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Original Article

Activation of the ERK signaling pathway is involved in CD151-induced angiogenic effects on the formation of CD151-integrin complexes

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Aim: To assess the roles of extracellular signal-regulated kinase (ERK), p38, and CD151-integrin complexes on proliferation, migration, and tube formation activities of CD151-induced human umbilical vein endothelial cells (HUVECs).

Methods: CD151, anti-CD151 and CD151-AAA mutant were inserted into recombinant adeno-associated virus (rAAV) vectors and used to transfect HUVECs. After transfection, the expression of CD151 was measured. Proliferation was assessed using the 3-[4,5-dimethylthiazol- 2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. Cell migration was evaluated in Boyden transwell chambers using FBS as the chemotactic stimulus. The tube formation assay was performed on matrigel. The potential involvement of various signaling pathways was explored using selective inhibitors.

Results: CD151 gene delivery increased the expression of CD151 at both the mRNA and protein levels. Overexpression of CD151 promoted cell proliferation, migration and tube formation *in vitro*, and phosphorylation of ERK was also increased. Further, CD151-induced cell proliferation, migration, and tube formation were attenuated by the ERK inhibitor PD98059 (20 μmol/L) but not by a p38 inhibitor (SB203580, 20 μmol/L). Moreover, there was no significant difference in CD151 protein expression between the CD151 group and the CD151-AAA group, but the CD151-AAA mutant abrogated cellular proliferation, migration, and tube formation and decreased the phosphorylation of ERK.

Conclusion: This study suggests that activation of the ERK signaling pathway may be involved in the angiogenic effects of CD151. Activation of ERK was dependent on the formation of CD151-integrin complexes. Therefore modulation of CD151 may be as a novel therapeutic strategy for regulating angiogenesis.

Keywords: CD151; cell migration; cell proliferation; angiogenesis; ERK; p38 MAPK; human umbilical vein endothelial cells

Introduction

As a member of the transmembrane 4 superfamily (TM4SF), CD151 (also known as platelet-endothelial cell tetraspan antigen 3, PETA-3) contains two extracellular loops, four hydrophobic transmembrane domains, and two short cytoplasmic tails[1, 2]. CD151 is broadly expressed in various cell types, but in endothelial cells it is characteristically localized to cell–cell junctions and endosomes[3, 4]. Previous studies have shown that CD151 is involved in regulating cell motility, adhesion, spreading, and morphogenesis[1–6]. Over the past decade, accumulating evidence has suggested that CD151 plays a crucial role in angiogenesis[7–10]. CD151 knock-out cells display markedly reduced endothelial events related to angiogenesis (migration, spreading, invasion, matrigel contraction, tube formation and spheroid sprouting)[7]. Our research group previously demonstrated that transfection with CD151 cDNA enhanced endothelial cell proliferation, migration and tube formation and significantly up-regulated endothelial nitric-oxide synthase (eNOS) expression via the PI3K/Akt signaling pathway[8]. Furthermore, we found that delivery of the CD151 gene increased the number of microvessels in a pig myocardial ischemia model and a rat ischemic hindlimb model, suggesting that CD151 is a potential target for therapeutic angiogenesis[9, 10]. However, the molecular mechanisms that govern the effects of CD151 in angiogenesis have not been well elucidated.

Several studies have demonstrated that CD151 forms

complexes with the integrins α3β1, α6β4 and α6β1 and that the CD151-α3β1 integrin complex has unusually high stoichiometry, proximity, and stability[3, 5, 11]. Notably, the specific QRD[194–196] motif of CD151 is necessary for α3β1 interaction. This motif is located in CD151's large extracellular loop[12]. When the key motif was mutated, CD151's ability to complex with integrin α3 was impaired[12, 13]. CD151 may also use the same site to form complexes with α6β1, α6β4, and other integrins[5, 12, 13]. In parallel, the intracellular domain of CD151 may determine the molecule's association with signaling molecules such as PtdIns 4-K and PKC[11, 14]. Hence, a "transmembrane linker" model for CD151 has been proposed, and CD151 signaling is thought to be mediated by CD151-integrin complexes[6, 14, 15].

Mitogen-activated protein (MAP) kinases play important roles in various cellular responses[16–18]. In this study, using a gene transfer technique with a recombinant adeno-associated virus (rAAV) vector, we examined the roles of MAP kinases, particularly extracellular signal-regulated kinase (ERK) and p38 MAP kinase (p38), in CD151–induced endothelial cell proliferation, migration and tube formation. In addition, we mutated the QRD[194–196] motif and observed the effects of this CD151 mutant. The purpose of the present study was to investigate the mechanism(s) by which CD151 induces angiogenesis.

