Original Research

Epicatechin gallate promotes vascularization in co-culture of human osteoblasts and outgrowth endothelial cells

Liyan Zhang[1]*, Miaoran Wang[1]*, Huiqing Qiu[1,2]*, Yusen Wei[1], Lu Zhou[1], Nannan Nian[1], Zhongli Shi[3,4], Dailun Hu[5] and Bin Ma[6]

[1]Graduate School, Hebei Medical University, Shijiazhuang 050017, China; [2]Department of Geriatrics, The First Hospital of Hebei Medical University, Shijiazhuang 050030, China; [3]Hebei Key Laboratory of Brain Science and Psychiatric-Psychologic Disease, The First Hospital of Hebei Medical University, Shijiazhuang 050030, China; [4]Central Laboratory, The First Hospital of Hebei Medical University, Shijiazhuang 050030, China; [5]Department of Pathogenic Biology, Hebei Medical University, Shijiazhuang 050017, China; [6]School of Medical, Molecular and Forensic Sciences, Murdoch University, Murdoch, WA 6150, Australia
*These authors contributed equally to this paper.
Corresponding authors: Dailun Hu. Email: 17300574@hebmu.edu.cn; Bin Ma. Email: B.Ma@murdoch.edu.au

Impact Statement

Tissue-engineered bone needs to achieve rapid and efficient vascularization/angiogenesis. We have previously used co-cultures of osteoblast and outgrowth endothelial cells (OECs; isolated and cultivated from peripheral blood) to promote angiogenesis and osteogenesis. Using our cell co-culture model, we now confirm the promoting effects of epicatechin gallate (ECG; the main component of green tea catechins with various physiological and pharmacological properties, e.g. anti-inflammation response, antioxidant activities, regulation of cell proliferation/apoptosis, and anticancer effects) in angiogenesis and osteogenesis. ECG thus has potential applications in the promotion of angiogenesis/vascularization and osteogenesis *in vitro* for the better production of bone tissue and many other constructs. In addition, it can be used *in vivo* for the further continuous stimulation of angiogenesis/osteogenesis and the survival of tissue constructs after implantation. Furthermore, ECG might be used to promote wound/bone fracture healing and to prevent bone tissue loss (e.g. caused by osteoporosis and aging).

Abstract

Prevascularization is crucial for the survival of tissue-engineered bone and further bone repair/regeneration. Since epicatechin gallate (ECG), the most abundant flavanol in green tea, shows potential beneficial effects on endothelial cells and bone cells, we decided to investigate whether it promotes vascularization/angiogenesis and osteogenesis using a co-culture system containing human primary osteoblasts (POBs) and outgrowth endothelial cells (OECs). We found that treatment with ECG (1) significantly enhanced microvessel formation in co-culture of POB and OECs, (2) improved cell viability/proliferation and the angiogenic/osteogenic capacities of OEC/POBs, (3) significantly increased the levels of E-selectin, IL-6, TNF-α, IFN-γ, VEGF, and PDGF-BB in co-cultures of POB and OEC, and (4) upregulated HIF-1α, HIF-2α, NF-κB, iNOS, GLUT1, VEGF, and Ang1/2 but downregulated PHD1 in monocultures of OEC or POB. Our findings demonstrate that ECG promotes angiogenesis and osteogenesis (probably via HIF signaling) in co-cultures of OECs and POBs. ECG thus has potential applications in the promotion of angiogenesis/vascularization in many tissue constructs including those of bone.

Keywords: Epicatechin gallate, vascularization, outgrowth endothelial cell, bone engineering, hypoxia-inducible factor, angiogenesis

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Introduction

A tremendous demand exists for tissue-engineered bone worldwide.[1–3] In clinical practice, large-segment bone defects caused by trauma, tumor resection, osteomyelitis, and congenital deformities are common and difficult to repair.[1–3] In addition, non-union and delayed fracture healing often occur in conventional fractures and intervertebral fusion.[3] These bone defects can seriously threaten the health and lives of patients. Hence, the rapid and "complete" repair of such bone defects remains a hot topic in both tissue engineering and clinical medicine. One aim of tissue engineering approaches (living transplantation) via the development of bioactive/biodegradable bone substitutes (i.e. tissue-engineered bone) to repair bone defects and to enhance the body's self-repair ability to achieve successful regeneration is

Table 1. Specificities and sources of primary and secondary antibodies.

Target (clone) [Cat. no.]	Conjugate	Species and isotype	Company
CD31 (2F7B2) [MA5-15336]		Mouse IgG1, monoclonal	Thermo Fisher Scientific (Waltham, MA, USA)
Ki67 (SP6) [MA5-14520]		Rabbit IgG, monoclonal	Thermo Fisher Scientific
Osteocalcin (N-terminal) [SAB1306277]		Rabbit IgG, polyclonal	Sigma (Burlington, MA, USA)
Human alkaline phosphatase /ALPL [MAB1448]		Mouse IgG1, monoclonal	R&D Systems (Minneapolis, MN, USA)
Osteopontin		Rabbit IgG, polyclonal	Affinity Biosciences (Changzhou, Jiangsu, China)
VEGFA (EP1176Y) [ab52917]		Rabbit IgG, monoclonal	Abcam (Cambridge, UK)
HIF-1 alpha [ab2185]		Rabbit IgG, polyclonal	Abcam
HIF-2 alpha [ab199]		Rabbit IgG, polyclonal	Abcam
Angiopoietin 1 [ab8451]		Rabbit IgG, polyclonal	Abcam
Angiopoietin 2 [ab8452]		Rabbit IgG, polyclonal	Abcam
Anti-PHD1/prolyl hydroxylase (EPR2746) [ab113077]		Rabbit IgG, monoclonal	Abcam
NF-κB p65 (E379) [ab32536]		Rabbit IgG, monoclonal	Abcam
iNOS (EPR16635) [ab178945]		Rabbit IgG, monoclonal	Abcam
Glucose transporter GLUT1 (EPR3915) [ab115730]		Rabbit IgG, monoclonal	Abcam
GAPDH [AP0063]		Rabbit IgG, polyclonal	BioWorld (Bath, UK)
Rabbit IgG [A23220]	Dylight 488	Goat polyclonal	Abbkine (Wuhan, Hubei, China)
Rabbit IgG [A23320]	Dylight 549	Goat polyclonal	Abbkine
Rabbit IgG [A23720]	Dylight 680	Goat polyclonal	Abbkine
Rabbit IgG [A23920]	Dylight 800	Goat polyclonal	Abbkine

to overcome the shortcomings and deficiencies of traditional repair methods (e.g. autologous/allogeneic bone transplantation and artificial material replacement).[1–4]

Nevertheless, one challenge that is faced when tissue-engineered bone is used in such approaches is the achievement of rapid and efficient vascularization/angiogenesis.[5,6] Currently, three common experimental approaches are used to promote the vascularization/angiogenesis of tissue-engineered bone. The first is to use the co-culture of osteoblasts/mesenchymal stem cells (MSCs) and vascular endothelial cells to promote the vascularization of tissue-engineered bone.[7–9] Recent studies have shown that the osteogenic ability and the degree of vascularization of the tissue-engineered bone grown with vascular endothelial cells are higher than those of the tissue-engineered bone constructed by osteoblasts only.[9] Endothelial progenitor cells from peripheral blood or cord blood are potential autologous cell sources for cellular therapies aimed at enhancing the neovascularization of tissue-engineered bone.[7–10] The second is the application of bioactive factors (e.g. growth factors or natural products) to promote vascularization.[4,11] The third is to construct vascularized tissue-engineered bone by vascular bundle implantation. Under the action of *in vivo* environment factors and local stimuli, many collateral capillaries can grow and fuse to form a functional capillary network.[12]

After implantation, tissue-engineered bone is exposed to hypoxic conditions before a functional vascular network can form to supply cells/tissues with nutrients and oxygen.[13,14] Therefore, hypoxia signaling pathways might be closely related to the angiogenesis/vascularization of tissue constructs. Indeed, in our previous study involving the use of a co-culture model, we have shown that short-term hypoxia can indeed promote vascularization.[8]

