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Human periodontal ligament stem cell seeding on calcium phosphate cement scaffold delivering metformin for bone tissue engineering

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ABSTRACT

Objectives: (1) develop a CPC-metformin scaffold with hPDLSC seeding for bone tissue engineering; and (2) investigate the effects of CPC-metformin scaffold on hPDLSC proliferation, osteogenic differentiation and bone matrix mineralization for the first time.

Methods: hPDLSCs were harvested from extracted teeth. CPC scaffolds (with or without metformin) were prepared. Three groups were tested: (1) control group (growth medium); (2) osteogenic group (osteogenic medium); (3) metformin + osteogenic group (CPC-metformin scaffold, cultured in osteogenic medium). hPDLSC viability, osteogenic differentiation and mineralization were measured. SEM was used to examine cell morphology.

Results: After culturing for 14 days, all three groups demonstrated excellent hPDLSC attachment and viability, as shown in live-dead staining, CCK-8 assay, and SEM examinations. The osteogenic group had 3–8 folds, 5 folds and 6 folds of increases in osteogenic gene expressions, ALP activity and mineral synthesis, compared to control group. Furthermore, the metformin + osteogenic group had 3-fold to 4-fold increases over those of the osteogenic group in osteogenic gene expressions, ALP activity and mineral synthesis.

Conclusions: hPDLSCs were demonstrated to be a potent cell source for bone engineering. The novel CPC-metformin-hPDLSC construct is highly promising to enhance bone repair and regeneration efficacy in dental, craniofacial and orthopedic applications.

1. Introduction

Bone defects often occur due to congenital defects, trauma, infection and other diseases that lead to the loss of bone tissues [1,2]. Due to an aging population, the need for bone repair and regeneration is rapidly growing. Although autografts are regarded as the gold-standard for repairing bone defects, their application is hindered by the invasive procedure and limited amount of bone harvest. Bone tissue engineering using stem cells and scaffolds is an exciting approach to meet this increasing need [3].

It is beneficial for the bone scaffold to mimic natural bone's extracellular matrix and be able to induce and promote bone regeneration. Calcium phosphate cement (CPC) has excellent biocompatibility, osteoconductivity, osteoinductivity and bioactivity [4–6]. CPC is a promising scaffold material for dental and craniofacial repairs [6]. The CPC powder contains one or more kinds of calcium phosphate powders such as tetracalcium phosphate (TTCP) and dicalcium phosphate-anhydrous (DCPA). The CPC paste is formed by mixing the CPC powder with a liquid. The paste can be molded to the desired shape and then self-set and harden *in situ* to become a nanostructured and bone mineral-mimicking apatite scaffold in the bone defect [7].

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Combining the scaffold with seed cells is promising to greatly enhance the tissue regeneration efficacy [8–10]. Three-dimensional scaffolds and cells can work synergistically to promote the tissue repair process [8,9,11,12]. The seed cells can enhance the bone tissue regeneration by differentiating into the osteogenic lineage and/or releasing molecules that increase the osteogenesis efficacy [7]. The most-frequently studied seed cells are human bone marrow mesenchymal stem cells (hBMSCs), which are regarded as the gold-standard cell source for bone tissue engineering research. However, hBMSCs require an invasive procedure to be harvested. Therefore, other stem cell sources are needed.

Human periodontal ligament stem cells (hPDLSCs) are mesenchymal stem cells (MSCs) isolated from the periodontal ligaments of extracted human teeth. Several studies revealed that hPDLSCs can be induced into the osteoblastic lineage, presenting osteogenic gene expressions and mineral synthesis [13–15]. hPDLSCs have several advantages, including the ease of harvesting from teeth that are extracted for reasons such as orthodontic treatments and removal of wisdom teeth, a strong self-renewal ability, and multipotency characteristics [16]. hPDLSCs can differentiate into bone, muscle, nervous tissues, periodontium-like connective tissues and cementoid tissues [16,17]. Therefore, hPDLSCs are highly promising to serve as seed cells for periodontal tissue regeneration, as well as craniofacial and orthopedic tissue engineering applications. However, to date, there has been no report on the use of hPDLSCs with CPC scaffold for bone regeneration.

