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# CREG1 promotes angiogenesis and neovascularization

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## TABLE OF CONTENTS



## 1. ABSTRACT

Angiogenesis has long been considered as an important strategy for ischemic injury. It has been reported that cellular repressor of E1A-stimulated genes (CREG1) promotes human umbilical vein endothelial cell (HUVEC) proliferation, migration, and protects endothelial cell (EC) from apoptosis. However, its potential effect on angiogenesis remains undefined. In the present study, we investigated the role and mechanisms of CREG1 in promoting angiogenesis. We found that adenovirus-transduced CREG1 expression in HUVECs increases EC tube formation in matrigel and promotes neovascularization in matrigel plugs grafted into wild type mice. In addition, adenoviral CREG1 expression enhances filopodia formation, which is accompanied by increased expression of integrin-linked kinase (ILK) and activation of its downstream effector Cdc42. Hindlimb perfusion was significantly reduced after femoral artery ligation in CREG1 heterozygous knockout mice. Finally, adenoviral CREG1 was injected intramuscularly in gastrochemius and partially restores ischemic hindlimb perfusion. Our results demonstrated that CREG1 increases EC filopodia formation and vascular assembly via ILK-Cdc42 activation and promotes neovascularization, which might be a therapeutic target for ischemic injury.

## 2. INTRODUCTION

Angiogenesis is a complex process involving endothelial cell (EC) proliferation, activation, sprouting and migration, which leads to vessel growth from primary plexus or existing vessels and functioning circulatory system generation (1). The discovery of the angiogenesis molecular mechanism helps to treat two diseases: 1) ischemia caused by vasoconstriction, thrombosis or embolism, where tissue damage can be repaired by therapeutic angiogenesis (2-5); and 2) diseases such as retinopathy and tumors that can be cured or delayed by inhibition of pathological angiogenesis (6-8). Large efforts of angiogenesis therapy have been focused on improving endothelial cell survival, proliferation, motility and function. Therapeutic angiogenesis are currently being tested in clinical trials to treat human ischemic diseases, such as limb ischemia, myocardial infarction, and stroke, using growth factor, diverse proteins with intrinsic proangiogenic activity, stem/progenitor cells and pharmacological molecules administration (9).

During angiogenesis, activated ECs degrade local basement membrane and the vessel begins to "sprout" with

migrating tip cells leading a column of proliferating stalk cells (10). It continually develops into a network and form vessel lumens. The "sprouting" is a key cellular process that acts as rate-limiting step in angiogenesis. Many genes, such as *VEGF*, *FGF2*, *NOTCH*, *Wnt* and *DLL4* have emerged as critical regulators of proper blood vessel sprouting (11-15). However, the underlying molecular mechanisms remain largely unknown.

Cellular repressor of E1A-stimulated genes (CREG1) is a glycoprotein which suppresses oncogene E1A transcription and cellular transformation (16), attenuates NTERA-2 (human teratocarcinoma cell) proliferation (17, 18), induces neuron differentiation and mediates glucocorticoid-induced proliferation in ileal epithelial cells (19). Our previous study demonstrated that CREG1 is expressed abundantly in the adult vascular endothelium and dramatically decreases during atherogenesis (20). Adenoviral-transduced CREG1 expression promotes re-endothelialization in balloon-injured arteries (21, 22), activates ECs, and increases EC permeability (23, 24). The observations suggest that CREG1 might play an important role in angiogenesis. In this study, we showed that adenoviral CREG1 promotes EC filopodia formation, angiogenesis and neovascularization. These effects are likely mediated by integrin-linked kinase (ILK)/Cdc42 signaling pathway.

## 3. MATERIALS AND METHODS

### 3.1. Animals

*Creg1*$^{+/-}$ mice were on a mixed C57BL/6×sv129 genetic background (1:1). Mice genotype was examined by PCR amplification of tail DNA (data not shown). Wild type mice (C57BL/6) were obtained from Department of Laboratory Animal Science, Peking University Health Science Center, China. Mice were maintained and handled in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals (Institutional Animal Care and Use Committee of Northern Hospital, Shenyang, China).

### 3.2. Primary human umbilical vein endothelial cells (HUVECs) culture

HUVECs were isolated from human umbilical cords and cultured in medium 199 (Invitrogen, Carlsbad, CA) containing 10% (v/v) fetal bovine serum (FBS, HyClone, UT) and EC growth supplements (rhVEGF: 5 ng/ml, rhEGF: 5 ng/ml, rhFGF basic: 5 ng/ml, rhIGF-1: 15 ng/ml) at 37°C in an atmosphere containing 5% $CO_2$. Endothelial cell identity was confirmed by immunostaining with CD31 and VE-cadherin antibodies. Passage 2-6 were used in all experiments (20). The murine EC line (SVEC 4-10) was purchased from American Type Culture Collection (Manassas, VA).

### 3.3. Adenoviral infection

Adenoviral CREG1 (AdCREG1) and its vector control (AdGFP) were created as described previously (21). These replication-deficient virus was propagated in 293 cells with DMEM supplemented with 10% (v/v) FBS. ECs were transduced with adenovirus (20 MOI) (multiplicity of infection) for 2 consecutive days. The expression of CREG1 was assessed by Western blot.

### 3.4. Western blotting

Proteins were separated on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were incubated with appropriate primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies. Specific binding was detected using enhanced chemiluminescence reagents from GE (Piscataway, NJ). Blots were quantified using a Bio-Rad gel documentation system (Hercules, CA). Experiments were performed in triplicate.

