CCL2 promotes proliferation, migration and angiogenesis through the MAPK/ERK1/2/MMP9, PI3K/AKT, Wnt/β-catenin signaling pathways in HUVECs

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Abstract. Severe bone trauma can lead to poor or delayed bone healing and nonunion. Bone regeneration is based on the interaction between osteogenesis and angiogenesis. Angiogenesis serves a unique role in the repair and remodeling of bone defects. Monocyte chemoattractant protein-1, also known as CC motif ligand 2 (CCL2), is a member of the CC motif chemokine family and was the first human chemokine to be revealed to be an effective chemokine of monocytes. However, its underlying mechanism in angiogenesis of bone defect repair remains to be elucidated. Therefore, the present study investigated the detailed mechanism by which CCL2 promoted angiogenesis in bone defects based on cell and animal model experiments. In the present study, CCL2 promoted proliferation, migration and tube formation in human umbilical vein endothelial cells (HUVECs) in a concentration-dependent manner. Western blot analysis revealed that treatment of HUVECs with CCL2 upregulated the protein expression levels of rho-associated coiled-coil-containing protein kinase (Rock)1, Rock2, N-cadherin, c-Myc and VEGFR2. Furthermore, CCL2 promoted the expression of MAPK/ERK1/2/MMP9, PI3K/AKT and Wnt/β-catenin signaling pathway-related proteins, which also demonstrated that CCL2 promoted these functions in HUVECs. Immunohistochemical staining of Sprague Dawley rat femurs following bone defects revealed that VEGF expression was positive in the newly formed bone area in each group, while the expression area of VEGF in the CCL2 addition group was markedly increased. Therefore, CCL2 is a potential therapeutic approach for bone defect repair and reconstruction through the mechanism of angiogenesis-osteogenesis coupling.

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Introduction

Bone defects caused by high-energy trauma, bone tumors, limb deformities and bone infections can lead to poor and delayed bone healing, and nonunion (1,2). Although bone tissue can be completely regenerated in the body with time, bone defects that are beyond the critical length range of self-repair experience difficulty in self-repair and reconstruction to restore normal bone length and stiffness (3). Bone is a highly vascularized, unique, complex and dynamic tissue with regenerative properties that can repair itself and restore its original structural integrity after injury or destruction, although bone damage can lead to a host of additional problems, such as ruptured blood vessels, hematoma formation and nerve damage, as it is accompanied by local tissue hypoxia with the release of systemic cytokines stimulating the formation of fresh blood vessels in the bone defect site (4). Angiogenesis is a complex process involving various cell interactions, growth factor stimulation and biomechanics. Angiogenesis originates from the production of matrix metalloproteinases in endothelial cells, which degrade the basement membrane to promote endothelial cell migration (5). Endothelial cell migration is a key component of the angiogenic response, and endothelial cells move toward areas with high concentrations of VEGF and other growth factors through the proteolytic basement membrane (6). When endothelial cells relocate into tissue, they reproduce and differentiate to construct new blood vessels. The regeneration of bone is based on the interaction between osteogenesis and angiogenesis, largely depending on angiogenesis, which serves a crucial role in bone repair and remodeling. Angiogenesis refers to the reproduction and migration of endothelial cells to the sites of original blood vessels to form a new crisscross vascular network, continuously providing sufficient nutrients, cytokines, neurotransmitters and oxygen for bone cells, bone tissue and the inner and outer membranes of bone (7). Therefore, understanding how to effectively promote

angiogenesis in bone defects may serve an important role in bone repair.

Monocyte chemoattractant protein-1, also known as CC motif ligand 2 (CCL2), an effective chemokine of monocytes, is a member of the CC chemokine family and was the first human chemokine to be discovered. It is produced or induced by multiple types of cells in the body in response to oxidative stress. It serves a valuable role in various pathophysiological processes, such as inducing macrophages, immune stress, recruitment, inflammatory response (8), angiogenesis, cell proliferation, migration and wound repair (9). It also stimulates directional or nondirectional cell migration (10). Although CCL2 has been reported to promote angiogenesis, the detailed mechanism of CCL2 has yet to be fully elucidated. In addition, examining the underlying mechanism by which CCL2 promotes angiogenesis will yield novel functional benefits to promote rapid reconstruction and repair of bone defects. Therefore, the present study investigated the mechanism of CCL2 in angiogenesis for bone defect repair and remodeling using cell and animal model experiments.

Materials and methods

Cell culture. Human umbilical vein endothelial cells (HUVECs) were obtained from ScienCell Research Laboratories, Inc (cat. no. 8000). HUVECs were grown in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin and streptomycin (Beijing Solarbio Science & Technology Co., Ltd.), and cultured in a cell incubator at 95% humidity at 37˚C with 5% carbon dioxide. Ethics approval for the use of HUVECs was not required according to our institute's guidelines (http://www.gdmuah.com/info/1851/8954.htm).

