Bone tissue engineering scaffolds with HUVECs/hBMSCs cocultured on 3D-printed composite bioactive ceramic scaffolds promoted osteogenesis/angiogenesis

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\section*{A B S T R A C T}

\textbf{Background: /Objective:} Tissue engineering involves scaffolds, cells and growth factors, among which growth factors have limited applications due to potential safety risks and high costs. Therefore, an alternative approach to exogenously induce osteogenesis is desirable. Considering that osteogenesis and angiogenesis are coupled, a system of human umbilical vein endothelial cells (HUVECs) and human bone mesenchymal stem cells (hBMSCs) coculture is more biologically adapted to the microenvironment in vivo and can mediate osteogenesis and angiogenesis via paracrine signalling. Hence, in this study, a HUVECs/hBMSCs coculture system with appropriate cell and medium proportions was established. The substrate for the coculture system was a 3D-printed composite bioceramic scaffold (\(\beta\)-TCP/CaSiO\(_3\)) based on a previous study. The aim of this study was to explore the potential of this system for bone tissue engineering.

\textbf{Methods:} Bioactive ceramic scaffolds for tissue engineering were fabricated via a 3D Bioplotter\textsuperscript{TM} system. The coculture system for \textit{in vitro} and \textit{in vivo} studies consisted of direct contact between HUVECs and hBMSCs cultured on the 3D-printed scaffolds.

\textbf{Results:} The proportions of HUVECs/hBMSCs and medium components were determined by cell viability, and the coculture system showed negligible cytotoxicity. CD31 secreted by HUVECs formed strings, and cells tended to aggregate in island chain-like arrays. Real-time cell tracking showed that HUVECs were recruited by hBMSCs, and the integrin expression by HUVECs was upregulated. Ultimately, osteogenic and angiogenic marker gene expression and protein secretion were upregulated. Moreover, the obtained bone tissue engineering scaffolds could induce early osteogenic protein secretion and capillary tube formation in nude rats.

\textbf{Conclusion:} These bone tissue engineering scaffolds without exogenous growth factors exhibited the ability to promote osteogenesis/angiogenesis.

\textbf{Translational potential of this article:} The fabricated 3D-printed bioactive ceramic scaffolds could provide mechanical, biodegradable and bioadaptive support for personalized bone regeneration. In addition, the bone tissue
engineering scaffolds exhibited the ability to promote osteogenesis/angiogenesis without the addition of exogenous growth factors, thus mitigating safety risks. Although application of the HUVECs/hBMSCs coculture system might be a time-consuming process, further development of cord blood storage could be beneficial for multicell coculture.

1. Introduction

Bone repair is a complex and long process, and bone tissue engineering scaffolds with excellent osteogenesis/angiogenesis could be potential candidates for bone regeneration applications [1]. Growth factors can bind to specific receptors on target cells and efficiently stimulate cells [2]; however, growth factors are limited by high costs, potential side effects, production processes, dose control, high batch variability and species-related problems [3,4]. Considering microenvironment bio-adaptability in vivo, suitable types of seed cells are compatible with the natural biological microenvironment [5,6]. Cell fate can be mediated by paracrine signalling [7], and secreted chemotactic factors can promote cell recruitment and gap junction formation [8]. As a result, cellular behaviours can be mediated to achieve bone regeneration.

Mesenchymal stem cells (MSCs) have the potential to differentiate into mesodermal cell types [9] and have been widely utilized in tissue engineering or as medicinal signalling cells [10]. MSCs can also supply exogenous factors that induce site- or tissue-specific resident stem cells to construct new tissue [11]. In addition, osteogenesis and angiogenesis are coupled since vessels provide nutrients and oxygen, remove waste [12] and maintain metabolism to balance homeostasis [13,14]. The paracrine signalling of vascular endothelial growth factor (VEGF) and bone morphogenetic protein 2 (BMP2) between human umbilical vein endothelial cells (HUVECs) and human bone mesenchymal stem cells (hBMSCs) has been reported to be a complementary factor in angiogenesis and osteogenesis [6]. Hence, HUVECs/hBMSCs coculture might promote osteogenesis and angiogenesis [15]. In this study, a coculture system with HUVECs and hBMSCs was utilized.

