Bioprinting of an osteocyte network for biomimetic mineralization

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Abstract

Osteocytes, essential regulators of bone homeostasis, are embedded in the mineralized bone matrix. Given the spatial arrangement of osteocytes, bioprinting represents an ideal method to biofabricate a 3D osteocyte network with a suitable surrounding matrix similar to native bone tissue. Here, we reported a 3D bioprinted osteocyte-laden hydrogel for biomimetic mineralization in vitro with exceptional shape fidelity, a high cell density (10^7 cells per ml) and high cell viability (85%–90%). The bioinks were composed of biomimetic modified biopolymers, namely, gelatine methacrylamide (GelMA) and hyaluronic acid methacrylate (HAMA), with or without type I collagen. The osteocyte-laden constructs were printed and cultured in mineralization induction media. After 28 d, increased dendritic cell connections and enhanced mineralized matrix production were observed after the addition of type I collagen. These results were further confirmed by the expression of osteocyte-related genes, markers of osteocyte morphology (Connexin43 and E11/Podoplanin), markers of mineralization (dentin matrix acidic phosphoprotein 1 (Dmp1)) and the cellular response to parathyroid hormone (PTH). Moreover, the 3D bioprinting constructs outperformed the 2D monolayer culture and they were at least comparable to 3D casted hydrogels in mimicking the natural osteocyte phenotype. All results indicated that the 3D bioprinting osteocyte network shows promise for mechanistic studies and pharmaceutical screening in vitro.

1. Introduction

Osteocytes are a group of quiescent cells aligned as three-dimensional (3D) networks embedded in mineralized bone matrix and are interconnected via cellular dendrites along the lacunocanalicular system. According to recent studies, osteocytes not only play an essential role in orchestrating bone remodelling physiologically but are also involved in a wide range of diseases, such as osteoporosis, osteoarthritis, osteosarcoma and tumour metastasis [1–3]. However, due to osteocytes’ inaccessible location within the bone matrix and their poor proliferative behaviour, an ideal osteocyte model is lacking, which has severely limited further relevant research. For this reason, the construction of a 3D osteocyte network for biomimetic mineralization in vitro would be desirable as a bone tissue model for molecular biology studies and preclinical high-flux drug screening. To date, only a few studies have explored the biofabrication of a 3D osteocyte network in vitro by assembling osteoblasts or osteocytes with microbeads [4–6]. Although Sun Q et al discovered that the phenotype of such 3D constructs was partly similar to that of native osteocyte networks, the time-consuming and costly fabrication process and the inevitable requirement for microfluidic devices substantially limit their application prospects [4].

The IDG-SW3 cell line, generated from long bone, could replicate the phenotype of mineralized osteocytes in vitro after being fully differentiated. During mineralization induction, IDG-SW3 cells express a full osteogenic profile and produce a calcified extracellular matrix. In addition, IDG-SW3 cells specifically express a green fluorescent protein under the control of the dentin matrix acidic
phosphoprotein 1 (Dmp1) promoter, which enables live monitoring of the mineralization levels [7, 8].

As a revolutionary technology, 3D bioprinting can deposit supporting biomaterials and living cells at the same time in a cell-friendly manner to create 3D constructs replicating the native tissue structure and in vivo microenvironment [9]. In addition to regenerative medicine, 3D bioprinting could also be applied in high-throughput drug screening, the development of in vitro functional tissue equivalents, cell-based sensors and tumour environment modelling [10–13]. Given the network spatial arrangement of osteocytes, 3D bioprinting represents an ideal biofabrication method for osteocyte network engineering to provide a 3D culture environment with a suitable surrounding matrix similar to native bone tissue.

Type 1 collagen is the major organic component of bone matrix and has been regarded as an ideal functional substance for bone tissue engineering. Zhao C et al reported that the production of sufficient levels of collagen was necessary for bone mineralization in vivo [14]. Gillette BM et al showed that type 1 collagen can promote bone tissue regeneration by providing biochemical signals inducing cellular mineralization and differentiation [15]. Interestingly, many recent studies have reported the feasibility of 3D bioprinting cell-laden hydrogels made of type I collagen-based bioinks, but whether type I collagen compromises the bioinks’ printability or promotes osteocyte mineralization in 3D culture is unclear [16, 17].

Many studies have focused on osteoblast differentiation of mesenchymal stem cells, but mineralization of early osteocytes has not been well studied. Mineralization has major implications for bone quality as well [18]. The major obstacle is the lack of an ideal experimental model to investigate osteocyte mineralization. In this study, we presented a method to biofabricate an osteocyte-laden 3D hydrogel with bone matrix analogue-based bioinks. We demonstrated that the bioprinted 3D constructs have exceptional shape fidelity and biocompatibility. A dendritic osteocyte network and mineralized matrix deposition were observed after 28 d of mineralization induction. More importantly, fluorescence immunohistochemistry and RT-qPCR confirmed that type I collagen significantly enhanced the osteocyte maturation and mineralization under 3D culture conditions.