Antibodies and reagents. CCL2 (cat. no. M1203-03S) was purchased from United States Biological. Antibodies against rho-associated coiled-coil-containing protein kinase (Rock)1 (cat. no. #4035), N-cadherin (cat. no. #13116), c-Myc (cat. no. #18583), phosphorylated (p-)ERK1/2 (cat. no. #4370), p-AKT (cat. no. #13038), AKT (cat. no. #4691), Wnt5a/b (cat. no. #2530), low-density lipoprotein receptor related protein 6 (LRP-6; cat. no. #3395), β-catenin (cat. no. #8480), p-PI3K (cat. no. #17366), PI3K (cat. no. #4257), GAPDH (cat. no. #5174) and α-tubulin (cat. no. #2125) and the secondary antibodies goat anti-rabbit IgG (cat. no. #7074) and anti-mouse IgG (cat. no. #7076) were purchased from Cell Signaling Technology, Inc. VEGFR2 (cat. no. ab134191) and MMP9 (cat. no. ab228402) antibodies were obtained from Abcam. Antibodies against Rock2 (cat. no. sc-398519), ERK1/2 (cat. no. sc-514302) and VEGF (cat. no. sc-57496) were obtained from Santa Cruz Biotechnology, Inc.

Cell proliferation assay. HUVECs ($2x10^3$ cells per well) were seeded into 96-well plates in ~100 μl total medium, incubated at 37˚C for 24 h, and then treated with various concentrations (0, 25, 50, 75, 100 and 150 ng/ml) of CCL2 at 37˚C for 0-96 h. Following addition of 10 μl Cell Counting Kit-8 (Beyotime Institute of Biotechnology) solution per well and incubation at 37˚C for 2 h, the OD value was determined by a microplate reader (MK3; Thermo Fisher Scientific, Inc.) at OD 450 nm.

Transwell migration assay. The fresh serum-free DMEM (100 μl) containing $2x10^4$ HUVECs and various concentrations (0, 50, and 100 ng/ml) of CCL2 was added to the upper chamber. The lower chamber was filled with 500 μl of medium containing 10% FBS and cells were incubated at 37˚C for 24 h. Subsequently, cells that remained in the upper chamber without successfully migrating were wiped away. Cells on the membrane were fixed in methanol at room temperature for 15 min and stained with crystal violet staining solution at 37˚C for 15 min. The cells were imaged in six random microscopic fields (magnification, x100) under a light microscope (Olympus Corporation). Migrated cells (% of control)=(migrated cells treated with 50 or 100 ng/ml CCL2-migrated cells treated with 0 ng/ml CCL2)/migrated cells treated with 0 ng/ml CCL2 x100.

Wound healing assay. HUVECs were cultured for 24 h in 6-well plates with lines at the bottom of the plate. When the cell fusion rate approached 100%, a 100-μl pipette tip was used to draw a line perpendicular to the mark line in the culture plate, and PBS was used to clean the culture well twice. Cells that had come loose were removed, 2 ml serum-free medium with various concentrations of CCL2 (0, 50 and 100 ng/ml) was added and cells were placed back in the incubator for further culture. Subsequently, the migration distance of HUVECs imaged under a light microscope (Olympus Corporation) at 0 and 24 h was compared, the migration area was calculated and the effect of different treatments on cell migration was analyzed. Migration area (%)=(migration area at 0 h-migration area at 24 h)/migration area at 0 h x100.

Tube formation assay. HUVECs ($1.5x10^4$) were cultured with different doses of CCL2 (0, 25, 50 and 100 ng/ml) in DMEM in Matrigel-precoated (37˚C; 30 min) angiogenesis slides at 37˚C with 5% CO_2. After 4 h of incubation, tube formation was observed under a light microscope (Olympus Corporation) and images were captured for five randomly selected microscopic fields and tube formation was analyzed using ImageJ software (v1.8.0; National Institutes of Health).

Western blotting. Total protein was collected with an appropriate amount of protein RIPA lysis buffer (PMSF:RIPA=1:100; Beyotime Institute of Biotechnology). BCA protein assay kit (Beyotime Institute of Biotechnology) was used to measure protein concentration and each sample adjusted to the same concentration. Proteins (20 μg/lane) were separated by SDS-PAGE on 10 and 12% separation gels. After gel electrophoresis (100 V; 2 h), membrane transfer (250 V; 2.5 h) and blocking (5% skimmed milk; room temperature; 2 h), the 0.2 μm pore size PVDF membranes (Merck KGaA, Darmstadt, Germany) were incubated with primary antibodies (diluted 1:1,000) overnight at 4˚C. Subsequently, the membranes were washed with TBS with 0.1% Tween-20 (three times; 10 min) before addition of the secondary antibody (diluted 1:3,000) at room temperature for 1 h. Finally, the immunoreactive membranes were imaged using a Chemiluminescent and Fluorescent Imaging System (Tanon 5200; Tanon Science and Technology Co., Ltd.), and the gray levels of the protein bands were determined using ImageJ software (v1.8.0, National Institutes of Health).