Various approaches are used for coculture [16]: direct contact includes 3D and 2D conditions, and indirect contact can be further divided into three approaches, namely, semipermeable membrane separation, conditioned medium and conditioned endothelial cell medium (ECM) [17,18]. Coculture with indirect contact is used to study the mechanism and relationships formed by paracrine signalling, while coculture with direct contact on scaffolds is used to study candidates for tissue engineering applications. In addition, 3D scaffolds with direct-contact coculture can be used to produce MSCs/HUVECs spheroids, which are crucial for exosome secretion by MSCs [19–21]. Additionally, interconnected porosity has been proven to be necessary for an implant to induce osteogenesis [22]. 3D printing techniques have the inherent advantage of fabricating interconnected porous scaffolds [23,24] and could play an important role in personalized bone regeneration [25]. Therefore, 3D-printed interconnected porous bioactive ceramic scaffolds were utilized as the substrate for HUVECs/hBMSCs culture. In our previous studies, 3D-printed composite ceramic scaffolds were fabricated using CaSiO3 [26], which promoted osteogenesis and angiogenesis [27], and the effects of the proportion of β-TCP/CaSiO3 on MSCs were studied. In addition, the shape, size and porosity of the scaffolds were selected based on our previous study [28].

In this study, a coculture system consisting of HUVECs and hBMSCs was established on porous β-TCP/CaSiO3 scaffolds fabricated by a 3D Bioplotter™ system (Regenovo, China). The direct coculture strategy was achieved via the successive seeding of HUVECs/hBMSCs onto the 3D printed scaffolds; then, the tissue engineering scaffolds were evaluated via in vitro and in vivo studies, the process was showed in Scheme 1. Cell and medium proportions were optimized based on cell viability assessments, and the established coculture system, which aimed to promote osteogenesis/angiogenesis, was also assessed via immunofluorescence and in vitro real-time tracking (0–48 h). After HUVECs/hBMSCs were cocultured on β-TCP/CaSiO3 3D printed scaffolds, the expression of genes and the secretion of proteins related to osteogenesis and angiogenesis were detected in vitro. For the in vivo study, nude rats were utilized to avoid an immune rejection response, and the extent of osteogenesis/angiogenesis after submuscular implantation was assessed to reveal the osteoinductive capacity of the bone tissue engineering scaffolds.

2. Materials and methods

HUVECs and ECM were purchased from Scienccell, USA. The ECM contained 5% fetal bovine serum (FBS, Catalogue No.0025), 1% endothelial growth additives (EGS, Catalogue No.1052), and 1% penicillin streptomycin solution (P/S, Catalogue No.0503). HUVECs were primary cultured. hBMSCs (passage 2) and complete medium (HUXMA-90011) were purchased from Cyagen Biosciences, USA. Complete medium contained 10% FBS, 1% l-glutamine and 1% penicillin streptomycin solution.

Scheme 1. Overview of the experiment. The HUVECs/hBMSCs were seeded onto the 3D printed scaffolds fabricated by a 3D Bioplotter™ system and the tissue engineering scaffolds were evaluated via in vitro and in vivo studies.
2.1. Scaffold fabrication

The scaffolds were fabricated based on our previous study [26]. Briefly, the raw materials, β-tricalcium phosphate and silicate calcium particles, were prepared by chemical precipitation. Ammonium polyacrylate (PAA-NH₄) and hydroxypropylmethyl cellulose (MV = 4000) were used as the dispersant and viscosifier, respectively. The dispersant and viscosifier were mixed with the raw materials with a planetary ball mill (Nanda Instrument Plant, Nanjing, China). Ultimately, the slurry used for rapid prototyping was endowed with liquidity and the ability to solidify. An extrusion rapid prototyping 3D Bioplotter™ system (Regenovo, Hangzhou, China) was used for rapid prototyping. The printed scaffolds were sintered at 1100 °C.

2.2. Characterization of scaffolds

The morphology and exterior of the scaffolds were observed by field emission scanning electron microscopy (SEM) (Zeiss Merlin, Germany). Before SEM observation, the samples were sputter-coated with platinum, and an electroconductive paste was twined laterally to link the upper surface with the platform. X-ray diffraction (XRD) and energy dispersive spectroscopy (EDS) were utilized to measure the crystal phase and elements in the scaffolds.

2.3. Cell culture and seeding

HUVECs and hBMSCs were cultured separately in an incubator at 37 °C and with 5% CO₂ before seeding. HUVECs and hBMSCs were seeded at passages 3–5 and 4–6. The culture medium was prepared as specified and refreshed every 2 days. Trypsin solution with 0.25% EDTA was utilized to detach cells from the culture dish. The working solution was 2.5 μg/mL 2 mL of CM-Dil solution was added to each well. Then, the plate was incubated for 1 h before analysis with a microplate reader at a wavelength of 450 nm. The cytotoxicity of the coculture system was assessed by detecting the concentration of lactate dehydrogenase, which was released only by damaged cells.

2.4. Viability of cocultured cells

Cell viability was evaluated for different ratios of cells and medium components. The CCK-8 (Dojindo, Kumamoto, Japan) assay was used to characterize the intracellular dehydrogenase density and assess cell viability after 24 and 48 h of coculture. HUVECs and hBMSCs were cocultured in medium containing a 10:1 ratio of medium and stock solution. The samples were observed by laser scanning confocal microscopy (Leica TCS SP8, Germany).