2. Methods

2.1. Bioink preparation and 3D bioprinting of cell-laden hydrogels

Gelatine methacrylamide (GelMA), hyaluronic acid methacrylate (HAMA) and phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) were purchased from StemEasy Biotechnology Company (Jiangsu, China). The type I collagen solution from rat tail was purchased from Sigma Chemical Co. (#C3867, St. Louis, MO, USA). Three different bioinks were used for bioprinting in this research. For a high degree of biomimicry of bone tissue in vivo, type I collagen was added to the bioinks at concentrations of 0% w v⁻¹, 0.06% w v⁻¹, and 0.18% w v⁻¹. The chemical composition and abbreviation of the bioinks are listed in table 1. First, sterile GelMA (5% w v⁻¹) and HAMA (1% w v⁻¹) were dissolved in phosphate-buffered saline (PBS), and then, LAP (0.5% w v⁻¹) was added as the radical photoinitiator. Next, the solution was stirred thoroughly at 37 °C overnight until it was completely dissolved and filtered through a filter (0.22 μm). Subsequently, type I collagen was added to the bioink and vigorously stirred in the dark at 37 °C for 3 h to obtain a homogenous pre-hydrogel bioink. The bioinks were neutralized with 1 M NaOH before the next steps were performed. The collagen-free bioink also was subjected to the same stirring and centrifugation steps as the collagen-containing bioink. Bubbles were removed by centrifugation (3000 rpm, 5 min). Before bioprinting, 50 μl of cell suspension with 1 × 10⁷ cells was added to 1 ml of prehydrogel solution and gently stirred avoiding light to achieve uniformity [19].

EnvisionTEC 3D-Bioplotter (EnvisionTEC, Germany) was used to print the 3D hydrogel constructs. Before printing, we designed a pyramid structure (1.2 cm×1.2 cm×1.2 cm) and a layer-by-layer lattice structure (10 mm×10 mm×2.4 mm, 8 layers). During printing, the fibre diameter was 300 μm, the printing speed was 15 mm s⁻¹, and the nozzle temperature was 20 °C. After the printing process, 3D scaffolds were UV crosslinked with a low light intensity (Io = 15 mW cm⁻², 20 cm far from the UV source) for 1 min and then incubated in mineralization-inducing media for 28 d.

The casted hydrogel was prepared by extruding 0.3 ml of pre-hydrogel into 24-well plates with a 1-ml syringe to form a hemispherical construct with a diameter of approximately 10 mm. After casting, hydrogels were crosslinked with UV at the same light intensity as 3D printing for 1 min and then incubated in mineralization-inducing media for 28 d.

| Table 1. Compositions of bioinks. |
| Sample | GelMa | HAMA | LAP | Collagen I |
| 3D-HaGel | 5 | 1 | 0.5 | – |
| 3D-HaGelColl-Low | 5 | 1 | 0.5 | 0.06 |
| 3D-HaGelColl-High | 5 | 1 | 0.5 | 0.18 |

2.2. Characterization of the bioinks and constructs

2.2.1. Scanning electron microscopy (SEM)

The surface structures of the 3D printed constructs were investigated by SEM. Briefly, the samples were fixed in 4% paraformaldehyde for 30 min at room temperature.
temperature and lyophilized. SEM (Hitachi SU8200, Japan) was used to observe the surface topography.

2.2.2. Rheology analysis
An Anton Paar MCR302 rheometer with a plate-plate geometry (diameter = 25 mm) was used to evaluate the rheological properties of bioinks. A shear rate ranging from 0.1 to 100 s⁻¹ was applied to assess the shear viscosity behaviour in the flow sweep assay at 25 °C. Temperatures from 20 °C to 45 °C were used to evaluate the viscosity in the temperature sweep assay.

2.2.3. Compression assay
An Instron 5697 universal material testing system (Instron, USA) was used to evaluate the mechanical properties of the 3D hydrogel constructs. All samples used in the compression assays were printed as a cuboid shape (5 mm × 5 mm × 5 mm), and the speed of the crosshead was set to 1 mm min⁻¹. Young’s moduli were defined as the slope of the initial linear part of the stress-strain curves. At least three constructs were tested in each group for statistical significance.

2.3. IDG-SW3 cell line culture and mineralization
Tissue culture media were purchased from HyClone Laboratories. Foetal bovine serum (FBS) was purchased from Gibco. Type I collagen was obtained from Becton Dickinson Lab. IFN-γ was purchased from Thermo Fisher (PMC4031). As described previously [7], the osteocyte cell line IDG-SW3 (kindly provided by Dr. Lynda Bonewald from the USA) was passaged at 33 °C in a 5% CO₂ atmosphere in permeable media (αMEM with 10% FBS, 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, and 50 U ml⁻¹ IFN-γ) on rat tail type I collagen-coated dishes. For induction of mineralization, the IDG-SW3 cells were cultured at 37 °C with 5% CO₂ in mineralization-inducing media (permeable media without IFN-γ, 50 µg ml⁻¹ ascorbic acid and 4 mM β-glycerophosphate) for 28 d.