Metformin, an antidiabetic biguanide drug, was approved by the United States Food and Drug Administration (FDA) in 1995 for treating type 2 diabetes, and is currently widely used by diabetic patients worldwide [18]. Metformin is a non-toxic and well-tolerated drug [19]. Intriguingly, recent studies indicated that metformin can also induce osteogenesis by promoting the differentiation of stem cells and pre-osteoblasts [20]. It was demonstrated that metformin enhanced the osteodifferentiation *via* the activation of the AMP-activated kinase (AMPK) signaling pathway [21]. Metformin showed the capability to increase the osteodifferentiation of several types of stem cells, including hBMSCs [22], human umbilical cord mesenchymal stem cells (hUMSCs) [23], human induced pluripotent stem cell-derived mesenchymal stem cells (iPSC-MSCs) [19], human dental pulp stem cells (hDPSCs) [24,25] and PDLSCs [26,27]. However, previous studies on the effects of metformin on hPDLSCs all directly added the metformin into the culture medium [26,27]. To facilitate potential clinical applications, it would be better to use a scaffold to deliver metformin which can be placed into a bone defect for local release. Our previous studies have shown that CPC scaffold containing chitosan is an effective delivery vehicle for metformin; however, hPDLSCs have not been seeded on CPC scaffold in previous studies [24,28]. To date, a literature search revealed no report on the combination of hPDLSCs with CPC-metformin scaffold for bone regeneration.

Therefore, the objectives of this study were to: (1) develop a CPC-metformin scaffold with hPDLSC seeding for bone tissue engineering; and (2) investigate the effects of CPC-metformin on hPDLSC proliferation, osteogenic differentiation and bone mineral synthesis for the first time. The following hypotheses were tested: (1) hPDLSCs seeded on CPC scaffold would be able to proliferate and successfully differentiate into the osteogenic lineage; (2) Compared to CPC scaffold without containing metformin, hPDLSCs seeded on CPC-metformin scaffold would have much greater osteogenic gene expressions, alkaline phosphatase activity and bone matrix mineral synthesis.

2. Materials and methods

2.1. Harvesting hPDLSCs from extracted teeth

Periodontal ligament (PDL) tissues were obtained from human adult premolars that were removed from individuals who had their teeth extracted due to orthodontic treatment (aged 12–26 years). The procedures were approved by the Institutional Review Board of the University of Maryland Baltimore. All patients or their guardians was informed with written consent. hPDLSCs were isolated and characterized following the methods in the previous studies with minor modifications [29]. The PDL tissues were isolated from the middle third of the root surface, cut into fragments under sterile conditions, then digested in 3 mg/mL collagenase I (Worthington Biochem, Freehold, NJ, USA) and 4 mg/mL dispase (Roche, Mannheim, Germany) for 1 h at 37 °C in a humidified atmosphere with 5% carbon dioxide (CO_2). PDL samples were placed into culture dishes (Costar, Cambridge, MA, USA) with growth medium which consisted of dulbecco's modified Eagle's medium (DMEM, GIBCO BRL, Grand Island, NY, USA) supplemented with 20% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin (P.S, GIBCO BRL), and incubated at 37 °C with 5% CO_2. Single cells were observed 3 days later, and cell colonies were formed at 7 days. The individual cell colonies were digested to a single cell suspension using filter paper (Whatman, TISCH Scientific, North Bend, Ohio, USA) with 0.25% Trypsin-EDTA (GIBCO BRL). The cells were moved into 24 well plates (Costar, Cambridge, MA, USA) and culture dishes. The medium was changed every three days and the cells were passaged when they reach 70% to 80% confluency. Cells between passage 3–5 were used for the present study.

2.2. Identification of primary hPDLSCs by immunostaining and flow cytometry analysis

Immunofluorescence (STRO-1 and CD34) staining was used to identify the cells. hPDLSCs (passage 2) cultured for 7 days were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 for 5 min, and blocked with 0.1% bovine serum albumin for 30 min. Subsequently, the cells were incubated with the primary mouse monoclonal antibody anti-human STRO-1 or CD34 (1:100, Invitrogen) overnight at 4 °C, followed by the secondary antibody mouse anti-mouse Alexa Fluor 488 (green fluorescence) (Invitrogen). The cell nuclei were stained with DAPI (1 μg/mL, Sigma, St. Louis, MO, USA) for 10 min. The samples were then examined under epifluorescence microscopy (Eclipse TE2000-S, Nikon, Melville, NY, USA).