2.5. Cell adhesion

Cell adhesion on scaffolds was quantified and characterized by measuring the expression of the integrin subunits α₂, α₅, α₁, and β₁ in the early stage (12 h) via real-time polymerase chain reaction (RT-PCR) analysis. The combinations of the subunits were listed below in Table 1. Before extracting RNA, Dynabeads (Dynabeads™ CD31 Endothelial Cell

### Table 1

| Combination | Synonyms | Ligands         |
|-------------|----------|-----------------|
| α₂β₅        | VLA-2    | Collagen, laminins |
| α₅β₃        | VLA-5, fibronectin receptor | Fibronectin, proteases |
| α₁β₁        | VLA-5    | Vitronectin; fibrinogen |

2.6. Real-time cell tracking in vitro

To distinguish the cell distribution by optical observation, the cell dye CM-Dil (C7001, Life, USA) and CellTracker Green CMFDA dye (C7025, Life, USA) were used to label HUVECs and hBMSCs. CM-Dil was diluted with Dulbecco’s phosphate-buffered saline, and the concentration of the working solution was 2.5 μg/mL. 2 mL of CM-Dil solution was added to the HUVECs culture flask, and then the culture flask was incubated at room temperature for 5 min before being placed in a 4 °C refrigerator for 15 min. CellTracker Green CMFDA dye at a concentration of 5 μg/mL was reacted with hBMSCs in the culture flask for 30 min at room temperature. Then, the samples were observed by scanning confocal microscopy.

2.7. Osteogenic and angiogenic gene expression

The expression levels of osteogenic and angiogenic genes were characterized by RT-PCR using the same process as that used for the analysis of integrin subunit expression. The housekeeping gene was GAPDH, and the osteogenic gene markers were the alpha 1 chain of type I collagen (Col-I), BMP2, osteocalcin (OCN) and osteopontin (OPN). The gene markers for angiogenesis were basic fibroblast growth factor (bFGF), platelet endothelial cell adhesion molecule (PECAM-1 or CD31), endothelial nitric oxide synthase (eNOS), kinase domain region receptor (KDR or VEGF-R2), transforming growth factor-β (TGF-β) and VEGF. The

### Table 2

| Gene      | Direction | Sequence (5’-3’) |
|-----------|-----------|-----------------|
| GAPDH     | Forward   | TGGTGCTGGCTGGATGGTGA |
| α₂        | Forward   | TTGGCTGGATAGCGAAGAGG |
| α₅        | Forward   | CAAAGCGTTGGAAAAAGG |
| α₁        | Reverse   | ATCCACAGTGGAGAGGAG |
| β₁        | Reverse   | CGTGGTGGACAGGAGAGA |

11155D, Life, USA) were utilized to isolate cells. Dynabeads bind to CD31 receptors on target cells, allowing the cells to be separated under a magnetic field. The process of RT-PCR was as follows [29]. (1) DNA extraction. TRIzol was used to lyse cells, chloroform was used to split the water from the oil phase, and isopropanol was used to precipitate RNA. (2) Reverse transcription. The obtained RNA was quantified by a NanoDrop2000 spectrophotometer (Thermo Scientific, USA) and then normalized according to the manufacturer’s protocol for the reverse transcription assay. The extracted RNA was reverse transcribed into cDNA using a PrimeScript RT reagent kit with gDNA Eraser(Takara Biotechnology, Japan). (3) Characterization. The cDNA was analysed with QuantStudio 6 Flex RT-PCR system software (Applied Biosystems, USA), and quantitative polymerase chain reaction (qPCR) was performed with a SYBR green assay (Iq Supermix, Bio-Rad). The housekeeping gene was GAPDH, and the primers for the integrin subunits α₂, α₅, α₁, and β₁ were listed in Table 2. Gene expression was compared by △△Ct analysis, where Ct represented the cycle time after the threshold was reached.
Table 3

