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ARTICLE

Synergy effects of copper and silicon ions on stimulation of vascularization by copper-doped calcium silicate

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Copper (Cu) has been reported to be able to stimulate vascularization/angiogenesis, which is critical for regeneration of vascularized tissue in tissue engineering. Silicate bioceramics have also been reported to have the stimulatory effects on vascularization due to the silicon (Si) ions released from silicate biomaterials. Therefore, we hypothesize that combination of Cu and Si ions may show synergy effects on vascularization. Then, a copper-doped calcium silicate bioceramic (Cu-$CaSiO_3$, Cu-CS) was designed and synthesized with the purpose to enhance the stimulatory effects of copper salts or pure silicate bioceramics on vascularization by combining the effects of Cu and Si ions. The cytocompatibility of Cu-CS was firstly assessed by testing the influence of Cu-CS ion extracts on proliferation of human umbilical vein endothelial cells (HUVECs). Thereafter, vascularization of HUVECs on ECMatrix™ gel or co-cultured with human dermal fibroblasts (HDFs) in Cu-CS extracts were evaluated and expression of angiogenic growth factors were analyzed. Results revealed that, as compared to CS extracts and media containing soluble $CuSO_4$, Cu-CS extracts possessed stronger stimulatory effects on upregulation of angiogenic growth factors, which finally resulted in better stimulatory effects on vascularization. During vascularization process, paracrine effects dominated in co-culture system. In addition, lower concentrations of Cu and Si ions released from Cu-CS than those released from pure CS or $CuSO_4$ were enough for stimulating vascularization, which indicated that there were synergy effects between Cu and Si ions during stimulation of vascularization by Cu-CS. Taken together, the designed Cu-CS may be suitable as a new biomaterial for regenerating blood vessels in tissue engineering.

1. Introduction

Vascularization is critical for tissue engineering, in which regenerated tissues need to form new active vessel networks with host tissue to supply enough oxygen and nutrients [1, 2]. However, it is very difficult to grow new vessels in large size tissue reconstructs, for the spontaneous growth of new blood vessels is only several tens of micrometers per day [3]. Therefore, it is of great importance to stimulate vascularization in tissue engineering constructs.

There are three main approaches to promote vascularization in tissue engineering, in which integration of angiogenic factors with bioengineered scaffolds have been widely investigated. For instance, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) were integrated into biomaterial matrixes to facilitate angiogenesis in many tissue engineering investigations [4]. However, these approaches are restricted because of the high cost, complex techniques, short half-lives of angiogenic growth factors, and the potential safety problems caused by the uncontrollable release behavior of growth factors *in vivo* [5, 6]. In addition, the angiogenic factors may also lose activity during the strict processing procedures, such as residual organic solvent and high temperature treatment [7].

Some biomaterials have been reported to be able to promote angiogenesis process without addition of angiogenic growth factors, which might be an alternative for directly using growth factors. For example, silicate based 45S5 Bioglass® could stimulate the secretion of angiogenic growth factors such as VEGF and bFGF from cells, so as to stimulate angiogenesis of endothelial cells *in vitro* [8, 9]. Recently, we have reported that calcium silicate (CS) could stimulate angiogenesis of human umbilical vein endothelial cells (HUVECs) in both mono-culture and co-culture systems. We found that Si ions released from CS (0.7-1.8 μg ml^{-1}) played a key role in the angiogenic stimulation process. In addition, we elucidated the mechanism through which the Si ions released from CS stimulated the vascularization. For the mono-culture system of HUVECs, Si ions of CS stimulated the HUVECs to highly express VEGF and subsequently activated the KDR by themselves to initiate the angiogenesis pathway. However, in the co-culture system of HUVECs and HDFs, Si ions stimulated abundant VEGF secretion from co-cultured HDFs (co-HDFs), which subsequently facilitated high expression of its receptor KDR from co-cultured HUVECs, in which paracrine effects played a key role [10].

Previously, an intimate relationship between copper levels and vascularization has also been demonstrated. Lack of copper has been confirmed to cause suppression of vascular formation [11,

[12], since several copper-binding proteins exist in serum, which have significant stimulatory effects on new vessel formation [11]. In addition, it was revealed that copper complexes or even copper salts, for example, copper sulfate, could stimulate endothelial cell migration and angiogenesis [13, 14]. Cu ions have been reported to be able to promote wound healing due to the stimulation effects of Cu ions on the VEGF expression of endothelial cells. The most recognized mechanism of the copper stimulates vascularization is that copper ions share some of the pathways utilized by hypoxia, which subsequently promotes VEGF expression from cells [15].

To take the advantages of Cu ions' stimulatory effects on vascularization for tissue engineering, $CuSO_4$ has been mixed with calcium phosphate scaffolds in order to improve *in vivo* micro vessel formation once the scaffolds were implanted [16]. However, the role of the Cu ions released from the Cu-containing composite scaffolds and the mechanisms of the stimulation of vascularization by the Cu-containing biomaterials were not elucidated. In addition, controlled concentration of the Cu ions released from the mixed biomaterials is very important, as mismanage of copper can cause potential damages in the cellular condition due to the adventitious redox reactions catalyzed by "free" copper [14]. Unfortunately, the release behavior of Cu ions from soluble copper salts or complexes is generally in a "burst" way, even they are mixed with biomaterials, which may result in high concentration of Cu ions in short term and incur potential risks.

Recently, Wu et al. incorporated copper into mesoporous bioactive glass scaffolds to obtain a copper-containing biomaterial with slow release of Cu ions. Results showed that the copper-containing mesoporous bioactive glass could induce hypoxia inducible factor-1α (HIF-1α) secretion from human bone marrow stromal cells (hBMSCs), and subsequently enhanced the expression of VEGF from cells [17]. The effective concentrations of Cu ions for upregulating the expression of HIF-1α and VEGF have been reported as in the range from 14 μg ml^{-1} to 56.6 μg ml^{-1}. In addition, Cattalini et al. developed an organic polymer-bioactive glass nanoparticles composite films loaded with Cu^{2+} ions (Cu-composite films) [18]. Results showed that the presence of Cu^{2+} stimulated the vascularization of HUVECs on the composite films and the effective concentration of Cu^{2+} for stimulating vascularization is 11 μg ml^{-1}. However, the specific role of individual ions and the mechanisms of the Cu-containing biomaterials in stimulation of vascularization were not elucidated due to the complex composition of bioglasses used in these studies, and in particular, the Cu effect in co-culture system of HUVEC and other cells such as fibroblasts and BMSCs, which is more close to the real situation of tissue regeneration process *in vivo*, have not been reported.

