Caveolin-1 interacts and cooperates with the transforming growth factor-β type I receptor ALK1 in endothelial caveolae

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**Aims** Activin receptor-like kinase (ALK1) is a transforming growth factor (TGF)-β type I membrane receptor restricted almost entirely to endothelial cells (ECs) and involved in vascular remodelling and angiogenesis. Previous reports have shown that the ubiquitous TGF-β type I receptor ALK5 and the type II receptor are located in cholesterol-rich membrane microdomains named caveolae. The aim of this work was to assess the location of ALK1 in endothelial caveolae as well as to study the role of caveolin-1 on the TGF-β/ALK1 signalling pathway.

**Methods and results** The subcellular distribution of ALK1 was analysed by confocal microscopy and co-fractionation experiments in human ECs. The association between human ALK1 and caveolin-1 was studied in caveolin-1-deficient human epithelial cells by co-immunoprecipitation. The functional role of caveolin-1 on the ALK1-mediated TGF-β signalling was elucidated using ALK1-specific luciferase reporters in human ECs, caveolin-1−/− mouse embryonic fibroblasts, and rat myoblasts. Confocal microscopy analyses, as well as cholesterol depletion experiments in the presence of cholesterol-depleting agents such as nystatin or methyl-β-cyclodextrin, demonstrated that ALK1 is located in endothelial caveolae. Also, co-immunoprecipitation assays showed that ALK1 associates with the main caveolae component caveolin-1. Mapping of the ALK1/caveolin-1 interaction revealed that the caveolin-1 scaffolding domain and the caveolin-1 binding motif in ALK1 are responsible for this association. Moreover, this hitherto not reported interaction had a functional consequence for the ALK1-dependent signalling. In contrast with the previously published ALK5/caveolin-1 interaction, caveolin-1 enhances the TGF-β/ALK1 signalling pathway, promoting the activity of the ALK1-specific reporters. Conversely, specific suppression of caveolin-1 abrogated the ALK1 signalling pathway.

**Conclusion** ALK1 is located in endothelial caveolae where it functionally interacts with caveolin-1 through its scaffolding domain, suggesting a joint contribution of ALK1 and caveolin-1 as key mediators of the TGF-β pathway in angiogenesis.

1. **Introduction**

Angiogenesis is the process by which new blood vessels are formed from pre-existing ones.1 Upon angiogenic stimuli, quiescent endothelial cells (ECs) proliferate and migrate in response to different cytokines such as the transforming growth factor-β (TGF-β).2 TGF-β triggers the signal to the nucleus through the heterodimeric association of two different receptors (types I and II) with cytoplasmic Ser/Thr-kinase activity.3 Activin receptor-like kinase (ALK)5 and ALK1 are TGF-β type I receptors expressed in cultured ECs and involved in angiogenesis.3,5 The almost endothelial-restricted ALK16 has been related to the activation phase of angiogenesis, promoting cellular proliferation and migration and basal membrane degradation.4,5 In contrast, the ubiquitous ALK5 has been linked to the resolution phase of angiogenesis, involving inhibition of proliferation and migration as well as activation of the extracellular matrix synthesis.3

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ALK5 and ALK1 propagate the TGF-β signalling by phosphorylating Smad proteins. Whereas Smad2/3 are the targets for ALK5 activity, Smad1/5/8 are phosphorylated by ALK1. Once phosphorylated, these Smad proteins associate with the common partner Smad4 and translocate into the nucleus to regulate gene expression. Within this TGF-β pathway, several components, including ALK1, have been identified as critical mediators of the angiogenic process.  

The ACVRL1 gene codes for ALK1 protein and mutations in this gene are responsible for an autosomal dominant vascular dysplasia termed Hereditary Haemorrhagic Telangiectasia (HHT) type 2, characterized by recurrent haemorrhages and cerebral, hepatic and pulmonary arteriovenous malformations. The pathogenic mechanism of this vascular disorder appears to be the haploinsufficiency of ALK1, a hypothesis supported by the fact that heterozygous knock-out mice for ALK1 (Acvrl1+/−) show an HHT phenotype. In addition, genetic inactivation in mice shows that embryos homozygous for ALK1 (Acvrl1−/−) die at 10–10.5 days postcoitum due to vascular and cardiac anomalies. Caveolae are plasma membrane microdomains enriched in cholesterol and glycosphingolipids closely related to lipid rafts but characterized by the presence of a protein family named caveolins. Caveolin-1 is the representative and most abundant member of this family. It is a small integral membrane protein, and the principal component of caveolae in many cell types, with particularly high levels in ECs. The region comprised between Asp82 and Arg101 is termed scaffolding domain and is implicated in protein–protein interactions. This domain can recognize a set of binding motifs with the consensus sequences (Phe92Ala, and Val94Ala), and the caveolin-1 deleted in the scaffolding domain (Cav1-001, where n = 2) has been developed. For RNA interference (siRNA) studies, the pRNA-U6-cav-1 vector, that suppresses the rat caveolin-1 gene, was used. Cell transfections were carried out with SuperFect Reagent (Qiagen).

