–GFP towards cell membranes, pericentrosomal caveosomes and perinuclear regions occurred upon treatment with GFP-tagged E44 or IbeA, suggesting that the co-localization of vimentin and caveolin-1 towards caveolae/caveosomes is required for IbeA-mediated bacterial entry. The patterns of vimentin and caveolin-1 co-localization were similar to the distribution patterns of vimentin and GM1 and vimentin and GM3. These results further demonstrated that caveolae/lipid rafts are the platform for IbeA–vimentin-induced signalling. The protein–protein interaction between caveolin-1 and vimentin was confirmed by immunoprecipitation assays using either antibody against these proteins (Supplementary Figure S3B). To define further the role of caveolin-1 in IbeA–vimentin-mediated invasion, caveolin-1 expression in HBMECs was knocked down by siRNA. After siRNA transfection, caveolin-1 expression was examined by Western blotting. The result showed that the protein expression in caveolin-1 siRNA (CAV1-KD)-transfected HBMECs was significantly reduced when compared with that of the control (Supplementary Figure S3C). The CAV1-KD effects were also examined by immunofluorescence staining (Supplementary Figure S3D). The caveolin-1 protein was homogeneously distributed in HBMECs transfected with the control siRNA (CAV+/CON) without GFP-tagged E44 treatment. A strong co-localization of clustered vimentin and caveolin-1 occurred in the control HBMECs upon IbeA+ E. coli K1 infection (CAV+/E44). Invasion assays were carried out to determine whether the caveolin-1 knockdown was correlated with decreased IbeA+ E. coli K1 entry into CAV1-KD HBMECs. As shown in Supplementary Figure S3E, caveolin-1 knockdown resulted in more than 50% inhibition of E44 invasion, whereas no blocking effect on ZD1-mediated invasion was observed. These results suggest that caveolin-1-enriched raft microdomains serve as a platform for IbeA–vimentin-induced signalling, which is required for E. coli K1 invasion of HBMECs.

DISCUSSION

IbeA is an important virulence factor that contributes to E. coli K1 invasion of both intestinal epithelial cells and BMECs in vitro and in vivo [8,42]. E. coli bacteraemia/sepsis is thought to arise from the gastrointestinal tract. Human and animal studies suggest that the development of sepsis and meningitis is correlated with the magnitude of bacteraemia, which is essential for E. coli crossing the BBB [1]. Our previous studies suggest that IbeA is important for translocation of E. coli K1 across the intestinal epithelial barrier and the BBB [8,42]. In our previous paper, vimentin was identified as an HBMEC surface protein that binds to IbeA through its head domain [10]. This protein, however, is not expressed in human intestinal epithelial cells, such as Caco-2, suggesting that different entry mechanisms may be responsible for E. coli K1 penetration across the gut barrier and the BBB. PSF may be a common co-receptor that contributes to IbeA+ E. coli K1 penetration across epithelial cells and HBMECs. In the present study, we have demonstrated further the important role of vimentin in IbeA-mediated E. coli K1 invasion of HBMECs with several lines of evidence. First, IbeA+ E. coli K1 invasion of HBMECs was specifically blocked by the inhibitors and the head domain peptide of vimentin. Secondly, IbeA-mediated E. coli K1 invasion was completely abolished by overexpression of GFP–VD/MD in HBMECs as compared with the entry capacity of the IbeA deletion mutant ZD1 in GFP-transduced HBMECs. Thirdly, phosphorylation of vimentin by CaMKII and activation of the ERK signalling pathway are required for IbeA+ E. coli invasion. Fourthly, IbeA and IbeA+ E. coli K1 were able to induce clustering of vimentin at the surface of HBMECs that is required for the invasion process. Fifthly, the entry capacity of IbeA-coated beads was correlated with vimentin binding and clustering at the cell surface, suggesting that IbeA is sufficient to mediate E. coli K1 invasion of HBMECs through its interaction with vimentin. Lastly, IbeA+ E. coli K1 invasion was inhibited by lipid-raft-disrupting agents (filipin and nystatin) and caveolin-1 siRNA, suggesting that caveolae/lipid rafts are signalling platforms for inducing IbeA–vimentin-mediated E. coli invasion of HBMECs.