In our latest study, Lin and Chang et al. designed a strontium-substituted calcium silicate bioactive ceramic for enhancing osteoporotic bone regeneration [19]. In that study, the combination of Si ions and Sr ions could stimulate osteogenesis and the two ions have synergy effects on osteoporotic bone regeneration. This idea indicates that it may be feasible to combine two or more elements to design a new bioceramic to stimulate vascularization/angiogenesis. As both Si and Cu ions were found to be able to stimulate vascularization, in this study, we hypothesize that the combination of Cu and Si ions may result in an addition of the stimulatory effects, which will further enhance vascularization in comparison to pure Cu ions or Si ions. Since the CS ceramic, which only contains Ca and Si elements, has the simplest composition among silicate bioceramics, we propose to dope Cu into CS ceramic to design a copper-containing calcium silicate bioceramics (Cu-CS), and to study the vascularization ability of Cu-CS. To dope Cu into CS is feasible as we have doped microelements, which exist in the human body, such as cobalt, strontium and zinc, into silicate bioceramics through chemical synthesis in our previous studies [19-21]. In order to test our hypothesis that the combination of Cu and Si ions can result in stronger stimulatory effects on vascularization than pure Cu or Si ions, the stimulatory effects of ionic products of Cu-CS extracts and CS extracts as well as media containing $CuSO_4$ were compared in both mono-cultures of endothelial cells and co-cultures endothelial cells with fibroblasts.

2. Materials and methods

2.1. Preparation and characterization of Cu-calcium silicate (Cu-CS) powders

The calcium silicate (CS) powders and copper substituted CS powders (Cu-CS) with 2.5 mol% of Ca replaced by Cu were synthesized by chemical precipitation method [22]. The amount of Cu doped into CS was determined according to the effective concentration of Cu ions reported in literatures. The solution of $Ca(NO_3)_2$ or the mixed solution of $Ca(NO_3)_2$ and $Cu(NO_3)_2$ were firstly prepared according to the expected substitution degree, and then were dropwisely added into Na_2SiO_3 solution at room temperature under rigorous stirring with (Cu+Ca) / Si molar ratio of 1.0. After completing addition, the precipitates were further stirred for 24 h. Then, the precipitates were filtered and washed by deionized water, followed by further washing with anhydrous ethanol for three times. The obtained precipitates were dried at 60 °C for 24 h, and finally calcined at 900 °C for 2 h to obtain CS and Cu-CS powders. The as prepared CS and Cu-CS powders were sieved to obtain powders with particulate size less than 200 mesh.

The phase composition of the obtained powders were characterized by X-ray diffraction (XRD: D/max 2550V, Rigaku, Japan) with mono-chromated CuKα radiation. The microstructure of the obtained Cu-CS bioceramic powders was observed with scanning electron microscopy (SEM: JSM-6700F, JEOL, Tokyo, Japan). The Cu content of the prepared Cu-CS powders was measured using inductively coupled plasma optical emission spectroscopy (ICP-OES: 710-ES, Varian, USA).

2.2. Ion extracts preparation and ion concentration determination

Ion extracts of copper substituted calcium silicate (Cu-CS) and calcium silicate (CS) were prepared according to the protocol recorded in the literatures [23], which were adapted from the standard procedure in ISO10993-1[24]. Briefly, 1 g bioceramic powders were immersed in 5 ml serum-free endothelial basal medium-2 (EBM-2; Lonza) in a culture dish with 10 cm diameter, and incubated in a humidified incubator containing 5% CO_2 at 37 °C for 24h. Then, the supernatant was collected by centrifuging and sterilized through a filter (Millipore, 0.22 μm). The obtained extracts were stored at 4 °C for further use.

To determine the optimum concentration range of the ceramic extracts for subsequent experiments, original extracts of Cu-CS and CS were diluted at ratios from 1/4 to 1/512 using Endothelial Cell Medium+5% FBS (Fetal Bovine Serum)+1%

ECGS (endothelial cell growth supplement)+1% P/S (penicillin/streptomycin) (ECM) (Sciencell, USA), which was used for culturing HUVECs in the present study. The preliminary results of HUVECs cell proliferation assays indicated that the Cu-CS extracts diluted at the ratios from 1/32 to 1/512 demonstrated non-cytotoxicity for HUVECs. Therefore, dilution ratios of 1/32, 1/64 and 1/128 were applied in following cell experiments. To further understand the influences of different ion concentrations on HUVECs, particularly copper ions, the Cu-CS and CS extracts at different dilution ratios of 1/32, 1/64 and 1/128 were analyzed by an inductively coupled plasma optical emission spectrometer (ICP-OES, Varian 715-ES, USA) to detect the ion concentrations of copper, as well as, silicon, calcium and phosphorus. Meanwhile, ECM was analyzed as the control medium. Culture media with same concentrations of Cu ions to those in the Cu-CS extracts at different dilution ratios of 1/32, 1/64 and 1/128 were also prepared by dissolved certain amount of $CuSO_4$ $5H_2O$ (Sigma), recorded as Cu 1.5, Cu 0.7 and Cu 0.3 in figures. The media were used for the HUVECs-HDFs direct co-culture model.

2.3. Cell isolation and culture

HUVECs were isolated from the human umbilical cord vein according to the descriptions of Jaffe et al.[25]. The isolated cells were dispersed and cultured on culture dish with ECM in a humidified incubator containing 5% CO_2 at 37 ^oC. The culture medium was refreshed every 3 days until the primary HUVECs became confluent and the confluent cells were routinely subcultured by trypsinization. The obtained cells were immunofluorescence stained with von Willebrand Factor (vWF) to confirm their endothelial cell characters. Only early passages (passage 2-7) of HUVECs were used in this study.

Human dermal fibroblasts (HDFs) were isolated from the superficial layer of adult human skin dermatomed at a depth of 400 μm according to the procedures in previous work [26]. Dulbecco's Modified Eagle Medium (DMEM, Gibco)-high glucose supplemented with 10% (vol/vol) FBS and 1% (vol/vol) P/S were used as HDFs' culture medium. HDFs below passage 10 were used in the co-culture system.

2.4. Cell proliferation assay

For evaluating the cytocompatibility of the Cu-CS ceramic powders, we evaluated the effects of Cu-CS and CS ceramic extracts diluted at ratios from 1/4 to 1/512 on HUVECs' proliferation. ECM was used as control medium.