Intracellular hypoxia-inducible factors (HIFs) are transcription factors that respond to decreases in the available oxygen in the cellular environment, namely hypoxia. Hypoxia-inducible factor-1α (HIF-1α) directly activates the expression of several hundred target genes to maintain cellular oxygen homeostasis and to mediate adaptive, protective, and pathological processes.[15] For example, HIF-1 activation can increase cell proliferation, differentiation, and survival.[16] In addition, HIF-1 activation can also increase angiogenesis/vascularization and osteogenesis under various conditions.[8,17,18] Therefore, HIF-1 has been used as a pharmacological target for the treatment and therapy of certain diseases (e.g. cancer).[19]

(-)-Epicatechin-3-gallate (ECG), the main component of green tea catechins, has various pharmacological and physiological properties (e.g. in the anti-inflammation response, in the mediation of antioxidant activities, in the regulation of cell proliferation/apoptosis, and in anticancer effects during angiogenesis, invasion, and the various stages of metastasis).[20] In the context of angiogenesis/vascularization, ECG can exert protective effects on human microvascular endothelial cells and can even promote neovascularization under certain conditions.[21,22] Furthermore, ECG (or (-)-epigallocatechin-3-gallate [EGCG]) can also promote the osteogenic differentiation of human bone marrow MSCs and stimulate osteoblast differentiation.[23,24] Because of these beneficial effects of ECG, it has been used to promote health/fitness and has been tested for the potential control/treatment of certain disorders and diseases.[20,25,26] We therefore decided to investigate whether ECG (an activator/regulator of the HIF-1 signaling pathway)[27] can promote the vascularization/angiogenesis of bone tissue constructs using a co-culture system[7,8] containing human primary osteoblasts (POBs) and outgrowth endothelial cells (OECs).

Materials and methods

Reagents

ECG ($C_{22}H_{18}O_{10}$; 99%, from green tea; Cat. No.: 426323) was supplied by J&K Scientific (San Jose, CA, USA). ECG was diluted in phosphate-buffered saline (PBS; Sigma) to make a stock solution. The specificities and sources of antibodies are described in Table 1.

Isolation and culture of OECs and POBs

For the isolation and culture of OECs, three healthy volunteers were enrolled (one man and two women; aged 28, 21, and 23 years, respectively). Mononuclear cells were isolated from their peripheral blood by Ficoll-Paque™ Plus (GE Healthcare, Chicago, Illinois, USA) density gradient centrifugation.[28] OECs were then prepared from these mononuclear cells and characterized by checking their surface markers (e.g. CD31, CD34, CD45, and CD14) with flow cytometry.[28] OEC was cultivated in endothelial growth medium (EGM)-2 (Lonza, Basel, Switzerland) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific), supplements from the EGM-2 BulletKit (Lonza), and penicillin-streptomycin (100 units/mL; Thermo Fisher Scientific). OECs from passages 8 to 13 were used for this study.

POBs were isolated from human cancellous bone fragments from six donors who underwent hip replacement surgery (without other disorders/infection). After isolation and culture, POBs were characterized using markers, such as osteocalcin and osteonectin, as previously described.[7,8,14] POBs were cultivated in Gibco Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12; Thermo Fisher Scientific) containing 10% FBS and penicillin-streptomycin (100 units/mL). POBs from passages 3 to 6 with osteogenic characteristics were used. OECs and POBs from at least three different donors were used for each monoculture experiment.

Treatment of co-culture of POBs and OECs with ECG

POBs (1×10^5/well) were seeded onto round glass coverslips (diameter 13 mm; precoated with rat tail collagen type I [Sigma]) in 24-well plates and were cultivated in GIBCO DMEM/F12 medium containing 10% FBS and penicillin-streptomycin (100 units/mL). At 24 h after incubation, 1×10^5 OECs were added to each well. The co-cultures were then cultivated for seven days in EGM-2 (with 10% FBS, supplements from the EGM-2 BulletKit, and penicillin-streptomycin [100 units/mL]). After 14 days of *in vitro* culture, cells/co-cultures were treated with ECG (experimental group) or PBS only (control group). After a medium change, the co-culture was further cultivated for three days. The supernatant and cells were then collected for further analysis.

Cell viability assay

Cell viability was assessed by Cell Counting Kit-8 (CCK-8; AR1160-100, Boster Bio, Wuhan, Hubei, China). Briefly, 2×10^3 OECs or POBs in 100 µL medium were plated into each well of 96-well plates and were cultured in a 37°C incubator (with 5% CO_2) for 24 h. Only PBS was added to the control group, whereas ECG was added at various concentrations to the experiment group. After treatment, cells were further cultivated for 24 h. Each group consisted of five replicate wells. The culture medium was then removed, and diluted CCK-8 solution (1:10) was added to each well. After the cells had been further incubated for 1 h, absorbance at 450 nm was recorded using the GloMax®-Multi Detection System (Promega, Madison, WI, USA).

Relative survival rate = Corrected absorbance values of the treatment group/Corrected absorbance values of the control group. The relative survival rate of control group was set to 1.0.

Wound healing assay

OECs (2×10^5) were plated into each well of six-well plates (with marking lines having been drawn onto the bottom of the plates in advance). After 24 h of culture, that is, once the cells had reached 100% confluency, a sterile 200 µL pipette tip was used to scratch the bottom of the cell culture and to create linear defects perpendicular to the predrawn marker lines. The exfoliated cells were removed by three washes of PBS (Sigma), and the OEC culture was further cultivated in EGM-2 medium (with and without ECG). Images of wound closure were taken at the exact location at 0 and 6 h using an inverted Nikon Y-IDP inverted light microscope equipped with a color camera (Nikon, Tokyo, Japan).

$$\text{Relative wound width} = \frac{(\text{Width 1 (0 h)} - \text{Width 2 (6 h)})}{\text{Width 1 (0 h)}} \times 100\%$$

Tube formation assay

BD Matrigel™ matrix was purchased from BD (Franklin Lakes, NJ, USA), and the assay was performed according to the manufacturer's instructions. Matrix gel (10 mg/mL) was thawed overnight on ice at 4°C in a refrigerator. Subsequently, 50 µL Matrigel was plated into precooled 96-well plates horizontally (allowing the Matrigel to distribute evenly) and incubated for 1 h at 37°C. OECs (2×10^4) in 100 µL culture medium with/without ECG were then loaded onto the top of the Matrigel in the 96-well plates. Care was taken to avoid the formation of air bubbles. After 6 h of incubation, images of the cells were taken using an inverted Nikon Y-IDP light microscope equipped with a color camera. After image acquisition, the total length of any tubules and the number of branches formed were counted. Each treated group comprised four to six wells.

Immunofluorescent staining

Washing (3×5 min) between each step was performed with PBS. Cells or co-cultures on 13-mm glass coverslips were fixed with 4% paraformaldehyde (PFA) at room temperature for 15 min. After being washed and treated with 0.3% Triton X-100 (Sigma) in PBS for 5 min, the cells received treatment with 2% goat serum (Solarbio, Beijing, China) in PBS for 20 min to block potential unspecific binding sites. After the blocking step, the cells were incubated with primary antibodies at 4°C overnight, followed by a 1-h incubation with the secondary antibodies. Following the final wash step, the coverslips were mounted upside-down on microscope slides (26×76 mm^2) with VECTASHIELD Antifade Mounting Medium (Vector Laboratories, Newark, CA, USA).