The expression of surface antigen profiles of hPDLSCs were characterized *via* flow cytometry. The hPDLSCs (passage 2) were harvested by 0.25% trypsin-EDTA and washed with cold phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA). Cell number at 1×10^5 cells/tube were incubated with the conjugated antibody against STRO-1-PE (Santa Cruz Biotech, Dallas, TX, USA), CD34-FITC (BD Biosciences, San Jose, CA, USA), PE-conjugated Mouse IgG control antibody (BD Biosciences), FITC-conjugated Mouse IgG control antibody (BD Biosciences) for 20 min on ice in the dark. The cell suspensions were washed twice and resuspended with cold PBS containing 1% BSA, and analyzed using a flow cytometry cell sorting Vantage cell sorter (BD Biosciences). Isotype control antibodies were used as negative controls. Data was analyzed with FACS software (FlowJo LLC, Ashland, OR, USA).

2.3. Fabrication of CPC scaffolds and seeding with hPDLSCs

TTCP was synthesized *via* a solid reaction of DCPA and calcium carbonate, which were mixed and heated at 1500 °C for 6 h in a furnace (Model 51333, Lindberg, Watertown, WI, USA). The reactant was quenched to room temperature then ground in a ball mill (Retsch PM4, Brinkman, NY, USA) and sieved to obtain TTCP particles with a median particle size of 5 μm. DCPA powder was ground for 24 h in the ball mill in 95% ethanol and sieved to obtain a median particle size of approximately 1 μm. TTCP and DCPA were mixed in a blender at a molar ratio of 1:3 to form the CPC powder [30,31]. A chitosan solution was used as the CPC liquid, at a chitosan/(chitosan + water) mass fraction of 15% [8,24,32]. To incorporate metformin into CPC, 50 μg of metformin were dissolved in the chitosan solution for each specimen. In our

previous study, the CPC specimen containing 50 μg of metformin continued to release metformin at a sustained rate for three weeks, and successfully enhanced the odontogenic differentiation and mineralization of hDPSCs [24]. The CPC powder and liquid were mixed at a mass ratio of 3:1 [8,24], and the paste was placed into a mold with a diameter of 10 mm and a thickness of 1 mm. After incubating in a humidor at 37 °C for 24 h, the CPC specimen was removed from the mold. The CPC disks were sterilized in an ethylene oxide sterilizer (Andersen, Haw River, NC, USA) for 24 h and then degassed for 7 days prior to cell seeding. Three groups were tested:

1) Control group (CPC + 15% chitosan liquid, cultured in growth medium);
2) Osteogenic group (CPC + 15% chitosan liquid, cultured in osteogenic medium);
3) Metformin + osteogenic group (CPC + 15% chitosan liquid + 50 μg metformin in each CPC specimen, cultured in osteogenic medium).

A density of 5×10^4 hPDLSCs was seeded into each well containing a CPC scaffold disk with 0.5 mL of medium in a 24-well plate. The osteogenic medium consisted of the DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 100 nM dexamethasone, 10 mM β-glycerophosphate, 0.05 mM ascorbic acid, and 10 nM 1α,25-dihydroxyvitamin (Millipore) [8].

2.4. Live/dead staining and CCK-8 cell proliferation

After culturing for 1, 7 or 14 days, a live/dead viability kit (Molecular Probes, Eugene, OR, USA) was used to evaluate cell attachment and viability on CPC. CPC disks were washed with PBS and incubated with 4 mM ethidium homodimer-1 (EthD-1) and 2 mM calcein-AM in PBS for 20 min. Disks were viewed *via* an epifluorescence microscopy. Three randomly chosen fields of view were captured for each specimen. Five specimens (n = 5) yielded 15 images for each time point for each group. The live (green) and dead (red) cells were counted. The live cell density (D) and the percentage of live cells (P) were calculated. D = number of live cells in the image/the image area [30]. P = number of live cells/(number of live cells + number of dead cells) [30].

In addition, a cell counting kit (CCK-8, Dojindo, Tokyo, Japan) was used to evaluate cell proliferation at 1, 4, 7, 10 and 14 days. Three replicates in each group were used for this assay (n = 3). After culturing in 300 μL of growth medium or osteogenic medium with 10% CCK-8 for 2 h, the cell proliferative rate was determined *via* measuring the absorbance at an optical density of 450 nm using a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA).

2.5. Scanning electron microscopy

CPC disks with hPDLSCs cultured for 14 days were examined using scanning electron microscopy (SEM, Quanta 200, FEI, Hillsboro, OR, USA). The constructs were fixed with 1% glutaraldehyde (Millipore) in PBS, dehydrated with a graded series of ethanol (30%–100%), and rinsed with hexamethyldisilazane (Millipore). The surfaces of specimens were sputter-coated with platinum and examined in SEM.