Vimentin is a well-known marker for mesenchymal cells such as endothelial cells [43]. It is normally expressed in cells of mesenchymal origin, but not in epithelial cells.
EMT (epithelial–mesenchymal transition) processes are usually associated with embryonic development and the malignant conversion of epithelial tumour cells [43]. As the invasive EMT phenotype of tumour cells is associated with expression of vimentin, this protein may also contribute to the adhesive or invasive phenotype of microbial pathogens. VHD is essential for binding of toxin proteins of *P. multocida* to the host cells [14]. ASFV infection leads to reorganization of vimentin into a cage structure by CaMKII-mediated phosphorylation of VHD at Ser*25* [16]. Vimentin rearrangements involving filament assembly/disassembly are regulated by phosphorylation of the head domain [12]. Concurring with these findings, the present study demonstrated that the head domain is essential for IbeA-mediated *E. coli* K1 invasion of HBMECs. Vimentin may be an important scaffolding protein during signal transduction as it associates with, and is also a substrate for, CaMII, protein kinase C, Yes, PRaf-1 and PAK (p21-activated kinase), Rho, Aurora B and cGMP [43]. Our study suggests that CaMII-induced phosphorylation of vimentin is necessary for IbeA+ *E. coli* K1-mediated invasion of HBMECs. We also demonstrated that activation of ERK by IbeA+ *E. coli* K1 is required for bacterial invasion. A peptide (ERK89) derived from the vimentin-binding domain of ERK acts as an antagonist to specifically block IbeA+ *E. coli* K1-induced phosphorylation of ERK and bacterial invasion, suggesting that the vimentin-binding domain is essential for the ERK signalling pathway. The association of vimentin with various signalling molecules such as ERK probably reflects its regulation during cell adhesion, migration and signal transduction [12]. As the IbeA-binding site for bacterial entry is located in VHD, this domain, which contains multiple phosphorylation sites, should be the most interesting part of this protein for further dissection of its role in the pathogenesis and therapeutics of *E. coli* K1 meningitic infection.

Recently, vimentin has emerged as an organizer of a number of critical proteins that contribute to attachment, migration and cell signalling [12]. This protein has been identified as a raft protein present in lipid raft proteomes of endothelial cells [22], suggesting that vimentin contributes to lipid-raft-dependent cellular signalling. In the present study, the involvement of vimentin-enriched lipid rafts/caveolae in IbeA+ *E. coli* K1 invasion of HBMECs was demonstrated by using cholesterol-depleting agents, raft fractionation analysis, immunofluorescence microscopy and siRNA-mediated knockdown of caveolin-1 expression. Disruption of raft micromembrane domains by depleting cholesterol resulted in a significant decrease in IbeA+ *E. coli* invasion of HBMECs. Normally, only a small amount of vimentin is present in lipid rafts of uninfected HBMECs, but the recruitment of vimentin to the raft micromembrane domains was significantly induced upon the entry of IbeA+ *E. coli* or IbeA-coated beads into HBMECs. The association of lipid rafts with bacterial internalization was demonstrated further by fluorescence microscopy, using specific probes for two commonly used markers (GM1 and GMA3) of lipid rafts. The strong co-localization and clustering of vimentin with the lipid markers were observed in HBMECs infected with IbeA+ *E. coli*. The association of lipid raft markers with intracellular IbeA+ *E. coli* in HBMECs was consistent with the previous work demonstrating microbial infection/invasion of host cells via raft micromembrane domains [29–31], and led us to examine the role of a known lipid raft protein, caveolin-1, in IbeA–vimentin-mediated *E. coli* invasion. Caveolin-1, which is required for the formation of caveolar structures, was found to be required for the invasion of *E. coli* into HBMECs, because blocking the expression of caveolin-1 by siRNA also inhibited invasion of HBMECs by IbeA+ *E. coli*. The exact mechanism through which caveolin-1 functions in this event is not known, but this protein is co-localized with vimentin in clusters upon IbeA+ *E. coli* invasion. There are three types of caveolar structures containing caveolin-1: (i) caveolae that are confined to the cell surface by cortical actin filaments, (ii) pericentrosomal caveosomes, and (iii) caveolar vesicles that travel in a bidirectional fashion along microtubules between the cell surface and the caveosome [44]. It has been demonstrated that vimentin-based filaments were responsible for the spatiotemporal fixation of caveolar clusters [45], suggesting that vimentin is involved with caveolar translocation. Concurring with these findings, the present study suggests that dynamic changes in the three types of caveolar structures and caveolar translocation are closely associated with clustering of vimentin upon *E. coli* K1 infection.