Materials and methods

Materials

All cell culture reagents were obtained from Invitrogen (Carlsbad, CA), including Dulbecco's modified Eagle's medium (DMEM), trypsin and fetal bovine serum (FBS). The restriction enzymes were purchased from TaKaRa (Dalian, China). Antibodies against ERK1/2 (also called p42/44 MAPK), phospho-ERK1/2, p38, phospho-p38, CD151, and β-actin were purchased from Santa Cruz Biotechnology, Inc (California, USA). PD98059 and SB203580 were supplied by Calbiochem Novabiochem (Darmstadt, Germany). The enhanced chemiluminescent (ECL) substrate was obtained from Pierce (Rockford, Illinois, USA). Hybrisol solution was purchased from Intergen (Purchase, NY). Tween-20, PMSF, aprotinin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich Chemical Co (St Louis, MO). Matrigel was purchased from B&D Biosciences (Heidelberg, Germany). [α-^{32}P]-dCTP (3000 Ci/mmol) was obtained from Rui Fu Biological Engineering Company Nucleic Acid Laboratory (Beijing, China). All other chemicals and reagents were purchased from Sigma-Aldrich unless otherwise specified.

Construction of pAAV-CD151, pAAV-anti-CD151, pAAV-CD151-AAA, and pAAV-GFP

The PzeoSV-CD151 plasmid was kindly provided by Dr Xin A ZHANG, Department of Molecular Science, University of Tennessee Health Science Center (Memphis, Tennessee, USA). The construction of pAAV-CD151, pAAV-anti-CD151, and pAAV-Green fluorescent protein (GFP) has been described previously[8–10, 19]. The CD151 mutant was generated by recombinant PCR. The pAAV-CD151 vector contained the full-length wild-type human CD151, and cDNA was used as the template. For the CD151-AAA mutant, the following primers were used: 5'-TGTGGAATTAATTTCCATGCCTCCAACATC-3' (internal sense primer to amplify the 3'-region) and 5'-GGCATGGAAATTAATTCCACAAAGAGCCAC-3' (internal antisense primer to amplify the 5'-region) of the CD151 template. Then we used either external sense (5'-GCTTAGATCTGCCACCATGGGTGAGTTCAACGAG-3') or external antisense (5'-GACGCGGCCGCTCAGGCGTAGTCGGG-3') primers. The final recombinant PCR was performed using purified PCR products and external sense and external antisense primers. The final PCR products were incised by the *Bgl* II and *Not* restriction enzymes, and then the incised products were purified and ligated into the AAV vector at the *Bam* H I and *Not* I restriction sites. Proper ligation was confirmed by sequencing.

Construction and preparation of recombinant adeno-associated virus

The rAAV vector pXXUF1, packaging plasmid pXX2, adenovirus helper plasmid pHelper, and a rAAV plasmid containing GFP cDNA were obtained from Dr Xiao XIAO (University of Pittsburgh, Pittsburgh, PA).

The packing and production of rAAV-GFP, rAAV-CD151, rAAV-antiCD151, and rAAV-CD151-AAA were carried out using a triple-plasmid cotransfection method in human embryonic kidney cells (293 cells)[10, 20]. The cells can be transfected at 70% to 80% confluence. A total of 85 mg of plasmid DNA (the molar ratios of dsAAV-GFP or dsAAV-CD151 or dsAAV-antiCD151 or rAAV-CD151-AAA to pXX2 and pHelper were 1:1:1) was dissolved in 0.25 mol/L CaCl$_2$, and the solution was quickly mixed with 2×BES [*N,N*-Bis(2-hydroxyethyl)taurine]-buffered saline and added to the cells in a 15-cm plate. The cells were harvested 48 to 72 h after transfection. After three cycles of freeze-thaw and centrifugation, the rAAVs remained in the supernatant, and single-step gravity-flow column purification was applied[21]. The eluted rAAVs were aliquoted and stored at -80 °C. The titers of vector particles were determined by quantitative DNA dot-blot hybridization using [α-^{32}P]-dCTP[20, 21].

Isolation and culture of endothelial cells

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical veins by collagenase treatment. The cells were grown in DMEM supplemented with 20% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 5 U/mL heparin at 37 °C under 5% CO$_2$ and 95% air. Only cells passaged less than five times were used for experiments.

Transfection of endothelial cells with rAAVs

Cells were grown to 60% confluence, the medium was removed, and the cells were washed three times with PBS and incubated with DMEM containing 0.5% FBS at 37 °C for 12 h. We directly added corresponding volumes of rAAV-

GFP, rAAV-CD151, rAAV-antiCD151 or rAAV-CD151-AAA to the medium (about 50 virions/cell) in 6-well plates, according to the titers of vector particles. For the control group, PBS was added to the medium. Then the cells were grown under 5% CO_2 and 95% air overnight. The next day, 1 mL DMEM supplemented with 20% fetal bovine serum was added to every group. Cells were incubated in the conditions above for 5 days and then subjected to the following assays.

Proliferation assay

HUVECs transfected with rAAV-CD151, rAAV-antiCD151, rAAV-CD151-AAA, or rAAV-GFP were trypsinized and seeded in 96-well plates (1×10^4 cells/well) . After attachment, the cells were exposed to DMEM with 0.5% FBS for 48 h and then the effects of CD151 on HUVEC proliferation were evaluated using the MTT colorimetric assay. Briefly, the medium was removed and replaced with medium containing 5 mg/mL MTT, and the cells were incubated for 4 h. The medium was then aspirated, and the product was solubilized with dimethyl sulfoxide (DMSO). Absorbance for each well was measured at 570 nm using a microplate reader (Bio-Tek Instrument, USA).