The HUVECs (5×10^3 cells per well) were seeded on 96-well plates and cultured in a humidified atmosphere containing 5% CO_2 at 37 ^oC incubator. After 12 h, the culture media were replaced by the prepared diluted Cu-CS and CS extracts at different dilution ratios form 1/4 to 1/512. Then, the cells were constantly cultured for 7 days, and a Cell Counting Kit (CCK)-8 assay (Beyotime) was applied on cells at 0, 1, 3 and 7 day according to the manufacturer's instruction. Briefly, at the end of each culture time point, the culture media were removed and replaced with 100 μl fresh medium containing 10 μl CCK-8, and the cells were continued to be cultured in 37 ^oC incubator for 2.5 h. The absorbance of the reaction product was measured spectrophotometrically using an enzyme-linked immunoadsorbent assay microplate reader (Synergy 2, Bio-TEK) at wavelengths of 450 nm. The readings represent the number and metabolic activity of cells.

2.5. In vitro angiogenesis assay

It has been demonstrated that the migration of endothelial cells (ECs) and the formation of tube-like network structures, which is called capillary cords, are critical steps for angiogenesis [27]. ECs are widely used as the model for the angiogenic property test of biomaterials. ECMatrix™ (Millipore, Cat. No. ECM625), which consists of laminin, collagen type IV, heparin sulfate proteoglycans, entactin and nidogen, was applied to evaluate the angiogenic capacity of Cu-CS and CS extracts. Briefly, 96-well culture plates were coated with ECMatrix™ in advance according to the manufacturer's instructions. Then, HUVECs (4×10^3 cells per well) were cultured in Cu-CS and CS extracts (1/32, 1/64 and 1/128) with ECMatrix™ underneath for 4, 7 and 12 h, and HUVECs cultured on ECMatrix™ with ECM were set as the control group. At each time point, cells in the 96-well plates were observed under bright field using an inverted fluorescence microscope (Leica DMI 3000B, German), and photographed from five random microscopic fields. The nodes, circles and tubes are symbols and parameters of the gradual regeneration process of angiogenesis, representing the primary, interim and later stages, respectively. Therefore, according to the manufacturer's instructions, the numbers of branch points in HUVECs lines (nodes), mesh-like circles (circles) and tube-like parallel cell line (tubes) were manually counted.

2.6. HDFs-HUVECs direct contact co-cultures

To further assess the angiogenic capacity of Cu-CS, HDFs-HUVECs direct co-culture model was applied. In this model, both HDFs and HUVECs were seeded in 24-well plates at the density of 2×10^4 cells per well. HUVECs and HDFs were also seeded at the same density in 24-well plates. For co-cultures, 500 μl ECM in addition to 500 μl DMEM-H + 10% FBS + 1% P/S were applied as culture medium. After being cultured for 24 h, cells were synchronized by culturing in serum-deprived medium for 12 h followed by changing the co-culture media with Cu-CS and CS extracts diluted with control medium at 1/32, 1/64 and 1/128 ratios or the culture media containing $CuSO_4$. The co-cultured cells were continuously cultured for 7 days, and the co-culture media were refreshed every 3 days. Finally, the cells were immunofluorescence stained with von Willebrand Factor (vWF) and DAPI. Thereafter, the capillary-like networks formed by HUVECs were subsequently observed using an inverted fluorescence microscope (Leica DMI 3000B, German). The numbers of capillary tube-like structure, which represents the late period of angiogenesis process, were manually counted.

2.7. Immunofluorescence

Immunofluorescence staining of vWF was applied on HDFs-HUVECs direct contact co-culture system or mono-cultured cells to reveal the formation of tubule networks of HUVECs. Briefly, after being co-cultured for 7 days, cell layers were washed twice with preheating phosphate-buffered saline (PBS) and fixed with 4% (wt./vol.) paraformaldehyde (PFA) at room temperature for 15 mins. After that, cell layers were permeabilized with frozen methanol for 5 mins at room temperature and blocked with PBS + 1% (wt./vol.) bovine serum albumin (BSA) for 1 h at 37 ^{o}C. Then, co-cultured cells were continued to be incubated for 2 h at 37 ^{o}C in primary antibody solutions containing rabbit anti-vWF (diluted with PBS + 0.5% BSA at 1/300). After the cells were washed with PBS for two times, Alexa 488 goat anti-rabbit IgG secondary antibody solutions (diluted with PBS + 0.5% BSA at 1/1000) were added in and the cells were incubated at 37 ^{o}C for another 1 h. DAPI was used to stain the nucleus in blue. Finally, capillary-like network formation in co-cultured cells was observed under a fluorescence microscope (Leica DMI 3000B, Germany).

2.8. Separation of HUVECs and HDFs after direct contact co-culture using magnetic beads

In order to measure the VEGF gene and KDR expression in co-HUVECs and co-HDFs, magnetic beads were applied to separate the two co-cultured cells. Briefly, after co-cultured cells were trypsinlized, co-cultured HUVECs were then separated from HDFs by magnetic beads combined with an antibody against CD31, a specific protein of endothelial cells, according to the method established by Guillotin et al. [28]. The separated HUVECs and HDFs were named co-HUVEC and co-HDF, respectively.

2.9. Quantitative real-time polymerase chain reaction (Q-RT-PCR)

For detecting the gene expression of VEGF, KDR, HIF-1α and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Q-RT-PCR technology has been applied. Q-RT-PCR was performed according to our previous studies [29]. Firstly, RNA was extracted from different cell samples. For RNA extracts, HUVECs and HDFs were seeded at the density of 4 × 10^5 per well in 6-well plates and mono-cultured or co-cultured with different media. After the cells were incubated for 7 days, mono-cultured cells or separated cells were washed twice with cold PBS and the cell samples were subjected to extraction of total RNA using an E.Z.N.A. Total RNA kit I (OMEGA, Bio-tek) according to the manufacturer's instruction. Then, the concentration of RNA was obtained by a nano-drop 1000 reader (Thermo Scientific) and cDNA was synthesized using a ReverTra Ace-α kit (Toyobo Co. Ltd, Japan). 1 μl aliquot of cDNA, which was diluted in sterilized Mill-Q water at the ratio of 1/10 was then mixed with 9 μl of SYBR-GREEN for Q-RT-PCR. 400 nM of primers of VEGF, KDR, HIF-1α and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (all from Sangon Biotech (Shanghai) Co. Ltd.) were respectively added into the mixtures, in which GAPDH was used as a housekeeping gene. The primers' sequences are summarized in Table 1. Real-time PCR Master Mix (ToYOBO Co. Ltd.) containing primers were loaded in a 384-well plate and the Q-RT-PCR analysis was performed using the 7900 Real-time PCR system (Applied Biosystems). The protocol for Q-RT-PCR was as following: The initial incubation step of denaturation is 1 min at 95 ^{o}C, which was followed by 40 cycles (95 ^{o}C for 15 s, 60 ^{o}C for 15 s, 72 ^{o}C for 45 s) of PCR. Data were analyzed with the SDS 2.3 software and compared by the ΔΔCt method, and each Q-RT-PCR was performed in triplicate for yield validation. Data were finally normalized to GAPDH mRNA expression of each condition and were quantified relative to the corresponding gene expression from control samples, which were standardized to 1.