2.1 Cell culture

The human microvascular EC line HMEC-1 (provided by Dr Edwin Ades, Centers for Disease Control and Prevention, Atlanta, USA) was cultured as described. The human epithelial cell line HEK 293T (American Type Culture Collection, CRL-11268), the rat Lei69 myoblasts (provided by Dr Joan Massague, Memorial Sloan-Kettering Cancer Center, NY, USA), and the mouse embryonic fibroblasts (MEFs) from WT and Cav1−/− mice were grown in DMEM medium plus 10% FCS. For caveolae disruption assays, cells were incubated with methyl-β-cyclodextrin (MβCD) or nystatin (Sigma) for 1 h in the absence of FCS. This 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 Plasmids and transfections

Expression vectors encoding HA-tagged full-length ALK1 wild-type (WT) and kinase dead (KR, Lys229Arg) in pcDNA3-HASL plasmid were provided by Dr Kohei Miyazono (University of Tokyo). ALK1-WT was used as a template in site-directed mutagenesis assays to generate ALK1-001 (Trp406Ala) and ALK1-011 (Phe401Gly and Trp406Ala) mutants. The myc-tagged caveolin-1 expression vectors WT and mutated in the scaffolding domain (SDmut, Phe92Ala, and Val94Ala), and the caveolin-1 deleted in the scaffolding domain (ΔS2–101) have been described. For RNA interference (siRNA) studies, the pRNA-U6-cav-1 vector, that suppresses the rat caveolin-1 gene, was used. Cell transfections were carried out with SuperFect Reagent (Qiagen).

2.3 Antibodies

ALK1 was detected with a rabbit (31) or goat (R&D Systems) polyclonal antibody (pAb) and caveolin-1 was recognized with a rabbit pAb (sc-894; Santa Cruz). Proteins with HA or myc epitopes were detected with Alexa-647 red-conjugated anti-rabbit IgG antibodies (Molecular Probes). Human 293T cells were transfected and, after 48 h, lysed as indicated in Supplementary Material. Total cell lysates were immunoprecipitated using specific antibodies and protein-G Sepharose (GE Healthcare). Samples were separated by SDS-PAGE under reducing conditions and electrotransferred to a PVDF membrane for immunodetection. Proteins were revealed with the SuperSignal chemiluminescent substrate (Pierce).

2.4 Immunofluorescence microscopy

HMEC-1 were fixed and permeabilized as described. Samples were incubated with anti-ALK1 or anti-caveolin-1 antibodies, followed by incubations with Alexa-488 green-conjugated anti-goat IgG or Alexa-647 red-conjugated anti-rabbit IgG antibodies (Molecular Probes), respectively. Nuclei were visualized by incubation with 4′,6-diamidino-2-phenylindole, DAPI (Sigma). Samples were mounted with ProLong Gold (Invitrogen) and observed with a spectral confocal microscope (Leica Microsystems).

2.5 Isolation of caveolae-enriched membrane fractions

HMEC-1 were lysed with MBS buffer (25 mM 2-[N-morpholino]ethanesulfonic acid pH 6.5, 150 mM NaCl) plus 1% Triton X-100. Cellular extracts were sheared by passing through a 30G syringe and mixed with 80% sucrose in MBS buffer and overlaid with a discontinuous sucrose gradient. Samples were subjected to the centrifugation at 200,000 g. Fractions were collected and proteins were precipitated with 40% trichloroacetic acid and resuspended in Laemmli buffer.