Migration assay

HUVECs transfected with rAAV-CD151, rAAV-antiCD151, rAAV-CD151-AAA, or rAAV-GFP were exposed to DMEM with 0.5% FBS for 48 h and then cell migration was assayed using a modified Boyden chamber technique. Briefly, 200 µL DMEM containing 10% FBS was added to the bottom well. Cells were resuspended in the appropriate buffer at a concentration of 10^6 cells/mL, and 800 µL of the cell suspension was added to the top well of the transwell chambers. A filter with 8 µm pore size separated the bottom and top wells. After incubation for 24 h at 37 °C in a 5% CO_2 atmosphere, the cells that had not migrated were removed from the upper surface, and those that migrated to the lower surface of the filters were fixed in methanol and stained with hematoxylin. Migrating cells adherent to the underside of the filter were enumerated using an ocular micrometer and by counting 10 high-powered fields (HPFs, ×200). Data are presented as relative migration (number of cells/HPF).

Tube formation

HUVECs transfected with different rAAV viruses were exposed to DMEM with 0.5% FBS for 48 h, and then the cells were plated in 24-well plates with Matrigel. Matrigel (0.5 mL) was polymerized on 24-well plates, and 5×10^4 cells were then plated in full-growth medium for 1 h. Once the cells were seeded, the medium was replaced with medium containing 0.5% serum. Tube formation was visualized using an inverted microscope (Nikon TE 2000) equipped with digital imaging. For each treatment, 10 field images were captured, and the area containing endothelial tubes and networks that had formed was quantified using the Scion Image Analysis System (windows version of scion image, NIH) with background subtraction.

RT-PCR analysis

HUVEC RNA was extracted using Trizol (Gibco) as described previously[5]. RT-PCR analysis specific for human CD151 (Genbank sequence NM 004357) with an HA tag (forward primer, 5′-ATGGGTGAGTTCAACGAG-3′; reverse primer, 5′-GCCGCTCAGGCGTAGTC -3′) and β-actin (forward primer, 5′-GGAGAAGGACCCAGATC-3′; reverse primer, 5′-GATCT-TCATGAGGTAGTCAG-3′) was then performed using an RT-PCR kit (Takara Biotechnology) according to the manufacturer's instructions. The conditions for PCR were one cycle of denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 45 s, 60 °C for 40 s, and 72 °C for 1 min, with a final extension at 72 °C for 7 min. PCR products were resolved in 1.5% agarose gels and stained with ethidium bromide.

Western blot analysis

HUVEC protein was extracted as follows. Briefly, the medium in six-well plates was discarded, and cells were gently washed three times with cooled PBS. Lysis buffer (500 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 0.02% sodium azide, 0.1% SDS, 100 µg/mL phenylmethylsulfonyl fluoride, 1 µg/mL aprotinin, 1% Nonidet P-40, and 0.5% sodium deoxycholate) was added to the cells (0.25 mL/well). After incubation on ice for 30 min, the lysate was centrifuged at 12 000×*g* at 4 °C for 10 min. The protein concentration of the supernatant was determined using the Bradford method. Lysates (25 µg protein/lane) were resolved by SDS-PAGE gel, transferred to nitrocellulose membranes, and blocked with 5% nonfat dry milk in 10 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl, and 0.1% Tween 20. The membranes were then incubated with primary antibodies against CD151, ERK1/2, phospho-ERK1/2, p38, and phospho-p38 overnight at 4 °C. Peroxidase-conjugated secondary antibodies were applied for 2 to 3 h. The ECL system was used to visualize the separated proteins. Blots were stripped and reprobed with β-actin as a loading control. The intensities of the various protein bands were quantified by densitometry (GeneTools analysis software).

Evaluation of signaling pathways

To examine the signaling mechanisms through which CD151 enhances endothelial cell proliferation, migration, and tube formation, inhibitors of ERK (PD98059, 20 µmol/L) and p38 (SB203580, 20 µmol/L) were added to cultured HUVECs for 8 h. The effects of these inhibitors on endothelial cell proliferation, migration, and tube formation were observed.

Statistical analysis

Data were analyzed using SPSS 13.0 statistical software (SPSS Inc, USA). Each variable was examined to determine whether it was normally distributed using the Shapiro-Wilk test. Data were expressed as means±SEM. Comparisons between two groups were performed using Student's *t*-tests. Three or more groups were compared by analysis of variance followed by the Newman-Keuls test. *P* values less than 0.05 were considered significant.

Results

Expression of CD151 in different groups

HUVECs transfected with rAAV-GFP were observed with an inverted fluorescence microscope (Figure 1A). In our experiment, CD151 was linked to an HA-tag, and RT-PCR analysis was specific for CD151 with this tag. Therefore, CD151 mRNA was detected in the CD151 group (positive), but not in the control, GFP and anti-CD151 groups (Figure 1B). Similar levels of β-actin mRNA were detected in all groups, confirming the integrity of prepared RNA.