Confocal microscopy and image analysis

Confocal microscopy was performed using a ZEISS LSM 900 laser scanning confocal microscope (Zeiss, Oberkochen,

Germany). Images were acquired and then saved in TIFF format. For each experiment, all images were acquired and processed using the same scales and parameters. The intensity index of images was calculated using the ImageJ software as previously described.[7] The area of microvessel was analyzed using the method described in our previous studies.[7,8]

Annexin V-fluorescein isothiocyanate Apoptosis Staining

The Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit I was purchased from BD, and the assay was performed according to the manufacturer's instructions.[11] After OECs had been cultivated to an appropriate density *in vitro*, they were treated with ECG at an appropriate concentration for 24 h. The cells were then harvested (by trypsinization), counted, and stained with Annexin V-FITC. Finally, the cells were analyzed by a Navios Flow Cytometer (Beckman Coulter, Brea, CA, USA) within 1 h.

Western blotting

Dilution and washing (4 × 10 min) between each step were performed with Tris-buffered saline with 0.1% Tween® 20 Detergent (TBST). Protein samples were extracted from the cells by 14,000 × g centrifugation at 4°C after cell lysis. The protein concentration of the samples was determined by the BCA Protein Assay Kit (Boster Bio, Pleasanton, CA, USA).

The TGX Stain-Free™ FastCast Acrylamide Gel Preparation Kit was purchased from Bio-Rad (Hercules, CA, USA). Proteins were separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% non-fat dry milk in TBST for 1 h and then incubated in the diluted primary antibody at 4°C overnight. After being washed, the membrane was incubated in the diluted secondary antibody (goat anti-rabbit IgG Dylight 800) for 1 h at room temperature. Finally, the blot was scanned and analyzed by an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous standard, and the relative expression rate of control cultures was set to 1.

Relative protein expression rate = (Protein intensity of experiment group/GAPDH intensity of experiment group)/(Protein intensity of control group/GAPDH intensity of control group).

Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) kits for human E-selectin, interleukin 6 (IL-6), Interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) were purchased from Multi Sciences Biotech (Hangzhou, Zhejiang, China). ELISA of the supernatant from co-cultures of OECs and POBs was performed according to the manufacturer's instructions.[11] Absorbance was measured using a Tecan Infinite M200 Pro Plate Reader (Tecan, Männedorf, Switzerland) at 450 and 570 nm.

RNA extraction and quantitative real-time polymerase chain reaction

Total RNA from OECs was extracted using the RNA-easy Isolation Reagent R701 (Vazyme, Nanjing, Jiangsu, China) according to the manufacturer's instructions.[11] The concentration and purity of the RNA samples were determined spectrophotometrically by the measurement of absorbance at 260 and 280 nm. Integrity was confirmed by agarose gel electrophoresis.

Synthesis of first-strand cDNAs, quantitative real-time PCRs, and data analysis was performed as previously described.[11] Quantitative real-time polymerase chain reaction (qRT-PCR) primers were synthesized by SaiBaiSheng Gene Technology (Beijing, China). The sequences for qRT-PCR primers were as follows: vascular endothelial growth factor (VEGF), forward 5′-CGA GGG CCT GGA GTG TGT-3′, reverse 5′-CCG CAT AAT CTG CAT GGT GAT-3′; platelet-derived growth factor-BB (PDGF-BB), forward 5′-GAT CCG CTC CTT TGA TGA TCT-3′, reverse 5′-TCC AAC TCG GCC CCA TCT-3′; GAPDH, forward 5′GGTGAAGGTCGGTGTGAACG3′, reverse 5′CTCGCTCCTGGAAGATGGTG3′. GAPDH was used as an endogenous standard, and the value of control cultures was set to 1.

Statistical analysis

All experiments were repeated at least three times ($n = 3$), and all data are expressed as means ± standard deviations. MS-Excel was used for statistical analysis. In cases of more than three parameter groups, one-way analysis of variance (ANOVA) was used for statistics. Statistical analysis was performed using GraphPad Prism Version 8.2.0 (GraphPad Software Inc., San Diego, CA, USA).

Statistically significant difference was evaluated using the paired Student's *t*-test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). Differences were considered statistically significant if the P value was smaller than 0.05.

Results

ECG enhances microvessel formation in co-cultures of OECs and POBs

We first tested the effect of ECG on microvessel formation in co-cultures of OECs and POBs. The days *in vitro* (DIV) 14 co-culture was stimulated with ECG for 24 h, and CD31 was used to assess the microvessel area. The results are shown in Figure 1. In all treated co-cultures (with ECG at 2, 4, and 8 μM), significant enhancement of microvessel formation was observed compared with corresponding control co-cultures. Among the three ECG concentrations, we observed that 4 μM gave the best stimulatory effect in all three donors (Figure 1[A]). This type of stimulatory effect was present in all three OEC donors (Figure 1[B]).

ECG promotes cell survival, proliferation, and migration of OECs

To explore the potential mechanisms of the ECG promotion of microvessel formation, we investigated the effects of ECG on monocultures of OEC. These monocultures were treated

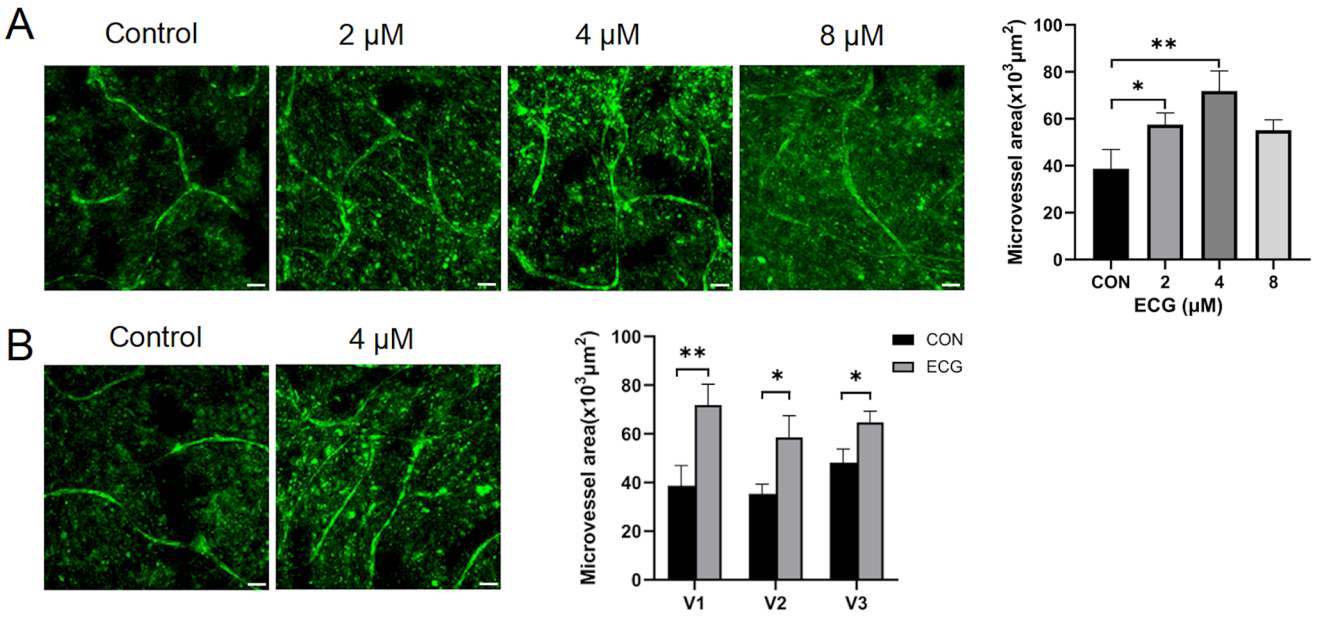

Figure 1. Effect of ECG treatment on microvessel formation in co-cultures of POBs and OECs. DIV14 co-cultures of POBs and OECs were treated with ECG for 24 h and cultivated further for three days before CD31 staining for microvessels. The quantification of the microvessel area is shown in the right panels. (A) Co-cultures of POBs and OECs (from donor V1) stimulated with ECG at various concentrations. (B) Co-cultures of POBs and OECs (from donors V1, V2, and V3) stimulated with 4 μM ECG. For each experiment, three representative images from co-cultures were acquired and analyzed. $^{*}P < 0.05$; $^{**}P < 0.01$; scale bars = 50 μm; Objective lens: 20×; CON: control.

with ECG at various concentrations, and the cell viability of the OEC was analyzed (Figure 2[A]). We found that high concentrations (62.5–1000 μM) of ECG reduced cell survival (indicating cytotoxicity), whereas low concentrations (0.5–8 μM) of ECG promoted cell survival.