### 3.5. *In vitro* angiogenesis assays

The formation of tube-like structures by HUVECs was performed as described previously (25). Briefly, 24-well plates were coated with matrigel (300 µl/well) and polymerized for 30 min at 37°C. HUVECs ($1\times10^5$) were suspended in 500 µl FBS-free DMEM and seeded onto polymerized matrigel in each well and incubated at 37°C for 16 h. Tube formation was observed by inverted phase contrast microscope and images were captured by a video graphic system. Tube formation was quantified by measuring the length of tubes in six randomly chosen fields in each well using Leica QWN imaging software (Leica, Germany). For filopodia protrusion assay, $3\times10^4$ HUVECs were plated on Nunc 4-well chamber slides surrounding precast three-dimensional (3D) matrigel. Cells migrated overnight and invaded into matrigel. Filopodia were counted in each well and their lengths were measured using imaging software.

### 3.6. *In vivo* matrigel plug assay

*In vivo* matrigel plug assay was performed as described previously (26). 0.6 ml matrigel containing $1\times10^5$ SVECs was infected by adenovirus and injected subcutaneously near the abdominal midline in 6-8 week old C57BL/6 mice (n=5 per group). After 14 days, the matrigel pellets were collected, fixed in 4% (w/v) paraformaldehyde for 1 h, infused in 7.5% (w/v) sucrose overnight and frozen in OCT embedding compound. Cryosections were stained with hematoxylin-eosin (HE) and anti-CD31 antibody to examine the formation of blood vessels. The vessel size was quantified at 10× magnifications by measuring vessel area in five randomly chosen fields each well using Leica QWN imaging software (Leica, Germany).

### 3.7. Immunofluorescent staining

Sections or cells were fixed with 4% (w/v) paraformaldehyde for 20 min. After washing three times with PBS, samples were permeabilized with 1% (v/v) Triton X-100 in PBS for 30 min and blocked with goat serum for 20 min at room temperature. Samples were then stained with CD31 primary antibody and Alexa Fluor-555 IgG secondary antibody (Invitrogen, Grand Island, NY) (1:300). Nuclei were counterstained with DAPI. Quantification was done by Leica QWN analysis system. Experiments were performed in triplicate.

### 3.8. Spheroid sprouting assay

The assay was performed as described previously (27) with modifications. HUVEC spheroids were generated by hanging-drop culture of 500 HUVECs in EBM-2 medium, 10 % FCS and 20% methylcellulose (Sigma

Biochemicals) overnight. Then the spheroid was embedded in matrigel and rapidly transferred into prewarmed 24-well plates and polymerized for 30 minutes. 100 µl endothelial basal medium was added on top of the spheroid and cultured for 72 hours. Pictures were taken using microscope and the cumulative sprout length was measured per spheroid using ImageJ software (National Institutes of Health, Bethesda, MD). n=10.

### 3.9. Affinity pull-down assay

Rho GTPase activity was determined by pull-down assay as previous described (28). Cells were washed with ice-cold PBS and incubated with lysis buffer (25mM HEPES, pH 7.5, 150mM NaCl, 1% NP-40, 10% glycerol, 25mM NaF, 10mM MgCl2, 1mM EDTA, 1mM sodium orthovanadate, 10µg/ml leupeptin and 10µg/ml aprotinin) for 30 min. After centrifugation (14,000 g for 10 min), the cell lysates were incubated with 50 µl GST-RBD (specifically binds to activated RhoA) or GST-PBD (binds to activated Rac1 and Cdc42) fusion protein beads at 4° C for 1 hour. The cells were washed 3 times in 500 µl wash buffer (25 mmol/L Tris pH7.5, 30 mmol/L EDTA, and 600 mmol/L $MgCl_2$) and immunoblotted with anti-RhoA, Rac1 or Cdc42 antibodies.

### 3.10. Real-time PCR

Total RNA was isolated using TRIzol solution (Invitrogen, Grand Island, NY) and reverse transcribed to cDNA using AMV reverse transcriptase (TAKARA). PCR primers used for detecting ILK in HUVECs are 5'-AGT CAA GCT TTC TAC CAT GGA CGA CAT TTT CAC TCA -3' (forward) and 5'-CGG AAT TCT TGT CCT GCA TCT TCT C-3' (reverse). Results were analyzed by using an ABI Prism 7300 Sequence Detector System (PE Applied Biosystems).

### 3.11. RNA interference

Short hairpin RNAs (shRNAs) against ILK and the scrambled control were purchased from Ambion (Austin, TX). 1 day prior to transfection, $2\times10^5$ HUVECs were plated in 35-mm dishes (Falcon, Las Vegas, NV) and transfected with ILK shRNA (40 µM final concentrations) in X-tremeGENE siRNA transfection reagent (Roche Diagnostics, Canada) for 48 h. SVECs were transfected with 20 nM of mouse Cdc42 shRNA (UUUGGGUCCCAACAAGCAAGAAAGG) (Invitrogen, Grand Island, NY) in Opti-MEM I medium for 24 h (29).

### 3.12. Hindlimb ischemic model and laser Doppler image analysis

To determine the role of CREG1 in neovascularization *in vivo*, we used a hindlimb ischemic mouse model. Femoral artery was ligated, AdCREG1 or AdGFP (200 MOI) was injected into gastrocnemius muscle and blood flow was measured pre- and post-operatively (day 14) by PeriScan PIM3 laser Doppler system (Perimed AB, Sweden) in 8-10 week old male mice (n=5 per group).