Western blot analysis showed that expression of CD151 protein increased significantly in the CD151 group compared with the control group and the GFP group, but it decreased dra-
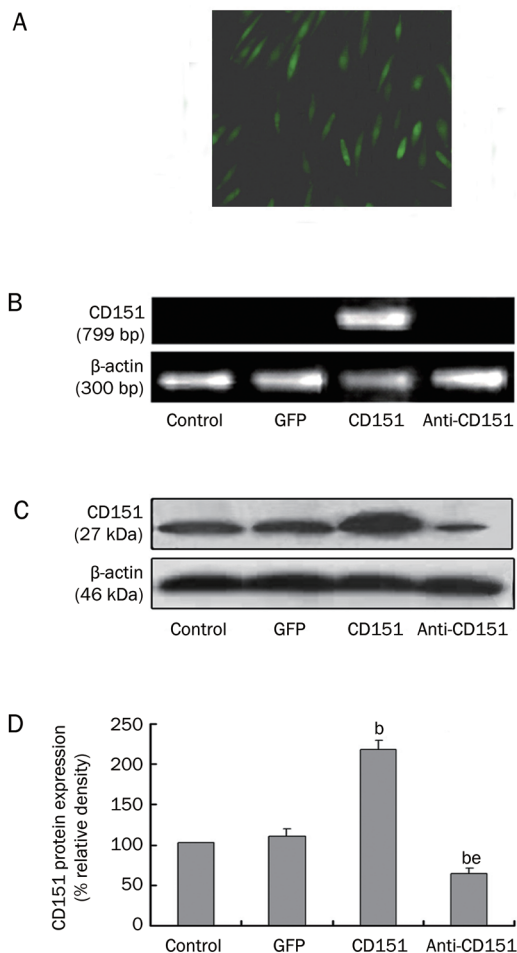
Figure 1. CD151 transfection increases the expression of human CD151 mRNA and protein. (A) HUVECs transfected with rAAV-GFP observed with inverted fluorescence microscope 7 days after transfection. (B) Detection of human CD151 gene expression by RT-PCR. The anticipated sizes of PCR products for CD151 and β-actin were 799 and 300 bp, respectively. The human CD151 mRNA was detected in CD151 group, but not in the control, GFP and anti-CD151 groups. (C) and (D) Western blot analysis for CD151 in HUVECs transfected with rAAV-GFP, rAAV-CD151 or rAAV-anti-CD151. β-actin was used as an internal loading control. The mean density of CD151 in control group was defined as 100%. $^bP<0.05$ *vs* the control group and GFP group. $^eP<0.05$ *vs* the CD151 group. *n*=3. Data are mean±SEM.

matically in the anti-CD151 group (Figures 1C and 1D). There were no significant differences in the expression of CD151 between the control group and the GFP group. These results indicate that rAAV-CD151 transfection increases CD151 expression.

Effects of CD151 on the proliferation, migration and tube formation of HUVECs

Endothelial cell proliferation, migration and tube formation are important processes in angiogenesis and vessel sprouting[22]. To determine the effects of rAAV-mediated CD151 gene transfection on HUVEC proliferation, migration and tube formation, we conducted experiments using the MTT assay, transwell Boyden chambers, and matrigel, respectively[8].

First, the MTT assays showed that rAAV-CD151 transfection significantly enhanced the proliferation of HUVECs, whereas proliferation was significantly inhibited in the anti-CD151 group (Figure 2A). Next, in Boyden chamber assays, we observed that the number of migrated cells on the underside of the filter in the CD151 group was much greater than that in the control group and the GFP group (Figures 2B). In contrast, the anti-CD151 group showed less migration. Finally, we examined the effect of CD151 on tube formation. Matrigel tests demonstrated that rAAV-mediated CD151 gene transfection significantly increased tube formation compared with the control group and the GFP group. In contrast, the anti-CD151 group exhibited decreased tube formation (Figure 2C and 2D).

These results suggest that CD151 transfection promotes endothelial cell proliferation, migration and tube formation, but anti-CD151 transfection inhibits those effects.

Effects of CD151 on the phosphorylation of ERK and p38

Western blot analysis revealed that transfection with rAAV-CD151 increased expression of phosphorylated ERK compared with the control group and the GFP group, whereas transfection with rAAV-anti-CD151 decreased expression of phosphorylated ERK (Figure 3A). However, there were no significant differences in the expression of phosphorylated p38 among the four groups (Figure 3B), suggesting that overexpression of CD151 may not influence the phosphorylation of p38. These data suggest that CD151 could activate the ERK signaling pathway, but it likely does not involve the p38 pathway.

Roles of ERK and p38 signaling pathways in CD151-induced cell proliferation, migration and tube formation

To investigate the roles of ERK and p38 in CD151-induced proliferation, migration, and tube formation of endothelial cells, we applied inhibitors of ERK (PD98059) and p38 (SB203580) to HUVEC cultures following transfection with rAAV-CD151.