Table 1 Primer sequences used in Real time RT-PCR

Gene	Gene Bank	Primer sequences	Tm (^{o}C)
$VEGF_{165}$	AB_021221	F:5'TGCGGATCAAACCTCACCA 3' R:5'CAGGGATTTTTCTTGTCTTGCT 3'	58
KDR	NM_002253	F:5' GTGATCGGAAATGACACTGGAG 3' R:5' CATGTTGGTCACTAACAGAAGCA 3'	60
HIF-1α	NM_001243084.1	F: 5'CCATGTGACCATGAGGAAAT 3' R: 5'CGGCTAGTTAGGGTACACTT 3'	60
GAPDH	NM_002046	F:5' GATTTGGTCGTATTGGGCG 3' R: 5'CTGGAAGATGGTGATGG 3'	60

2.10 Protein extraction and Enzyme-linked immunosorbent assay (ELISA)

To measure the protein concentration of HIF-1α, total protein from cells were extracted. For protein extraction, both HDFs and HUVECs were seeded at 8×10^4 cells/well in 6-well plates and cultured with different media for 7 days. The co-culture media were refreshed every 3 days. At predetermined time point, cells were removed from cultured plates by trypsin and cell pellets were obtained by centrifuging. The co-cultured cells were then separated. The separated cells and mono-cultured cells were washed twice with cold PBS before adding 300 μl of RIPA buffer containing 50 mM Tris ⊣HCl, pH 8, 150 mM NaCl, 0.1% (vol/vol) Nonidet P-40, 10 μg ml^{-1} aprotinine, 10 μg ml^{-1} leupeptin, and 1 mM phenylmethanesulfonyl fluoride (Sigma). Then, the cells were agitated and sonicated with an ultrasonic processor to fully extract the total protein from cells. The mixture was then centrifuged at 16000 rpm for 10 mins and the supernatant was collected and frozen at -80 ℃ for future analysis. Quantification of the protein was performed using bicinchoninic acid protein assay kit (Pierce-Perbio Science).
HIF-1α from the cell total protein was quantified using specific HIF-1α ELISA kits (Ray Biotec). Briefly, the cell total protein cultured with different culture media were submitted to the immuno-enzymatic detection, according to the protocol described by the supplier. Results are expressed in ng/ml.

2.11. Statistical analysis

All the data were expressed as mean ± standard deviation. Three independent experiments conducted and at least five samples from each test were taken for statistical analysis. Differences were considered significant when $p < 0.05$ (*or # or @) or $p < 0.01$ (**). In addition, a one-way ANOVA with Tukey's post hoc test was used for statistical analysis of multiple comparisons.

3. Results

3.1. Characterization of Cu-CS powders

Fig. 1a represents the XRD patterns of the Cu-CS and CS powders, which were shown in black and red curves, respectively. It is clear that all of the products were identified as pure β-CS phase (JCPDS card: No. 84-0654), and the shape of the sharp diffraction peaks indicated that the obtained CS and Cu-CS powders were well crystallized. Moreover, the XRD scanning results clearly confirmed that the corresponding peaks of the synthetic Cu-CS powders significantly shifted to higher degree when compared with the pure CS materials. SEM results, which are shown in Fig. 1b, indicated that the particle size of the obtained CS (down) and Cu-CS (up) powders were irregular in shape and non-uniform in size with a range of 100 nm-2 μm. The ICP-OES analysis result revealed that the replacement percent of Ca by Cu was 2.86 mol.% in Cu-CS powders, which was slightly higher than that of the theoretical value.

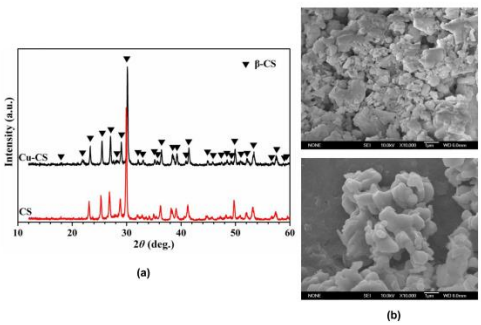

Fig.1 Composition and morphology of calcium silicate (CS) and Cu-doped calcium silicate (Cu-CS) obtained in the present study. (a) X-ray diffraction of CS and Cu-CS, which indicated that Cu-CS powders had significant higher degree of corresponding diffraction peaks as compared to the pure CS powder . (b) SEM images of Cu-CS particles (up) and CS particles (down), showing that the particle size of Cu-CS ranges from 100 nm to 2 μm.

3.2. Proliferation of HUVECs cultured in extracts

HUVECs were cultured in Cu-CS, CS powder extracts (dilution ratios from 1/4 to 1/512) and control medium and the proliferation of HUVECs were evaluated. As shown in Fig. 2, the results revealed that the Cu-CS and CS extracts at dilution ratios of 1/32 to 1/512 were non-cytotoxic. CS extracts at dilution ratios of 1/64, 1/128 and 1/256 significantly stimulated HUVECs proliferation as compared to the control medium (Fig. 2a). The proliferation profiles of HUVECs cultured with Cu-CS extracts diluted from 1/32 to 1/512 were almost the same to that of HUVECs cultured with control medium, which indicated that Cu-CS extracts at those dilution ratios were non-cytotoxic but had no obvious stimulatory effects on HUVEC proliferation (Fig. 2b). On the whole, we concluded that both CS and Cu-CS extracts demonstrated good cytocompatibility for HUVECs at certain concentration range from 1/32 to 1/512.

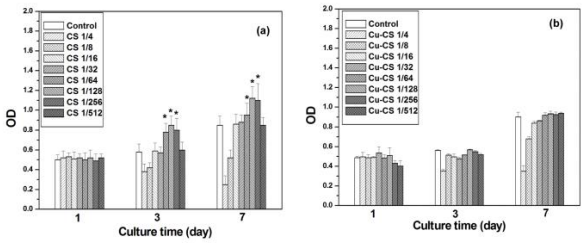

Fig.2 Proliferation study of HUVECs cultured with different media for 1, 3 and 7 days. (a) The proliferation profile of HUVECs cultured with control medium and CS extracts diluted with control medium at different dilution ratios ranging from 1/4 to 1/512. CS extracts with dilution ratios at 1/64, 1/128 and 1/256 stimulated proliferation of HUVECs. (b) The proliferation profile of HUVECs cultured with control medium and Cu-CS extracts diluted with control medium at different dilution ratios ranging from 1/4 to 1/512. The Cu-CS extracts are cytocompatible but showed no stimulatory effects on HUVEC proliferation at all concentration.