2.4. Live/Dead assay
Live/Dead assays were carried out according to the manufacturer’s protocol. Briefly, the hydrogels were gently rinsed with PBS 5 times and then immersed in Live/Dead staining dye (Biovision, K501-100) at 37 °C for 20 min while avoiding light. Subsequently, the 3D constructs were rinsed in PBS 5 times. Laser scanning confocal microscopy (CLSM, Leica TCS SP8) with Image-Pro-Plus 6.0 was used to observe and count the number of dead cells (red fluorescence) and live cells (green fluorescence). The cell viability was calculated by dividing the number of total cells by live cells. The osteocyte density was calculated by dividing the hydrogel area by the number of live cells. Three independent fields per sample were used for quantification.

2.5. Inductive coupled plasma (ICP) emission spectrometer
The contents of calcified deposits in the 3D printed constructs and casted hydrogel were investigated using ICP emission spectroscopy. Briefly, 3D printed constructs and casted hydrogel were incubated in mineralization media for 28 d. The samples were fixed with 4% paraformaldehyde for 30 min at room temperature, lyophilized and weighed with a digital balance. Then, 0.2 g of lyophilized samples from each group were dissolved in 10 ml of concentrated nitric acid. Next, the contents of calcified elements were determined using ICP emission spectroscopy.

2.6. Fluorescence immunocytochemistry (ICC)
The samples were fixed in ice-cold 4% paraformaldehyde at 4 °C for 30 min and treated with 0.3% Triton for 20 min. Next, the samples were rinsed with PBS 3 times, blocked with 10% goat serum at room temperature. Nuclei were stained using 4% paraformaldehyde for 30 min at room temperature, lyophilized and weighed with a digital balance. Then, 8 μl of lyophilized samples from each group were dissolved in 10 ml of concentrated nitric acid. Next, the contents of calcified elements were determined using ICP emission spectroscopy.

2.7. Osteocyte morphology observation
For observation of the osteocyte morphology in the 3D hydrogel and in the 2D-single layer (which served as the control) after 28 d of culture, FITC-phalloidin (Sigma-Aldrich, P5282) and DAPI (Sigma-Aldrich, E11/Podoplanin CGACCAGTTTCTAACACCTGCCTTCT CTGTCCCAGCAACACTGAGTCCC
FGF23 GATCTCCACGGCAACATT TGCTTCTGCGACAAGTAG
SOST TCCTCCTGAGAACAACCA CTGTACTCGGACACATCTT
DMP1 TCATTCTCCTTGTGTTCCTT TCTTCTGATGACTCACTGTT
OPG GCCTCCTGCTAATTCAGAA GGTATAATCTTGGTAGGAACAG
Connexin 43 TACCACGCCACCACCGGCCCA GGCATTTTGGCTGTCGTCAGGGAA
RANKL CAGCATCGCTCTGTTCCTGTA CTGCGTTTTCATGGAGTCTCA
PHEX GAAAGGGGACCAACCGAGG AACTTAGGAGACCTTGACTCACT
E11/Podoplanin CGACCAGTTTCTAACACCTGCCTTCT CTGTCCCAGCAACACTGAGTCCC

| Gene     | Forward sequence(5′-3′) | Reverse sequence(3′-5′) |
|----------|-------------------------|-------------------------|
| PHEX     | GAAAGGGGACCAACCGGAGG    | AACTTAGGAGACCTGCAGTCAG |
| RANKL    | CAGCATCGCTCTGTTCCTGTA   | CTGGTCTTTCTATGGAGTCTCA |
| Connexin 43 | TACACCGCCACCCAGGCCCA   | GGCATTTTGGCTGCTGCTAGGAA |
| OPG      | GCCTCTGCTAAATTCAAGAA   | GGTTAATCTTGGTAGGAACAG  |
| DMP1     | CTACCTCTGCTGCTGTTCCTT  | TCTTCTGATGACTCTAATTCAT |
| SOST     | TTACCTGAGAACACAACCA    | CTGTACCTGCGAGCAACATCTT |
| FGF23    | GATCTCCAGGCGCAACATT    | TGCTTCTGCGAGCAACATCTT |
| E11/Podoplanin | CGACCAGTTTCTAACACCTGCCCTT | CTGTCCCGAGCAAACACTCAGTCCC |

Table 2. Primer’s sequences used for RT-qPCR.
in this research are listed in Table 1. All the primers used (Sigma-Aldrich) for 24 hr at room temperature, and 450 nm. was used to measure the absorbance of each well at 37°C for 24 hr. The plates were incubated at 37°C, and the cell viability of osteocytes in the 3D bioprinted hydrogel and casted hydrogel. The plates were placed in 12-well plates and immersed in 1 ml of complete medium containing 10% of the CCK-8 reagent according to the manufacturer’s instructions. Briefly, hydrogels were formed to evaluate cell viability using a CCK-8 assay. The CCK-8 (Bimake, Houston, USA) assay was performed as previously described.

2.8. Fluorescence imaging of Dmp1-GFP and Alizarin red staining
IDG-SW3 cells were isolated from 8Kb-Dmp1-GFP mice crossed with the immortomouse, and GFP was expressed under the control of the Dmp1 promoter. Herein, Dmp1-GFP fluorescence was evaluated at 7 d, 14 d, 21 d and 28 d after mineralization induction using CLSM at an excitation wavelength of 488 nm. For Alizarin red staining, 3D hydrogel constructs were fixed in 4% paraformaldehyde for 30 min at room temperature, stained in 1 µg ml⁻¹ Alizarin red (Sigma-Aldrich) for 24 hr at room temperature, and then rinsed with PBS 5 times.