### 3.13. Reagents

Reconstituted basement membrane matrigel was purchased from BD Biosciences (Bedford, MA). Anti-Cdc42, RhoA and Rac-1 antibodies are from Cell Signaling Technology Inc (Beverly, MA). Mouse monoclonal anti-CREG1, anti-CD31 and anti-VE cadherin antibodies are from R&D Systems (Minneapolis, MN). Mouse monoclonal anti-β-actin is from Abcam (Hong Kong, China).

### 3.14. Statistical analysis

Data are expressed as mean±SD. All data were analyzed using SPSS 13.0 statistical software (Chicago, IL). Differences between two groups were compared using unpaired Student's *t*-tests. Differences among three or more groups were compared using one-way analysis of variance. Statistical significance was defined as $p<0.05$ (two-tailed).

## 4. RESULTS

### 4.1. Adenovirus-transduced CREG1 expression promotes angiogenesis

Adenovirus-transduced CREG1 expression leads to a 3.4-fold increase in CREG1 expression in HUVECs and promotes endothelial tube formation in matrigel compared to the AdGFP transduced group (Figure 1A, B, C, D&E). To confirm this finding *in vivo*, we transduced mouse SVECs with AdCREG1 or AdGFP in matrigel and subcutaneously injected the matrigel into the flanks in C57BL/6 mice. After 4 weeks of matrigel injection, the matrigel plugs were retrieved and a high density of blood vessels was observed by gross examination in AdCREG1 plugs but not in AdGFP controls (Figure 1F, G&H). Blood vessel formation is significantly increased in the plugs by AdCREG1-transduction in SVECs. These results suggest that adenovirus-transduced CREG1 expression in ECs promotes angiogenesis.

### 4.2. Adenovirus-transduced CREG1 expression stimulates filopodia protrusions and angiogenic sprouting

Endothelial sprouting guided by tip cell filopodia is an early step of angiogenesis (30). To assess the effect of adenoviral CREG1 on endothelial sprouting, we cultured endothelial spheroids in matrigel for 3 days. Phase-contrast micrographs of live cultures showed that AdCREG1-transduced spheroids send out multiple sprouts with filopodia at the leading edge (Figure 2A). Only a few of short sprouts were observed in the AdGFP-transduced spheroids. AdCREG1 increases endothelial sprouting by 2.3-fold (Figure 2B). To facilitate filopodia quantitation, we cultured AdCREG1-transduced HUVECs on a thin-layer of matrigel. Adenoviral CREG1 increases both the number and the length of filopodia in the front migrating cells compared with AdGFP-transduced HUVECs (Figure 2C, D&E). These results suggest that adenoviral CREG1 stimulates filopodia protrusions and angiogenic sprouting, which may contribute to endothelial network assembly.

### 4.3. Cdc42 activation is required for CREG1-induced filopodia formation and endothelial network assembly

In our previous study, we found that adenoviral CREG1 enhances Cdc42 expression in mRNA level in HUVECs transduced with AdCREG1 compared with AdGFP control (data not shown). In this study, to