Subsequently, monocultures of OEC were treated with 4 μM ECG to check the effect of ECG on cell apoptosis (Figure 2[B]). Compared with the control group, apoptosis was significantly reduced after treatment with 4 μM ECG for 24 h. Monocultures of OEC were also treated with 4 μM ECG, and the cell proliferation was analyzed by Ki67 immunostaining (Figure 2[C]). We observed that OEC proliferation was enhanced after stimulation with 4 μM ECG for 24 h. Finally, the wound scratch assay revealed that treatment of OECs with 4 μM ECG for 24 h enhanced the directed cell migration ability of OECs (Figure 2[D]).

ECG increases the angiogenic ability of OECs

Observation of the *in vitro* formation of capillary-like tubes by endothelial cells on a basement membrane matrix is a rapid and robust method for screening various factors that promote or inhibit angiogenesis. To study the effect of ECG on their angiogenic ability, OECs were treated with 4 μM ECG for 24 h, and tubule formation was analyzed. As shown in Figure 3(A), ECG treatment enhanced tubule formation significantly compared with the control group.

We also checked the effect of ECG on the expression levels of several angiogenic factors, such as VEGF, angiopoietin 1 (Ang1), and angiopoietin 2 (Ang2). As shown in Figure 3(B), ECG upregulated the expression of VEGF, Ang1, and Ang2 in OECs compared with the control group.

ECG promotes cell proliferation/survival and upregulates osteogenic factors

We also investigated the effect of ECG on osteogenesis, in addition to angiogenesis. Monocultures of POB were treated with ECG at various concentrations for 24 h, and the cell viability was analyzed (Figure 4[A]). We found that high concentrations (62.5–1000 μM) of ECG reduced cell survival (indicating cytotoxicity), whereas low concentrations (0.5–8 μM) of ECG promoted the cell survival of POBs. Subsequently, monocultures of OEC were treated with 4 μM ECG for 24 h, and the cell proliferation was checked by Ki67 immunostaining (Figure 4[B]). We observed that POB proliferation was enhanced after stimulation with 4 μM ECG. To investigate the osteogenic effects of ECG, POBs were also treated with 4 μM ECG for 24 h, and immunofluorescence staining was performed for two essential osteogenic factors (osteocalcin and osteopontin) and bone alkaline phosphatase (ALP). The results are shown in Figure 4(C). The expression levels of osteocalcin, osteopontin, and ALP increased significantly after ECG treatment.

ECG upregulates inflammatory and angiogenic factors in co-cultures

To analyze the molecular mechanisms by which ECG promotes angiogenesis and osteogenesis in co-culture, we explored several inflammatory and angiogenic factors related to angiogenesis. At 24 h after ECG treatment, the supernatants from the co-cultures of OECs and POBs were examined by ELISA, and the results are shown in Figure 5(A). We observed that the expression level of E-selectin had increased at 24 h after ECG treatment. In addition, the expression levels of three cytokines, namely IL-6, TNF-α, and INF-γ

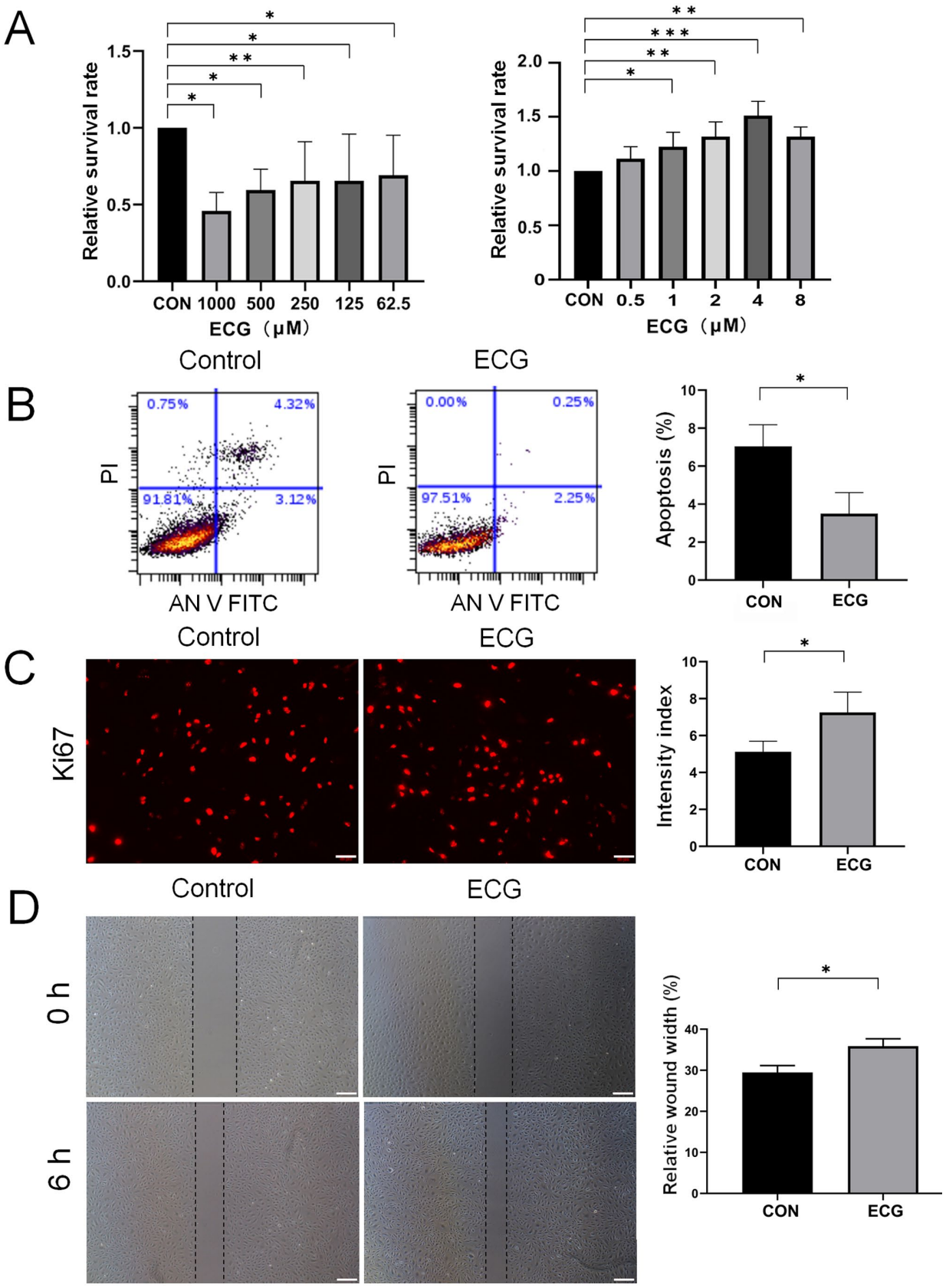

Figure 2. Effect of ECG on cell viability, apoptosis, proliferation, and directed migration ability of OECs. (A) The left panel shows the cell viability of OECs treated with ECG at high concentrations (1000, 500, 250, 125, or 62.5 μM) for 24 h. The right panel shows the cell viability of OECs treated with ECG at low concentrations (0.5, 1, 2, 4, or 8 μM). (B) to (D) OECs were treated with 4 μM ECG for 24 h and analyzed. (B) The effect of ECG on apoptosis was evaluated by Annexin V-FITC Apoptosis Staining. (C) OECs were stained for Ki67 (red) to check the effect of ECG on cell proliferation. (D) Wound scratch assay to study directional OEC migration *in vitro* (without and with ECG treatment). The black dashed lines show the wound/wound closure. For each experiment, three representative images from OECs were acquired and analyzed. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; Objective lens: 20×; scale bars = 50 μm; CON: control.