2.9. RNA isolation and RT-qPCR
Total RNA was purified from the 3D hydrogels using the RNeasy Mini Kit (Qiagen, 74 104), and cDNA was synthesized with the GoScript™ Reverse Transcription Kit (Promega, A276A) according to the manufacturer’s instructions. Real-time qPCR was conducted with an ABI Prism 7500 system (Applied BioSystems, Foster City, CA, USA) using 2× SYBR Green qPCR Master Mix (Bimake, B21202). All the primers used in this research are listed in Table 2. The 2⁻ⁿ⁻ΔΔCt method was used for quantification of each target gene expression with normalization to β-actin [20].

2.10. Parathyroid hormone (PTH) treatment
At day 28, IDG-SW3 cells in mineralization-inducing media were treated with 50 nM PTH (bovine fragment 1–34, Sigma-Aldrich, P3671) or PBS for 24 hr. Then, total RNA was isolated, and RT-qPCR was performed as previously described.

2.11. Cell counting Kit-8 (CCK-8) assay
The CCK-8 (Bimake, Houston, USA) assay was performed to evaluate cell viability according to the manufacturer’s instructions. Briefly, hydrogels were placed in 12-well plates and immersed in 1 ml of complete medium containing 10% of the CCK-8 reagent to compare the cell viability of osteocytes in the 3D bioprinted hydrogel and casted hydrogel. The plates were incubated at 37 °C for 1 h. A microplate reader was used to measure the absorbance of each well at 450 nm.

2.12. Statistical analysis
Each group contained at least three independent samples, and statistical significance was determined from at least three independent experiments. All data in this study are presented as the mean ± standard deviation (SD). Student’s t-test or two-way analysis of variance (ANOVA) with Dunnett’s multiple comparison test in SPSS 13.0 software was used for analysis, and a P-value <0.05 was considered statistically significant.

3. Results

3.1. Characterization of the bioinks and constructs
As shown figure 1(A), the IDG-SW3 cell line with the Dmp1-GFP reporter gene was printed into 3D hydrogel constructs with various concentrations of type I collagen, and the cell viability and osteocytic functions were determined after mineralization induction. Printing parameters, e.g. the bioink composition, nozzle temperature, extrusion speed and UV crosslink time, were optimized to increase the formability and cell viability. As shown in figures 1(B) and (C), optical images of the hydrogel constructs were used to evaluate the printability. The shape fidelity of all three groups was high: the pyramid construct was 40 layers tall (layer thickness = 300 µm, total height = 1.2 cm), and the lattice construct was 8 layers tall (layer thickness = 300 µm, total height = 2.4 mm) with a distance between fibres of 800 µm. The microstructures of the 3D constructs were further observed using stereomicroscopy (figure 1(D)). We found that all scaffolds showed clear grid shapes, but the overall size decreased to approximately 98.75% of the designed size due to UV crosslinking.

Subsequently, SEM was conducted to observe the surface topographical features of the 3D constructs. As shown in figure 2(A), type I collagen was observed within the hydrogel with good dispersion. After 28 d of culture, the cells in the 3D-HaGel group showed poor spreading and spherical morphology, but the cells in the 3D-HaGelCol1-Low and 3D-HaGelCol1-High groups were well spread and distributed with many cell-cell contacts. These results suggest that type I collagen in the hydrogel might enhance osteocyte adhesion and cell-cell contact formation.

Next, the rheological behaviours of the three bioinks were studied. Temperature sweep analysis indicated that the three hydrogels were thermosensitive, and incorporation of type I collagen did not change this property, making it suitable for 3D bio printing at room temperature (figure 2(D)). In the range of shear rates we studied (0.01–10 s⁻¹), all three bioinks exhibited shear thinning behaviours, and the viscosity decreased with the increased shear rate (figure 2(E)). Figure 2(F) shows the plots of the elastic modulus (G’), viscous modulus (G’’), and shear viscosity in a log-log plot.
Figure 1. Schematic diagram of the 3D bioprinting process. (A) IDG-SW3 cells were blended with biomimetic bioinks and then loaded into the bioprinter. The bioprinting parameters were optimized based on structure stability. After printing, the samples were cultured in mineralization induction media for 28 d, and then, cell viability, mineralization conditions and osteocyte function were evaluated. Optical images (B), (C) and stereomicroscopy images (D) of the 3D bioprinting constructs of the 3D-HaGel, 3D-HaGelCol1-Low and 3D-HaGelCol1-High groups.

3.2. Cell viability in the 3D constructs during mineralization

To gain insight into hydrogel biocompatibility and whether the bioprinting process affected cell viability, we performed Live/Dead assays 7 d, 14 d and 28 d after mineralization induction. As shown in figure 3, cell viability only slightly decreased over time and was always higher than 80% for all the samples. Quantitatively speaking, the viability of the IDG-SW3 cells after 28 d of incubation was 83.869 ± 1.981%, 87.738 ± 3.918% and 87.734 ± 1.093% for the 3D-HaGel, 3D-HaGelCol1-Low and 3D-HaGelCol1-High groups, respectively, with no significant differences. Based on these data, all three hydrogels display high biocompatibility, indicating that the bioprinting process and UV crosslinking are compatible with the cells.