2.6 Immunoprecipitation and western blot analyses

Human 293T cells were transfected and, after 48 h, lysed as indicated in Supplementary Material. Total cell lysates were immunoprecipitated using specific antibodies and protein-G Sepharose (GE Healthcare). Samples were separated by SDS-PAGE under reducing conditions and electrotransferred to a PVDF membrane for immunodetection. Proteins were revealed with the SuperSignal chemiluminescent substrate (Pierce).
2.7 Reporter assays

For GAL transactivation assays, plasmid encoding GAL4-Smad1 was co-transfected with the GAL4-luciferase reporter pFr5-Luc (Stratagene). Reporter assays with TGF-β-responsive promoter constructs were performed using p(CAGA)_{12}-Luc and p(BRE)_{12}-Luc constructs as described. The p(BRE)_{12}-Luc construct contains a repeated sequence of the Id1 promoter that is activated via ALK1-Smad1/5/8. Relative luciferase units were determined in a TD20/20 luminometer (Promega). Experiments were performed in triplicates at least three times and representative experiments are shown in the figures. Statistical analysis was carried out using the Student’s t-test and significant differences were determined (P < 0.01).

3. Results

3.1 ALK1 co-localizes and co-fractionates with caveolin-1 in caveolae-enriched domains of ECs

The presence of ALK1 in caveolae was assessed by confocal microscopy. As revealed in HMEC-1, caveolin-1, a marker for these microdomains, co-localized with ALK1 in a well-defined dotted pattern (Figure 1A). When cholesterol was depleted from the membrane, by means of MβCD treatment, the co-localization of both proteins was drastically reduced. Thus, caveolae disruption by cholesterol sequestering altered the subcellular distribution of ALK1, suggesting that it was located in caveolae. To confirm this compartmentalization, HMEC-1 were grown to confluency, lysed and subjected to a discontinuous sucrose gradient. In control untreated cells, both ALK1 and caveolin-1 were immunodetected in fractions #4 and #5, although they were also detected in the so-called non-raft fractions (#6–12) (Figure 1B). In contrast, caveolae disruption by MβCD treatment inhibited the localization of ALK1 and caveolin-1 in the caveolar fractions #4 and #5, and leading to a redistribution of these proteins along the non-rafts fractions. In addition, immunogold staining with specific antibodies demonstrated the presence of ALK1 at discrete microdomains of the membrane in human ECs (Supplementary Material, Figure 1). These results support the presence of ALK1 in caveolae.

![Figure 1](https://academic.oup.com/cardiovascres/article-abstract/77/4/791/335299)

Figure 1 Localization of ALK1 in caveolae. (A) Confocal microscopy. HMEC-1 were treated or not with methyl-β-cyclodextrin (MβCD), fixed, permeabilized, and immunostained with anti-ALK1 (green) or anti-caveolin-1 (red) antibodies. Irrelevant antibodies were used as negative controls (last row). Nuclei were revealed with DAPI staining (blue). (B) Immunodetection of ALK1 and caveolin-1 in the caveola fraction. HMEC-1 treated or not with MβCD, lysed and total extracts subjected to a discontinuous sucrose gradient ultracentrifugation. Fractions were separated by SDS-PAGE under reducing conditions and ALK1 and caveolin-1 were immunodetected by western blot (WB). As a control, an aliquot of total cellular lysates (TCL) was included.
3.2 The caveolin-1 scaffolding domain is responsible for the interaction with ALK1

As ALK1 was located in caveolae, we hypothesized that it may be interacting with caveolin-1. Thus, 293T cells lacking endogenous caveolae were co-transfected with WT versions of HA-tagged ALK1 and myc-tagged caveolin-1, and subjected to immunoprecipitation experiments. Antibodies to ALK1 were able to co-immunoprecipitate caveolin-1 (Figure 2A), suggesting that ALK1 directly interacts with caveolin-1. Co-immunoprecipitation experiments were also performed in ECs, demonstrating the association between endogenous ALK1 and caveolin-1 (Figure 2B). As

![Figure 2](https://academic.oup.com/cardiovascres/article-abstract/77/4/791/335299)