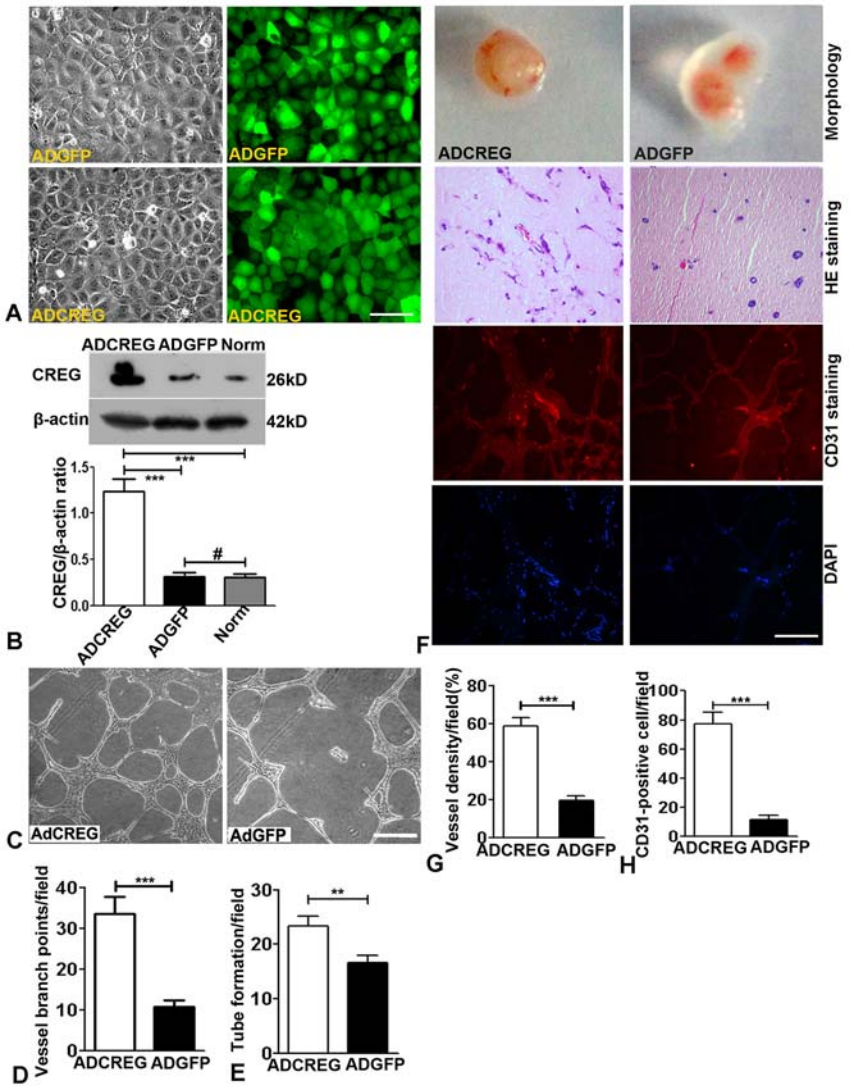


**Figure 1.** Adenoviral CREG1 accelerates angiogenesis. (A) Phase-contrast and fluorescent micrographs show HUVECs transduced with adenoviruses carrying CREG1 (AdCREG1) or GFP (AdGFP). Bars=50 μm. (B) Cell lysates were collected and CREG1 levels were analyzed by immunoblotting. The blots were quantified by densitometry and plotted as the ratio of CREG1 to beta-actin. Data are presented as mean±SD. n=3. Norm: normal HUVECs. (C, D) HUVECs transduced with CREG1 or GFP adenoviruses were seeded onto 3D matrigel and tube formation was observed after 24 h. Three images were taken per well from randomly chosen fields and branching points and vessel densities were quantified. Each experiment was performed in triplicate. Bars=100 μm. (F, G) SVECs were transduced with AdCREG1 or AdGFP, mixed with matrigel, and implanted under the mouse skin for 14 days. Blood vessel growth in matrigel plugs was examined by morphology (hematoxylin-eosin and CD31 staining). Quantification of vascular density was performed in randomly chosen triplicate fields. Bars=100 μm. $^{\#}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.

determine whether Rho GTPase is involved in CREG1-mediated filopodia formation and endothelial network assembly, we first examined their activation after adenoviral transduction in HUVECs. Affinity pull-down assay using GST-PBD agarose beads that specifically bind to activated RhoA, Rac1, and Cdc42 demonstrated that Cdc42 is selectively activated by adenovirus-transduced CREG1 expression (Figure 3A&B). SiRNA-mediated Cdc42 silencing (Figure 3C&D) almost abolishes filopodia formation and tube formation in HUVECs compared with the scrambled control (Figure 3E, F&G). These results suggest that Cdc42 activation is required for CREG1-induced filopodia formation and endothelial network assembly.

### 4.4. CREG1 induces Cdc42 activation and angiogenesis through ILK

To determine whether integrin linked kinas (ILK) is involved in CREG1-induced Cdc42 activation and angiogenesis, we first analyzed the expression of ILK and

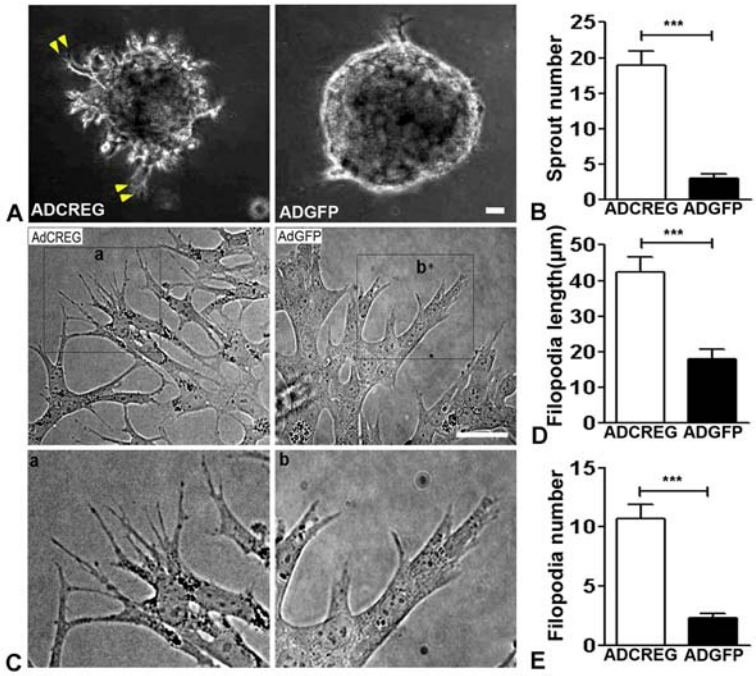


**Figure 2.** Adenoviral CREG1 increases endothelial filopodia protrusions and sprouts. (A, B) Endothelial spheroids were cultured on matrigel for 3 days and multiple vessel sprouts from the spheroids were counted, n=3. Bars=100 μm. Arrows show the capillary sprouts. (C) Phase-contrast micrographs show angiogenic sprouting in the ECs onto a thin-layer matrigel after 48 h. Quantification of length (D) and numbers of filopodia (E) were performed from randomly chosen fields in triplicate. Scale bars=25 μm. Data are presented as mean±SD (n = 6). $^{\#}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.

integrin related protein involved in cell mobilization. Adenoviral CREG1 increases ILK protein by more than two-fold in HUVECs, but no effects on integrin beta1 protein expression (Figure 4A&B). To determine if the increase of ILK occurs at the transcription level, we performed quantitative real-time (RT)-PCR and found a twofold increase of the ILK mRNA in AdCREG1-transduced HUVECs (Figure 4C). These results suggest that adenoviral CREG1 upregulates ILK expression likely through transcriptional regulation.

