Aggretin Venom Polypeptide as a Novel Anti-angiogenesis Agent by Targeting Integrin alpha2beta1

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VEGF and VEGFR antibodies have been used as a therapeutic strategy to inhibit angiogenesis in many diseases; however, frequent and repeated administration of these antibodies to patients induces immunogenicity. In previous studies, we demonstrated that aggretin, a heterodimeric snake venom C-type lectin, exhibits pro-angiogenic activities via integrin α2β1 ligation. We hypothesised that small-mass aggretin fragments may bind integrin α2β1 and act as antagonists of angiogenesis. In this study, the anti-angiogenic efficacy of a synthesised aggretin α-chain C-terminus (AACT, residue 106–136) was evaluated in both in vitro and in vivo angiogenesis models. The AACT demonstrated inhibitory effects on collagen-induced platelet aggregation and HUVEC adhesion to immobilised collagen. These results indicated that AACT may block integrin α2β1 – collagen interaction. AACT also inhibited HUVEC migration and tube formation. Aortic ring sprouting and Matrigel implant models demonstrated that AACT markedly inhibited VEGF-induced neovascularisation. In addition, induction of FAK/PI3K/ERK1/2 tyrosine phosphorylation and talin 1/2 associated with integrin β1 which are induced by VEGF were blocked by AACT. Similarly, tyrosine phosphorylation of VEGFR2 and ERK1/2 induced by VEGF was diminished in integrin α2-silenced endothelial cells. Our results demonstrate that AACT is a potential therapeutic candidate for angiogenesis-related diseases via integrin α2β1 blockade.

Angiogenesis is the growth of blood vessels from pre-existing vasculature and plays an important role in wound healing, tumour growth/metastasis and inflammation-related diseases. Accordingly, there has been considerable interest in the use of novel anti-angiogenic agents as adjuncts to cancer therapies. Endothelial cells interact with the extracellular matrix (ECM) through cell surface adhesion receptors that mediate the neovascularisation processes. β1 and αv integrins have been reported to modulate neovascularisation processes, and αvβ3 has also been implicated in angiogenesis due to its high level of expression in angiogenic vessels. The role of these adhesion molecules in angiogenesis is demonstrated by the in vivo anti-angiogenic efficacy of αvβ3 monoclonal antibodies and αvβ3 antagonists including the snake venom disintegrin, which has demonstrated anti-angiogenic efficacy in vivo.

Collagen is one of the ECM and is crucial for cell migration. Integrin α2β1, one of several collagen receptors, is expressed on endothelial cells and platelets. Upon integrin α2β1-expressing cell adhesion to collagen, many physiological functions are activated, including extracellular matrix remodelling and the ERK pathway. α2β1 integrin has been implicated in extracellular matrix remodelling in addition to endothelial cell migration, proliferation and neovascular formation. Snake venoms contain many enzymes and polypeptides which can affect the matrix and cell interaction. We previously demonstrated that a C-type lectin-related protein, aggretin, exhibits pro-angiogenic activities through interaction with endothelial integrin α2β1 as a collagen-like agonist. Using binding and functional studies, we demonstrated that integrin α2β1 is the major receptor of aggretin on human umbilical vascular endothelial cell (HUVECs). In vivo vascular endothelial growth factor (VEGF)-driven angiogenesis was selectively reduced by integrins α1 and α2 inhibition without affecting any pre-existing vasculature. In addition, one selective α1β1 integrin inhibitor, obtustatin, has been reported to inhibit in vivo angiogenesis. These data indicate that integrin α2β1 and α1β1 antagonism may inhibit signalling pathways involved in angiogenesis.