To quantitatively describe osteocyte density in 3D hydrogels, we calculated the cell density based on Live/Dead fluorescent images of different groups at day 7. As shown in supplementary figure 1 (available...
online at stacks.iop.org/BF/12/045013/mmedia), osteocyte density in 3D bioprinted hydrogels ranged from $135.5 \pm 9.7 \text{ mm}^{-2}$ to $143.0 \pm 23.69 \text{ mm}^{-2}$ but the difference among these three groups was not statistically significant.

### 3.3. Biomimetic mineralization in the 3D constructs

Dentin matrix protein 1 (Dmp1), a mineralized extracellular matrix protein secreted by mature osteocytes, plays a central role in bone mineralization [22]. In mineralization media, IDG-SW3 cells expressed Dmp1 with a green fluorescent protein as a tag (Dmp1-GFP), which functions as a marker for osteocyte mineralization. To test whether type I collagen promoted osteocyte mineralization in the 3D hydrogel scaffolds, we monitored the fluorescence intensity of Dmp1-GFP under mineralization induction. As shown in figures 4(A) and (B), the IDG-SW3 cells in all constructs presented negative GFP expression 7 d after mineralization. From days 14 to 21, increased GFP-positive cells were observed in the 3D-HaGelCol1-Low and 3D-HaGelCol1-High groups. At day 28, the 3D-HaGelCol1-High group showed the highest relative GFP intensity, which was significantly higher than that of the 3D-HaGelCol1-Low ($P < 0.01$) and 3D-HaGel groups ($P < 0.01$). To further determine calcium deposition and mineralized nodule formation in the 3D hydrogel constructs, we performed Alizarin red S staining 28 d after mineralization induction. In figure 4(C), 3D reconstruction images demonstrated that more calcium deposition was observed in the collagen-containing hydrogels than in the hydrogels without collagen. Additionally, we found that most mineralized nodules were colocalized with GFP-positive cells. These results indicated that type I collagen enhances IDG-SW3 mineralization in the 3D hydrogel constructs.

### 3.4. Osteocyte morphology in the 3D constructs

Cx43 and E11/Podoplanin are essential osteocyte biomarkers. In figures 5(A) and (B), a panel of images obtained from ICC using anti-Cx43 and anti-E11 antibodies after mineralization culture for 28 d is shown. Strong Cx43 expression was observed in all samples, but the relative amount of Cx43 that colocalized with DAPI and Dmp1-GFP did not differ significantly among our three constructs (figure 5(C)). Surprisingly, E11 expression colocalized with DAPI and Dmp1-GFP, and exhibited an obvious dose-dependent increase compared to the hydrogels lacking type I collagen (figure 5(D)). Then, to gain insight into the morphological change in the cells within the printed hydrogels, we stained the cytoskeleton and nuclei of the IDG-SW3 cells in the 2D single layer and in the 3D hydrogel constructs 28 d after mineralization induction. As shown in figure 6(A), the IDG-SW3 cells in the
Figure 3. Cell survival rate in the 3D constructs during mineralization. (A) Live/Dead assays were carried out 7 d, 14 d and 28 d after mineralization induction. (B) The cell survival rate was quantified by image analysis of the scaffolds. The ‘ns’ indicates no statistical significance between two groups of data.

2D single layer had a flattened, irregular branched morphology with few elongated dendritic processes during culture. The cells in the 3D-HaGel hydrogel showed an irregular cobblestone-like shape, and the cells in 3D-HaGelCol1-Low and 3D-HaGelCol1-High groups presented a typical dendritic morphology with dense dendrites aligned tightly, forming a network within the hydrogels. Interestingly, the volume of the cell body, the number of osteocytic dendrites and the average dendrite length of cells in the hydrogels increased as the type I collagen concentration increased (figures 6(B)–(D)). Compared with cells in the 2D single layer, osteocytes in 3D-HaGelCol1-High showed a significantly larger cell volume, along with longer and denser dendrites. These data indicate that our formulated bioinks offer an ideal mechanical and chemical microenvironment for the mineralization of IDG-SW3 cells and that incorporation of type I collagen within bioinks enhances E11 expression and dendrite formation.

3.5. Gene expression in the 3D constructs during mineralization

As shown in figure 7(A), we measured the expression of osteocyte biomarkers, including Phex, Rankl, Opg, E11, Connexin43, Dmp1, Sost and Fgf23, using RT-qPCR with actin serving as an internal control to evaluate the differentiation of immature osteocytes into completely mineralized and mature osteocytes. The expression of the Phex, Opg, E11, Dmp1 and Fgf23 mRNAs was significantly increased in the 3D-HaGelCol1-High group, while the expression of the
Rankl and Sost mRNAs was decreased in the 3D-HaGelCol1-High group. This gene expression pattern highly mimicked the process of in vivo mineralization. Intriguingly, significant difference in the gene expression of the Connexin43 mRNA was not observed among the three groups, consistent with the ICC results shown in figure 5(C).