3.3. In vitro angiogenesis

An *in vitro* angiogenesis assay kit containing ECMatrix™ gel was used to evaluate the angiogenic abilities of HUVECs. We can see from Fig. 3a (left column) that, after 4 h culture, HUVECs cultured on ECMatrix™ gel in all media gradually self-assembled to form branched nodes (node) and mesh-like circles (circle), which represents the typical morphology of HUVECs in the initial and interim stages of angiogenesis. It can be seen from the analysis in Fig. 3b that HUVECs cultured in 1/64 Cu-CS extracts formed the most branched nodes and circles, which reached around 1.1 times more than that cultured in CS extracts with the same dilution ratio.

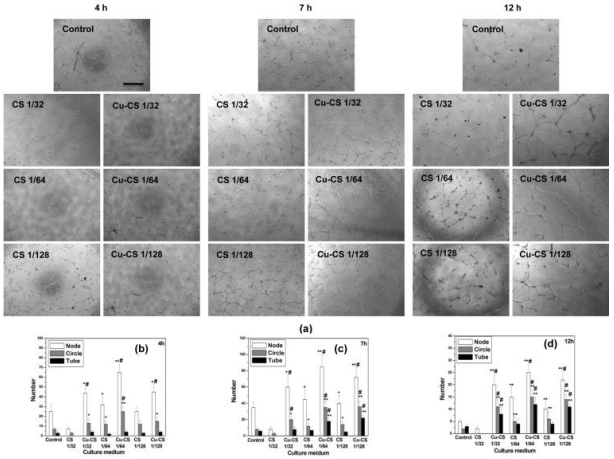

Fig.3 In vitro angiogenesis study of HUVECs cultured with different media on ECMatrix gel. (a) Optical images of HUVECs cultured on ECMatrix in the presence of CS and Cu-CS extracts at 1/32, 1/64 and 1/128 dilution ratios for 4 h (left column), 7 h (middle column) and 12 h (right column). Bar = 200 μm. (b) The statistics of the number of nodes, circles and tubes formed in the culture after 4, 7 and 12 h, respectively. Data represent means ± SD (n = 5). The symbols * and ** indicated that the node number, circle number and tube-like number in the samples are significantly higher than those in the HUVECs cultured with control medium. The symbols # indicated the node number, circle number and tube-like number in the cell cultured with Cu-CS extracts are significantly higher than those in the HUVECs cultured with CS extracts.

After being cultured for 7 h, the HUVECs cultured in the Cu-CS extracts still presented more notable angiogenic patterns (such as nodes and circles) than those cultured in CS extracts or controls, and the diameter of the circles became bigger as well (Fig. 3a (middle column)). At this time point, HUVECs cultured with 1/64 Cu-CS extracts formed the most nodes and 1/128 Cu-CS formed the most circles and tubes among the overall groups, which were statistically demonstrated in Fig. 3c. In addition, the mesh-like structure and the tube structure, which represent the later stage of the angiogenesis process, gradually increased as culture time went by.

After HUVECs were cultured for 12 h, the mesh-like structure gradually collapsed in the CS and control group (right column), whereas HUVECs cultured in 1/64 Cu-CS extracts still maintained the most nodes, circles and tubes among all the groups (Fig. 3d) even though the numbers of these angiogenic patterns decreased in comparison to those at 7 h.

3.4. In vitro angiogenesis in HUVECs-HDFs co-culture model

A HUVECs-HDFs direct contact co-culture model was further applied to investigate the angiogenic stimulatory capability of Cu-CS compared to CS extracts. As we concluded from Fig. 4a, co-cultured HUVECs with the media containing Cu-CS, CS extracts and $CuSO_4$ (Cu 1.5 and Cu 0.7) elongated and formed more tubules than those co-cultured in control medium. Moreover, HUVECs co-cultured in Cu-CS extract group (Fig. 4a, middle column) formed most abundant branched points and highly anastomosed among all media although the CS extracts (Fig. 4a, left column) and the medium containing $CuSO_4$ with 1.5 and 0.7 μg ml^{-1} of Cu^{2+} (Fig. 4a, right column) also stimulated the vascularization of HUVECs as compared to the control group. Interestingly, the medium containing $CuSO_4$ with only 0.32 μg ml^{-1} Cu^{2+} showed no stimulatory effects on the vascularization of co-HUVECs. Corresponded to the fluorescence images, the numbers of tubule networks formed by co-cultured HUVECs, representing the degree of self-assembly angiogenesis in different media, were manually counted and presented in Fig. 4b. It demonstrated that the number of tubules formed by HUVECs co-cultured in Cu-CS extracts was significantly more than those co-cultured in CS extracts at the same dilution ratios, media containing $CuSO_4$ or control group. In addition, the maximum quantity of tubule-like networks was achieved when HUVECs co-cultured with HDFs in 1/64 Cu-CS extract, presenting about 2 times more than that in the control group. No obvious vascularization was observed in mono-cultured cells (data not shown).

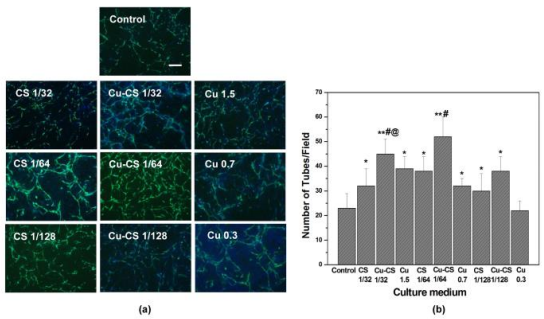

Fig.4 Tubule formation by co-HUVECs in direct contact co-cultures was stimulated by CS and Cu-CS extracts diluted with control medium at 1/32, 1/64 and 1/128, which was indicated by the vWF stained co-HUVECs and statistical study at the end of the cultures (7 days) (a). Immunofluorescence staining of vWF was in green and nucleus was in blue. Nuclear was stained in blue with DAPI. Bar=200μm. (b) The quantification and statistical analysis of tubule formation was obtained by counting and comparing the number of tubes in the images. * and ** represent $P < 0.05$ and $P<0.01$, respectively, when compared with control. # indicated that the co-HUVECs cultured with Cu-CS extracts formed more tubes than those cultured with CS extracts at same dilution ratios. @ indicated that the co-HUVECs cultured with Cu-CS extracts at the dilution ratio of 1/32 formed more tubes than those cultured with CS extracts at dilution ratio of 1/128 although these two extracts contain same concentration of Cu ions.