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VEGF has been established to be involved in many stages of angiogenesis in malignant diseases via its multi-functional effects in activating and integrating signalling pathway networks\(^\text{14}\). VEGF signalling blockade reduces new vessel growth and induces endothelial cell apoptosis. Thus, the use of tyrosine kinase inhibitors or VEGF/VEGF receptor (VEGFR) antibodies to inhibit crucial angiogenic steps represents a practical therapeutic strategy for the treatment of neovascularisation diseases\(^\text{15}\). E7280, a potent angiogenesis inhibitor, has been shown to reduce integrin \(\alpha_2\) mRNA expression and inhibit basic fibroblast growth factor/VEGF-induced HUVEC proliferation and tube formation\(^\text{16,17}\). Integrin \(\alpha_2\beta_1/\alpha_2\beta_3\) expression is reportedly regulated by VEGF, and an inhibitory antibody against \(\alpha_2\beta_1/\alpha_2\beta_3\) has been shown to inhibit angiogenesis and tumour growth in VEGF-overexpressing tumour cells\(^\text{12,18}\). Therefore, we hypothesised that peptide-based integrin \(\alpha_2\) blockade may have potential anti-tumour effects by inhibiting angiogenesis.

In this study, we demonstrate that aggretin \(\alpha\)-chain C-terminal (AACT, 31 amino acid residues) inhibits collagen-induced platelet aggregation and HUVEC adhesion predominantly via integrin \(\alpha_2\beta_1\) ligation. The ability of endothelial cells to adhere to collagen was also diminished by integrin \(\alpha_2\) silencing. Thus, we hypothesised that aggretin-derived integrin \(\alpha_2\) antagonism may inhibit angiogenesis in response to VEGF. In this study, we unveiled the anti-angiogenic activities of AACT by demonstrating its inhibitory effects on HUVEC proliferation and tube formation\(^\text{16,17}\). VEGF-stimulated focal adhesion kinase (FAK), Phosphoinositide 3-kinase (PI3K) and Extracellular Signal-regulated Kinase 1/2 (ERK 1/2) phosphorylation were attenuated by AACT. The talin1/2 associated with integrin \(\alpha_2\beta_1\) was also abolished by AACT. Similarly, VEGF-induced VEGFR2 and ERK1/2 activation were abolished by integrin \(\alpha_2\) siRNA transfection. These results demonstrate that AACT inhibits angiogenesis in response to VEGF via \(\alpha_2\beta_1\) integrin blockade.

Results

Effects of AACT on collagen-induced platelet aggregation and HUVEC-collagen interaction. Since the integrin \(\alpha_2\beta_1\)-type lectin-like receptor 2 (CLEC-2) were demonstrated as the binding targets of AACT\(^\text{19}\) and there are lack of CLEC-2 expression in HUVECs\(^\text{20}\), the integrin \(\alpha_2\beta_1\) may be the binding target in HUVECs. To investigate the inhibitory effect of AACT on integrin \(\alpha_2\beta_1\) activation, we examined the effect of AACT on collagen-induced platelet aggregation. As shown in Fig. 1A, AACT (25 and 50 \(\mu\text{g/ml}\), equivalent to 6.75 and 13.5 \(\mu\text{M}\), respectively) significantly inhibited collagen-induced aggregation (approximately 50\% inhibition). Furthermore, to confirm integrin \(\alpha_2\beta_1\) as the major target for AACT-mediated HUVEC-collagen attachment, we next examined the involvement of integrin \(\alpha_2\) in cell adhesion. Endothelial cell adhesion to collagen was inhibited by integrin \(\alpha_2\) mAb and AACT (50 \(\mu\text{g/ml}\)). Similarly, knockdown of \(\alpha_2\) also inhibited cell adhesion to collagen (Fig. 1B). Moreover, we investigated the binding of AACT to integrin \(\alpha_2\). HUVECs treated with or without AACT (50, 100 and 300 \(\mu\text{g/ml}\)) were cultured with anti- \(\alpha_2\) antibodies. As shown in Fig. 1C, AACT inhibited the binding of integrin \(\alpha_2\) mAb to endothelial cells as measured by flow cytometry, but not the binding of anti- Glycoprotein VI (GPVI) or anti-Glycoprotein Ib (GPIb) antibodies (Fig. 1D and E). We also used the HUVECs membrane receptor to explore the binding site of AACT on HUVECs. HUVECs membrane proteins bound to biotinylated AACT were isolated and eluted. Only one membrane receptor was recognized by integrin \(\beta_1\) (Fig. 1F). These results indicate that AACT inhibits platelet and HUVEC-collagen adherence, predominantly via integrin \(\alpha_2\) blockade.