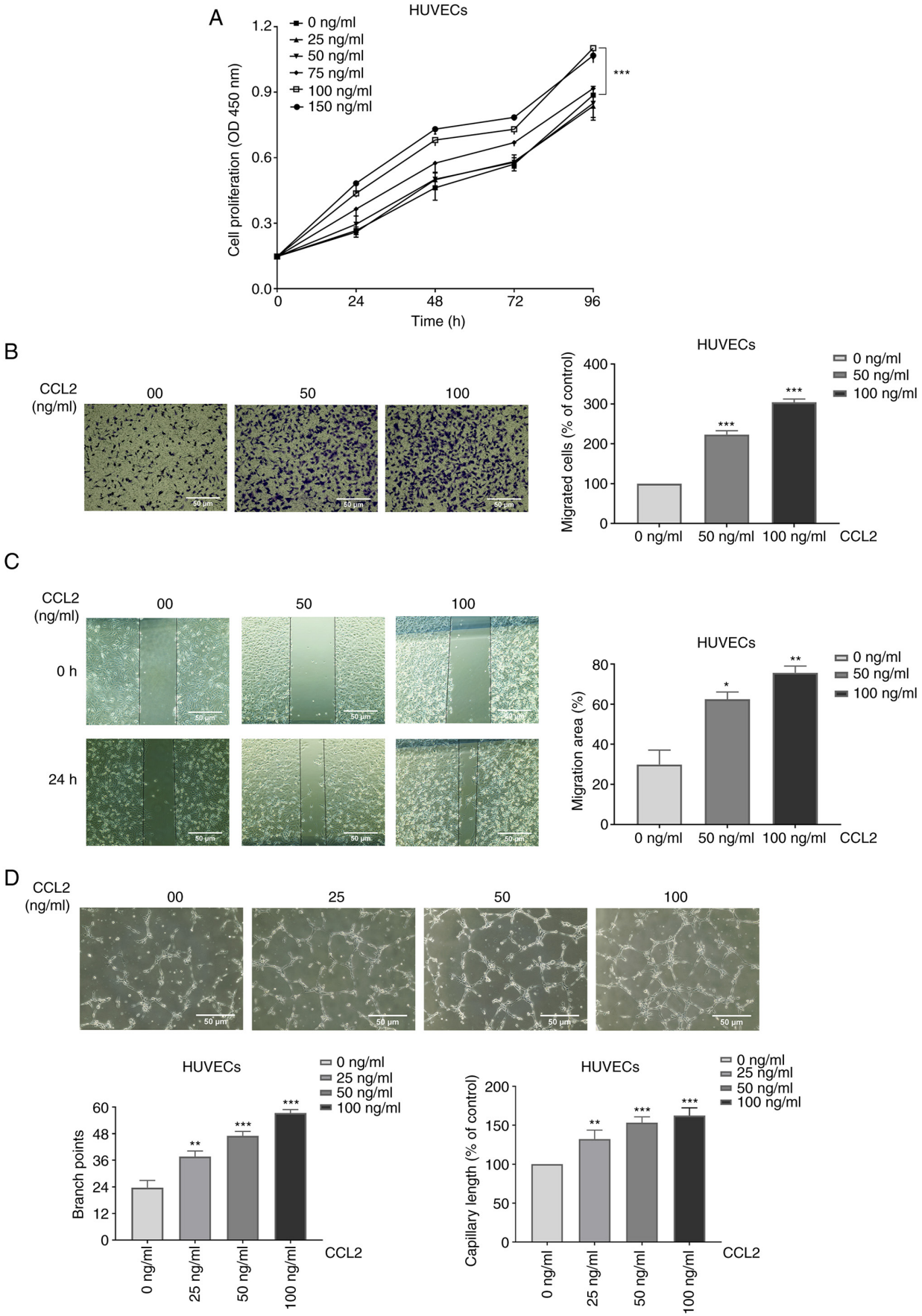

Figure 1. CCL2 promotes the growth, migration and angiogenesis of HUVECs. (A) HUVECs were incubated with 0-150 ng/ml CCL2, followed by determination of cell proliferation using a Cell Counting Kit-8 assay. (B) Transwell migration assays were used to assess the migration of HUVECs. (C) Representative images of the wound healing of HUVECs treated with CCL2 (0, 50 and 100 ng/ml) at a magnification of x100. (D) The tube formation assay. Representative images (x100) of tube formation improvement in HUVECs treated with CCL2 (0-100 ng/ml). *P<0.05, **P<0.01, ***P<0.001 vs. control group. CCL2, CC motif ligand 2; HUVECs, human umbilical vein endothelial cells.