In summary, we have demonstrated for the first time the important role of vimentin-mediated signalling in IbeA-mediated *E. coli* K1 invasion of HBMECs. IbeA+ *E. coli* K1 invasion of HBMECs appears to play an important role in the pathogenesis of *E. coli* meningitis [4–11]. Further work will define how CaMII and ERK contribute to the IbeA-induced signalling network and how vimentin and PSF function together to promote uptake of *E. coli* K1 into HBMECs. Dissecting the roles of the host receptors and raft micromembrane domains in the invasion of IbeA+ *E. coli* into HBMECs should allow for a better understanding of how to break the cycle of meningitic infection.

**AUTHOR CONTRIBUTION**

Feng Chi designed and performed the experiments on vimentin-mediated signalling, blockage of bacterial invasion, lipid rafts, siRNA-mediated knockdown of caveolin-1, IbeA-coated beads and immunofluorescence analysis of HBMECs. Timothy D. Jong made the vimentin constructs. Lin Wang prepared cell cultures and carried out protein expression and purification. Yannan Ouyang assisted on the confocal microscopy study. Feng Chi participated in the preparation of the original and final manuscripts. Chun-Hua Wu and Wei Li participated in the experimental design. Sheng-He Huang planned and approved the experimental design, supervised the study, and prepared the original and final manuscripts.

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SUPPLEMENTARY ONLINE DATA
Vimentin-mediated signalling is required for IbeA+ E. coli K1 invasion of human brain microvascular endothelial cells

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Figure S1  PSF distribution in lipid rafts in IbeA-mediated E. coli K1 invasion of HBMECs

PSF distribution in the lipid rafts of HBMECs treated as indicated was detected with a mouse anti-PSF antibody. HBMECs were pre-incubated with or without filipin (1 μM) and triggered by E44, ZD1 or IbeA-coated beads. Floatation was performed in OptiPrep gradients and nine fractions were collected. Fractions 1–9 represent the gradients from top to bottom. Fraction 2 marked with an asterisk (*) consisted of caveolin-1-enriched lipid rafts. CON, control; WB, Western blot.

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Figure S2 Localization of vimentin with GM1 and GM3 in HBMECs infected with IbeA+ E. coli K1 (E44)

(A) HBMECs were infected with (E44) or without (CON) E44 and stained with combinations of anti-vimentin antibody (V9) conjugated to Cy5 (blue) and CTxB–FITC (green). (B) HBMECs were infected with or without E-44 and stained with combinations of anti-GM3 mouse monoclonal antibody (IgM) conjugated to rhodamine (red) and anti-vimentin antibody (V9) conjugated to FITC (green). Immunofluorescence analysis was performed using confocal microscopy. The merged images are shown in the right-hand panels (Merge). All of the side views were acquired from the Z-stack of images. The orthogonal projections of the optical section were viewed from XZ and YZ angles along the horizontal and vertical broken lines respectively. Scale bar, 25 μm.

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Figure S3  IbeA+ E. coli K1 (E44)-induced vimentin surface recruitment is caveolin-1-dependent

(A) HBMECs transduced with lentivirus GFP–VIM were then transfected with caveolin-1–RFP. Confocal microscopy was used to examine the co-localization of vimentin and caveolin-1 with different treatments. Arrows indicate GFP–E44 or GFP–ZD1. Scale bar, 25 μm. (B) HBMEC total lysates were immunoprecipitated (IP) using the mouse anti-vimentin (V9) antibody and rabbit anti-caveolin-1 antibody and subjected to Western blotting using rabbit anti-vimentin antibody and rabbit anti-caveolin-1 antibody respectively. (C) HBMECs were transfected with CAV1-KD or control siRNA (CON). The protein samples were subjected to Western blotting using rabbit anti-caveolin-1 antibody and mouse anti-actin antibody as an internal loading control. (D) Immunofluorescence microscopy was used to examine the effects of caveolin-1 knockdown on GFP–E44-induced clustering and reorganization of vimentin/caveolin-1. HBMEC were triple-stained with the V9 antibody conjugated to Cy5 (blue), rabbit anti-caveolin-1 antibody conjugated to rhodamine (red) and DAPI (4′,6-diamidino-2-phenylindole) (black/white). The merged images are shown in the right-hand panels except for DAPI (Merge). Scale bar, 50 μm. (E) Effects of caveolin-1 knockdown on bacterial invasion. The results are expressed as relative invasion. *P < 0.05; **P < 0.01.