We have shown that CD151 can promote endothelial cell proliferation, migration and tube formation. Treatment with the ERK inhibitor PD98059 resulted in attenuated effects of CD151 on proliferation, migration and tube formation (Figure 4A–4C). This suggests that ERK is involved in CD151-mediated cell responses. In contrast, treatment with the p38 inhibitor SB203580 did not significantly inhibit CD151-induced cell
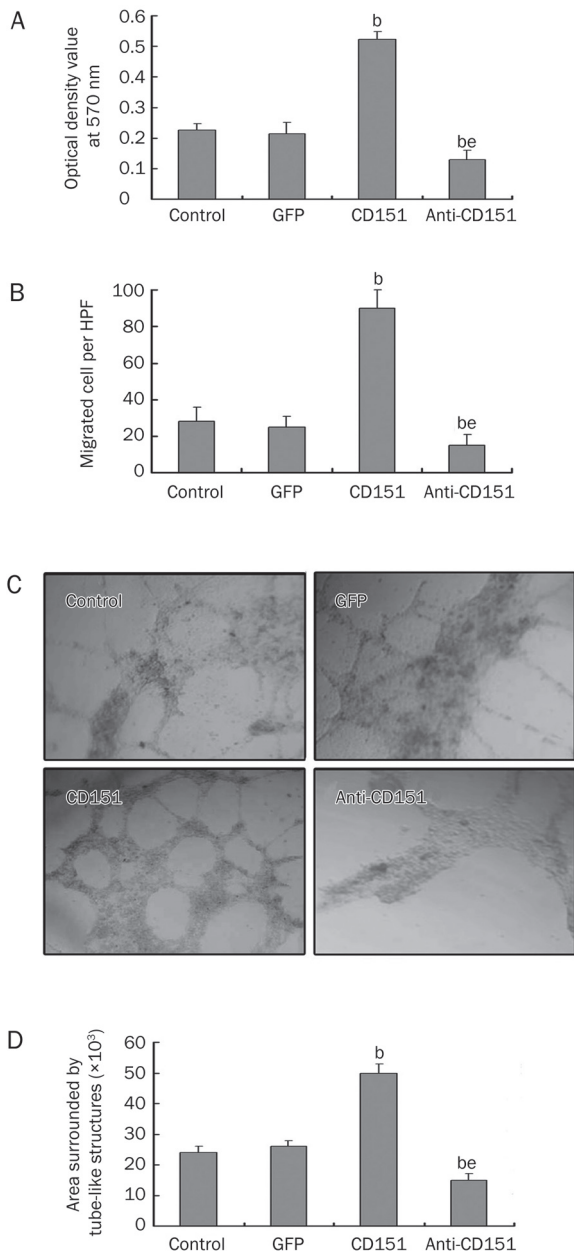
Figure 2. CD151 gene delivery promotes the proliferation, migration, tube formation of HUVECs. (A) MTT assay results after HUVECs transfected with rAAV-CD151, rAAV-anti-CD151 and rAAV-GFP. rAAV-CD151 transfection significantly enhanced HUVECs proliferation as compared with the control and GFP groups. (B) Transwell boyden chamber assays showed rAAV-CD151 transfection promoted the migration of HUVECs. (C) and (D) Matrigel test demonstrated that rAAV-CD151 transfection dramatically increased the tube formation of HUVECs. (C) Representative photomicrographs showing effect of CD151 on tube formation (×100). (D) Quantitative analysis of the effect of CD151 on tube formation. $^{b}P<0.05$ *vs* the control group and GFP group. $^{e}P<0.05$ *vs* the CD151 group. *n*=3. Mean±SEM.

proliferation, migration and tube formation (Figure 4A–4C). Combined, these data suggest that CD151 enhances endothelial cell proliferation, migration and tube formation via the
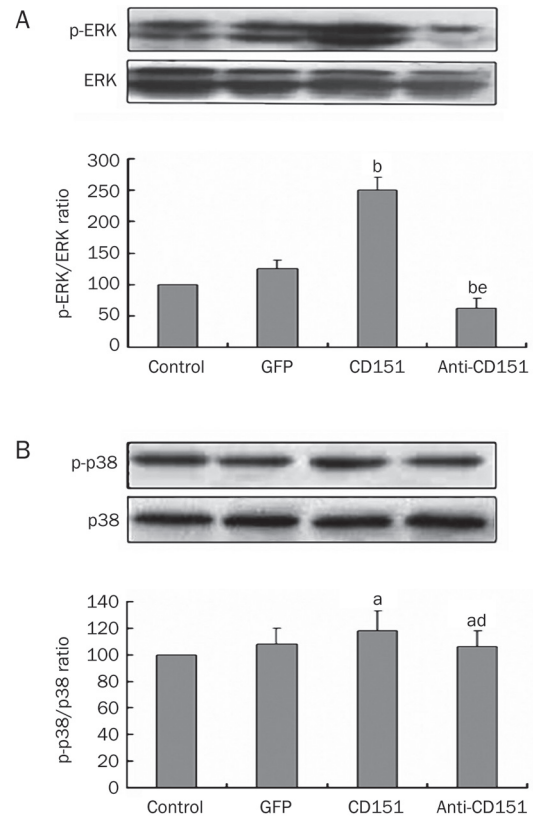