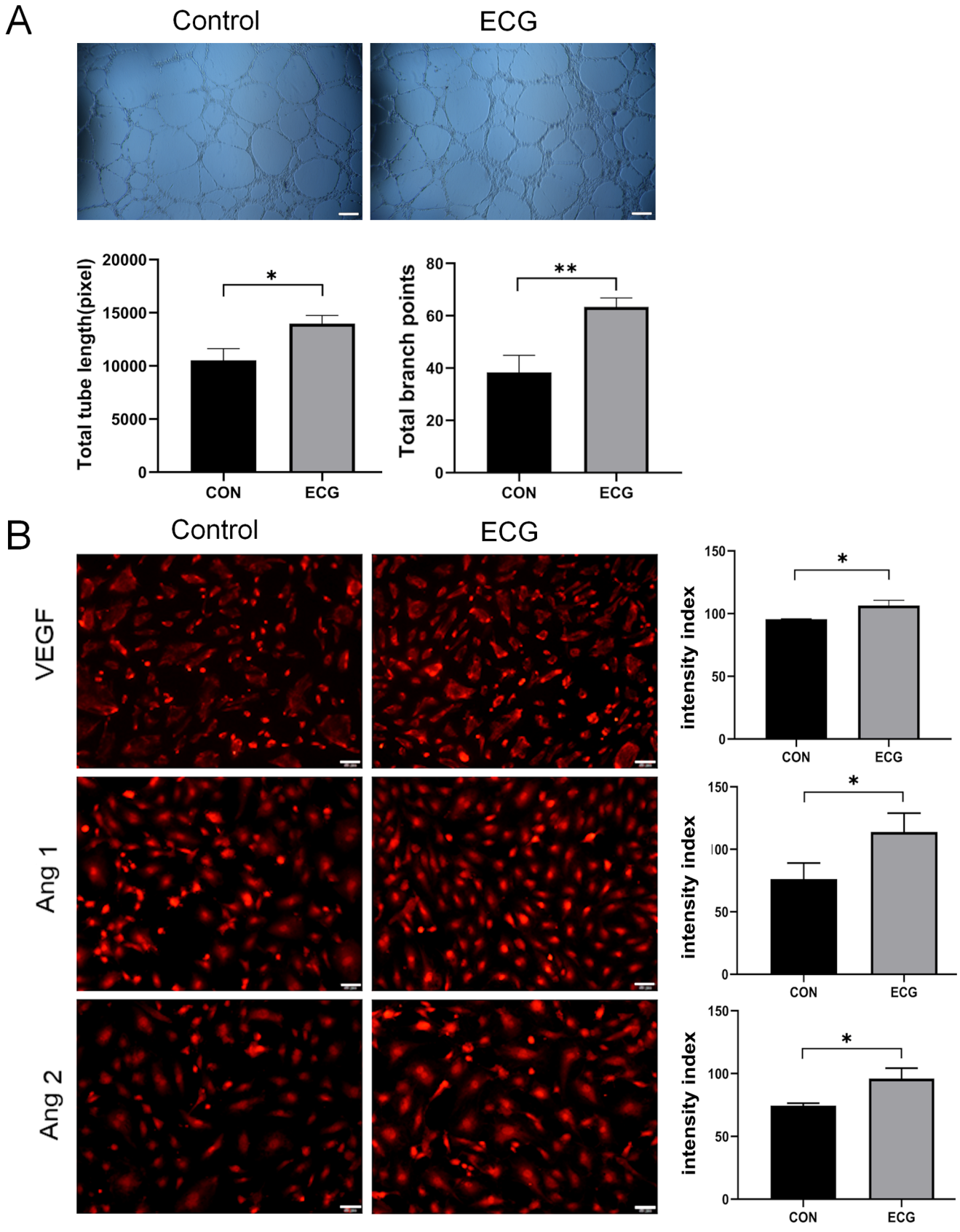

Figure 3. Effect of ECG treatment on the angiogenic capacities of OECs. (A) Representative images (upper panels) showing the formation of tubular structures with/without ECG treatment (4 μM, 24 h). Quantitative analysis (lower panels) showing the total length of tubules and the number of branches. (B) OECs were treated with 4 μM ECG for 24 h and were stained for angiogenic factors-VEGF, Ang1, and Ang2 (red). Three images from co-cultures were taken and analyzed for each experiment. Quantitative analyses are shown in the right panels. *$P < 0.05$; **$P < 0.01$; VEGF: vascular endothelial growth factor; Ang1: angiopoietin 1; Ang2: angiopoietin 2; CON: control; Objective lens: 20×; scale bars = 50 μm.