**Figure 2** ALK1 associates with caveolin-1. (A) Caveolin-1 deficient 293T cells were transfected with HA-tagged ALK1 and myc-tagged caveolin-1, as indicated. Aliquots of TCL or anti-HA immunoprecipitates (IP) were separated by SDS–PAGE and analysed by WB using a pAb to caveolin-1. The mature (asterisks) and unglycosylated (arrow head) forms of ALK1 are indicated. The levels of α-tubulin were used as a loading control. (B) HMEC-1 were lysed and immunoprecipitated with a rabbit pAb to ALK1 or an irrelevant species-matched antibody. Immunoprecipitates were separated by SDS–PAGE and analysed by WB using a pAb to endogenous caveolin-1. Aliquots of TCL were separated by SDS–PAGE and analysed by WB using anti-ALK1 or anti-caveolin-1 antibodies. (C) Role of caveolin-1 scaffolding domain in the interaction with ALK1. Human 293T cells were co-transfected with HA-tagged ALK1 and the WT or scaffolding domain mutant (SDmut) versions of myc-tagged caveolin-1. Aliquots of TCL were separated by SDS–PAGE and analysed by WB using anti-ALK1 or anti-caveolin-1 antibodies. (D) Schematic representation of ALK1. Sequences corresponding to the putative consensus motif within the wild-type ser/thr-kinase domain and the generated mutants are shown. (E) Human 293T cells were co-transfected with myc-caveolin-1 and different versions of HA-tagged ALK1. Aliquots of TCL or anti-myc immunoprecipitates were separated by SDS–PAGE and analysed by WB. The mature (asterisks) and unglycosylated (arrow head) forms of ALK1 are indicated. Densitometry of the mature ALK1 bands from three different experiments was carried out and the mean of the IP/TCL ratio is shown.

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most of caveolin-1 interactions are mediated by its scaffolding domain, comprised between Asp82 and Arg101, we analysed the involvement of this region. Thus, when the scaffolding domain of caveolin-1 was deleted (Δ82–101), the co-immunoprecipitation of ALK1 yield decreased ~5-fold compared to the WT construct, as quantified by densitometry of the bands (Figure 2C, histogram). This result points out that the caveolin-1 scaffolding domain is the key region in the association with ALK1. A detailed analysis of the ALK1 amino acid sequence showed that the peptide WAFGLVLW (Trp399–Trp406) fits well with the reported consensus motif FXXFXXXXF for the binding to the caveolin-1 scaffolding domain. To study the involvement of these residues, ALK1 mutants termed 001 (Trp406Ala) and 011 (Phe401Gly and Trp406Ala) were generated (Figure 2D) and then co-transfected with myc-tagged caveolin-1 in 293T cells. As shown in Figure 2E, anti-myc antibodies co-immunoprecipitate ALK1 WT, whereas a substantial decrease was obtained with both ALK1 mutants, confirming the key role of the caveolin-1 binding motif in ALK1. As a loading control, the expression levels of all ALK1 versions were equivalent.

3.3 Caveolae are involved in the ALK1 signalling pathway

Once demonstrated the presence of ALK1 in caveolae, we wondered whether this subcellular localization affected the functional activity of ALK1. First, as caveolae are cholesterol-rich microdomains, the effect of varying cholesterol levels was analysed in HMEC-1. Thus, cholesterol treatment was able to significantly increase the basal activity of the ALK1-specific reporter vector p(BRE)2-Luc (Figure 3A). In contrast, cells treated with cholesterol sequestering agents such as nystatin or MβCD led to a significant decrease of the ALK1-specific pathway. Furthermore, when disrupted caveolae were reconstituted by the addition of cholesterol, the p(BRE)2-Luc activity was efficiently restored. Taken together, these data demonstrate that the ALK1 signalling pathway is significantly modulated by the presence of caveolae.

Figure 3 ALK1 localization in caveolae induces its signalling pathway. (A) The ALK1-specific reporter vector p(BRE)2-Luc was transfected in HMEC-1. After transfection, cells were treated or not with MβCD or nystatin. Culture media were replaced by fresh medium containing or not cholesterol in order to re-establish caveolae. The transcriptional activity was measured using a luciferase reporter assay. Significant differences were observed between cholesterol treated and untreated cells (*P < 0.01). (B–D) Caveolin-1 promotes the TGF-β/ALK1 signalling pathway. (B) HMEC-1 were co-transfected with caveolin-1 or empty vector (EV) and the reporter construct p(BRE)2-Luc and treated or not with 10 ng/ml TGF-β for 24 h. Caveolin-1 over-expression gives rise to an induction in the p(BRE)2-Luc reporter activity when cells are treated or not with 10 ng/ml TGF-β for 24 h. Transcriptional activity was measured using the luciferase reporter assay. (C) Increasing amounts of caveolin-1 were co-transfected with the p(BRE)2-Luc in HMEC-1 in absence or presence of TGF-β (1 ng/ml for 4 h). Transcriptional activity was measured using the luciferase reporter assay. (D) Induction of GAL4-Smad1 transactivation depends on caveolin-1. Wild-type ALK1 and ALK1 kinase death mutant (KR) were co-transfected with caveolin-1 and the GAL4-Smad1 system in HMEC-1, as indicated. Smad1 phosphorylation activity was measured using the luciferase reporter assay. Significant differences were observed between caveolin-1 expressing (+) and non-expressing (−) cells (*P < 0.01).
pathway depends on its subcellular localization in cholesterol-rich microdomains.