3.6. Responses to PTH treatment

Mature osteocytes were reported to respond to PTH stimulus by altering gene expression. We determined the fold changes in the expression levels of osteocyte-related biomarkers in response to PTH treatment to further confirm that the 3D bioprinting of osteocytes represents a valid model for biomimetic mineralization in vitro, as shown in figure 7(J). As expected, 50 nM PTH treatment at day 28 for 24 hr significantly downregulated the Dmp1, Sost and Opg mRNAs and slightly upregulated the expression of the E11 and Rankl mRNAs. The ratio of Rankl/Opg was significantly increased. In particular, the 3D-HaGelCol1-High group exhibited the most sensitive and dramatic response to PTH treatment among the three hydrogel groups, with the exception of the expression of the Sost mRNA.

To further evaluate the advantage of culturing osteocytes in the 3D network over the 2D single layer, we compared the fold change in the gene expression of IDG-SW3 cells after PTH treatment in the 3D hydrogel with that in the 2D single layer at day 28. As shown in figure 8(A), both the 3D cultured and 2D cultured osteocytes presented a reduction in Sost and Opg mRNA expression with an increase in Rankl and Fgf23 expression. In comparison, the changes in gene expression observed in the 3D cultured osteocytes were much stronger than those in the 2D single layers. Then, we evaluated the levels of the SOST protein, a major biomarker of completely mature osteocytes, using ICC in the 3D cultured and 2D cultured osteocytes (figure 8(B)). Sclerostin-positive cells were difficult to detect in the 2D cultured cells, while most 3D cultured osteocytes highly expressed sclerostin. These data demonstrated that the 3D osteocyte network outperforms the 2D culture in biomimetic mineralization in vitro.

3.7. The advantages of 3D bioprinted scaffolds over casted hydrogels during mineralization

Optical images of 3D bioprinted scaffolds and casted hydrogels are shown in Supplementary figure 2(A). We
first analysed osteocyte viability in 3D bioprinted and casted hydrogels 28 d after mineralization induction to determine the advantages of 3D bioprinting over casting in the biofabrication of osteocyte-laden constructs. In Supplementary figure 2(B), 3D reconstructions of images revealed that more dead cells (red) were observed in the casting group, particularly in the deep layer of the casted hydrogel (white arrowhead). The quantitative analysis also showed a significantly higher relative osteocyte viability in 3D bioprinted scaffolds (84.54 ± 0.65%) than in the casted hydrogel (75.93 ± 1.07%, P < 0.01) (Supplementary figure 2(D)). Additionally, this result was also confirmed by the CCK-8 assay, in which the relative cell viability in the bioprinting group was significantly higher than in the casting group beginning on day 14 after mineralization induction (P < 0.01) (Supplementary figure 2(C)).

Analyses of Dmp1-GFP expression, Alizarin red S staining and ICP emission spectroscopy were performed 28 d after mineralization induction to compare the biomimetic mineralization level in 3D bioprinted scaffolds with the casted hydrogels. As elucidated in Supplementary figure 3(A), the bioprinting group showed a significantly higher relative GFP intensity than casting group. In Supplementary figures 3(B) and (C), more calcified nodules and increased deposition of mineralized elements were observed in 3D bioprinted scaffolds.

Figure 5. Levels of the Connexin43 and E11/Podoplanin proteins in cells cultured in the 3D constructs. ICC staining of osteocyte-related markers after 28 d of culture in mineralization induction media. Connexin43 (A) and E11/Podoplanin (B) are shown in red, while Dmp1-GFP is shown in green, and nuclei are shown in blue. White arrowheads indicate cells positive for the osteocyte marker (red), DAPI (blue) and Dmp1-GFP (green) at the same time. The relative percentages of Connexin43 (C) and E11/Podoplanin (D) colocalization were quantified by conducting an image analysis. The asterisks (**) represent statistical significance at P < 0.01.
Figure 6. Osteocyte morphology in the 3D constructs. (A) Confocal microscopy images of printed osteocytes stained with F-actin (green) and a nuclear stain (blue) within the hydrogels on day 28. The relative volume of the cell body (B), number of dendrites per cell (C) and average dendrite length (D) were quantified in an image analysis using image-pro plus 6.0 software. The asterisks (* *) represent statistical significance at P < 0.01.

4. Discussion

Osteocytes, the major cell type of bone cells, function as multifunctional regulators controlling bone remodelling, phosphate homeostasis and mechanotransduction in the skeleton [23–25]. The recent surge of interest in osteocyte function has led to an increased demand for new research tools to replicate the native osteocyte network in vitro [7, 26]. This study presented a new 3D bioprinting biomimetic osteocyte network that was constructed to expand the toolkit for osteocyte biological research.