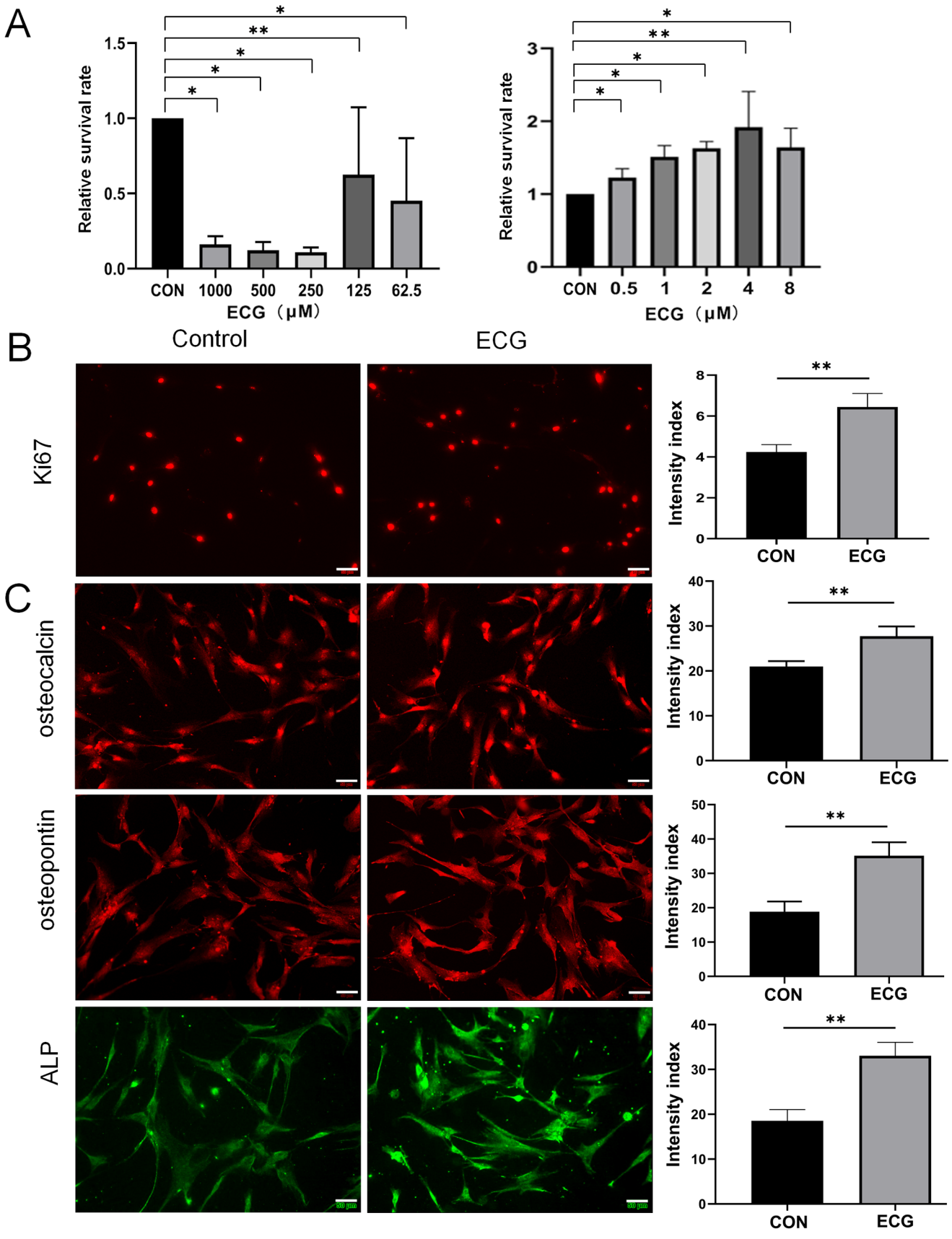

Figure 4. Effect of ECG treatment on monoculture of POBs. (A) The left panel shows the cell viability of POBs treated with ECG at high concentrations (1000, 500, 250, 125, or 62.5 μM) for 24 h. The right panel shows the cell viability of POBs treated with ECG at low concentrations (0.5, 1, 2, 4, or 8 μM). (B) and (C) POBs were treated with 4 μM ECG for 24 h, and the cells were immunostained for Ki67 and osteogenic markers (osteocalcin, osteonectin, and ALP). Representative images are shown in the left panels, and the quantitative analyses are shown in the right panels. $n = 3$; $^{*}P < 0.05$; $^{**}P < 0.01$; CON: control; ALP: alkaline phosphatase; Objective lens: 20×; scale bars = 50 μm.

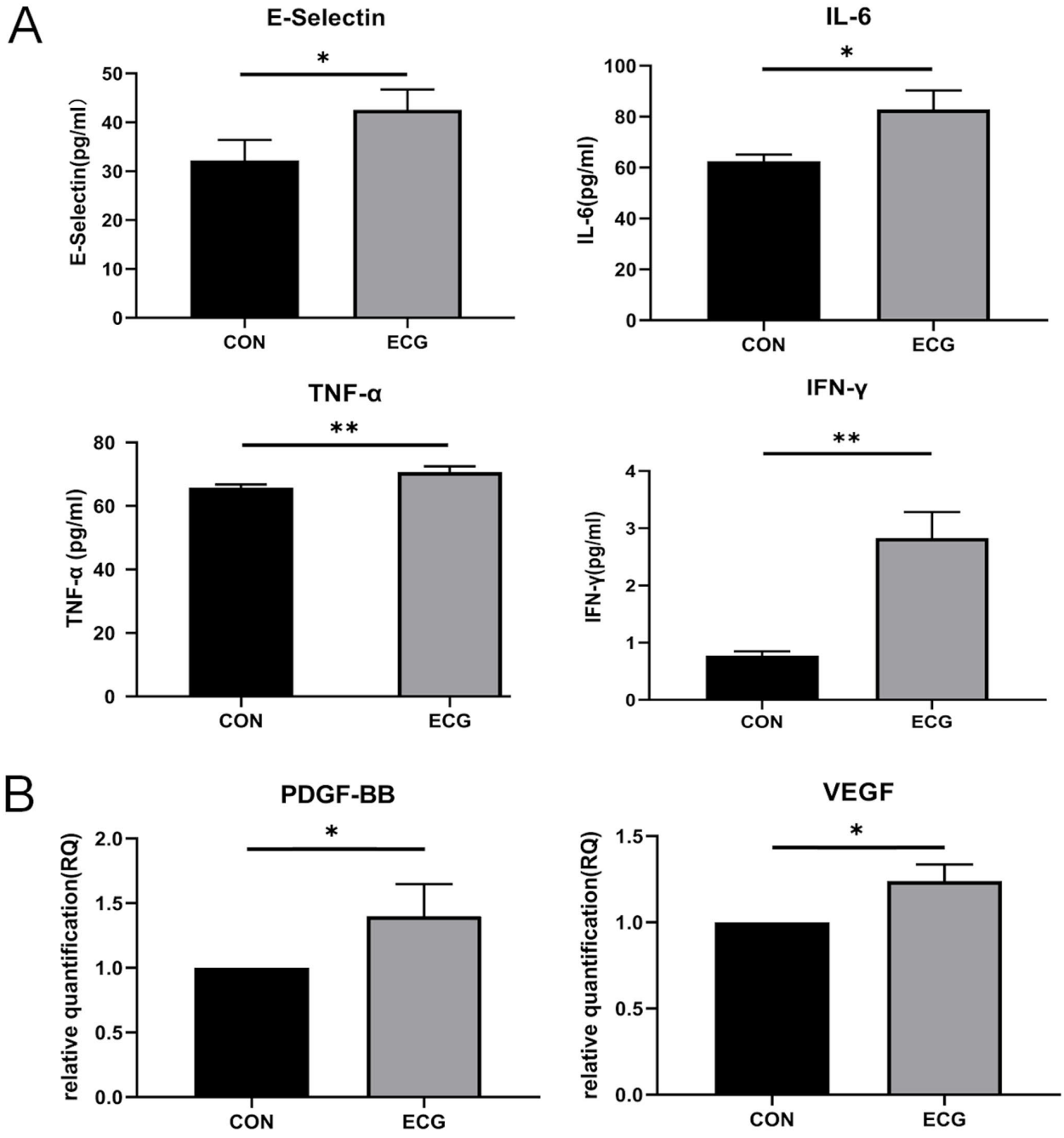

Figure 5. Effect of ECG on the expression levels of several inflammatory and angiogenic factors in co-cultures of POBs and OECs after treatment with 4 μM ECG for 24 h. (A) The levels of E-selectin, IL-6, TNF-α, and IFN-γ were analyzed by ELISA. (B) Relative gene expression of VEGF and PDGF-BB was measured by qRT-PCR. $n = 3$. $^{*}P < 0.05$; $^{**}P < 0.01$; CON: control.

(which play essential roles in endothelial cell survival and exert proangiogenic effects on endothelial cells) were also significantly upregulated after a 24-h treatment with 4 μM ECG (Figure 5[A]). Subsequently, qRT-PCR showed that the levels of VEGF and PDGF-BB in co-cultures of OECs and POBs were upregulated after a 24-h treatment with 4 μM ECG (Figure 5[B]).

Effect of ECG on expression of hypoxia signal pathway molecules

To explore the molecular mechanisms by which ECG promotes angiogenesis and osteogenesis, we analyzed several factors related to HIF signaling pathways in monocultures of POB or OECs

We first treated OEC monocultures with 4 μM ECG for 24 h and determined the expression levels of HIFs and their targeted signal pathway molecules. The results are shown in Figure 6(A). We found that, in OECs, ECG treatment upregulated HIF-1α/HIF-2α, while downregulating prolyl hydroxylase 1 (PHD1). In addition, ECG treatment increased the levels of glucose transporter 1 (GLUT1), nuclear factor κ-B (NF-κB), and inducible nitric oxide synthase (iNOS).

We also treated POB monocultures with 4 μM ECG for 24 h and assessed the expression levels of HIFs and their targeted signal pathway molecules. We observed similar regulatory