| Gene   | Direction | Sequence (5’-3’)                |
|--------|-----------|---------------------------------|
| GAPDH  | Forward   | TGTGCCCTGGTGTGATCTGA            |
|        | Reverse   | TTGGCTGTGAAGAGCGAGGAG           |
| BMP2   | Forward   | TGGTGAAGGTTGGTGGTGA             |
|        | Reverse   | TTCAACCTGGGTTGGTTTTTT          |
| OPN    | Forward   | TAGGACACCGTTGTAACAAAGCG         |
|        | Reverse   | TGCAAGGGCGTTTTGTCACTTT          |
| OCN    | Forward   | AGACGTGGCGGCAAGCCTA             |
|        | Reverse   | TAGGGGGAAGGTCTGTCACCTAC         |
| Col-I  | Forward   | GCTTGGCCACTGTTGGAAGA            |
|        | Reverse   | CGACTTGTTTCCAGGGCACTCTCT        |
| bFGF   | Forward   | GTTGTCTAACTGGTACGTTGCAAAGCG    |
|        | Reverse   | CCAGTCTGTTAGTGCCACTCT           |
| CD31   | Forward   | CCGCATATCCAAGGTGACCA            |
|        | Reverse   | CCCCTGTTTGCAAGTGTTGGA           |
| eNOS   | Forward   | CTAGAGGCTGGCACTGCGGA            |
|        | Reverse   | CATTTAACTCTGTTGCGACTCT          |
| KDR    | Forward   | AGCCACCTCCTGGATTTGGGA           |
|        | Reverse   | CATGCCCTTACCACTGGCCAA           |
| TGF-β  | Forward   | TCCGGCAAGATACCTCA               |
|        | Reverse   | GCTAGGGCCAGAGCGCTCAA            |
| VEGF   | Forward   | CTACAACTCGGAGACTCCTGG           |
|        | Reverse   | TGGTGAGGCTTGGTGCCAGCAT          |

primer sequences for the osteogenic and angiogenic genes were listed in Table 3.

2.8. Immunofluorescence

Protein secretion was detected by immunofluorescence using OCN and CD31 as markers. The primary CD31 antibody was a monoclonal CD31 antibody (JC/70A) (Life, USA) with a goat anti-mouse IgG (H+L) highly cross-adsorbed secondary antibody (Alexa Fluor 594, Life, USA). The primary antibody against OCN was a polyclonal rabbit antibody (23418-1-AP, Proteintech, Wuhan, China) with a donkey anti-rabbit IgG highly cross-adsorbed secondary antibody (Alexa Fluor 594, Life, USA). The crystal phase and the elements of the scaffolds were measured via XRD and EDS. The surface layer consisted of β-TCP and CaSiO₃ (Fig. 1G). The circles correspond to the clear peak positions of β-TCP at 27.95° (131), 31.2° (4 22) and 34.6° (0 40). A peak at 30.06° corresponding to wollastonite (302) was also detected. The weights and atomic percentages of Ca, P and Si were shown in Fig. 1H.

3. Results

3.1. Scaffold morphology

The bioactive ceramic scaffolds were fabricated by the 3D Bioplotter™ system, as shown in Fig. 1A. The thermally treated scaffolds contained interconnected pores and micro pores on the surface, as shown in Fig. 1B. The scaffolds for the in vitro experiments were 8-mm-diameter cylinders with a height of 2.5 mm (Fig. 1C). Fig. 1E showed the rectangular scaffold used for submuscular implantation. The crystal phase and the elements of the scaffolds were measured via XRD and EDS. The surface layer consisted of β-TCP and CaSiO₃ (Fig. 1G). The circles correspond to the clear peak positions of β-TCP at 27.95° (131), 31.2° (4 22) and 34.6° (0 40). A peak at 30.06° corresponding to wollastonite (302) was also detected. The weights and atomic percentages of Ca, P and Si were shown in Fig. 1H.

3.2. Coculture system determination

The proportions of cells in the coculture system were selected by measuring cell viability. The number of cells was set as 6 × 10⁴. The results were summarized in Fig. 2. The number of hBMSCs was 0 (red column), 2 × 10⁵ (blue column), 3 × 10⁵ (cyan column), 4 × 10⁵ (pink column), and 6 × 10⁵ (brown column), and the corresponding proportions of hBMSCs to HUVECs were 0:1 (0%), 1:2 (33%), 1:1 (50%), 2:1 (66%) and 1:0 (100%). The HUXMA contents were 0%, 33%, 50%, 66% and 100%, and the proportions of conventional ECM to HUXMA were 0:1, 1:2, 1:1, 2:1 and 1:0. The results showed that the optical density (OD) of hBMSCs was higher than that of HUVECs, while the coculture with a 1:2 ratio of HUVECs to hBMSCs (4 × 10⁵ hBMSCs, pink column) had similar OD value with hBMSCs cultured alone. As the culture time was prolonged to 48 h, the coculture with a 1:2 ratio of HUVECs to hBMSCs showed the fastest proliferation. Other principles and strategies were also taken into consideration. Firstly, the purpose of this coculture is to induce osteogenesis, and angiogenesis is a crucial and indispensable process, the amounts of hBMSCs for osteogenesis should maintained considerable proportion. Secondly, the viability of HUVECs relied on ECM (especially ECGS in the medium); hence, the concentration of ECGS remained the same as that in the ECM. Thirdly, the proliferation rate of hBMSCs was inhibited under culture conditions with low FBS content (ECM containing 5% FBS, HUXMA containing 10% FBS). Herein, the coculture system with a 1:2 ratio of HUVECs to hBMSCs and a 1:2 ratio of ECM:HUXMA (HUXMA 66% content) was established to ensure an appropriate content of FBS, and the viability of cells in the coculture system substantially increased from 24 h to 48 h.