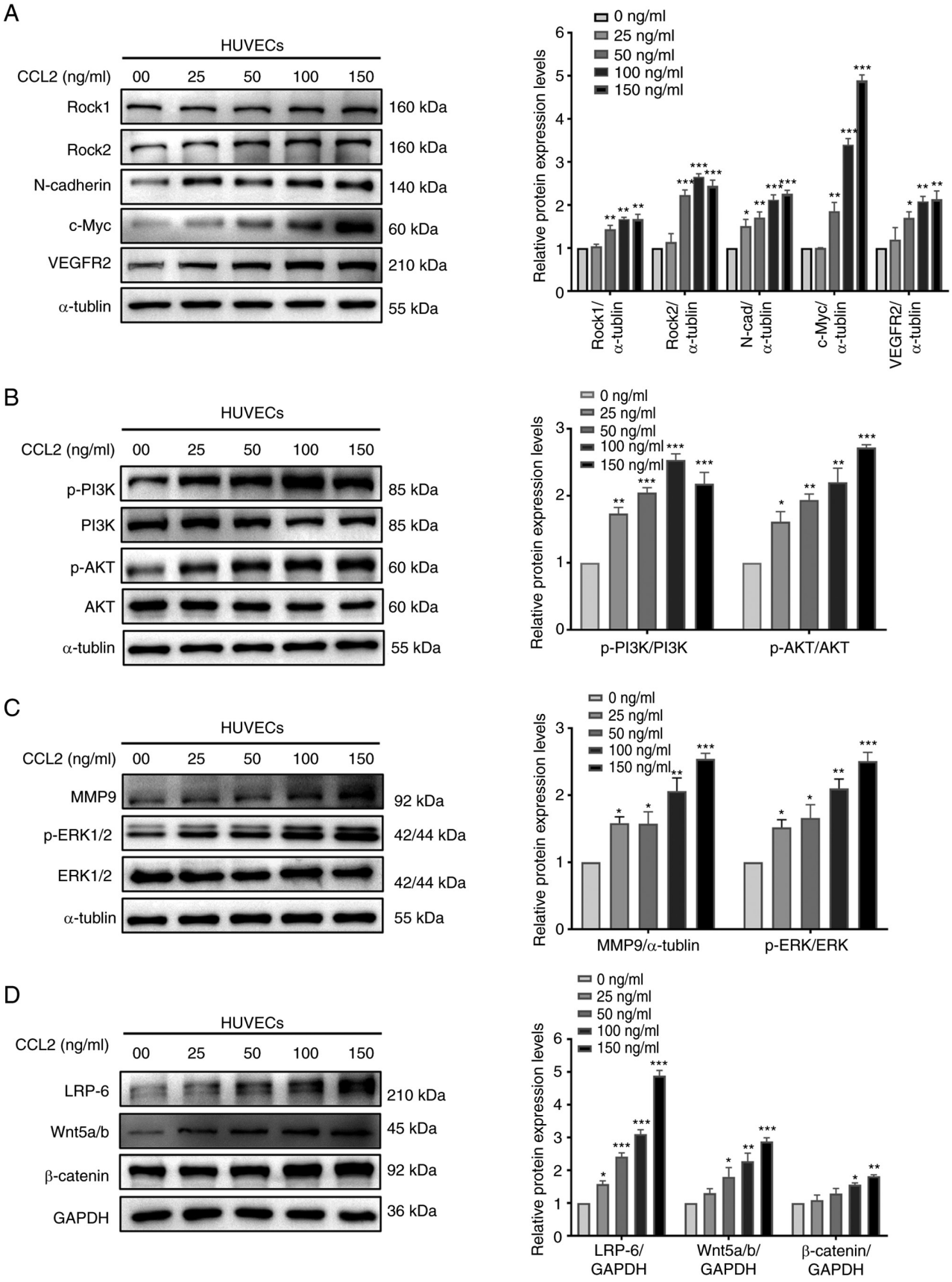

Figure 2. CCL2 promotes proliferation, migration and angiogenesis via the PI3K/AKT, MAPK/ERK1/2 and Wnt/β-catenin signaling pathways in HUVECs. (A) Western blot analysis of Rock1, Rock2, N-cadherin, c-Myc, VEGFR2 and α-tubulin in HUVECs treated with CCL2 at the indicated doses for 48 h. (B) Western blot analysis of p-PI3K,PI3K, p-AKT, AKT and α-tubulin in HUVECs treated with CCL2 at the indicated doses for 48 h. (C) Western blot analysis of p-ERK1/2, ERK1/2, MMP9 and α-tubulin in HUVECs treated with CCL2 at the indicated doses for 48 h. (D) Western blot analysis of LRP-6, Wnt5a/b, β-catenin and GAPDH in HUVECs treated with CCL2 at the indicated doses for 48 h. Gray analysis of protein bands was performed using ImageJ. *P<0.05, **P<0.01, ***P<0.001 vs. control group. CCL2, CC motif ligand 2; HUVECs, human umbilical vein endothelial cells; Rock, rho-associated coiled-coil-containing protein kinase; p-, phosphorylated; LRP-6, low-density lipoprotein receptor related protein 6.