Figure 3. Effects of rAAV-CD151 transfection on the phosphorylation of ERK and p38. (A) Western blot analysis for ERK, phosphorylated ERK in control group and the rAAV-GFP, rAAV-CD151 and rAAV-anti-CD151 groups. rAAV-CD151 transfection increased the phosphorylation of ERK. (B) Western blot analysis showed rAAV-CD151 transfection did not influence the phosphorylation of p38. $^{a}P>0.05$, $^{b}P<0.05$ *vs* the control group and GFP group; $^{d}P>0.05$, $^{e}P<0.05$ *vs* the CD151 group. *n*=3. Mean±SEM.

ERK signaling pathway, but p38 is likely not involved.

Effects of CD151-AAA mutant on CD151-induced cell proliferation, migration and tube formation

To investigate the function of CD151-integrin complexes, we first mutated the QRD[194–196] motif of CD151 (glutamine, arginine, aspartic acid) to AAA[194–196] (three consecutive alanines), resulting in a mutant to which integrins cannot bind (Figure 5A). Western blot analysis showed that there was no significant difference in the expression of CD151 between the CD151 group and the CD151-AAA group, suggesting that the CD151-AAA mutant does not influence the expression of CD151 protein (Figure 5B). However, the level of phosphorylated ERK was significantly decreased in the CD151-AAA group compared with the CD151 group (Figure 5C). These data suggest that the CD151-AAA mutant results in reduced activation of ERK via phosphorylation.

Next, we further studied the proliferation, migration and tube formation of HUVECs transfected with rAAV-CD151-AAA. CD151-induced proliferation of HUVECs was impaired in the CD151-AAA group (Figure 5D). Similarly, transwell
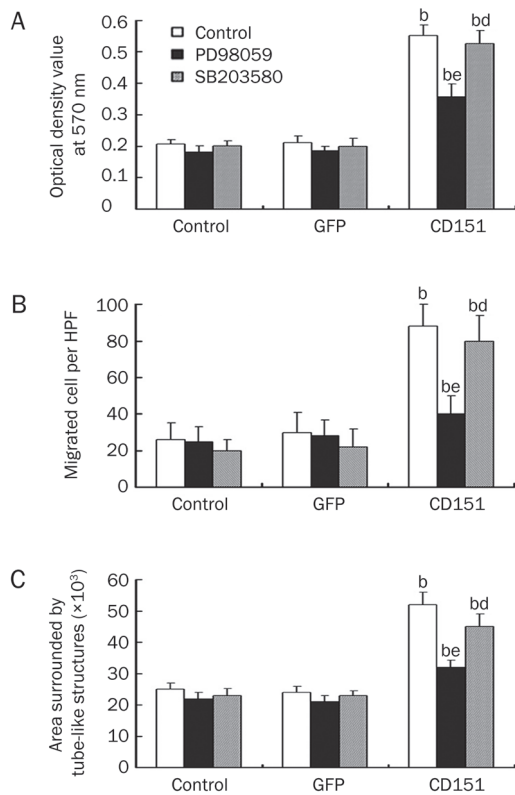
A

B

C

Figure 4. rAAV-CD151 transfection promotes the proliferation, migration, tube formation of HUVECs via ERK signaling pathway, but not via p38. (A) MTT assays showing effects of inhibitors of ERK (PD98059, 20 μmol/L), and p38 inhibitor (SB203580, 20 μmol/L) on CD151-induced HUVECs proliferation. (B) Transwell Boyden chamber assays showing effects of inhibitors of ERK (PD98059, 20 μmol/L) and p38 inhibitor (SB203580, 20 μmol/L) on CD151 enhancement of HUVECs migration. (C) The effects of inhibitors of ERK (PD98059, 20 μmol/L), and p38 inhibitor (SB203580, 20 μmol/L) on CD151-induced HUVEC tube formation on matrigel. $^bP<0.05$ *vs* the control and GFP groups. $^dP>0.05$, $^eP<0.05$ *vs* the CD151 group without inhibitors. $n=3$. Mean±SEM.

Boyden chamber assays demonstrated that the increase in migration observed in HUVECs treated with CD151 was also impaired in the CD151-AAA group (Figure 5E). CD151-AAA transfection decreased tube formation on matrigel compared with the CD151 group (Figure 5F and 5G). Together, these data suggest that delivery of the CD151-AAA mutant abrogates endothelial cell proliferation, migration and tube formation, which are promoted by CD151 gene delivery.