The cytotoxicity of the selected coculture system was characterized

2.10. Pathology and histology

Harvested implants were in 4% paraformaldehyde and decalci

ified with EDTA, followed by dehydration, permeation, wax saturation and embedding. Ultimately, implants were sectioned for haematoxylin-eosin (H&E) and Masson’s trichrome staining to examine new bone matrix and collagen deposition. The expression of CD31 and OCN was characterized by immunohistochemical staining.

2.11. Statistical analysis

Experimental results are shown as means with standard deviations as error bars. Data were evaluated by one-way analysis of variance (ANOVA) followed by Tukey’s test, with statistical significance set at p < 0.05 (marked as #).
by detecting lactate dehydrogenase released from damaged cells, and the results, shown in ESI Fig. 1, indicated that the cytotoxicity of the coculture system could be negligible.

3.3. Cell adhesion and real-time tracking on scaffolds

Based on the optical observation shown in Fig. 3A, the outline of HUVECs resembled cobblestone, while the larger hBMSCs were shaped like shuttles. The HUVECs and hBMSCs were arranged in an island chain-like array at an early stage and ultimately coalesced as a film when cultured in vitro. In addition, cell labelling and immunofluorescence were used to evaluate the cell distribution and preliminarily examine protein secretion in the coculture system. As shown in the cell-tracking results in Fig. 3C, cocultured cells initially adhered to the matrix and tended to cluster with other cells of the same type. Cell adhesion was observed after 12 h of culture, and clear communities were observed after 24 h of culture.
cultivation. Ultimately, the cells were arranged in island chain-like arrays, with HUVECs surrounded by hBMSCs. When the cells were cultured on plates for 7 d, HUVECs secreted PCAM-1 (also known as CD31), which formed a string, hBMSCs showed marked proliferation but little OCN secretion as shown in Fig. 3D.

When cells were seeded on the 3D-printed scaffolds, they could form a film on the surface, as shown in ESI Fig. 2, and could migrate between the extruded fibres. The results of the real-time tracking of HUVECs and hBMSCs on the scaffold were shown in Fig. 4A, and complete videos were provided in ESI Videos 3 and 4. HUVECs and hBMSCs were indicated by red and green fluorescence. hBMSCs were cultured on the scaffold first, as they could adhere to and spread on the scaffold; then, HUVECs were cultured and seemed to be recruited by the hBMSCs, as shown in ESI Video 3.

3.4. Expression of osteogenic differentiation and angiogenic genes in vitro

The expression levels of genes related to osteogenesis and
Figure 4. (A) Tracking of HUVECs and hBMSCs on the scaffold. HUVECs and hBMSCs were indicated by red and green fluorescence. hBMSCs were cultured on the scaffold for 12 h, and then HUVECs were added. The observations were carried out after incubating HUVECs for 0-5 h; the scale bar was 200 μm (B) RT-PCR of HUVECs and hBMSCs on the scaffold. Relative gene expression levels of the angiogenic markers TGF-β, bFGF, CD31, BMP2, VEGF, KDR and OCN. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Figure 5. (A) Immunofluorescence of cocultured cells on scaffolds. OCN was labelled with green dye; the red dye represented CD31; and DAPI was used to stain the nucleus. CO, EC and HB represented the cocultured cells, monocultured hBMSCs and monocultured HUVECs, respectively. The scale bar was 200 μm (B) Gene expression levels of integrin subunits α2, α5, αv, and β1 after 12 h of culture. Before extracting RNA, Dynabeads (Dynabeads™ CD31 Endothelial Cell 11155D, Life, USA) was utilized to isolate hBMSCs and HUVECs. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
angiogenesis were examined via RT-PCR. The group of HUVECs/hBMSCs cocultured on scaffolds was named CO group. The control groups consisted of hBMSCs cultured on scaffolds (named HB group) and HUVECs cultured on scaffolds (named EC group). The tested gene markers were bFGF, OCN, BMP2, PECAM-1 (CD31), eNOS, KDR (VEGF-R2), TGF-β and VEGF. No significant difference in the bFGF or TGF-β level was observed between the EC and HB groups; however, compared with the other groups, the CO group showed notably upregulated gene expression. VEGF and its receptor KDR were also markedly upregulated in the CO group compared with the EC group, but these genes were not expressed in the HB group. Low CD31 expression was detected in the HB group, and the CD31 expression level in the EC group was higher than that in the CO group. Regarding the osteogenic gene markers BMP2 and OCN, there was no significant difference in the expression of BMP2 between the EC and HB groups, while the CO group showed greatly upregulated BMP2 gene expression. The OCN gene expression level in the CO group was upregulated much more than that in the HB group, and there was no OCN expression in the EC group.