Effects of AACT on HUVEC viability and proliferation. As integrin \(\alpha_2\beta_1\) activation is involved in endothelial cell growth, we evaluated the inhibitory effects of AACT on cell viability using 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assays. As shown in Fig. 2A, AACT reduced serum induced HUVEC viability by 63.8\% at a concentration of 50 \(\mu\text{g/ml}\). Furthermore, in order to confirm the inhibitory effects of AACT on endothelial cell growth, we performed bromodeoxyuridine assays. As expected, AACT was found to significantly inhibit HUVEC proliferation (Fig. 2B).

AACT inhibits migration of HUVECs in vitro and ex vivo. As HUVECs migration is essential for angiogenesis, the effect of AACT (10, 25 and 50 \(\mu\text{g/ml}\)) on HUVEC haptotaxis migration with Transwell was assayed. As shown in Fig. 3A, a 4.72-fold increase was observed in the number of HUVECs in the lower filter membrane coated with collagen. Under similar conditions, AACT significantly inhibited HUVEC migration. Furthermore, we evaluated chemotactic migration with Transwell to determine the effect of AACT on HUVEC migration in response to VEGF. As shown in Fig. 3B, a 7.45-fold increase in the number of HUVECs was observed following VEGF stimulation, with AACT found to inhibit HUVEC migration. In addition, the vessels sprouting of the rat aortic ring induced by VEGF was also significantly decreased in AACT treated group (Fig. 3C). These results showed that AACT is capable of inhibiting VEGF-induced HUVECs migration in vitro and ex vivo.

Effects of AACT on Matrigel tube formation. HUVECs had significantly greater numbers of branching tube networks after 16 h of 20\% FBS incubation (20\% FBS treatment Fig. 4B as compared to serum free Fig. 4A), and this tube branching was attenuated by VEGF Ab treatment (Fig. 4C). AACT (10, 25 and 50 \(\mu\text{g/ml}\)) also attenuated serum-induced HUVEC tube formation (Fig. 4D–F). Moreover, to confirm the involvement of integrin \(\alpha_2\) in the tube formation process, we examined the inhibitory effect of integrin \(\alpha_1\) and \(\alpha_2\) Ab in our in vitro angiogenic model. Integrin \(\alpha_2\beta_1\) mAb treatment, but not integrin \(\alpha_2\) mAb treatment, significantly decreased VEGF-induced tube formation (Fig. 4G–J). These results indicate that AACT inhibits VEGF-stimulated angiogenesis predominantly via integrin \(\alpha_2\) blockade, as shown in Fig. 4K.

Effect of AACT on angiogenesis in response to Matrigel implantation. An in vivo model containing Matrigel premix with VEGF (200 ng/ml) was used to determine the inhibitory effect of AACT on angiogenesis. Matrigel (in the presence or absence of AACT (10, 25 and 50 \(\mu\text{g/ml}\)) was then subcutaneously
At 7 days after inoculation, capillary network formation was observed in implanted plugs. In the AACT-treated group, less vessel growth and less red blood cell infiltration was observed in implanted plugs (Fig. 5). Haemoglobin levels were significantly lower in AACT-treated mice. These results suggest that AACT also inhibits angiogenesis in vivo.

Effect of AACT on FAK/PI3K/ERK1/2 activation and talin 1/2 associated with integrin β1.