2.6. Quantitative real time-polymerase chain reaction (qRT-PCR)

At 1, 7, and 14 days, qRT-PCR (7900 H T, Applied Biosystems, Foster City, CA, USA) was used to measure the osteogenic gene expressions of hPDLSCs on CPC. The total cellular RNA of the cells was extracted using TRIzol reagent (Invitrogen, Grand Island, NY, USA) and reverse-transcribed into cDNA using a High-capacity cDNA Reverse Transcription kit (Applied Biosystems) in a thermal cycler (GenAmp PCR 2720, Applied Biosystems). RT2 SYBR® Green qPCR Mastermix (Qiagen, Germantown, MD, USA) was used to quantify the transcript levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), alkaline phosphatase (ALP), runt-related transcription factor-2 (Runx2), osteocalcin (OCN) and osterix (OSX). The human specific primers were synthesized commercially (Millipore Sigma), and the sequences of the primers are listed in Table 1. Relative expressions were calculated using the $2-\Delta\Delta Ct$ method and normalized by the Ct value of the housekeeping gene GAPDH. The Ct value of control group for 1 day served as the calibrator with a value of 1 [30].

Table 1
List of primer sequences used in qPCR.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
ALP	TCAGAAGCTAACACCAACG	TTGTACGTCTTGGAGAGGGC
Runx2	TCTGGCCTTCCACTCTCAGT	GACTGGCGGGGTGTAAGTAA
OCN	GCAAAGGTGCAGCCTTTGTG	GGCTCCCAGCCATTGATACAG
OSX	TCTCCATCTGCCTGACTCCT	AGCGTATGGCTTCTTTGTGC
GAPDH	TCAACGACCCCTTCATTGAC	ATGCAGGGATGATGTTCTGG

2.7. ALP activity assay

At 1, 7 and 14 days, cells were lysed in 0.2% Triton X-100 (Millipore Sigma) solution. The ALP activity of the cell lysate was measured by using an Alkaline Phosphatase Assay kit (QuantiChrom, BioAssay Systems, Hayward, CA, USA) with p-Nitrophenylphosphate (pNPP) as a substrate. The ALP activity was determined by measuring the absorbance at an optical density of 405 nm using a microplate reader (SpectraMax M5). The protein of cell lysate was quantified using Protein Assay Kit (Pierce BCA, Thermo Scientific, Rockford, IL, USA) following the manufacturer's protocol. The ALP activity was normalized to the protein amount and reported as mU/mg protein.

2.8. Alizarin red staining (ARS) and cell-synthesized bone matrix mineral assay

Alizarin Red S (ARS, Millipore) staining was used to detect mineral synthesis by hPDLSCs. After 1, 7 and 14 days, hPDLSCs on CPC were fixed with 4% paraformaldehyde for 30 min and stained with ARS. After staining for 30 min, the ARS solution was removed, the disks were rinsed with PBS to remove any loose ARS, and the disks were imaged. Six disks were tested for each group at each time period for mineral synthesis (n = 6). For quantification, the stained CPC disks were destained in 10% cetylpyridinium chloride (Millipore) for 1 h and the concentration was measured at optical density of 652 nm using a microplate reader (SpectraMax M5). Blank CPC disks with the same compositions, but without the cells, were measured at the same time. Blank disks were cultured in growth medium or osteogenic medium and treated in the same manner (n = 6). The ARS concentration of the blank CPC disks was subtracted from that of the disks with cells [30,33]. The ARS concentration of the control group at 1 day served as the calibrator.

2.9. Statistical analyses

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS 19.0, Chicago, IL, USA). All data were expressed as the mean value ± standard deviation (SD). Statistical significance was analyzed by using the one-way or two-way analyses of variance (ANOVA), followed by post hoc LSD (least significant difference) tests. All statistical analysis was considered significant when $p < 0.05$.