Discussion

Angiogenesis is the formation of new blood vessels from pre-existing vessels. It is a physiological or pathological neovascularization process in response to tissue ischemia and/or tumor growth[22]. Angiogenesis is a complex process involving extracellular matrix degradation, endothelial cell proliferation and migration, formation of tube structures and morphological differentiation[22, 23]. Recently, mounting evidence supports the hypothesis that CD151 plays an important role in angiogene-

sis, in addition to its recognized ability to regulate the motility and adhesion of cells[7–10].

CD151 is broadly expressed in various cell types[3, 4]. Using an *in vitro* study, our group demonstrated that rAAV-mediated CD151 gene transfer increased the expression of CD151. To differentiate between endogenous and exogenous CD151, we constructed an HA-tag linked to the transfected CD151, and the reverse primer for RT-PCR was designed specifically for the HA-tag. Therefore, the CD151 mRNA was only detected in the CD151 group, but not in other groups (Figure 1B). This interesting technique could be used to differentiate between the endogenous gene and the transfected gene, which is also expressed in basal conditions. In the present study, we also observed that CD151 gene delivery promoted endothelial cell proliferation, migration and tube formation, which is consistent with our previous data[8, 24]. However, the molecular mechanisms underlying these effects are not known.

Previous studies have shown that activation of ERK is generally associated with cell proliferation, angiogenesis and tumor metastasis[17, 25]. p38 is usually activated by inflammatory cytokines and leads to cell apoptosis[16, 18]. Interestingly, several recent papers revealed different data about the activation of ERK or p38 in the CD151 signaling pathway[6, 7, 26]. In MelJuSo melanoma cells, inhibitors and small interfering RNAs targeted to p38 were shown to abrogate CD151-enhanced cell migration and adhesion[6]. However, another study found that absence of CD151 did not affect phosphorylation of components of the MAPK pathway, such as p38 and ERK[7]. It seems likely that ERK and p38 would show different responses in the CD151-related signaling pathway, depending on the cellular context. The present work demonstrated that overexpression of CD151 increased the expression of phospho-ERK, indicating that CD151 gene transfection could activate the ERK pathway.

To further determine the role of ERK in CD151-mediated biological processes, we examined the influence of the ERK inhibitor PD98059. The addition of PD98059 to cultures of HUVECs attenuated the CD151-induced proliferation, migration and tube formation of these cells. Therefore, ERK upregulation may mediate, at least in part, the angiogenic effects of CD151. In contrast, we found that CD151 gene transfection did not influence the activation of p38, and an inhibitor of p38 (SB203580) did not significantly attenuate the CD151-mediated angiogenic effects.