To further elucidate the role of ILK in CREG1-mediated angiogenesis, we used a RNA-interference approach to knock down ILK expression in HUVECs. ILK short hairpin shRNA (shRNA2) nearly abolishes ILK protein expression compared to the scrambled control (Figure 4D&E). Affinity pull-down assay showed that silencing of ILK blocks Cdc42 activation in both AdCREG1- and AdGFP-transduced HUVECs (Figure 4F&G). ILK shRNA significantly decreases HUVEC filopodia extension and tube formation (Figure 4H, I, J&K). These results suggest that ILK is required for Cdc42 activation, filopodia formation and endothelial vessel assembly both in normal and AdCREG1-transduced ECs.

### 4.5. CREG1 increases neovascularization in ischemic hindlimb

To examine the loss of function of *CREG1* gene on neovascularization *in vivo*, we used *CREG1* heterozygous deficiency mice generated in our laboratory. Immunofluorescent analysis of normal mouse aortas revealed that CREG1 is highly expressed in the endothelium and the adventitia and moderately expressed in the media (Figure 5A). CREG1 protein and mRNA are largely reduced in aorta from *Creg1*$^{+/-}$ mice (Figure 5A&B). To determine whether the reduced CREG1 expression impacts neovascularization, we measured blood-flow recovery by laser Doppler image analysis in hindlimbs after left femoral artery ligation in *Creg1*$^{+/-}$ mice. The ratio of left-to-right leg blood-flow recovery is significantly decreased in *Greg1*$^{+/-}$ mice (14.54 ± 2.358% vs wild-type 71.34 ± 6.5% in calf; 38.03 ± 5.5% vs wild-type 52.83 ± 2.358% in thigh; $p<0.001$) (Figure 5C&D). Immunostaining for CD31 revealed that neovascularization in ischemic tissue is markedly reduced in *Creg1*$^{+/-}$ mice (Figure 5E). These results indicate CREG1 contributes to blood flow recovery after ischemic injury *in vivo*, likely due to increased neovascularization.

To test whether AdCREG1 has a beneficial effect on blood flow recovery, we injected AdCREG1 or AdGFP into the gastrocnemius muscle in *Creg1*$^{+/-}$ mice. Laser Doppler image analysis showed AdCREG1 improves hindlimb perfusion ratio (ischemic/control hindlimb) on day 14 in *Creg1*$^{+/-}$ mice compared to AdGFP receiving group (Figure 5F&G). In addition, immunostaining for CD31 showed a significant increase in capillary density in ischemic limbs transduced with AdCREG1 (Figure 5H). Furthermore, AdCREG1 increases the expressions of ILK and activated Cdc42 proteins in adenovirus CREG1