FAK and PI3K phosphorylation are involved in many cell responses to VEGF. In this study, we found that VEGF-induced FAK and PI3K p85α phosphorylation were significantly inhibited by AACT (Fig. 6). AACT
pre-treated HUVECs also demonstrated significantly decreased ERK1/2 activation (Fig. 6). Integrins are well established to be activated by clustering and binding of talin to integrin β-tail, we further tested the talin 1/2 association with integrin β1. Talin 1/2 and integrin β1 association was significantly abolished by AACT treatment. These results suggest that AACT inhibits integrin α2β1 activation and reduces VEGF-stimulated FAK, PI3K and ERK signalling.

**Role of integrin α2 involved in VEGF signalling.** The integrin α2-subunit siRNA was used to evaluate the involvement of integrin α2β1 in VEGF signalling. Expression levels of integrin α2 markedly decreased in siRNA-transfected cells compared to non-targeted control siRNA-transfected HUVECs (Fig. 7). VEGF-induced VEGF Receptor 2 and its downstream signalling molecular ERK1/2 phosphorylation were significantly inhibited in both integrin α2 siRNA transfected endothelial cells and cells pre-treated with AACT (Fig. 7). Collectively, these results indicate that integrin α2 is involved in VEGF signalling and that AACT inhibits angiogenesis via endothelial integrin α2 ligation.

**Discussion**

HUVEC expression of integrin αVβ3, αVβ5 and α2β1 has been implicated in angiogenesis21,22. Several snake venoms interact with platelets via α2β1, GPVI and GPIb; however, few demonstrate significant specificity. Many snake venoms have binding sites for several platelet targets. Recombinant techniques allow the production of specific polypeptides which bind integrin α2β1 without involving GP Ib or GPVI. In previous studies, we demonstrated that aggretin induces platelet activation through binding integrin α2β1 and GP Ib leading to FAK and PLC-γ phosphorylation23. Aggretin also induces HUVEC-dependent angiogenesis by interacting with integrin α2β1 through the PI3K/Akt/ERK1/2 pathways, with increased expression of VEGF11. Therefore, in this study we synthesised a small-mass aggretin fragment, AACT, and examined its effects on platelet aggregation and angiogenesis. Interestingly, we demonstrated that AACT blocks platelet aggregation induced by collagen (Fig. 1A), HUVEC-collagen attachment and HUVEC-integrin α2 mAb binding (Fig. 1B,C). These results indicate that this aggretin fragment acts as α2β1 antagonist rather than an intrinsic α2β1 agonist such as intact aggretin. Similarly, AACT was found to inhibit collagen/VEGF-induced HUVEC migration, FBS-induced Matrigel tube formation and VEGF-induced aortic ring sprouting (Figs 2–4). Since integrin α2β1 but not GPIb, GPVI and CLEC-2 is the major target of aggretin on endothelial cells (Fig. 1D–F), we hypothesised that AACT blocks in vitro or ex vivo angiogenesis via integrin α2β1 ligation. Furthermore, AACT abolished VEGF-induced angiogenesis in a Matrigel plug implant assay, suggesting that AACT may be utilised as an anti-angiogenic peptide for inhibiting angiogenesis in vivo (Fig. 5). Although the AACT exhibited a potent antiangiogenic activity, most results in our study is not dose-dependent. According to the previous studies, there are several sites for collagen/snacle binding and induced α2β1 activation24–28. It may be the reason why AACT was failed to show dose-dependent effect via competitive inhibition. We also found other native peptides derived from integrin α2β1 inhibitor were more likely through the on/off pattern to inhibit integrin α2β1 activity27,28. The detail integrin α2β1 binding site for AACT still needs further investigation.