3.5. Expression of VEGF,KDR and HIF-1α in cells

It can be seen from Fig.5 that Cu-CS, CS extracts and the media containing $CuSO_4$ stimulated the VEGF and KDR gene expression of mono-cultured HUVECs as compared to control medium while the media containing $CuSO_4$ with 0.3 μg ml^{-1} of Cu^{2+} showed no such effects. In addition, among the above

media, Cu-CS extracts showed strongest stimulatory effect on VEGF and KDR gene expression of mono-cultured HUVECs. HUVECs mono-cultured in the Cu-CS extracts diluted at 1/64 expressed the highest VEGF and KDR gene productivity, which were nearly 3 times more than those cultured in control medium.

However, VEGF and KDR gene expression in co-culture system were much higher than those in mono-cultured HUVECs. In addition, Cu-CS extracts also had strongest stimulatory effect on angiogenic factor expression from co-cultured cells as compared to CS extracts and the media containing $CuSO_4$ with 1.5 and 0.7 μg ml^{-1} of Cu^{2+}, although CS extracts and the media containing $CuSO_4$ with 1.5 and 0.7 μg ml^{-1} of Cu^{2+} also showed certain stimulatory effects when compared with control medium. Interestingly, the stimulatory effect on VEGF gene expression was for co-cultured HDFs but not for co-cultured HUVECs. It can be seen from Fig. 5a that the Cu-CS extracts significantly upregulated the VEGF gene expression of co-cultured HDFs as compared to CS extracts, the media containing $CuSO_4$ and control. Subsequently, we can see from Fig. 5b that its receptor KDR gene expression of co-cultured HUVECs was then activated and also upregulated by Cu-CS, CS extracts and the media containing $CuSO_4$ with 1.5 and 0.7 μg ml^{-1} of Cu^{2+}. In addition, the Cu-CS extracts also has strongest stimulatory effect on KDR expression among the above media. It is worth noting that the Cu-CS extracts with dilution ratio at 1/32 showed higher stimulatory effects on VEGF and KDR than the CS 1/128 even they had same Si ion concentration and the Cu-CS extracts showed higher stimulatory effects than the media containing $CuSO_4$ with the same concentration of Cu^{2+} to those in Cu-CS extracts. In addition, the VEGF and KDR expression from the cells treated with media containing $CuSO_4$ with 0.3 μg ml^{-1} Cu^{2+} was not upregulated. However, the Cu-CS extracts at the dilution ratio of 1/128 with 0.3 μg ml^{-1} Cu^{2+} still showed stimulatory effects.

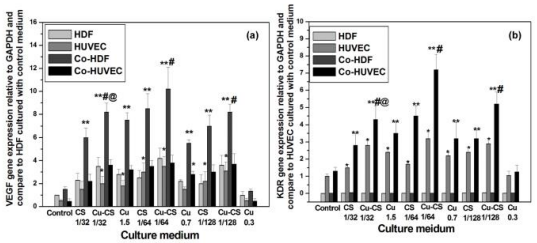

Fig.5 Gene expression of VEGF and its receptor KDR from cells cultured with CS, Cu-CS extracts and media containing $CuSO_4$ for 7 days. Data of VEGF gene expression was quantified relative to the same gene expression in HDF and data of KDR gene expression was quantified relative to the same gene expression in HUVEC cultured with control medium for 7 days. (a) Cu extracts, Cu-CS extracts and media containing $CuSO_4$ showed stronger stimulatory effects on the VEGF expression from mono-cultured HUVECs (*) and co-HDFs than those cultured with control medium (**). # indicated that indicated that the co-HDFs cultured with Cu-CS extracts expressed more VEGF than those cultured with CS extracts at same dilution ratios. @ indicated that the Cu-CS extracts at the dilution ratio of 1/32 showed higher stimulatory effects on VEGF expression from co-HDFs than CS extracts at dilution ratio of 1/128 although these two extracts contain same concentration of Cu ions. (b) The KDR expression showed the same profile to that of the VEGF

To further understand the specific roles of ionic products from CS and Cu-CS extracts in the vascularization stimulation, gene expression and protein production of HIF-1α had been detected. Interestingly, it can be seen from the Fig.6 that the CS, Cu-CS extracts and the media containing $CuSO_4$ with 1.5 and 0.7 μg ml^{-1} of Cu^{2+} had stimulatory effects on HIF-1α expression from co-HDFs. However, the Cu-CS extracts significantly augmented the gene expression (Fig.6a) and protein production (Fig.6b) of HIF-1α in co-HDFs. Cu-CS extracts with dilution ratio at 1/32 showed higher stimulatory effects on HIF-1α than the CS 1/128 even they had same Si ion concentration. Similarly, the Cu-CS extracts showed higher stimulatory effects on HIF-1α than the media containing $CuSO_4$ with the same concentration of Cu^{2+} to those in Cu-CS extracts. The HIF-1α expression from the cells treated with media containing 0.3 μg ml^{-1} Cu^{2+} was not upregulated. However, the Cu-CS extracts containing 0.3 μg ml^{-1} Cu^{2+} (at 1/128 dilution ratio) still showed stimulatory effects on HIF-1α.

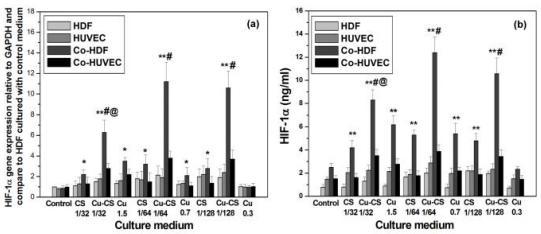

Fig.6 Expression of HIF-1α from cells cultured with CS, Cu-CS extracts and media containing 1.5, 0.7 and 0.3μg ml^{-1} Cu ions for 7 days. (a) Gene expression of HIF-1α from cells. Data of gene expression was quantified relative to the HIF-1α gene expression in HDFs cultured with control medium. Cu extracts, Cu-CS extracts and media containing 1.5 and 0.7 μg ml^{-1} Cu ions showed stronger stimulatory effects on the HIF-1α expression from co-HDFs than those cultured with control medium (* or **). # indicated that indicated that the co-HDFs cultured with Cu-CS extracts expressed more HIF-1α than those cultured with CS extracts at same dilution ratios. @ indicated that the Cu-CS extracts at the dilution ratio of 1/32 showed higher stimulatory effects on HIF-1α expression from co-HDFs than CS extracts at dilution ratio of 1/128 although these two extracts contain same concentration of Cu ions. (b) The HIF-1α protein production was detected by ELISA, which showed a same profile to that of the its gene expression.