3. Results

3.1. Identification of hPDLSCs

Fig. 1A shows an extracted tooth, and the PDL tissues were obtained

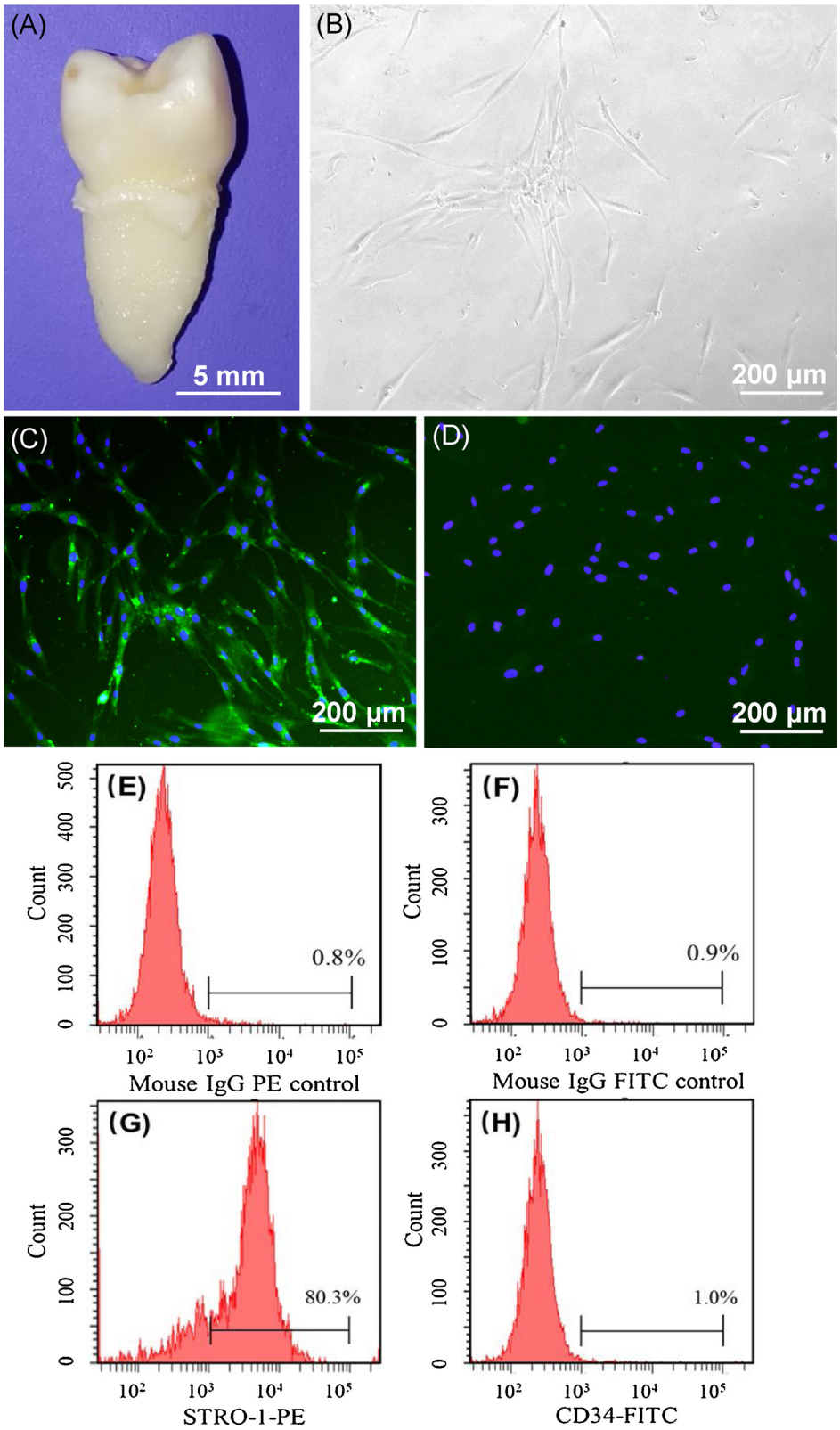

Fig. 1. Harvesting hPDLSCs from extracted teeth. (A) A extracted premolar with periodontal ligament. (B) A colony formed by prime hPDLSCs after culturing for 7 days. (C) After culturing for 7 days, hPDLSCs (second passage) were identified by immunostaining with STRO-1 antibody in green, and the nuclei were counterstained with DAPI in blue. (D) After culturing for 7 days, hPDLSCs (second passage) were immunostained with CD34 antibody, no green was shown, and the nuclei were counterstained with DAPI in blue. (E)-(H) show the immunophenotyping of human hPDLSCs by flow cytometry. (E)-(F) show the expressions of negative controls (IgG PE and IgG FITC). (G)-(H) present that the cells highly expressed MSC surface marker STRO-1 and were negative for hematopoietic and endothelial cell marker CD34. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

from the middle third of the root surface. Cell colonies formed at 7 days after culturing PDL fragments in the growth medium (Fig. 1B). The results of immunofluorescence staining demonstrated that the STRO-1 was positive (Fig. 1C), while the CD34 was negative (Fig. 1D). This indicated that these colony-forming cell populations possessed the markers of MSCs, and they were not contaminated by hematopoietic stem cells or endothelial cells. Fig. 1E-H show the results of flow cytometry. IgG PE (Fig. 1E) and IgG FITC (Fig. 1F) are negative controls. MSC surface marker STRO-1 (Fig. 1G) was expressed at 80.3%. On the other hand, the expression of hematopoietic and endothelial cell marker CD34 (Fig. 1H) was only 1.0%. In addition to those shown in Fig. 1, other surface markers were also identified by flow cytometry: 91.6% CD146-positive cells, 99.6% OCT4-positive cells, and only 5.9% Nanog-positive cells and 1.4% CD45-positive cells. These results