In the present research, we showed that UV exposure and mechanical shear stress did not obviously decrease osteocyte viability during bioprinting. Consistent with our experimental evidence, a recent paper reported that UV exposure as high as 43 mW cm\(^{-2}\) did not significantly reduce the cell viability of mesenchymal stem cells; this value was higher than the one we used in this work (15 mW cm\(^{-2}\)). In microextrusion printing, the printability is defined as the ease with which a hydrogel can be printed at a good resolution and its structure is maintained after printing [27]. The printability of the bioink is normally affected by its viscosity, yield stress, hydrogel strength, shape fidelity and ability to support living cells [27]. As shown in the rheological analysis, compared with the 3D-HaGel bioink, type I collagen-modified bioinks exhibited lower viscosity at the printing temperature and speed, forming soft cream types of gels with a low yield stress that enabled them to be reshaped and remolded during processing. After crosslinking, the mechanical strength of hydrogel constructs increased as the collagen concentration increased, which contributed to the high shape fidelity. This finding was also supported by the data shown in figure 1(D) in which the fibre diameter of 3D-HaGelCol1-High was thinner than the other constructs. Taking all these data into account, 3D-HaGelCol1-High presented the optimal bioink formulation with good gelling and high biocompatibility.

Osteocyte density is an important indicator of bone health and the metabolic rate of bone remodelling. According to the study by Mullender MG et al, osteocyte density varies in a specific range for each species and is inversely correlated with the age of the animal [28]. The osteocyte density ranges from approximately 942 mm\(^{-2}\) in young rats to approximately 150 mm\(^{-2}\) in elderly humans [29, 30]. In the present study, the data revealed a similar osteocyte density in the 3D bioprinted constructs to cortical bone from young human (about 130 to 200 mm\(^{-2}\)) [29]. In the future, if we attempt to develop biomimetic mineralization aged human or other animals, we could adjust the osteocyte density in hydrogels to the corresponding native level.

During osteocyte mineralization, several osteocyte biomarkers (Phex, Rankl, Opg, E11, Connexin43, Dmp1, Sost and Fgf23) are expressed sequentially [31]. As markers of early osteocytes, E11/Podoplanin positively controls the formation of osteocytic processes, maintaining osteocyte shape, and Connexin43 is a gap junction protein that transfers signals among osteocytes in response to
Figure 7. Gene expression and PTH responses of osteocytes embedded in 3D constructs during mineralization. (A) Biomarkers, including Phex, Rankl, Opg, E11, Connexin43, Dmp1, Sost and Fgf23, were chosen to evaluate osteocyte differentiation from the immature state to the completely mineralized state. (B)–(I) Gene expression levels relative to β-actin were determined by RT-qPCR and compared among the three groups. The data were plotted as a function of days after mineralization induction. (J) The fold change in the mRNA expression of osteocyte-related genes (Dmp1, E11, Sost, Rankl and Opg) after osteocytes cultured in different 3D hydrogels were treated with 50 nM PTH for 24 hr on day 28 are shown. The asterisks (*) and ** represent statistical significance at P < 0.05 and P < 0.01, respectively.

In the present study, the addition of type I collagen significantly enhanced E11 expression at both the mRNA and protein levels. This finding was also supported by the cell morphology analysis (figure 6(A)), which showed that osteocytes embedded in 3D-HaGelCol1-High displayed the densest and longest dendritic processes of the cells in all groups. However, the expression of the Connexin43 mRNA was similar for all constructs, indicating that type I collagen might not be involved in the functions of gap junctions between osteocytes. In addition, the progressively mineralized 3D osteocyte network exhibited increased levels bone remodelling markers (Rankl and Opg) and mineralization markers (Dmp1 and Phex), followed by a late osteocyte marker, Fgf23. Similar to previous studies,
this gene expression pattern indicated that the 3D-bioprinted osteocyte network can progress gradually throughout the final stages of osteocyte mineralization [31].

Sost is another important biomarker that is commonly expressed in mineralized mature osteocytes. In this study, osteocytes embedded in the 3D-HaGelCol1-High hydrogel showed expressed Sost at much higher levels than cells in the 2D single layer, suggesting differences in the gene expression profiles of osteocytes cultured in the 3D gel and the traditional 2D environment. This result was also confirmed by previous studies using MLO-A5, primary human osteoblastic cells and OmGFP66 [26, 34, 35]. Surprisingly, osteocytes with inhibited proliferation were found in the 3D construct. The proliferation of osteocytes in 2D culture conditions leads to distorted cell aggregates, but natural in vivo osteocytes are quiescent cells aligned in the network [36], so the gel entrapment culture may be superior to the monolayer culture in studying osteocyte function.

Multiple studies have discovered that responsiveness to PTH stimulation is an essential characteristic of functional osteocytes. As shown in the study by Prideaux M et al., the addition of PTH induced osteocytes to revert from a highly mature phenotype with obviously decreased Sost expression through calcium signalling [37]. Here, in addition to the similar gene expression patterns of the 3D bioprinted osteocyte network constructs to native bone, the 3D bioprinted osteocyte network was also responsive to PTH, showing upregulation of osteoblastic marker genes and downregulation of Sost and Dmp1 expression. Compared with the 2D cultured osteocytes, the 3D bioprinted osteocytes presented a better response to PTH stimulation. Furthermore, this 3D bioprinting construct can offer a valuable tool for elucidating the PTH functions in osteocytes. According to Keller H et al., Sost downregulation might directly mediate the effects of PTH on bone [38].