3.6. Ion concentration of Cu-CS and CS extracts

To quantitatively interpret the effective ions for HUVECs' pro-angiogenesis, ion concentration of Cu-CS ceramic extracts

diluted with ECM at the ratios (1/32, 1/64 and 1/128) which stimulated the angiogenesis of HUVECs and co-HUVECs were determined through ICP-OES with CS extracts and HUVECs' culture medium as controls. The results were shown in Table 2, from which we concluded that there was no obvious difference of the Ca and P ion contents between ceramic extracts (Cu-CS and CS), media containing $CuSO_4$ and control group. However, Cu-CS extracts with the presence of Cu ions had merely about half Si ion content compared to CS extracts at the same diluted ratios. The concentration of Si ions in Cu-CS extracts diluted at 1/32, 1/64 and 1/128 were 1.35 μg ml^{-1}, 0.62 μg ml^{-1} and 0.34 μg ml^{-1}, respectively, which were significantly lower than those in CS extracts at same dilution ratio (2.45 μg ml^{-1}, 1.83 μg ml^{-1}, 1.32 μg ml^{-1} for 1/32, 1/64 and 1/128, respectively). The concentrations of Cu ion in Cu-CS extracts diluted at 1/32, 1/64 and 1/128 were 1.5 μg ml^{-1}, 0.7 μg ml^{-1} and 0.32 μg ml^{-1}, respectively.

Table 2 Ion concentrations of ceramic extracts diluted at the ratio range that stimulated the angiogenesis of HUVECs and co-HUVECs

	Cu (μg/ml)	Si (μg/ml)	Ca (μg/ml)	P (μg/ml)
Control	0.00	0.06 ±0.02	65.73 ±0.14	17.21 ±0.28
Cu-CS 1/32	1.50 ±0.21	**1.35 ±0.14**	67.57 ±0.54	16.81 ±0.12
Cu-CS 1/64	0.71 ±0.15	0.62 ±0.11	65.59 ±0.72	16.65 ±0.32
Cu-CS 1/128	0.32 ±0.09	0.34 ±0.07	64.45 ±0.88	16.57 ±0.24
CS 1/32	0.00	2.45 ±0.17	64.58 ±0.36	16.85 ±0.12
CS 1/64	0.00	1.83 ±0.16	65.20 ±0.52	16.91 ±0.14
CS 1/128	0.00	**1.32 ±0.11**	64.14 ±0.48	16.72 ±0.24

4. Discussion

In present study, novel copper substituted calcium silicate powders (Cu-$CaSiO_3$, Cu-CS) were successfully prepared by ion substitution chemical precipitation. Previously, different elements, such as magnesium and zinc, have been doped into calcium silicate to stimulate osteogenesis [21, 30]. Results in this study also confirm that it is feasible to obtain a biomaterial with expected properties by designing a biomaterial through combining different elements. Copper has been added into bioglasses with the expectation of stimulating vascularization [17]. However, it is hard to investigate the specific role of different ions and elucidate the mechanism through which the bioglass stimulated vascularization due to the complex chemical composition of the glasses. Calcium silicate bioceramic is a good candidate for doping copper as it has simple composition, which facilitates the investigation of specific roles of different ions. More importantly, CS has been reported to be able to stimulate vascularization because of the critical Si ions in its ionic products [10, 31]. Therefore, it is reasonable to try to combine Cu ions and Si ions in order to obtain higher stimulatory effects on vascularization than pure Cu ions or Si ions by doping Cu into calcium silicate ceramics.

After an element was doped into CS, the crystallite structure of CS will be changed in addition to the chemical composition. In our study, the synthesized Cu-CS powders had significantly higher degree of corresponding diffraction peaks in XRD scanning pattern as compared to the pure CS powder. The characteristic XRD peaks depend on the factors such as crystallinity and crystallite size, which were determined by synthesis parameters such as reaction time, aging, temperature and so on [32]. During the synthesis process, the Ca ions in lattice of CS were replaced by Cu ions. Since Ca and divalent Cu ion diameters are 0.099 nm and 0.072 nm, respectively [33, 34], the lattice distortion was increased because of the reduced unit crystallite size. Therefore, the XRD peak shifting is attributed to the replacement of the Ca ions by Cu with smaller atomic diameter, which probably increased the deviation of lattice due to the reduced cell crystal size caused by the substitution . In addition, the incorporation of copper into CS framework might slow down the release of ions in lattice because of the smaller inter-planar distance and crystal volume of Cu-CS, which could inhibit the transport movement of atoms. This was reflected by the lower Si ionic concentration in Cu-CS extracts compared to CS extracts with the same dilution ratios.

Previous studies demonstrated that VEGF and its receptor KDR play a critical role in the development and progression of angiogenesis process in normal physiological and pathological conditions [35-37]. Therefore, it is believed that the stimulatory effects of Cu-CS on vascularization is due to the upregulation of VEGF expression in HUVECs or co-HDFs and the KDR activation in HUVECs or co-HUVECs after those cells were treated with Cu-CS extracts. It is worth noting that, the stimulatory effects of Cu-CS on the expression of VEGF and KDR were similar to the mechanism through which the CS affects the angiogenesis.

In addition, it has been widely accepted that HIF-1α plays a vital role in stimulating cells to express VEGF [38, 39]. VEGF is produced once hypoxia is activated, while the HIF-1α is the main indictor for cell hypoxia status as it is stabilized in cells under hypoxia condition. In this study, all co-HDFs cultured with Cu-CS extracts showed significantly higher expression of HIF-1α as compared to the mono-cultured or co-cultured HUVECs. The CS extracts also stabilized the HIF-1α in cells, resulting in the higher expression of HIF-1α in cells cultured with CS extracts, especially in co-HDFs. However, we found that, as compared to Cu ions, more Si ions were needed for achieving same stimulatory effects on HIF-1α. These findings indicate that Si ions have lower stimulatory effects on HIF-1α expression than Cu ions.

Previous studies have shown that some transition metal cations such as Co^{2+}, Cu^{2+}, and Ni^{2+} could cause the increase of the HIF-1α level in endothelial cells by inhibiting the degradation of HIF-1α [40, 41]. Wu et al. also reported that the doping of Cu^{2+} into bioactive glass induced a hypoxia microenvironment for hBMSCs and stimulated the expression of HIF-1α and VEGF from the cells [17]. In our previous study, we found that Si ions

released from CS also caused slight increase of HIF-1α in mono-cultured HUVECs [31]. Here, we found that the pure CS extracts also upregulated the expression of HIF-1α in co-HDFs and mono-cultured cells, which was in accordance with our previous study [31], but the effects were not as strong as those of Cu-CS extracts. In addition, in the Cu-CS extracts 1/64, the Si and Cu ion concentrations were only 0.62 μg ml^{-1} and 0.71 μg ml^{-1}, respectively. Our previous investigations confirmed that Si ions play an important role in the stimulation of proangiogenesis by pure CS and CS extracts with Si ion concentration of 0.7-1.8 μg ml^{-1} were found to be able to up-regulate the expression of proangiogenic factors [31]. In this study, the effective concentration of Si ions in Cu-CS extracts were almost half lower than those in CS extracts with same stimulatory activity. However, the expression of HIF-1α in those cells stimulated by Cu-CS 1/64 was significantly higher than that in the cells simulated by all other media. In addition, the expression of HIF-1α in those cells stimulated by Cu-CS 1/64 (12 ng ml^{-1}) was even higher than the sum of HIF-1α expression in cells cultured with CS 1/64 (~5 ng ml^{-1}) and Cu 0.7 (~5 ng ml^{-1}). Therefore, it can be reasoned that the Cu ions in Cu-CS extracts dominated the HIF-1α stabilization effects and played a key role in the induction of hypoxia microenvironment.