To complement the gene expression level analysis, OCN and CD31 protein secretions were examined via immunofluorescence staining (Fig. 5A). The results were consistent with the RT-PCR results. Notable fluorescence corresponding to secreted OCN was observed in the CO group, while weak fluorescence was observed in the HB group, and no fluorescence was observed in the EC group. The red fluorescence clearly indicated that CD31 was secreted in the CO and EC groups, while trace amounts of CD31 were secreted in the HB group, which might be attributed to the stimulation of hBMSCs by the scaffolds.

The gene expression levels of the integrin subunits α2, α5, αv and β1 were quantitatively measured, as shown in Fig. 5B. The cocultured cells were isolated via Dynabeads, and then the integrin expression levels were compared. The gene expression levels of integrin α2, α5 and αv were upregulated in HUVECs, while the gene expression of integrin αv was upregulated in hBMSCs. This finding indicated that HUVECs adhesion was improved with the establishment of the coculture system. Notably, integrin α5 expression was upregulated in both hBMSCs and HUVECs, while integrin β1 expression was downregulated in the coculture compared to the monocultures.

### 3.5. Osteogenesis and angiogenesis in vivo

The fabricated scaffolds were cultured with cells in vitro for 7 d beforehand and then implanted into female nude rats. The cocultured cells formed a film on the scaffold (ESI Fig. 3A). The extent of early osteogenesis and angiogenesis after 2 weeks implantation was measured through H&E and Masson’s trichrome staining combined with CD31 and OCN immunohistochemical staining. Little mineralized bone matrix was observed in the EC and HB groups, and secreted collagen I was rare on these scaffolds. Although more collagen I was present on the scaffolds in the CO group than that in the other groups based on Masson's trichrome staining (Fig. 6A2), new bone matrix was not observed by H&E staining. These results suggested that the tissue engineering scaffolds with cocultured cells could induce early collagen secretion and capillary tube formation; however, no mature bone formation was observed.

CD31 immunohistochemical staining revealed signs of lumens or capillary tubes on the scaffolds, which confirmed CD31 secretion in the CO group. Semiquantitative analyses were the average OD calculated by the ratio of the integrated OD to the stained area (Fig. 7D and E). This revealed that the CO group had the ability to quickly form vessels while erythrocytes could gather and form vessel-like structures without a large amount of CD31 protein in the HB and EC groups. Immunohistochemical staining revealed that the area of OCN staining was larger and that average OD was more intense than that of CD31. Although the stained area was widespread in the monoculture and coculture groups, the average OD was more intense in the CO group than in the EC and HB groups, which corresponded with the H&E and Masson staining results. The tendency towards osteogenesis/angiogenesis was more obvious in the CO group than in the EC and HB groups. In summary, tissue engineering scaffolds with cocultured cells could induce early protein secretion (Col-I, CD31 and OCN) and the generation of capillary tubes that secreted CD31. In addition, after 4 and 8 weeks, the scaffolds seemed to degrade, and signs of osteogenesis and angiogenesis became blurred after 4 (ESI Figs. 4) and 8 (ESI Fig. 5) weeks. These observations might be the result of incompatibilities between the nonhomologous seed cells and host cells.

**Figure 6.** (A–C) Pathological sections from the CO, EC and HB groups after 2 weeks submuscular implantation. The numbers 1 and 2 represented H&E and Masson staining, respectively. The scale bar was 50 μm.
4. Discussion

Angiogenesis and osteogenesis are indispensable for bone regeneration and remain challenges [4]. Traditional tissue engineering approaches utilize exogenous growth factors that can induce osteogenesis but might cause side effects [3]. The scaffolds for cell culture were 3D printed bioactive ceramic scaffolds consisting of β-TCP and CaSiO3, as described in our previous study [26]; this scaffold substrate can be biodegradable, and silicate has been proven to be capable of inducing angiogenesis by upregulating nitric oxide synthase [31] and osteogenesis [6,8]. In addition, the structure of the scaffolds consisted of interconnected pores to facilitate tissue ingrowth.