Tumour-induced angiogenesis is critical for nutrition and oxygen supply via blood to local tumour and tumour metastases to other organs. Anti-angiogenesis therapy provides many benefits, including broad applicability to different tumour types, less tumour cell resistance and reduced chemotherapeutic dosages. A C-type lectin-like selective α2β1 integrin inhibitor, vixapatin, has demonstrated the ability to inhibit angiogenesis29. E7820 inhibits the proliferation and tube formation of HUVECs through suppression of endothelial integrin

![Image](https://example.com/image.png)

**Figure 2. Effects of AACT on HUVEC viability and proliferation.** HUVECs were seeded overnight for attachment. After a further 16 h of starvation, cells were incubated with medium in the absence (M199) or presence of 20% FBS. Cells were either treated with 20% FBS only as a control or with the indicated concentration of AACT (10, 25, 50 μg/ml) for assay. With 48 h treatment, cells were added (A) MTT or (B) BrdU reagent. All experiments were conducted in triplicate at least four times and similar results were obtained. Data are presented as mean ± S.E.M. (n = 4). **P < 0.01; ***P < 0.001 compared with the control.
α2 mRNA expression. These studies indicate that integrin α2/β1 mediated angiogenesis may represent a novel pharmacological target.

VEGF is the major angiogenesis regulatory factor and induces neovascularisation via interaction with endothelial cells. To regulate angiogenesis-related processes, VEGF activates many signal transduction networks, such as FAK, PI3K/AKT, ERK1/2, Src and PLCγ. The inhibitory effect of AACT may be through ligation to integrin α2/β1, resulting in blockade of the VEGF signal transduction pathway. VEGF-induced PI3K and ERK1/2 activation were markedly inhibited by pre-treatment of HUVECs with AACT in this study. Several β1 integrins have been found to regulate angiogenesis and VEGFR2 activity. β1 integrins and VEGFR2 interaction plays a role in infante hemangiomata pathogenesis and matrix-bound VEGFA signalling. The CD36 and β1 integrin association was reported to cooperate with VEGFR2 in promoting angiogenesis. Integrin α2/β1 also physically associates with EGFR and functions regulate EGFR activation. These findings support a role for integrin α2/β1 as a mediator for VEGFR2 signalling. To confirm the action of integrin α2/β1 in VEGF induced signalling, we transfected integrin α2 siRNA and evaluated the effect of VEGF, where VEGF-induced ERK1/2 activation was found to be significantly inhibited by integrin α2 siRNA (Fig. 7A). These results suggest that the existence of a crosstalk or a positive feedback loop between integrin β1 and VEGFR signaling.

The modulatory role of integrin α2/β1 in angiogenesis observed in in vitro experiments illustrates its involvement in supporting VEGF signalling and HUVEC migration. Studies from other researchers have provided additional support for the involvement of α2/β1. Although the significance of the integrin α2/β1 in tissue angiogenesis remains undetermined, our findings support the concept that integrin α2/β1 contributes to the regulation of VEGF signalling. Integrin-linked kinase (ILK) has been identified as an integrin β1 tail binding protein.
Silencing ILK with siRNA significantly suppressed tube formation and reduced the tube lengths\(^3^9\). Loss of ILK signalling may be involved in decreasing the responses of integrin α2β1-silenced HUVEC to VEGF.

Integrin/ECM interactions are one of the major mediators of cell adhesion-mediated drug resistance\(^6\). β1 integrins reportedly play a crucial role in head and neck squamous cell carcinoma cell radioresistance\(^4^0\). Kanda et al. also reported increased β1, α2 and/or α5 integrin expression in refractory tumours following treatment with gefitinib and/or erlotinib\(^4^1\). Furthermore, they demonstrated the integrin β1/Src/Akt signalling pathway as a key mediator of acquired resistance to EGFR-targeted anti-cancer drugs (gefitinib or erlotinib)\(^4^1\). Combined treatment with E7820, an integrin α2 expression blocker, and erlotinib significantly decreased microvessel density and increased apoptosis of tumour-associated endothelial cells compared with use of only one of the agents\(^4^2\). Ligation of integrin α2β1 may modulate EGFR-mediated endothelial cell functions. The combination of an integrin α2β1 blocker with a growth factor inhibitor may represent an alternative strategy for overcoming drug resistance in cancer treatment.