Furthermore, we found that the effective concentrations of Cu ions released from Cu-CS for stimulating vascularization were much lower than those used in literatures. Cu ion concentration used in previous literatures for angiogenesis stimulation of endothelial cells generally ranged from 1 to 100 μmol L^{-1} [13, 42-44], which equals to 0.064 to 6.4 μg ml^{-1}. Wu et al. reported that the effective concentrations of Cu and Si ions released from Cu-containing mesoporous bioactive glass was 14.2 μg ml^{-1} and 11.9 μg ml^{-1}, respectively. In the present study, the effective concentrations of Cu ion in Cu-CS extracts ranged from 1.5 μg ml^{-1}l to 0.35 μg ml^{-1}, which were in the low range of the Cu ion concentrations reported in literatures [13], and much lower than those used in Wu's study [17]. With the same concentration of Si ions in Cu-CS 1/32 and CS 1/128 extracts, 1.5 μg ml^{-1} Cu^{2+} was enough for stimulation of vascularization although it was not the optimal concentrations of Cu and Si ions. In addition, the Cu-CS extracts with 0.3 μg ml^{-1} Cu ions obviously stimulated the expression of angiogenic growth factors, while media containing $CuSO_4$ with 0.3 μg ml^{-1} Cu ions did not. Since high concentration of Cu ions may cause certain cytotoxicity [14], and lower Cu concentrations may reduce the risk of cytotoxicity, our results indicated that the Cu-CS bioceramics have more effective stimulatory effects on vascularization than copper complexes or salts with lower potential cytotoxicity. All these results indicate that the Si ions may play a synergy effect with Cu ions in stimulation of angiogenesis. In this case, the effective Cu ion concentration was not necessary to be as high as those in water soluble divalent copper salts reported previously as the stimulatory effects of Cu ions were augmented by Si ions. Similarly, the effective Si ion concentration was not necessary to be as high as those in CS extracts as Cu ion played a key role in inducing a hypoxia microenvironment to stimulate vascularization.

As we know that Cu ions can stabilize the HIF-1α by preventing its degradation through the ubiquitin-proteasome pathway, which is mediated by proline hydroxylation of HIF-1α catalyzed by three HIF prolyl hydroxylases (PHDs) [45, 46]. It has been also reported that the Cu ions can enhance the formation of the HIF-1 transcriptional complex with p300 and increase the HIF-1 binding to the hypoxia responsive elements (HRE) sequence target genes [47]. It can be seen that, under the hypoxia status, the reactions between HIF-1α with other factors are complex. Therefore, the hypoxia microenvironment might be enhanced by Si ions released from Cu-CS, which may exist as SiO_3^{2-} and augment the stimulatory effects of Cu ions on vascularization. Although more detailed studies are required to provide a comprehensive understanding of the mechanism of synergy effects of Cu and Si ions, the present study sheds the light for designing bioceramic materials through combining different functional elements.

Therefore, the whole mechanism through which the Cu-CS stimulated vascularization in co-culture system can be elucidated as following: HIF-1α was upregualted in co-HDFs by Cu-CS extracts, and the overexpression of HIF-1α further resulted in the upregulation of VEGF expression in co-HDFs. The high expression of VEGF then acted on co-HUVECs and initiated the vascularization. This whole process indicates that Cu-containing calcium silicate bioceramics also stimulated the vascularization process through paracrine effects, which is similar to that of calcium silicate bioceramics. During this process, Cu ions played a key role in the induction of hypoxia microenvironment and Si ions played an important synergy effects to augment stimulatory effects of Cu ions. The process has been schematically illustrated by Fig. 7.

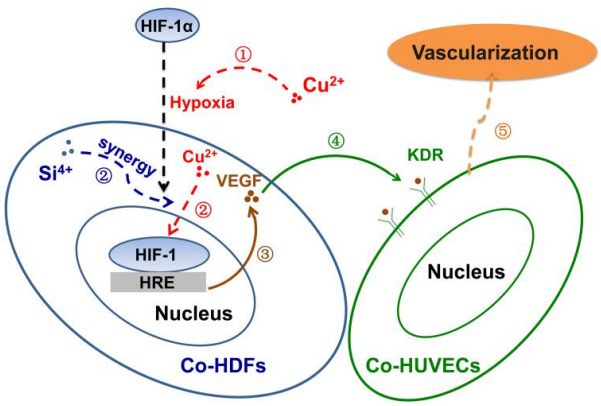

Fig.7 Illustration of the mechanism through which the Cu-CS stimulates vascularization in a HDF-HUVEC co-culture model. Paracrine effects in the communications between HDFs and HUVECs for angiogenesis can be seen.

5. Conclusion

In this study, a copper-doped calcium silicate (Cu-CS) was designed in order to obtain a bioceramic with higher stimulatory effects on vascularization than pure Cu or Si ions. The Cu-CS extracts presented significantly better proangiogenic capacity than CS extracts with same concentration of Si ions and the media containing $CuSO_4$ with same concentrations of Cu ions. In addition, significantly lower concentrations of Cu and Si ions from Cu-CS bioceramic extracts were needed to stimulate vascularization than that from the water soluble copper salts and CS extracts, respectively. We confirmed that optimal concentrations of the Cu and Si ions were 0.7 μg ml^{-1} and 0.6 μg ml^{-1}, respectively, in the Cu-CS bioceramic extracts, which is significantly lower than the optimal concentration of the single ions of Cu or Si, and suggests a synergic stimulatory effect of combination of Cu and Si ions from Cu-CS bioceramic during vascularization. In addition, in a HUVEC-HDF co-culture model, Cu ions in the Cu-CS extracts stabilized the HIF-1α in co-HDFs and further stimulated the VEGF expression of co-HUVECs, suggesting a paracrine effect of the combination of Cu and Si ions. All of these results indicate that, it is feasible to elaborately design a bioceramic with combination of two or more specific functional elements for stimulating vascularization in tissue engineering.

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