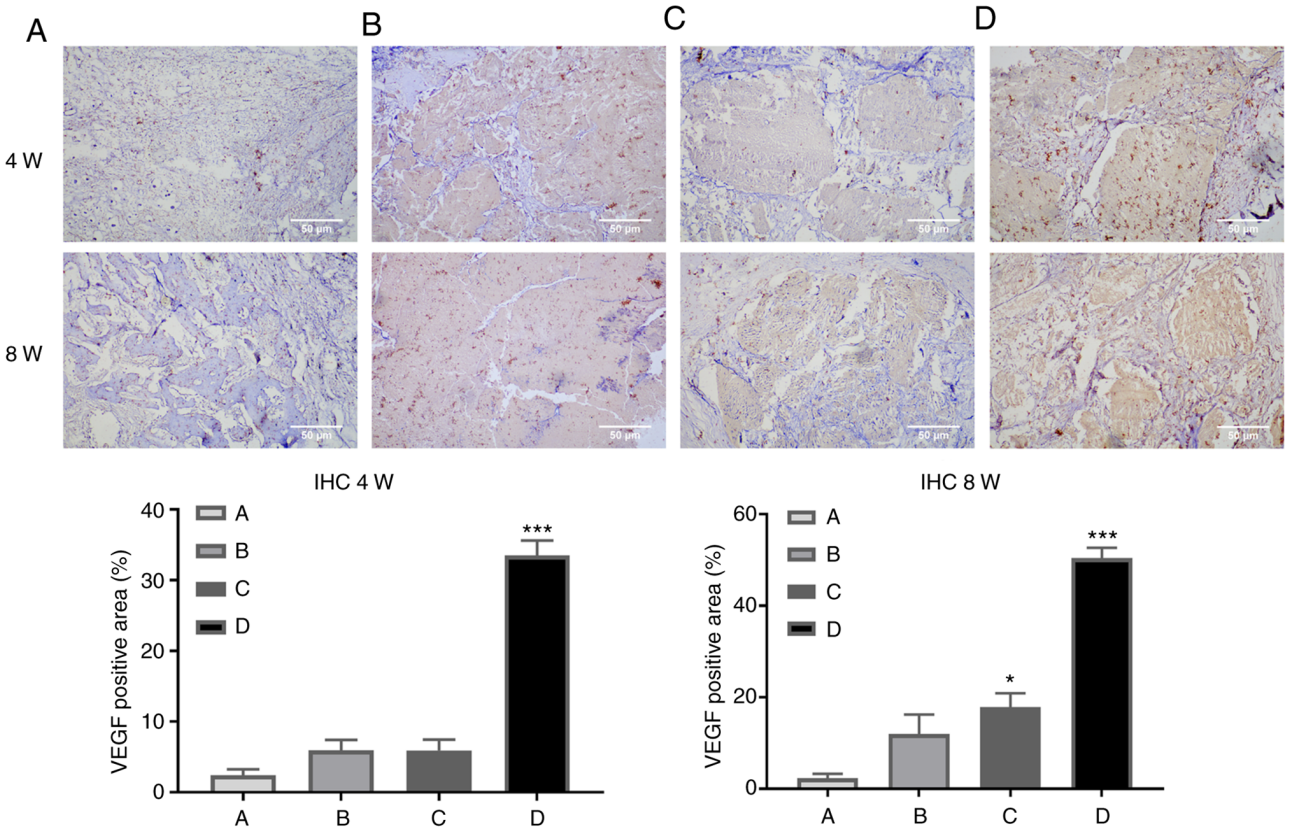

Figure 3. IHC results. Group A (nothing filled in), Group B (PLGA/TCP porous scaffold; 5x5x5 mm^3 filled in), Group C (PLGA/TCP porous scaffold + Gelma hydrogel filled in) and Group D (PLGA/TCP porous scaffold + Gelma hydrogel + 1 μg CCL2 filled in). *P<0.05, ***P<0.001 vs. control group. CCL2, CC motif ligand 2; PLGA/TCP, poly (lactic-co-glycolic acid)/tricalcium phosphate; IHC, immunohistochemistry; W, week.

Establishment of bone defects in a Sprague Dawley (SD) rat model. All animal experiments were approved by the Laboratory Animal Ethics Committee of Guangdong Medical University (Zhanjiang, China; ID Number: GDY1902126; date: 25.05.2019). The SD rats (Guangdong Medical Laboratory Animal Center) were maintained at 2 or 3 rats/cage under a 12-h light/dark cycle with adequate water and standard food, at temperatures of 22-24˚C and relative humidity of ~45%. Depending on the types of intervention, 12-week-old male SD rats (n=32) weighing 350-400 g were selected and randomly divided into four groups: Group A (simple bone defect not filled in), Group B [poly (lactic-co-glycolic acid)/tricalcium phosphate (PLGA/TCP) porous scaffold; 5x5x5 mm^3 filled in], Group C (PLGA/TCP porous scaffold + Gelma hydrogel filled in) and Group D (PLGA/TCP porous scaffold + Gelma hydrogel + 1 μg CCL2 filled in). The number of rats in each group was eight. The rats were anesthetized by intraperitoneal injection with pentobarbital sodium at a concentration of 1% at a dose of 30 mg/kg. The appropriate degree of anesthesia was assessed by the characteristics of stable breathing, sluggish corneal reflex, generalized muscle relaxation and loss of skin pinch response. After group assignment, the left femur of each rat was subjected to 3 mm midline osteotomy under anesthesia, and the bone defect area was filled or not filled according to the experimental group allocation. Furthermore, the left femur was fixed using an external fixator (Fig. S1). The bone defects establishment of each SD rat lasted ~40-60 min. The specific criteria for SD rat sacrifice were that rats were close to succumbing, with persistent dyspnea, inability to ingest food, significant loss of appetite, weight loss of >20% (the maximum percentage of body weight loss observed in the present study was ~10%), severe ulceration, heavy bleeding and limb paralysis. Penicillin sodium (80,000 units/kg/d;) was intramuscularly injected after surgery to prevent infection for 1 week and the surgical incision was disinfected with iodophor every day. The rats were monitored daily for surgical incision, mental status, diet, body weight, activity and excretion. Subsequently, each group was observed for 4 weeks, at which point half of the specimens were collected, and 8 weeks, at which point the other half of the specimens were collected. The SD rats were sacrificed with 10% sodium pentobarbital at a dose of 200 mg/kg by intraperitoneal injection. Mortality was verified by confirming the arrest of breathing, the disappearance of heartbeat and light reflex, and the dilation of pupils in SD rats. Finally, the femurs of the left leg were removed and fixed in 10% neutral formalin solution for 48 h and then stored in 75% ethanol.