3.4 Caveolin-1 stimulates the TGF-β/ALK1 signalling pathway

To assess the role of caveolin-1 on the ALK1 signalling pathway, HMEC-1 were transfected with caveolin-1 expression vector and the p(BRE)_2-Luc activity was assayed with or without TGF-β treatment. As expected, treatment with 10 ng/ml of TGF-β led to an inhibition of the p(BRE)_2-Luc activity in mock transfectants (Figure 3B). In contrast, when caveolin-1 was transfected, the ALK1-specific reporter underwent a clear increase of both basal and TGF-β-induced activities, suggesting a functional cooperation between caveolin-1 and the TGF-β/ALK1 pathway. Similar results were obtained in primary cultures of human dermal microvascular ECs (data not shown). As opposed to the inhibitory effects of high doses of TGF-β (10 ng/ml), low doses of TGF-β (1 ng/ml) during short time periods can increase the p(BRE)_2-Luc activity. Therefore, we used these experimental conditions to further assess the role of caveolin-1. Upon transfection with increasing amounts of caveolin-1, a dose-dependent induction of both basal and TGF-β-induced activities of the p(BRE)_2-Luc reporter was observed (Figure 3C). Moreover, caveolin-1 clearly enhanced the transactivation activity of the GAL4-Smad1 reporter that was further stimulated upon cotransfection with WT ALK1, whereas it was inhibited by the kinase death ALK1 mutant KR (Lys229Arg) (Figure 3D). This result excludes the possible involvement of other receptors of the TGF-β family also present in caveolae, and confirms the specificity of the caveolin-1 activating role on the ALK1 signalling pathway.

3.5 The functional cooperation of caveolin-1 on the TGF-β/ALK1 signalling depends on its scaffolding domain

As shown in Figure 2, the interaction between caveolin-1 and ALK1 is mediated by the scaffolding domain of caveolin-1 and the consensus motif Trp399–Trp406 of ALK1. To assess the functional involvement of these particular regions, caveolin-1 (SDmut and Δ82–101) and ALK1 (001 and 011) mutants were used in reporter assays. As expected, WT caveolin-1 increased the basal and TGF-β-induced p(BRE)_2-Luc activities respect to the empty vector in ECs (Figure 4A). In contrast, none of the caveolin-1 mutant versions was able to stimulate the p(BRE)_2-Luc reporter activity either in the absence or in the presence of low doses of TGF-β (Figure 4A). On the other hand, transfection experiments in caveolae deficient 293T cells showed that, as compared to the WT ALK1, the ALK1 mutants 001 and 011 were unable to functionally synergize with caveolin-1 to induce the p(BRE)_2-Luc reporter activity (Figure 4B). Taken together, these results demonstrate that the scaffolding domain of caveolin-1 and the consensus motif Trp399–Trp406 of ALK1 are functional interacting motifs in the ALK1 signalling pathway.

3.6 Caveolin-1 suppression abrogates the ALK1 signalling pathway

To assess the functional effects of caveolin-1 suppression, the small interfering RNA (siRNA) technique was employed to specifically knock-down endogenous caveolin-1 in L6E9 cells. This cellular system was chosen because it is a useful model to analyse TGF-β signalling, and shows endogenous expression of caveolin-1. Upon transient transfection with a siRNA expression vector specific for caveolin-1, a marked decrease on caveolin-1 protein levels, as compared to three different control siRNA vectors, was observed (Figure 5A). Next, the effect of caveolin-1 suppression on the ALK1 pathway was analysed. Transfection of ALK1 allowed the functional collaboration between the recombinant ALK1 and the endogenous caveolin-1, leading to the induction of the p(BRE)_2-Luc reporter activity (Figure 5B). However, when caveolin-1 expression was specifically suppressed by siRNA, the ALK1-dependent induction of the p(BRE)_2-Luc activity was drastically abolished. Similar inhibitory effects were observed with the SDmut and Δ82–101 versions of caveolin-1 that behaved as dominant negative for the ALK1-dependent activity. As a control, the ALK1-dependent induction of the