The mechanisms by which CD151 could activate ERK are not currently known. Earlier studies have shown that CD151 forms multimolecular complexes with many other transmembrane proteins[11, 14, 27]. In particular, CD151 forms strong complexes with some integrins via the QRD motif[194–196] [12, 13]. The formation of CD151-integrin complexes is reported to function as a "signal bridge" linking the "outside-in" signal transduction[6, 28–30]. Based on these data, we hypothesized that CD151 may also exert angiogenic effects through the formation of CD151-integrin complexes. Therefore, we mutated the QRD[194–196] motif of CD151 to AAA[194–196] in the present study, interfering with the formation of complexes between CD151
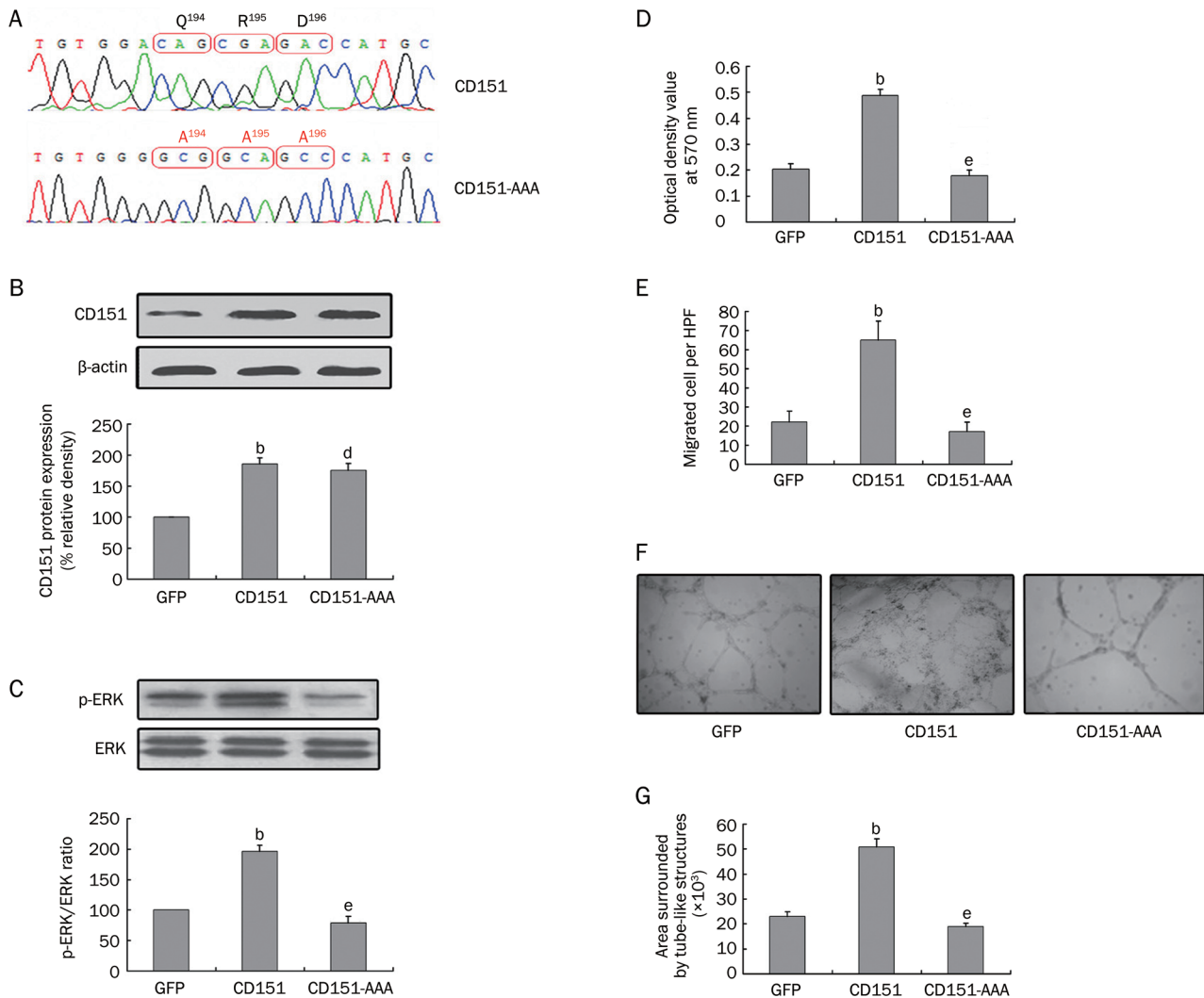
Figure 5. The effects of CD151-AAA on the proliferation, migration and tube formation of HUVECs. (A) The gene sequences of CD151 and CD151-AAA. The CD151 mutation (CD151-AAA) was undergone as follows: change the motif of QRD$^{194-196}$ to AAA$^{194-196}$. (B) Western blot analysis demonstrated there was no significant difference of CD151 protein expression between the CD151 group and the CD151-AAA group. (C) The effect of CD151-AAA on the phosphorylation of ERK. CD151 promoted the phosphorylation of ERK, but the CD151-AAA mutant abrogated this effect. (D) MTT assay showed that the CD151-induced proliferation of HUVECs was impaired in the CD151-AAA group. (E) Transwell Boyden chamber assays demonstrated the promoted migration of HUVECs was impaired in the CD151-AAA group. (F) and (G) The rAAV-CD151-AAA transfection abrogated the tube formation on matrigel. (F) Representative photomicrographs of tube formation. (G) Quantitative analysis of HUVECs tube formation. $^{b}P<0.05$ *vs* the GFP group. $^{d}P>0.05$, $^{e}P<0.05$ *vs* the CD151 group. *n*=3. Mean±SEM.

and integrins[12, 13]. Surprisingly, we found that the level of phospho-ERK in the CD151 mutant group was significantly decreased, but the level of CD151 protein was not significantly different in the CD151 and CD151 mutant (CD151-AAA) groups. These results provide perhaps the clearest evidence that impaired CD151-integrin complexes could significantly attenuate the phosphorylation of ERK, indicating that the formation of CD151-integrin complexes may be necessary for the activation of ERK.

Furthermore, the present study also revealed that cell proliferation, migration, and tube formation in the CD151 mutant group (CD151-AAA) were all decreased compared with the CD151 group. When CD151 could not complex with integrins, the effects of CD151 were all diminished. It seems that CD151-mediated promotion of angiogenesis depends on the formation of CD151-integrin complexes. Taken together, these data suggest that CD151 may activate the ERK signaling pathway and promote angiogenesis by forming complexes with integrins.

In summary, our data demonstrate that CD151 overexpression promotes endothelial cell proliferation, migration and tube formation via activation of the ERK signaling pathway. Furthermore, these effects are likely dependent on the formation of CD151-integrin complexes. To our knowledge, this is the most recent evidence showing ERK activation and the effects of CD151-integrin complexes in CD151-induced angio-

genesis. This study provides clues to better understand the function and molecular mechanisms of CD151. Our observations may also lend insight that could assist in the development of CD151 as a novel therapeutic strategy for regulating angiogenesis.

Acknowledgements

Author contribution

Zheng-xiang LIU designed the research and handled funding; Hou-juan ZUO, Jing-yang LIN, Zhao-yu LIU performed the research, analyzed data and drafted the manuscript; Wei-feng LIU and Tao LIU performed the research; Jun YANG and Yu LIU analyzed data and drafted part of the manuscript; Dao-wen WANG contributed new reagents and analytical tools and revised the manuscript.

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