In summary, we identified an important functional cooperativity between integrin α2β1 and VEGF. In particular, the findings of this study indicate that integrin α2β1 provides crucial support not only for endothelial cell migration but also for VEGF signal transduction and pro-angiogenic functions. In contrast to the intact aggretatin, a pro-angiogenic α2β1 agonist, AACT, was found to act as an antagonist of integrin α2β1 and suppresses VEGF-driven angiogenesis in \textit{ex vivo} and \textit{in vivo} models. Many angiogenesis inhibitors have been approved for clinical use and have been studied in clinical trials; however, side effects are reportedly associated with increased risk of arterial thromboembolism\(^4^3\). AACT has anti-angiogenic potential and exhibits anti-thrombotic activity by blocking collagen-induced platelet activation. This may also represent an alternative advantage of AACT for patients receiving anti-angiogenesis therapy at a risk of thromboembolism. Recently, our group found that AACT also blocks the interaction of platelet receptor CLEC-2 with the tumour receptor, podoplanin\(^1^9\). CLEC-2, a type II transmembrane receptor, is highly and selectively expressed in the liver and some myeloid subsets\(^4^1\). As CLEC-2 is not expressed on the surface of HUVEC\(^2^0\), the possibility of AACT interacting with CLEC2 can be
excluded, indicating that integrin α2β1 is the major target of AACT affecting angiogenesis. Thus, optimisation of small-mass CLP-derived integrin α2β1 antagonists contributes to future drug development.

Materials and Methods

Materials. Anti Antiphospho-ERK1/2 (Tyr 204, sc-101761), ERK1/2 (sc-514302), Antiphospho-PI3K-p85α (Tyr 508, sc–12929), PI3K-p85α (sc–1637), p-FAK (Tyr 397, sc-11765-R), FAK (sc-1688), VEGFR-2 (sc–6251) and secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti Antiphospho-VEGFR 2 (Y1175) was purchased from Cell Signaling (Danvers, MA). Beta actin (NB600–501) was purchased from Novusbio. Integrin α2 (ab24697), α1 (ab78479) and β1 (ab30483) were purchased from ABCAM. MTT and toluidine blue O were purchased from Sigma (St Louis, MO). M199, fetal bovine serum (FBS) and other cultured reagents were purchased from Gibco (Grand Island, NY). Endothelial cell growth supplement was purchased from Upstate Biotechnology. Recombinant human VEGF was purchased from R&D Systems (Minneapolis, MN). Aggretin α–chain C-terminus (AACT) 106–136 (CGALEKLTGFRKWVNYYCEQMHAFVCKLLPY) were synthesized by MDBio, Inc., Taiwan. All protocol were approved by Institutional Review of Board, National Taiwan University Hospital or Laboratory Animal Center, College of medicine, National Taiwan University. All experiments were performed in accordance with College of medicine, National Taiwan University regulations and the the written informed consent was obtained from all subjects for human platelet suspension preparation.

Preparation of human platelet suspension and platelet aggregation assay. Platelet suspensions were prepared as previously described45.

HUVECs culture. HUVECs were provided by the National Research Program for Biopharmaceuticals, Taiwan and approved by its Institutional Review of Board. HUVECs were maintained in M199 (with FBS (20%), ECGS (30μg/mL), L-glutamine (4 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL)) and incubated at 37 °C in 5% CO2. The cells were used between second to fourth passages.

Adhesion Assay. HUVECs were labeled BCECF-AM and then incubated with or without AACT (50 μg/ml) for 37 °C 30 min. HUVECs were applied to plates which were pre-coated with collagen or gelatin, 50 μg/ml and were subjected to adhesion as previously described11.