![Figure 4](https://example.com/figure4.png) Role of caveolin-1 scaffolding domain in the ALK1 signalling pathway. (A) HMEC-1 were co-transfected with the p(BRE)_2-Luc reporter vector, an empty vector (EV) and the wild-type (WT), the scaffolding domain mutant (SDmut) or the scaffolding domain deleted (Δ82–101) caveolin-1 versions. Cells were treated or not with 1 ng/ml TGF-β for 4 h and the transcriptional activity was measured using the luciferase reporter assay. Significant differences were observed between TGF-β-treated and untreated cells (*P < 0.01). (B) The reporter activity of the p(BRE)_2-Luc vector was analysed in 293T cells co-transfected or not with caveolin-1 and the WT or mutant versions (001 and 011) of ALK1. Significant differences were observed between caveolin-1 expressing (+) and non-expressing (–) cells (*P < 0.01).
reporter activity was further increased upon co-transfection with WT caveolin-1 (Figure 5B). Furthermore, to assess the effect of genetic inactivation of caveolin-1, MEFs from Cav1<sup>−/−</sup> were transfected with the p(BRE)<sub>2</sub>-Luc reporter vector in presence or absence of ALK1. As shown in Figure 5C, basal and ALK1-induced reporter activities were markedly decreased in caveolin-1 knock-out MEFs as compared to WT controls. In addition, ectopic expression of caveolin-1 in Cav1<sup>−/−</sup> MEFs yielded an ALK1-induced activity comparable to that of WT MEFs in the absence of recombinant caveolin-1.

Caveolin-1/ALK5 interaction gives rise to an inhibition on the Smad2 pathway, but little is known about the Smad3 pathway. Thus, we analysed the effect of caveolin-1 suppression on the transcriptional activity of the ALK5/Smad3 pathway specific reporter vector p(CAGA)<sub>12</sub>-Luc. As expected, down-regulation of caveolin-1 in Cav1<sup>−/−</sup> MEFs results in an enhancement of the p(CAGA)<sub>12</sub>-Luc reporter activity in response to TGF-β, supporting the view that caveolin-1 interferes with the ALK5 pathway (Figure 5D).

Taken together, these data corroborate the stimulating effect of caveolin-1 on the TGF-β/ALK1 signalling pathway, and suggest an inhibitory role on the TGF-β/ALK5 route.

4. Discussion

TGF-β is a multifunctional cytokine that controls proliferation, differentiation, apoptosis, homeostasis, and other functions in many cell types. In ECs, TGF-β can propagate the signal to the nucleus through two different and non-redundant type I receptors named ALK5 and ALK1. Here, we report that ALK1 is located in caveolae where it interacts with caveolin-1, the major protein component in caveolae, leading to an activation of the ALK1 signalling pathway. Although ALK5 can also be found in caveolae and interacts with caveolin-1, in this case the functional consequence is the inhibition of TGF-β/ALK5 cellular responses mediated by Smad2. Similar inhibitory effects have been described for almost all proteins with enzymatic activity that interact with caveolin-1, such as endothelial nitric oxide synthase, epidermal growth factor receptor, or protein kinase-C α and ζ isoforms. To the best of our knowledge, the only reported exception to this rule is the activation of
by caveolin-1 of insulin receptor signalling. Thus, the caveolin-1/ALK1 association described in this paper constitutes a special case of a functional cooperation of a cell surface receptor with caveolin-1.