Immunohistochemistry. After 10% formalin fixation at 4˚C for 48 h and EDTA solution (Beijing Solarbio Science & Technology Co., Ltd.) decalcification (2 months), bone tissue was dehydrated with gradient ethanol and xylene (75% ethanol for 1 h; 85% ethanol for 1 h; 95% ethanol for 1 h; 100% ethanol for 1 h; xylene I for 30 min; xylene II for 1 h) at room temperature. Subsequently, the bone tissue was embedded in paraffin (65˚C). After tissue sectioning (4 μm), bone callus sections were hydrated, blocked with 5% BSA (Beyotime Institute of

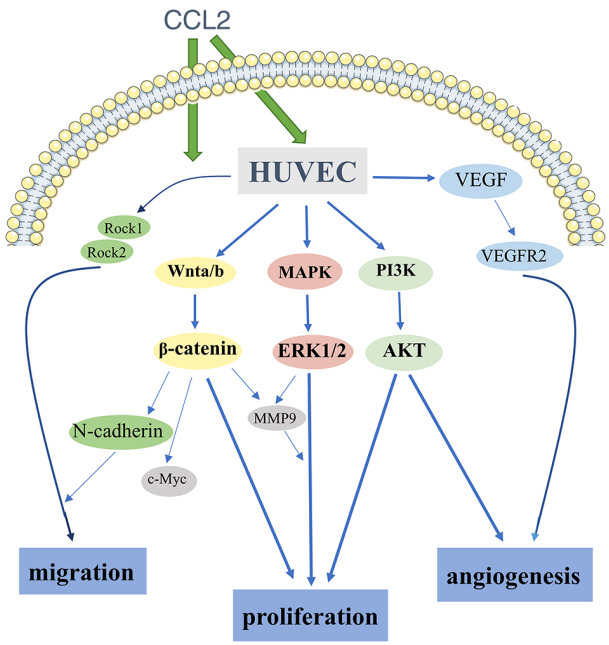

Figure 4. Mechanism of CCL2-induced proliferation, migration and angiogenesis in HUVECs. CCL2, CC motif ligand 2; HUVECs, human umbilical vein endothelial cells; Rock, rho-associated coiled-coil-containing protein kinase.

Biotechnology) for 1 h, and incubated with the VEGF primary antibody (diluted 1:200) at 4˚C overnight. After subsequent secondary antibody (diluted 1:200) incubation (room temperature; 30 min), 3,3'-diaminobenzidine dyeing solution (5 min) and hematoxylin (2 sec) were added for staining at room temperature. The images were captured under a light microscope (Olympus Corporation).

Statistical analysis. All experimental results in the present study were obtained from experiments repeated in at least three replicates. All data are expressed as the mean ± standard deviation. ImageJ (National Institutes of Health), SPSS Statistics 19.0 software (IBM Corp.) and GraphPad Prism 8.0 software (GraphPad Software, Inc.) were used to analyze the data and create the graphs, respectively. Unpaired Student's t-test was used for comparisons between groups. Statistical differences between ≥3 groups were determined using one-way ANOVA followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

CCL2 promotes proliferation of HUVECs. Cell proliferation assays revealed that CCL2 increased HUVEC proliferation in a concentration- and time-dependent manner (Fig. 1A).

CCL2 boosts the migration of HUVECs. The migration of HUVECs was measured using Transwell migration assay and wound healing assay. After treatment with different doses of CCL2 (0, 50 and 100 ng/ml) for 24 h, the cells that pierced the polycarbonate membrane and the coalescing wound areas were observed under a microscope at a magnification of x100. The results of the cell migration assay revealed that the numbers of migrated cells (% of control) of HUVECs were 100, 223±7 and 304±6% respectively (Fig. 1B). As shown in Fig. 1C, the migration area of HUVECs was compared among different treatment conditions. In addition, the results demonstrated that the migration areas of HUVECs were 30±5, 62±3, and 76±2%, respectively. The experiments revealed that CCL2 boosted the migration of HUVECs.