In this work, a coculture system containing endothelial cells and BMSCs was established to promote osteogenesis and angiogenesis. Moreover, the coculture system resembles that in vivo [16]. Initially, the cell and medium ratios needed for the coculture system were undetermined [5,8,32,33] due to the materials and intended purpose of the coculture system. Different cells demand different complete media (mainly different volumes of FBS) and additive factors, such as ECGS. Li et al [6] studied coculture system of HUVECs and hBMSCs cultured at a ratio of 2:1 in 1:1 ECM:HUXMA [34], which showed excellent osteogenesis/angiogenesis. Additionally, a study proved that HUVECs and hBMSCs cultured at a 5:1 ratio can form microcapillary-like structures with upregulated gene expression of CD31 and BMP2, while HUVECs and hBMSCs cultured at a 1:5 ratio tended to favour osteogenesis via the upregulation of Runx-2, ALP, OPN and BSP [5]. In addition, to maintain the viability of HUVECs, cocultures are sometimes maintained using ECM (with ECGS additive factors) [32]. In this work, the coculture conditions were selected based on cell viability, and the cocultured cells were more vigorous with a remarkable proliferation rate, when HUVECs and hBMSCs were cultured at a ratio of 1:2 in HUVECs and hBMSCs media at a ratio of 1:2 (Fig. 2). Since HUVECs were cultured under conditions of low FBS, which led to a low hBMSCs proliferation rate, the content of FBS should be moderate. Additionally, the purpose of this coculture is to promote osteogenesis, and angiogenesis is a crucial and indispensable procedure; hence, the coculture system used a 1:2 ratio of HUVECs and hBMSCs and a 1:2 ratio of HUVECs and hBMSCs medium. In addition, factors added to the ECM (e.g., ECGS) are important for the proliferation of HUVECs; therefore, the content of ECGS in the medium for the coculture system remained the same as that in the ECM. The in vitro observations showed that the cells in the established coculture system tended to aggregate in island chain-like arrays and formed cell–cell films (Fig. 3), the process of which could be finished within 2 d. The HUVECs seemed to be recruited by the hBMSCs, as observed by real-time cell tracking, and the integrin expression of the HUVECs was upregulated. Considerable CD31 was secreted and connected linearly. Furthermore, the in vivo studies showed that cell–cell films formed on the 3D scaffolds.

Analysis of the expression levels of osteogenic and angiogenic genes indicated that the coculture system favoured both angiogenesis and osteogenesis. VEGF is a key growth factor in angiogenesis that can mediate the proliferation, migration and differentiation of endothelial cells and induce endothelial cell blood vessel sprouting and cavity formation [12]. Its receptor, KDR, also known as VEGF-R2, plays a role in regulating lymphatic and vascular endothelial cells [6]. The expression of these genes was far more upregulated in the coculture system than in the monocultures in our study. bFGF is another multifunctional factor in the early stage of angiogenesis that can regulate the proliferation, migration and differentiation of endothelial cells and promote the formation of capillaries [12]. TGF-β is known as an important cytokine in the process of bone formation that regulates the release of VEGF and bFGF; Additionally, it is a chemotactic factor involved in the recruitment and proliferation of peripheral mesenchymal cells [14]. bFGF and TGF-β gene expression was far more upregulated in the CO group than in the EC and HB groups, with no significant variation between the EC and HB groups. CD31 (PECAM-1) is a widely investigated protein involved in angiogenesis that regulates endothelial cell adhesion and participates in endothelial cell migration, angiogenesis and integrin activation [35,36]. In our study, CD31 gene expression was upregulated in the EC group.

Figure 7. (A–C) Results of immunohistochemical staining in the CO, EC and HB groups after 2 weeks submuscular implantation. The numbers 1 and 2 represented CD31 and OCN staining, respectively. (D) was the average OD values calculated by the ratio of the integrated OD value to the stained area. (E) was the stained area proportion, and the scale bar was 50 μm.
compared with the CO group, which was attributed to the presence of RNA from hBMSCs. Regarding osteogenic gene markers [37], BMP2 is also a widespread growth factor belonging to the TGF-β superfamily and has been proven to efficiently promote osteogenesis [38]. In our study, BMP2 gene expression was far more upregulated in the CO group than in the EC and HB groups, while BMP2 gene expression was higher in the HB group than in the EC group. The late-stage osteogenic marker OCN is associated with bone mineralization [39,40]. OCN gene expression was also far more upregulated in the CO group than in the EC and HB groups, but OCN was not expressed in the EC group.

As a complement to the analysis of gene expression at the transcript level, the levels of CD31 and OCN protein secretion were analysed through immunofluorescence. The tendency of OCN secretion was consistent with the RT-PCR results. The fluorescence intensity of OCN in the CO group was much stronger than that in the HB group, while no secreted OCN was detected in the EC group. CD31 fluorescence in the CO and EC groups was much more intense than that in the HB group.