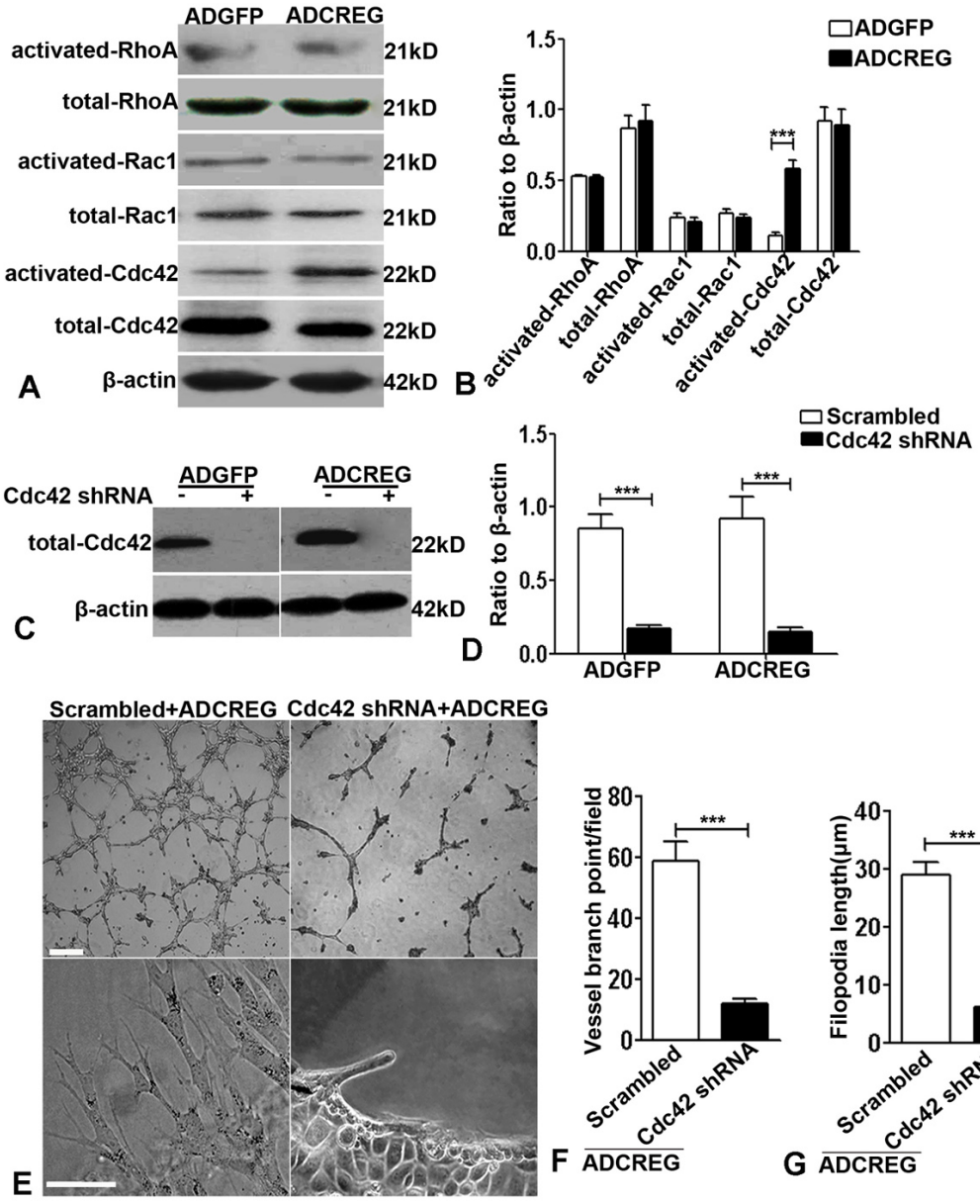


**Figure 3.** Cdc42 activation mediates CREG1-induced filopodia formation and endothelial network assembly. (A) Protein expression and activity of RhoA, Rac1 and Cdc42 were analyzed in HUVECs cultured on matrigel by immunoblotting and affinity pull-down assay, respectively. (B) Blots were quantified by densitometry and plotted as the ratio to beta-actin. (C) Silencing Cdc42 expression using Cdc42 shRNA was evaluated by immunoblotting analysis in AdCREG1- or AdGFP-transduced HUVECs. (D) Blots were quantified by densitometry and plotted as the ratio to beta-actin. (E) HUVECs with or without Cdc42 shRNA transfection were seeded onto matrigel to investigate tube formation. Branch points of capillary-like networks (F) and the filopodia length (G) were quantified by image analysis. Each experiment was performed in triplicate. Bars=50 μm. $^{\#}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.

infection compared with in adenoviral CREG1 transduced group (Figure 5I&J). These results support the role of CREG1 in promoting neovascularization.

## 5. DISCUSSION

In the present study, we demonstrate that (1) adenoviral CREG1 increases filopodia formation and endothelial network assembly in matrigel in HUVECs and SVECs. (2) ILK and its downstream effector Cdc42 activation mediate CREG1-induced filopodia formation and tube formation in HUVECs and SVECs. (3) adenoviral CREG1 partially increases neovascularization and tissue perfusion in ischemic hindlimb. These results suggest that CREG1 may be a new therapeutic target for angiogenesis and neovascularization.