CCL2 promotes tube formation in HUVECs. One of the most essential steps in the processes of angiogenesis is endothelial cell proliferation, and the regeneration of new blood vessels is required for bone tissue growth. Vessel formation serves a key role in tissue rebuilding (11). HUVECs exposed to different concentrations of CCL2 were cultured in Matrigel, and then the construction of capillary-like structures was imaged by microscopy at a magnification of x100. Tube formation was assessed based on the number of branch points and capillary length indexes. Following treatment with different doses of CCL2 (0, 25, 50 and 100 ng/ml) for 4 h, the branch points of tube formation were 23±3, 38±2, 47±2 and 57±2. In addition, the capillary lengths (100% of control) of tube formation were 100, 132±9, 153±6, and 162±9% respectively (Fig. 1D). The results revealed that CCL2 at a suitable concentration had a positive effect on tube formation.

CCL2 promotes the expression levels of proteins related to proliferation, migration and vascularization in HUVECs. A number of studies have reported that Rock1, Rock2, N-cadherin and c-Myc are closely associated with proliferation and migration, and VEGFR2 is associated with vascularization (12-15). According to the results of western blotting, HUVECs treated with CCL2 exhibited marked expression of these markers (Fig. 2A). Therefore, we hypothesized that CCL2 serves a significant role in the proliferation, migration and vascularization of HUVECs.

CCL2 upregulates the PI3K/AKT, ERK1/2 and β-catenin signaling pathways to promote the proliferation and migration of HUVECs. The PI3K/AKT, ERK1/2 and β-catenin signaling pathways are related to cell proliferation and migration (16-18). To gain further insights into the molecular mechanism whereby CCL2 alters HUVEC behavior, the present study investigated the effect of CCL2 on the PI3K/AKT, ERK1/2 and β-catenin signaling pathways. Western blotting revealed that CCL2 treatment of HUVECs increased the levels of p-AKT, p-PI3K, p-ERK1/2, MMP9, Wnt5a/b, β-catenin and LRP-6 (Fig. 2B-D). Therefore, it was hypothesized that CCL2 may exert a positive regulatory effect on proliferation and migration in HUVECs by inducing these signaling pathways.

Immunohistochemistry. Femoral samples collected at 4 and 8 weeks after the bone defect operation underwent EDTA decalcification and immunohistochemical staining. The results revealed that there was an increase in the expression area of VEGF in CCL2-treated groups (Fig. 3).

Discussion

Large bone defects caused by trauma, bone tumors, limb deformity and bone infection are major clinical challenges in

orthopedics and have become a focus of research in the field of orthopedics (2,19). In the context of bone defects, angiogenesis and bone defect reconstruction are closely related in space and time and achieving adequate vascular development within regenerating bone tissue remains a significant challenge (20). The formation of blood vessels regulates the function of osteoblasts during bone regeneration and is an important regulator of bone regeneration (21). This interconnection between osteogenesis and angiogenesis is called 'angiogenesis-osteogenesis coupling', which promotes the repair and reconstruction of bone defects (22,23). VEGF, which is involved in the possible mechanisms of angiogenesis and osteogenic coupling, serves an important role between angiogenesis and bone remodeling (14,24). VEGF binds to three types of receptor tyrosine kinases in mammals, VEGFR1, VEGFR2 and VEGFR3 (25), which mediate endothelial cell regeneration, angiogenesis and regulation of vascular permeability (26). The VEGF family, including VEGFA-D serve an influential role in angiogenesis and development, especially VEGFA, which is the major factor in angiogenesis and works primarily through VEGFR2 (27). In present study, the tube formation assay revealed that CCL2 promoted angiogenesis in HUVECs. High expression levels of the angiogenesis-associated protein VEGFR2 following CCL2 treatment also suggested that CCL2 promoted angiogenesis. The results of immunohistochemical staining of the collected femoral specimens demonstrated that VEGF expression was markedly increased in the group supplemented with CCL2, which also confirmed that CCL2 induced angiogenesis associated with bone defect repair and reconstruction. These results demonstrated that CCL2 promoted vascular formation in HUVECs, and drove angiogenesis and osteogenic coupling-related protein VEGF expression.