Flow cytometry. The inhibitory effect of AATC on integrin α2, GPIb and GPVI interaction with HUVECs was measured by flow cytometry. HUVECs were pretreated with AACT, and incubated with anti-integrin α2, anti-GPVI or anti-GPIb antibody at room temperature, then were analyzed byFACS Calibur (Becton Dickinson, San Diego, CA, USA).

MTT assay. HUVECs were starved with M199 contain 2% FBS and then grown in M199 with 20% FBS in absence or presence of AACT (10, 25 or 50 μg/ml). HUVECs were subjected to adhesion as previously described11.

BrdU proliferation assay. HUVECs were starved and then treated with or without AACT (10, 25 or 50 μg/ml) for 48 hours. Cell proliferation was measured by BrdU Cell Proliferation Assay Kit (Chemicon, Temecula, U.S.A), and followed by the manufacturer’s protocol.

Figure 5. AACT inhibits VEGF-induced angiogenesis in mouse Matrigel-plug assay. Matrigel 500μl containing 200 ng/ml VEGF with or without AACT (10, 25, 50 μg/ml) was subcutaneously injected into C57BL/5 C mice (5–9 mice/group). After 7 days, plugs were taken and photographed (Scar bar = 2 mm). Hemoglobin was measured as an indication of blood vessel formation, using the Drabkin method. Data are presented as mean ± S.E.M of at least 3 mice per group. **P < 0.01, ***P < 0.001 compared with control.
Binding assays. HUVECs were fixed with 1% para glutaraldehyde and then pretreated with AACT. Treated cells then incubated with integrin α2 antibody and were subjected to adhesion as previously described. Matrigel capillary tube formation. HUVECs (1.2 × 10^5 cells) treated with or without AACT (10, 25, 50 μg/ml) in presence of 20% FBS. Cells were subjected to tube formation as previously described. Total length in each condition was quantified by using Kurabo Angiogenesis Image Analyzer (Kurabo, Japan).
Aortic ring sprouting assay. Sprague-Dawley rats aortic rings were treated with or without AACT (10, 25 or 50 μg/ml) and placed in pre-coated Matrigel. Aortic rings were subjected to sprouting assay as previously described. The sprouting area was measured by the area of endothelial cell out-growth.

Matrigel-implant angiogenesis assay. Matrigel containing VEGF (200 ng/ml) was premix with or without AACT were subjected to matrigel implant assay as previously described.

Migration assay. Transwell (8.0 μm pore size, Costar) were coated with type I collagen (0.2 μg)/BSA (20 μg) or filled absence or presence of VEGF (20 ng/ml) as a chemoattractor in lower chamber. HUVECs were subjected to migration assay as previously described.

Protein-based affinity pulldown. These studies were performed as previously described.

Transfection of small interfering RNA (siRNA). Integrin α2 siRNA or non-targeting siRNA (negative siRNA) were transfected into HUVECs. The integrin α2 sense sequences of these three siRNA sequences were as follows: UGAAUUGUCUGGCGUAATT, CAACUGGGAUCUGUUCUGATT and GCCAAUGAGCCGAGAAUATT. The sequence for the negative siRNA is UAAGGCUAUGAAGAGAUAC

Immunoprecipitation and immunoblot analysis. HUVECs were pretreated with or without AACT (25 or 50 μg/ml) for 30 min, and VEGF (20 ng/ml) was added to the cells as basal or control for another 10 min. Cells were subjected to immunoprecipitation and immunoblot as previously described.

Statistical analysis. Data are presented as mean ± SEM. Groups were assessed by one-way ANOVA and Newman–Keuls multiple comparison test. P values less than 0.05 (P < 0.05) were considered to be significantly different.
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Author Contributions
C.-H. Chung. and T.-F. H. designed the research, analyzed results, and wrote the manuscript. C.-H. Chung, C.-H. Chang, C.-C.H., K.-T. L. and H.-C.P. performed the experiments, and analyzed results. T.-F. H. supervised the whole study. All authors reviewed the manuscript.

Additional Information
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