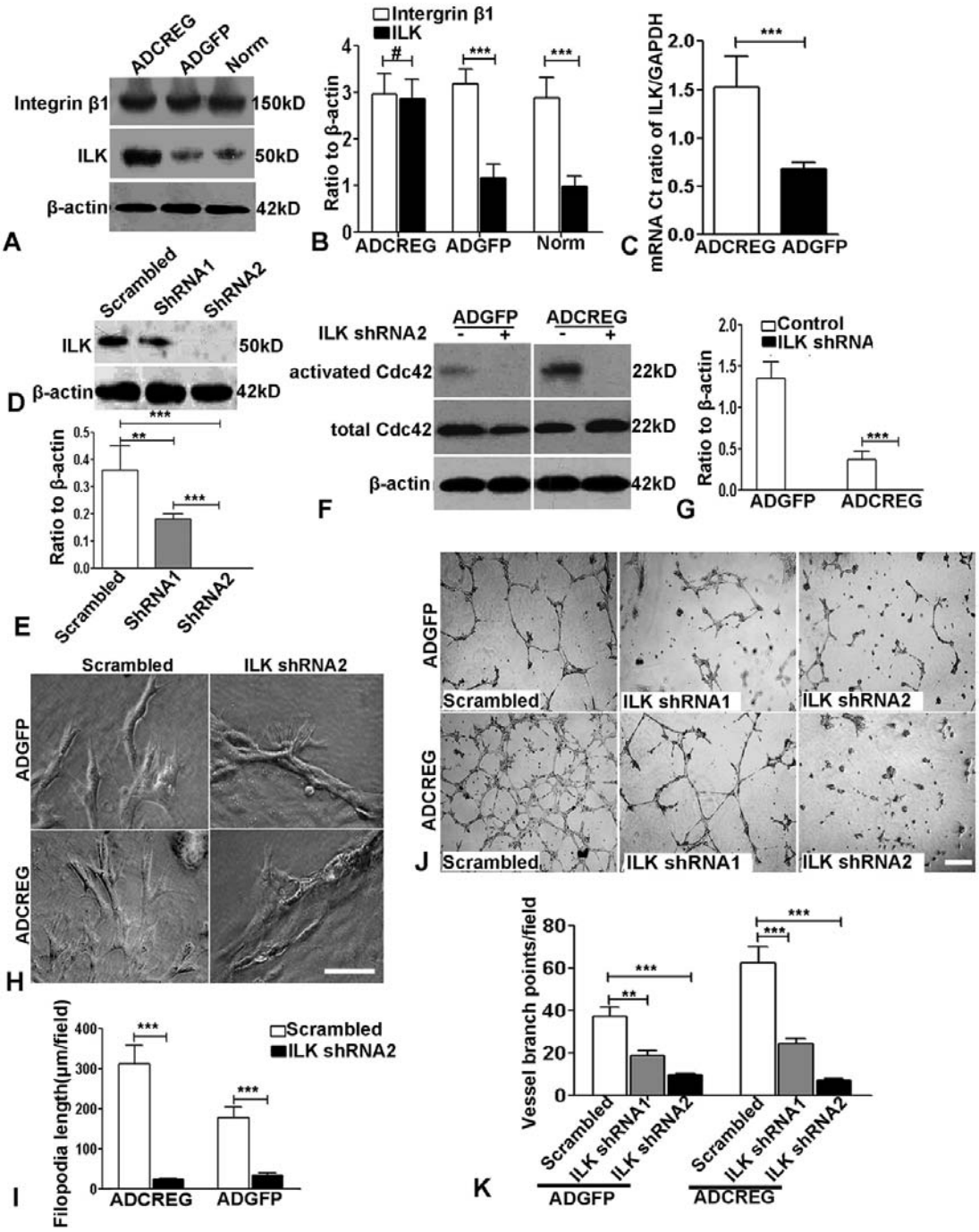


**Figure 4.** CREG1 increases ILK expression and Cdc42 activation, filopodia formation and vascular assembly. (A) Protein expression of integrin beta1 and ILK was analyzed by immunoblotting in HUVECs transduced with AdCREG1 or AdGFP. (B) Blots were quantified by densitometry and plotted as the ratio to beta-actin. (C) RT-PCR showed that adenoviral CREG1 increases ILK mRNA level. (D) Immunoblotting showed reduced ILK expression by human ILK shRNAs in HUVECs. (E) Blots were quantified by densitometry and plotted as the ratio to beta-actin. (F) Affinity pull-down assay and immunoblotting show that ILK shRNA significantly inhibits Cdc42 activity in both AdCREG1- and AdGFP-transduced HUVECs. (G) Blots were quantified by densitometry and plotted as the ratio to beta-actin. (H) Phase-contrast micrographs show ILK shRNA inhibits angiogenesis sprouting of AdCREG1- and AdGFP-transduced cells. Bars=50 μm. (I) Quantification of filopodia number in randomly chosen fields in triplicate wells. (J) ILK shRNA decreases tube formation in both AdCREG1- and AdGFP-transduced after 24 h seeded in matrigel. Bars=100 μm. (K) Quantification of vessel branch points in randomly chosen fields (n=5). Data are shown as mean±SD (n=5). $^{\#}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.