Cadherin may activate some transduction pathways, including the Wnt/β-catenin pathway, which serves a crucial role in the growth and development of cells and tissues (28). N-cadherin is a type of transmembrane glycoprotein that mediates the adhesion between endothelial cells and pericytes and is closely related to the formation and maintenance of blood vessels (29). N-cadherin promotes cell migration, mobility and polarity through intracellular signaling at cell-cell junctions (30,31). Rock is known as an effector switch that regulates a range of cell biological processes, including cell adhesion, proliferation, migration and gene expression (32,33). The most typical downstream receptors of ras homolog family member A are the serine/threonine kinases Rock1 and Rock2, which are two subtypes of the Rock gene (34). Rock1 participates in regulation of the signaling pathways of cell migration (35) and promotes formation of the actin network (36). Rock1 may mediate the recruitment and adhesion of circulating leukocytes and inflammatory cells to sites of vascular injury to form a new intima (37). Similar to Rock1, Rock2 is a key gene for biological functions related to the transfer process, including the destruction of adhesion, remodeling of the actin cytoskeleton, enhancement of cell activity and regulation of signaling pathways (38), such as cell proliferation and migration. A study reported that c-Myc regulates the expression of thousands of downstream genes related to the regulation of cell proliferation, migration and apoptosis, affecting biological metabolism, transcriptional expression, protein synthesis and cell cycle regulation (39). Furthermore, as a key regulator of tissue growth, angiogenesis and the expression of angiogenic regulatory factors, c-Myc participates in the dominant regulation of the angiogenic network architecture. By contrast, c-Myc deficiency decreases the expression of VEGF (40), which affects the construction of vascular tissue. The results of the present study revealed that CCL2 treatment promoted the proliferation and migration of HUVECs in a concentration-dependent manner as demonstrated by the increased expression of the proliferation- and migration-related proteins Rock1, Rock2, N-cad and c-Myc in HUVECs. These results demonstrated that CCL2 promoted proliferation and migration in HUVECs.

Although various physiological and pathological functions of angiogenesis in bone remodeling have been extensively studied, little is known about the detailed mechanisms by which CCL2-activated signaling pathways are involved in the proliferation, migration and vascularization of vascular endothelial cells. AKT is a specific serine/threonine protein kinase downstream of the PI3K signaling pathway that regulates key cellular processes such as glucose metabolism, energy transformation, cell proliferation, cell growth and cell death (41,42). It also increases the secretion of VEGF and the phosphorylation of endothelial nitric oxide synthase, which promotes vasodilation and angiogenesis to induce growth factors, blocking apoptosis and increasing the cell survival rate (43). Phosphorylation of AKT promotes cell proliferation, migration and angiogenesis, which can help cells adapt to hypoxia and acidosis (44). In addition, ERK is a member of the MAPK family, and is the key molecule for transferring signals from cell surface receptors to the nucleus (45) and is known for its promotion of proliferation and differentiation (46). The ERK signaling pathway in endothelial cells mediates various cellular processes, such as proliferation, migration, survival and differentiation (47). p-ERK promotes the expression of MMP9, which is one of the Zn^{2+}-dependent endopeptidases downstream of ERK signal transduction (48). MMP9 can degrade denatured collagen and lytic extracellular matrix to facilitate remodeling of the extracellular matrix (49), which is significant in a variety of biological and molecular processes, including tissue repair, wound healing, cell differentiation and metastasis (50). Wnt5a is one of the Wnt family members whose signaling pathways regulate most cell growth and development processes, including cell proliferation, migration and survival (51). Wnt5a is involved in the regulation of angiogenesis by activating the Wnt/β-catenin signaling pathway. Upregulation of Wnt5a/b promotes the accumulation of β-catenin and mobilizes the classic Wnt signaling pathway to promote the expression of downstream target genes, such as vascular endothelial cadherin and MMP9, which are implicated in accelerating angiogenesis (52). In the classic Wnt/β-catenin signaling pathway, the connection of Wnt ligands to frizzled transmembrane receptors and LRP-6 to form protein complexes, ensures cell survival. It is also associated with the promotion of bone formation (53). The present study revealed that CCL2 treatment enhanced the levels of p-PI3K, p-AKT, p-ERK1/2, MMP9, LRP-6, Wnt5a/b and β-catenin in HUVECs. Regrettably, the lack of evaluation for ERK, PI3K, and β-catenin levels in tissue is a limitation of the present study. In summary, these results suggested that CCL2 served a positive regulatory role in the proliferation, migration

and angiogenesis of HUVECs by upregulating the PI3K/AKT, MAPK/ERK1/2/MMP9 and Wnt/β-catenin signaling pathways.

In conclusion, to the best of the authors' knowledge, the present study was the first to demonstrate that CCL2 promoted proliferation, migration and angiogenesis in HUVECs. In addition, the present study investigated the high expression of molecular markers linked to these functions induced by CCL2, including Rock1, Rock2, N-cadherin and c-Myc. Next, using reliable and sufficient methods, it was demonstrated that CCL2 promoted proliferation, migration and angiogenesis by activating the PI3K/AKT, MAPK/ERK1/2/MMP9 and Wnt/β-catenin signaling pathways. In addition, the SD rat bone defect model confirmed that CCL2 promoted the expression of the angiogenesis-osteogenesis coupling associated protein VEGF in bone defect reconstruction. Due to its mechanism of inducing angiogenesis (Fig. 4), CCL2 may be a novel angiogenesis-osteogenesis coupling agent for bone defect repair and reconstruction.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BW and SL conceived the current study. ZP, HW, XP and HP performed the experiments. ZP, HP, XP, QT and HW analyzed the results. ZP, HP, and QT drafted the manuscript. HW, BW and SL and HP revised the manuscript. HP and ZP confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments are approved by the Laboratory Animal Ethics Committee of Guangdong Medical University. (ID Number: GDY1902126; date: 25.05.2019).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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