Nevertheless, it is worth mentioning that weak CD31 fluorescence was observed in the HB group, which indicated the induction of angiogenesis by the scaffolds. These results indicated that both osteogenesis and angiogenesis were promoted by the coculture of HUVECs and hBMSCs on the 3D-printed bioactive ceramic scaffolds.

An in vivo study was performed with submuscular implants in nude rats. The submuscular region of the biceps femoris could favour osteogenesis because of the large number of cells and high blood supply [41]. In the early stage, although little new bone matrix was observed, the capillary tube formation and protein secretion upregulation were confirmed with the coculture system (Fig. 6). However, protein secretion decreased over prolonged time periods. At 4 and 8 weeks, the harvested implants showed only some blurred OCN staining without signs of CD31 staining (ESI Figs. 4 and 5). The reasons for the lack of ectopic bone formation might be related to species differences between the seed and host cells.

MSCs have been reported to recruit pericytes instead of functioning as stem cells [11]; thus, seed cells might be able to recruit pericytes while host cells support tissue regeneration. The seed cells on the scaffolds could recruit pericytes and promote osteogenic protein secretion but the protein secretion were subject to the ectopic microenvironment and the species differences between the seed and host cells. As a result, little new bone matrix deposition was observed in the nude rats, but osteogenic protein secretion (Col-1, OCN), angiogenic protein secretion (CD31) and capillary tube formation were observed early after implantation.

Additionally, it should be noted that bone regeneration is a complex and long-term process that comprises multiple components, such as osteogenesis, angiogenesis, and osteoimmunology, which are regulated by the physicochemical and mechanical microenvironment [42,43]. Bone healing and cell fate can be controlled by physicochemical and mechanical stimuli, especially under 3D conditions [44]. This work confirmed the osteogenic and angiogenic effects of the obtained 3D-printed bioactive ceramic scaffolds and coculture system and demonstrated several advantages of clinical translation [45,46]. However, the underlying mechanisms promoting osteogenesis and angiogenesis require further investigation. A systematic exploration is also required to reveal how physicochemical and mechanical cues are recognized by cells and mediate cell fate under the conditions of HUVECs/hBMSCs coculture and then screen for the crucial molecular events [47] and cytokines [48,49]. In addition, in cocultures of cells from different species, the regulatory effects of biomaterials on the cells and the mechanisms underlying these effects might be more complicated compared to those in traditional in vitro monocultures. In coculture systems, the related signalling pathways might be cascades of osteogenic/angiogenic pathways, and the involved cytokines could be more diverse. Hence, further studies are needed to discover how physicochemical and mechanical cues can be recognized by cocultured cells and mediate the synergistic coupling of osteogenesis/angiogenesis.

5. Conclusions

In the present study, potential tissue engineering scaffolds for bone regeneration without exogenous growth factors were constructed. The 3D-printed bioactive ceramic scaffolds were composed of β-TCP and CaSiO3. A coculture system consisting of HUVECs and hBMSCs was utilized to promote angiogenesis and osteogenesis after identifying the appropriate cell and medium proportions. The resulting coculture system showed negligible cytotoxicity, and cells gathered into island chain-like arrays on the scaffolds in vitro. HUVECs formed CD31 strings in vitro, and the presence of hBMSCs favoured cell adhesion of HUVECs. The bone tissue engineering scaffolds, with an appropriate proportion of HUVECs/hBMSCs cultured onto 3D-printed bioactive ceramic scaffolds, promoted the expression of genes related to osteogenesis and angiogenesis in vitro. In addition, protein secretion (OCN, CD31 and Col-1) and capillary tube formation were observed during the initial period after implantation of the bone tissue engineering scaffolds in the submuscular region in vivo. Thus, this 3D-printed bioactive scaffold and coculture system has potential as a candidate for bone tissue engineering applications.

Credit author statement

Xiao Liu: Conceptualization, Methodology, Investigation, Writing-original draft, Data curation. Naru Zhao: Methodology, Investigation, Writing-review&editing. Haifeng Liang: Investigation (in vivo), Writing-review&editing. Bizhi Tan: Writing-review&editing. Fangli Huang: Writing-review&editing. Hao Hu: Writing-review&editing. Yan Chen: Writing-review&editing. Gang Wang: Writing-review&editing. Zemin Ling: Writing-review&editing. Chun Liu: Writing-review&editing. Yali Miao: Conceptualization, Resources, Writing-review&editing. Yingjun Wang: Conceptualization, Resources, Writing-review&editing. Xueqiang Zou: Conceptualization, Writing-review&editing. Supervision, Funding acquisition, Project administration.

Data availability

The data used in the present study are available from the corresponding author on reasonable request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jot.2022.10.008.

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