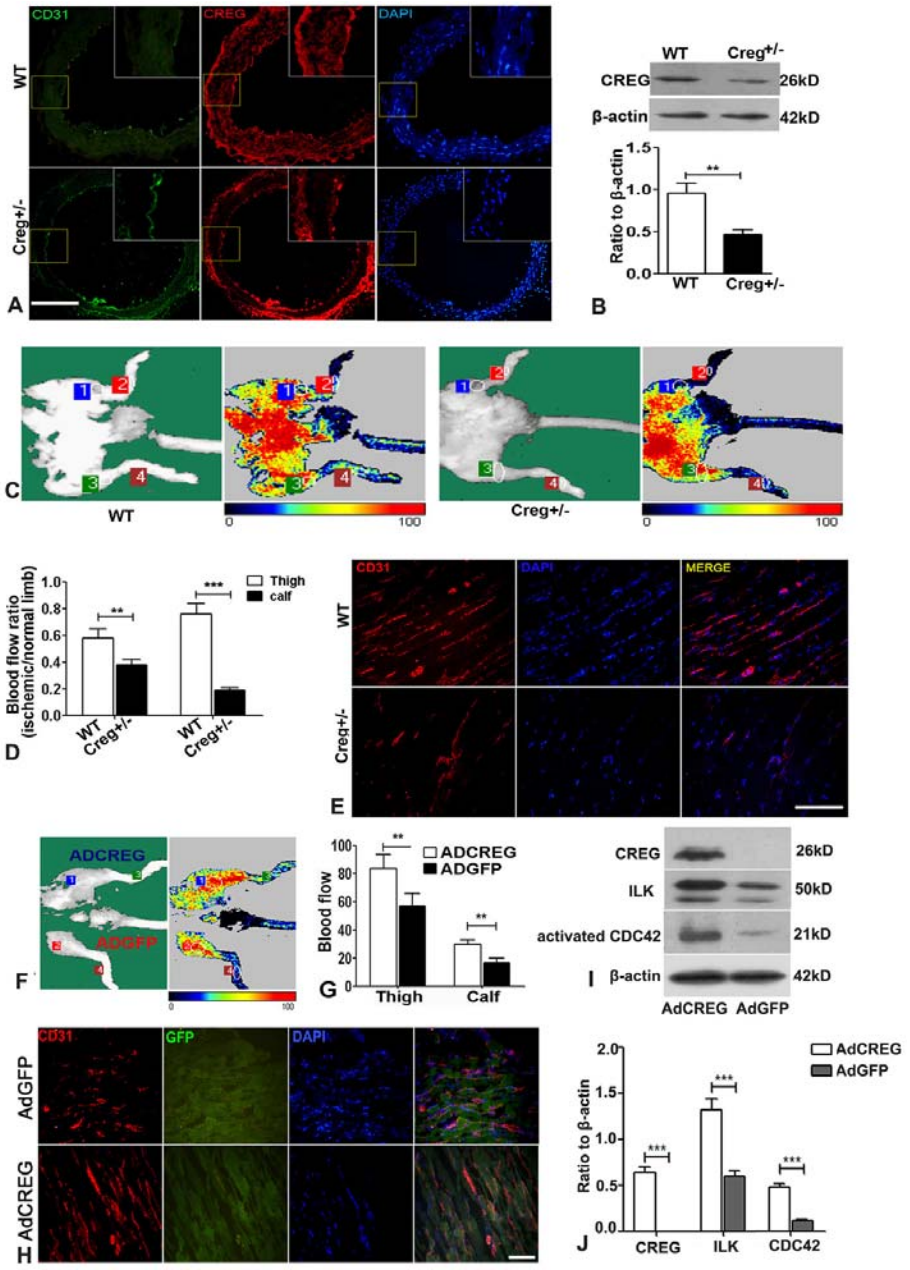


**Figure 5.** CREG1 promotes neovascularization *in vivo*. (A) Representative images of mice aortic sections immunostained for CD31 and CREG1 in wild-type and *CREG1*$^{+/-}$ mice. Bars=25 μm. (B) Immunoblotting shows reduced CREG1 expression in *CREG1*$^{+/-}$ mouse aortas. The level of CREG1 expression were quantified by densitometry and normalized against beta-actin expression level. Data are presented as mean±SD (n=6). (C) Mice were subjected to femoral artery ligation and the blood flow to the ischemic (left) and nonischemic (right) limbs was compared 14 days after surgery. Representative laser Doppler images show the *CREG1*$^{+/-}$ mice have less blood flow distribution in the ischemic limbs. (D) The levels of perfusion were quantified and expressed as the ratio to the control. n=5. (E) The capillary density of the ligated limb calf muscle was assessed by immunostaining for CD31 (red). Nuclei were counterstained with DAPI (blue). Scale bar=100 μm. (F) Male *CREG1*$^{+/-}$ mice were subjected to femoral artery ligation in both hindlimbs. After 3 days, the mice were injected intramuscularly with 200 MOI of AdCREG1 in the left hindlimb and AdGFP in the right hindlimb (n=6 per group). (G) The level of perfusion was quantified and expressed as the ratio to the control. (H) The ligated limb calf muscle was immunostained for CD31 (red). Fluorescent micrographs show HUVECs transduced with adenoviruses (Green). Nuclei were counterstained with DAPI (blue). Scale bar=100 μm. (I) Immunoblotting shows adenoviral CREG1 in *CREG1*$^{+/-}$ mouse hindlimbs enhances CREG1, ILK and activated Cdc42 expression. (J) Blots were quantified by densitometry and plotted as the ratio to beta-actin. Data are presented as mean±SD (n=6). $^{\#}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.

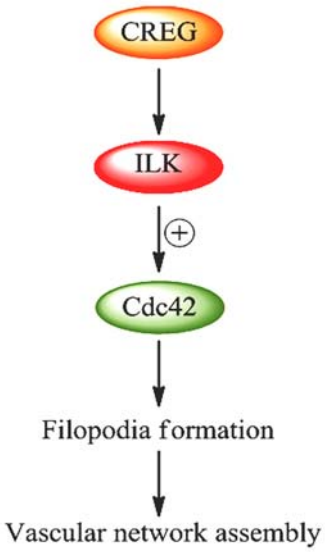


**Figure 6.** Model of CREG11 mechanisms in angiogenesis. CREG11 upregulates the expression of ILK and Cdc42 activation to promote EC filopodia formation and angiogenesis.

ILK is a multidomain protein that binds to the cytoplasmic tail of beta1/beta3-integrins. It serves as a scaffolding protein and transmits signals from the extracellular matrix to a variety of intracellular signaling pathways that regulate cytoskeletal organization, adhesion and migration (31). Increase in ILK is associated with activation of the small Rho GTPase, Cdc42 and Rac1, which are important regulators of cell protrusion and actin dynamics during migration (32). We showed that adenoviral CREG1 increases ILK transcription and selectively activates Cdc42 in HUVECs. ILK forms a tight complex with PINCH1/2 and alpha/beta-parvins (33-35). Beta-parvin has been shown to bind to Cdc42/Rac1-specific guanine nucleotide exchange factor ARHGEF6 (alpha-PIX) and activate Cdc42/Rac1 (36). These interactions may bring Cdc42 activators and effectors together at the integrin-ECM interface and promote actin cytoskeletal reorganization and cell migration.

In the present study, we show that adenoviral CREG1 increases ILK protein expression. How CREG1 upregulates ILK expression is currently unknown. Our quantitative PCR analysis suggests the regulation occurs at the transcriptional level. CREG1 has initially been shown to interact with transcription machinery and affect the expression of the adenoviral protein E1A-activated genes (16). Recently we found that the non-glycosylated CREG1 GFP fusion protein is localized at the nucleus (data not shown). It is possible that CREG1 acts as a transcription activator for ILK transcription.

In summary, our study demonstrates that CREG1 possesses angiogenic activity via ILK/Cdc42 activation-induced endothelial network assembly. CREG1, as a secreted glycoprotein involved in angiogenesis, might be a therapeutic target for ischemic injury.

## 6. ACKNOWLEDGEMENT

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