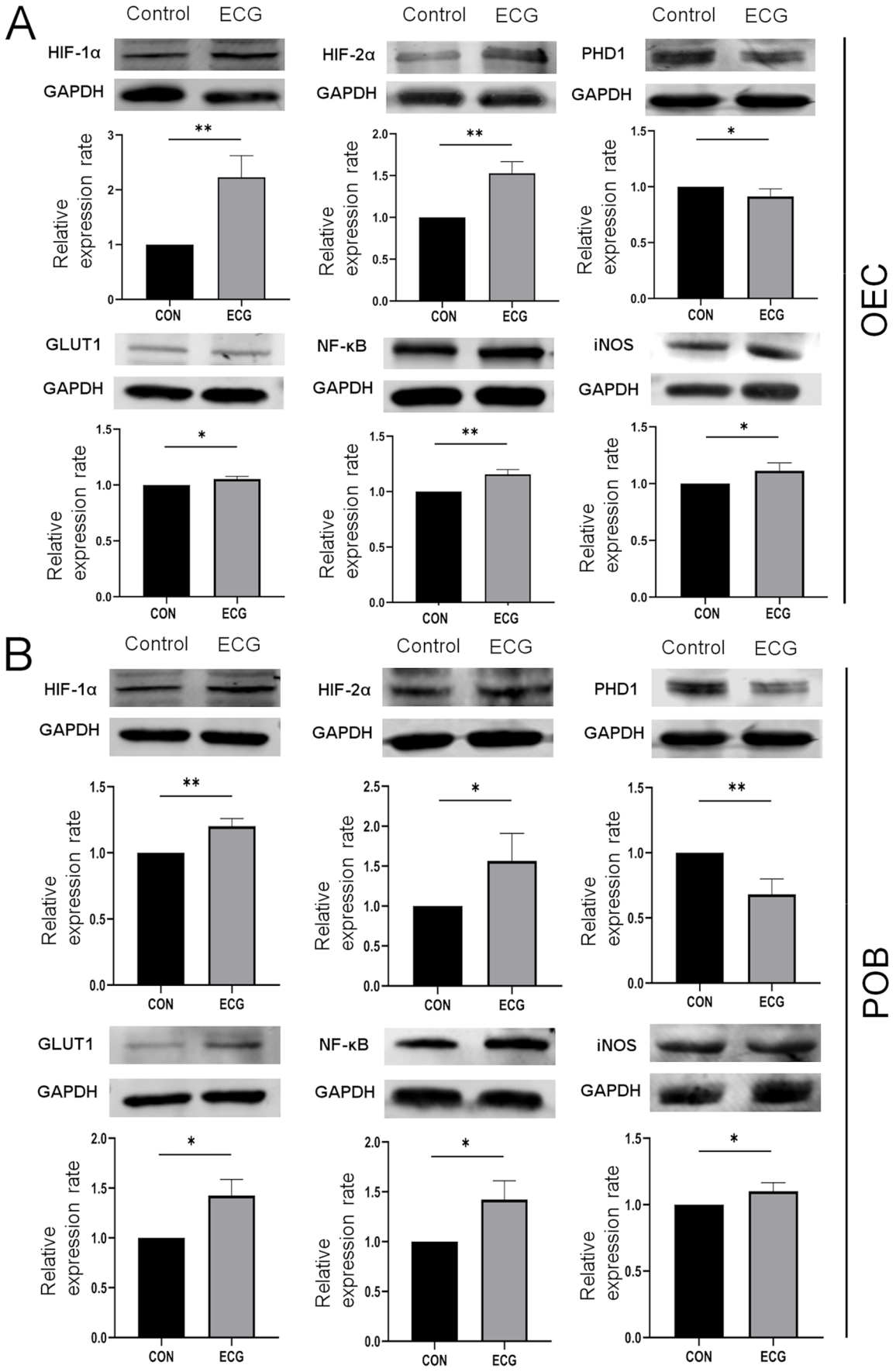

Figure 6. ECG treatment affects the expression of HIF and related factors in OECs and POBs. (A) Expression levels of HIF-1α, HIF-2α, PHD1, GLUT1, NF-κB, and iNOS in OECs after treatment with 4 μM ECG for 24 h. (B) Expression levels of HIF-1α, HIF-2α, PHD1, GLUT1, NF-κB, and iNOS in POBs after treatment with 4 μM ECG for 24 h. *$P < 0.05$; **$P < 0.01$; CON: control.

effects of ECG on POBs compared with its effects on OECs. The results are shown in Figure 6(B). We found that, in POBs, ECG treatment upregulated HIF-1α/HIF-2α, while downregulating PHD1. In addition, ECG treatment increased the expression levels of GLUT1, NF-κB, and iNOS in POBs.

Discussion

During the last few decades, various innovative strategies have been developed for the rapid and successful vascularization/angiogenesis in tissue-engineered constructs *in vitro* (before implantation) and *in situ* (after implantation).[9] Using a co-culture system containing POBs and OECs in this study, we have shown that ECG, a natural product from green tea, can promote angiogenesis/vascularization and osteogenesis at suitable concentrations.

ECG enhances angiogenic and osteogenic capacities of OEC and POB

Recent studies have demonstrated that ECG exerts protective effects on human microvascular endothelial cells and even promotes neovascularization under various conditions.[21,22,29] To explore the potential mechanisms of the ECG promotion of angiogenesis/vascularization, we analyzed the effects of ECG on monocultures of OECs. We have shown that ECG at appropriate concentrations (e.g. 4 μM) can promote cell viability, cell proliferation, and the directed migration of OECs, namely three essential factors for angiogenesis/vasculogenesis.

VEGF (a subfamily of PDGF) and the angiopoietins (members of a family of vascular growth factors) are essential molecules for new vessel formation and stabilization in development and in tissue repair.[30] VEGF induces the proangiogenic activation (facilitated by Ang2) of endothelial cells, leading to the destabilization of cell–cell and cell–matrix interactions of endothelial cells.[31] These steps play significant roles in vascularization and angiogenesis. Studies have also shown that Ang2 promotes angiogenesis through integrin signaling the endothelial-specific receptor, tyrosine kinase with immunoglobulin-like loops, and epidermal growth factor homology domains-2 [Tie2]).[31] In addition, Ang1 can contribute to blood vessel stabilization by recruiting other cells, such as smooth muscle cells.[32] Our findings demonstrate that ECG at the designated concentrations improves the angiogenic potential/capabilities of OECs.

ECG (or EGCG) also has beneficial effects on osteogenic differentiation and osteogenesis.[23,24] In addition, it can also prevent bone tissue loss and promote bone healing after fracture.[33,34] Indeed, some recent studies have confirmed that green tea consumption can decrease the risk of osteoporosis and osteoporosis-related fractures.[35,36] In this study, we have shown that ECG at appropriate concentrations (e.g. 4 μM) can promote POB proliferation and upregulate two osteogenic factors, namely osteocalcin and osteonectin. Our findings have demonstrated that ECG stimulation at the designated concentrations might also enhance the osteogenic capabilities of POBs.

ECG promotes vascularization and osteogenesis

In advanced tissue engineering, prevascularization strategies use the co-culture of endothelial cells with parenchymal cells, stem cells, or angiogenic factor-producing cells.[9,37] Before implantation, capillary networks that form throughout the construct can join up functionally with the capillaries/blood vessels of the host. The most efficient vascularization technique for 3D tissue constructs should combine prevascularization *in vitro* with vascularization via the angiogenic sprouting of a vascular pedicle.[38] In this study, we have found that ECG treatment enhances the formation of microvessels in co-cultures of POBs and OECs. The pathways that lead to this enhancement of microvessels have also been investigated.

First, the level of E-selectin, which can promote the survival of microvessels and angiogenesis,[39] increased significantly after 24 h of ECG treatment. Second, ECG treatment of the co-cultures enhanced the expression of three cytokines (IL-6, TNF-α, and IFN-γ), all of which might contribute to vascularization/angiogenesis.[7,40–42] Third, the levels of VEGF and PDGF-BB mRNAs in the co-cultures were also upregulated after ECG treatment. In our study, we found that VEGF was also expressed in POB monocultures and upregulated after ECG treatment. Our findings thus provide further evidence that POBs in co-cultures contribute to angiogenesis/vasculogenesis. Interestingly, since VEGF can also stimulate osteogenesis, as shown in some previous studies,[43] OECs, which can secret VEGF, might also contribute to osteogenesis or bone repair/regeneration through the VEGF signaling pathway.

HIF pathway is involved in the angiogenic/osteogenic promotion of ECG

Since ECG can stimulate the production of HIFs that subsequently induce angiogenic/osteogenic effects,[44–46] we have investigated whether HIF pathways are involved in the beneficial effects of ECG shown in our study. Our analyses of the HIF pathway in monocultures of POB or OEC have revealed that ECG upregulates the levels of HIF-1α, HIF-2α, NF-κB, iNOS, GLUT1, VEGF, Ang1, and Ang2, while downregulating the level of PHD1. Based on our experimental data, we propose a model for HIF and NF-κB signaling pathways in promoting angiogenesis and osteogenesis (Figure 7).