Studies have indicated that the chief constituent of the human bone matrix is type I collagen, which maintains a stable microenvironment for mineralized osteocytes [39]. In this study, we blended type I collagen with photocurable polymers (GelMA and HAMA) to form extracellular matrix (ECM) analogue-based bioinks. Recently, numerous studies have reported that orthopaedic implants functionalized with a layer of covalently bound type I collagen strongly promoted bone regeneration and osteointegration [40–42]. Regarding biofabrication, blending agarose with type I collagen significantly increased bioink’s shear modulus, and the fully developed type I collagen network enhanced endothelial cell adhesion and angiogenesis [43]. However, whether type I collagen regulates osteocyte maturation and mineralization requires further study. Consistent with these previous studies, type I collagen significantly enhanced osteocyte mineralization by increasing the expression of mineralization-related genes (Dmp1) and promoting calcified matrix deposition. Then, osteocytes in the type I collagen hydrogels showed more dendritic connections in morphology and a better response to PTH treatment than the cells in the other groups. These functional improvements might be explained by a mechanism in which type I collagen not only provides more Arg-Gly-Asp (RGD) motifs for cell adhesion but also directly activates integrin receptors on the osteocyte membrane [44].

Currently, cell biology research and preclinical drug evaluations rely primarily on 2D culture models or costly animal models. Previous studies generated 3D bone tissue constructs mainly by assembling cells with BCP microbeads, seeding cells on polymer composites or embedding cells in casted gels [17, 45–48]. Notably, 3D bioprinting has more advantages in
terms of the large scale, complex structure, and high-throughput biofabrication than casting. Simple casting of a hydrogel is only a small-scale laboratory method used to produce solid constructs. In the present study, we successfully fabricated 3D bioprinted osteocyte-network scaffolds with higher accuracy, higher productivity and better reproducibility than traditional methods. Depending on the exceptional printability of the formulated bioinks, biomimetic osteocyte-laden hydrogel scaffolds with complex topological structures were reconstructed based on clinical finite element analysis data, which might be helpful to replicate the natural bone microenvironment and further translate bioprinting to the clinic.

In this study, increased osteocyte viability and better mineralization were observed in 3D bioprinted constructs compared to casted hydrogels (supplementary figures 2–3). After reviewing relevant papers, the nutrition exchange efficiency might help explain the advantage of bioprinting. Nutrition and gas exchange are performed via free diffusion through the hydrogel to embedded osteocytes. According to the diffusion law, diffusion efficiency is proportional to the diffusion surface area and the pressure gradient [49]. Regarding the 3D osteocyte cultures in vitro, 3D bioprinted lattice constructs provide a larger diffusion surface area than solid casted hydrogels to facilitate nutrient diffusion during the 28-day incubation. Therefore, increased osteocyte viability and mineralization were observed in the 3D bioprinted hydrogel compared with the solid casted hydrogel. A low diffusion efficiency is always the ‘bottleneck’ limiting the biofabrication of large-scale in vitro functional models, but our study elucidated the ability of 3D bioprinting to overcome this ‘bottleneck’ by increasing diffusion surface area [50].

This study also had several limitations. First, we used the IDG-SW3 cell line for printing instead of primary human osteocytes. To date, only 3 different methods for the isolation of primary osteocytes have been published, including sequential digestion, magnetic cell sorting and osteoblast induction [51–53]. However, none of these methods was able to obtain an adequate number of purified primary osteocytes for bioprinting, since primary osteocytes are typical quiescent cells that are unable to be expanded ex vivo. According to previous studies, the gene transcription response and cell signalling activity of the IDG-SW3 cell line are very similar to those of primary osteocytes, so it is appropriate to use the IDG-SW3 cell line for mineralization studies [54, 55]. In future studies, we plan to coculture other bone cells with osteocytes based on this hydrogel construct to study intercellular communication in the bone microenvironment. Second, we did not blend inorganic substances of bone matrix, such as hydroxyapatite, into our bioinks. In this study, we noted that blending bioinks with hydroxyapatite resulted in turbidity in the transparent hydrogels, and it was difficult to detect fluorescence signals from cells embedded in opaque hydrogels. Further innovative analytical methods need to be established to further characterize the osteocytes in ceramic particle-impregnated hydrogels.

5. Conclusions

In this study, 3D osteocyte-network constructs were bioprinted with ECM analogue-based bioinks containing type I collagen. The hydrogels presented suitable rheological features for 3D bioprinting with excellent biocompatibility after crosslinking. The addition of type I collagen not only improved the construct mechanical properties but also enhanced the gene expression of osteocyte-related biomarkers. Obvious osteocyte dendritic connections and calcified nodules were found in the 3D-HaGelCol1 hydrogel. Moreover, compared with the cells in the 2D single layer and the casted hydrogel, the 3D bioprinted constructs presented increased mineralization and osteocyte functions. This 3D bioprinted osteocyte network offers a feasible new approach to investigate biomimetic mineralization as well as to perform pharmaceutical screening in vitro.

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