The presence of ALK1 in caveolae appears to be mediated, at least, by its interaction with caveolin-1. Thus, caveola disruption by cholesterol depletion significantly changed both the ALK1/caveolin-1 distribution at the cell surface in confocal experiments, as well as their co-fractionation in sucrose gradient assays. A detailed analysis of ALK1 amino acid sequence showed a consensus motif in the Trp399- Trp406 region for the interaction with the caveolin-1 scaffolding domain. Accordingly, specific mutations on this consensus motif in ALK1 as well as on the scaffolding domain of caveolin-1, considerably reduced the total amount of the respective co-immunoprecipitated proteins. However, we cannot discard the implication of other caveolin regions or other related proteins in this interaction. For example, since the TGF-β/ALK1 signaling pathway would be free from caveolin-1 inhibition. The presence of ALK1 in caveolae appears to be mediated, likewise, either the caveolin-1 mutations mentioned above or the caveolin-1 knock-down substantially abolish the TGF-β/ALK1 signalling pathway. Given the differential functional effects of caveolin-1 on the ALK1/ALK5 signalling balance, it would be predicted that mouse Cav-1−/− ECs would display a reduction in the ALK1 pathway, whereas the ALK5 route would be free from caveolin-1 inhibition. This hypothesis agrees with the reported fibrosis observed in caveolin-1 null mice. In this context, TGF-β would be signalling through the non-inhibited ALK5, whereas ALK1 pathway would be impaired. In fact, increased synthesis of extracellular matrix is a pathological process widely related to high circulating levels of TGF-β and with the ALK5 signalling pathway.

Taken together, the functional cooperation between ALK1 and caveolin-1 in ECs caveolae suggests that both proteins are involved in common cellular and/or physiological processes. In this regard, one is to note the reported involvement of both proteins in vascular remodelling and angiogenesis. Thus, caveolin-1 knock-out mice exhibit vascular abnormalities associated with increased endothelial permeability due to a tight junctions alteration, augmented acetylcholine-induced relaxation of arteries, and thickening of alveolar septa caused by uncontrolled EC proliferation and fibrosis. On the other hand, ALK1 haploinsufficiency in humans leads to HHT, a vascular disorder characterized by an impaired vascular remodelling/angiogenesis that leads to telangiectasias and arteriovenous malformations, and these vascular abnormalities are also reproduced in ALK1 knock-out heterozygous (Acvr1+/−) mice. Accordingly, ECs from HHT patients with mutations in ACVR1 show a deficient capacity to form capillary-like structures. Similarly, the specific caveolin-1 blockage results in an impaired formation of endothelial capillary-like tubules in vitro, whereas overexpression of caveolin-1 accelerates EC differentiation/tube formation. Therefore, the inability of forming capillary-like tubules exhibited by caveolin-1 null mice is in agreement with the caveolin-1-dependent activity of the ALK1 signalling pathway reported here. Thus, it can be postulated that the regulated expression of caveolin-1 modulates ALK1 activity, which in turn regulates the angiogenic process. Interestingly, caveolin-1 appears to be necessary in the early stages of endothelial tubulogenesis and it has been observed that caveolin-1 is up-regulated within the first 8 h just before the beginning of tubulogenesis.

Given the postulated balance between ALK1 and ALK5 in controlling the angiogenic switch and the experimental data described above, we propose that the endothelial tubulogenesis depends on the functional interplay between ALK1/caveolin-1 and ALK5/caveolin-1 in caveolae (Figure 6). In this hypothetical model, ALK1 is present in ECs caveolae, where caveolin-1 potentiates TGF-β/ALK1, while it represses TGF-β/ALK5 responses, leading to the activation of the angiogenic process. Supporting this model, caveolin-1 expression has been reported to be up-regulated during the early stages of angiogenesis. This regulatory role of caveolin-1 suggests that the association between caveolin-1 and ALK1 is a target for the treatment of diseases where the angiogenesis process is involved.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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Conflict of interest: none declared.

References

Figure 6 Hypothetical model for the functional cooperation of caveolin-1 in the ALK1 signalling pathway. In quiescent ECs, ALK1 and ALK5 interact with caveolin-1 in caveolae leading to a basal signalling mediated by Smad1 and Smad2/3, respectively. Upon an angiogenic stimulus, caveolin-1 is up-regulated and the equilibrium is displaced favouring the functional interaction with ALK1 in caveolae, while inhibiting the ALK5 pathway. Consequently, processes such as cellular migration and proliferation, and tubulogenesis are enhanced, promoting the activation phase of angiogenesis.
Caveolin-1 interacts with ALK1 in caveolae


6. Seki T, Hong KH, Oh SP. Nonoverlapping expression patterns of ALK1 and ALK5 reveal distinct roles of each receptor in vascular development. Lab Invest 2006;86:116-129.