Since the discovery of HIF, many studies have focused on HIF-1, which is induced with the onset of hypoxia, whereas the activation of HIF-2 is usually slower than that of HIF-1 and is maintained for longer. Despite being activated through a common hypoxia response element, HIF-1 and HIF-2 can activate distinct target genes depending on various cellular conditions.[47]

ECG can increase and stabilize HIF-1α by inhibiting PHD1–3, which targets HIF-1α for ubiquitylation and subsequent proteasomal degradation.[48] These steps may later activate genes that regulate glycolysis/glucose metabolism (phosphoglycerate kinase 1 [PGK1], GLUT1, and aldehyde dehydrogenase [ALDA]) and promote glycolytic flux, the sprouting of new capillaries, and angiogenesis (through VEGF, PDGF, and Ang1/2).[48] ECG can also increase the expression of HIF-1-targeted genes, such as GLUT1 and VEGF directly[20]; this increase then contributes to angiogenesis. In our study, we have shown that ECG treatment upregulates the level of GLUT1 (in POB and OEC monocultures) and VEGF (in OEC monocultures and in OEC/POB co-cultures).

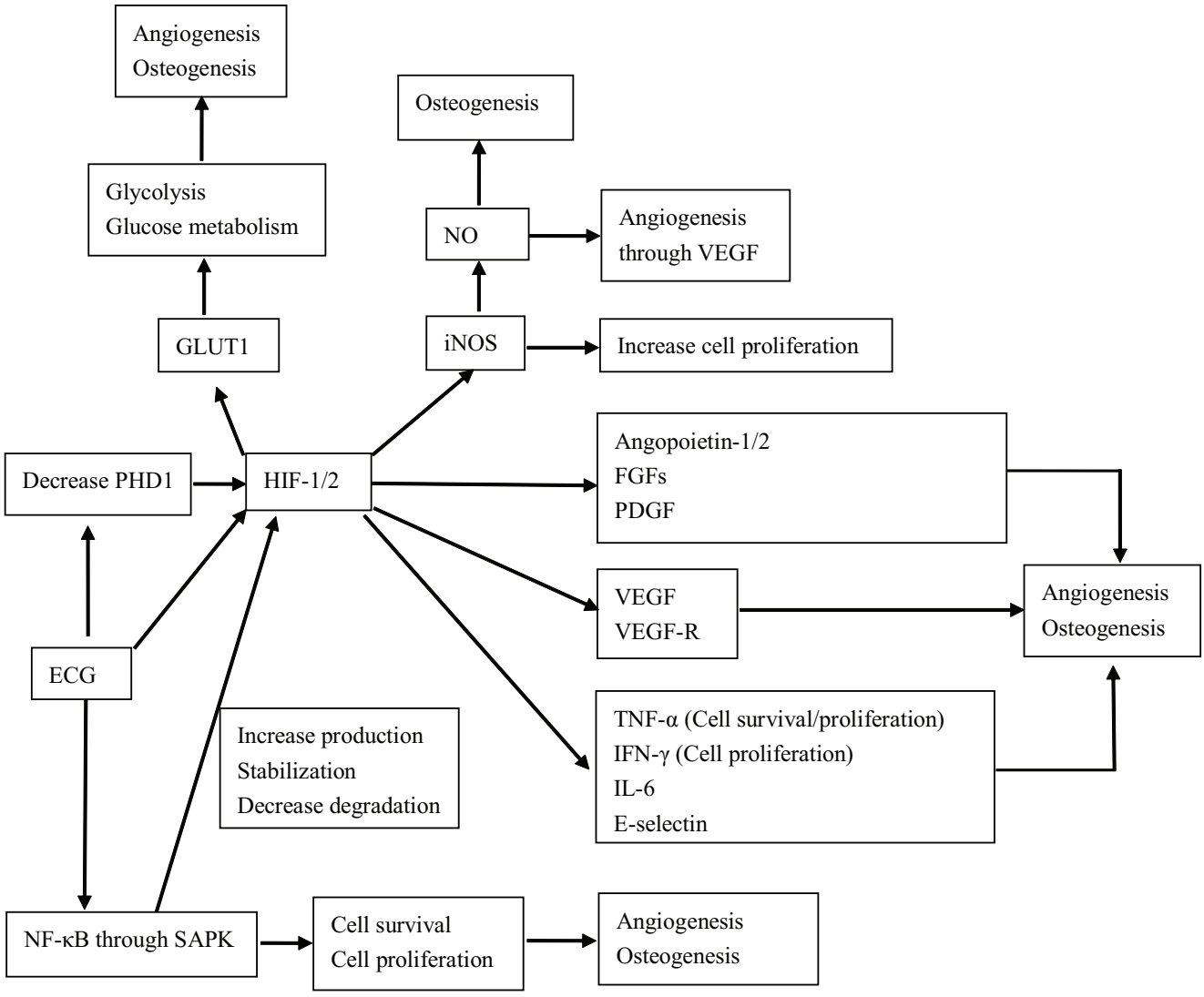

Figure 7. Potential pathways by which ECG promotes angiogenesis and osteogenesis. iNOS: inducible nitric oxide synthase; NO: nitric oxide; FGFs: fibroblast growth factors; PDGF: platelet-derived growth factor; SAPK: stress-activated protein kinases.

A recent study has also demonstrated that the iNOS pathway contributes to inflammation and angiogenesis.[49] Here, we have observed that the iNOS in POB and OEC monocultures is upregulated after ECG treatment indicating that the iNOS pathway may also be involved in the ECG's promotion of angiogenesis and osteogenesis.

Since the NF-κB pathway might also promote angiogenesis[50] and osteogenesis,[51] we additionally analyzed the levels of NF-κB after ECG treatment. We observed that, after ECG treatment, NF-κB is upregulated in POB and OEC monocultures. ECG possibly upregulates NF-κB to antagonize apoptosis induced by TNF-α, a protective activity that involves the suppression of the Jun N-terminal kinase (JNK) cascade for cell survival/proliferation and the promotion of angiogenic/osteogenesis.[51] For example, NF-κB directly binds to the iNOS promoter region to activate the transcription of iNOS,[52] which leads to an increase in the production of nitric oxide that promotes angiogenesis and osteogenesis. The NF-κB pathway induced by ECG can also increase HIFs (by increasing their production, support stabilization, and decreasing degradation),[53] which might further contribute to angiogenic/osteogenesis.

One limitation of our study is the low number of the donors of OECs. We have, however, previously used three donors to obtain OECs in our previous studies because of limited access to the OEC donors. Certainly, an increase in the number of donors would make the study more convincing.

In summary, our study has demonstrated that ECG at appropriate concentrations promotes angiogenesis/osteogenesis in a co-culture system consisting of OECs and POBs *in vitro*, possibly by modulating HIF signaling pathways. Although further *in vitro* and *in vivo* studies are needed, our results should facilitate the better construction of tissue-engineered bone for bone repair *in vitro* (by promoting prevascularization/osteogenesis before implantation). In addition, after implantation, ECG might also be used to continue the stimulation of vascularization/angiogenesis and osteogenesis/bone repair/healing. This type of additional

stimulation/promotion can be performed either through ECG (preadded in the scaffolds) slowly being released from bone tissue constructs or through the oral administration of ECG.

AUTHORS' CONTRIBUTIONS

DH, ZS, and BM conceived the study and designed the experiments. LZ, MW, and HQ performed experiments and analyzed the data. YW, LZ, and NN analyzed the results and provided comments. DH and BM wrote the article. All authors reviewed and approved the article.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICAL APPROVAL

This study was approved by the Institutional Review Board of the First Hospital of Hebei Medical University (Permit No. 20-22-530). Informed consent was obtained from all subjects involved in this study.

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ORCID IDS

Lu Zhou https://orcid.org/0000-0002-0823-6347
Zhongli Shi https://orcid.org/0000-0001-8319-7213
Bin Ma https://orcid.org/0000-0001-7053-0355

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