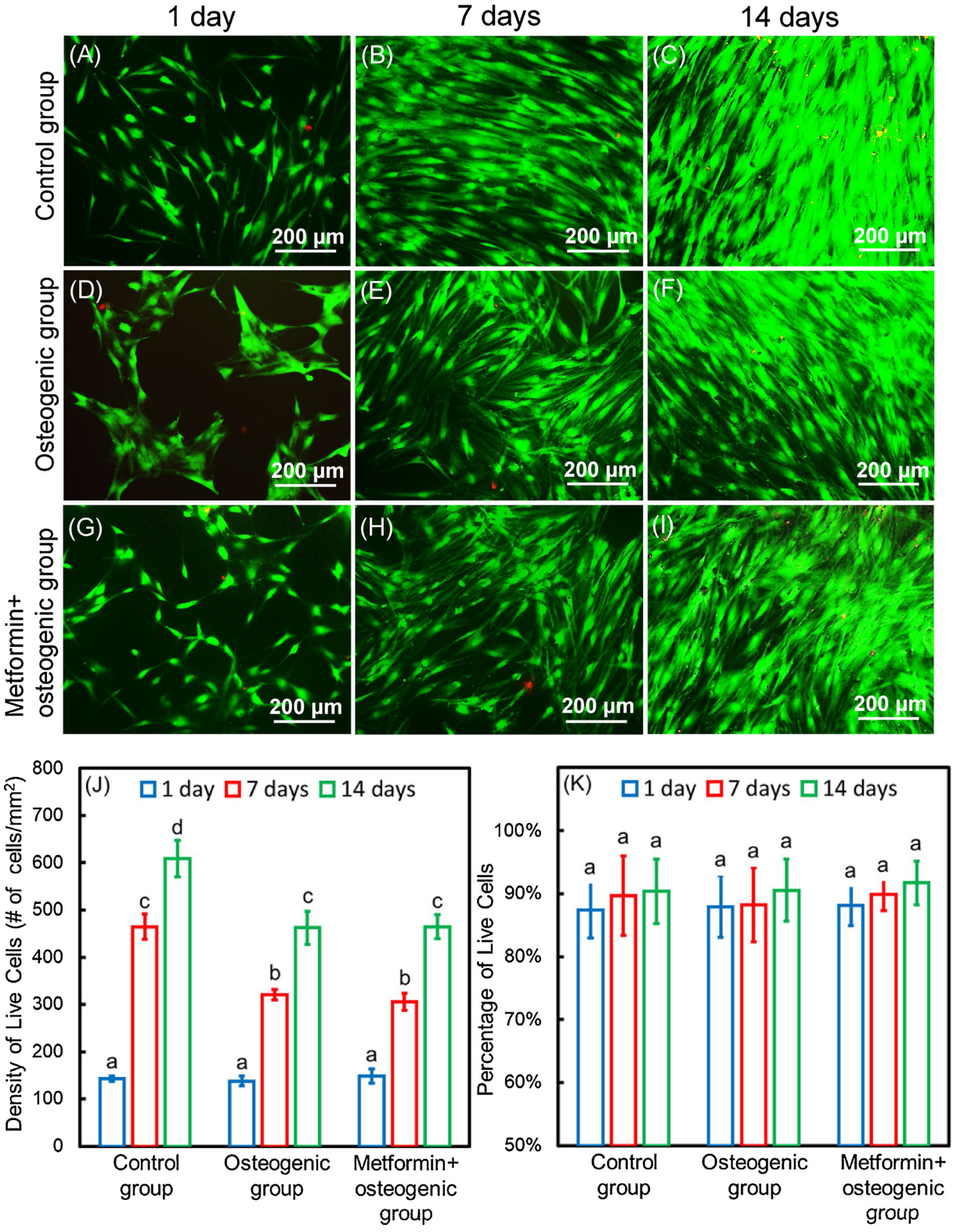

Fig. 2. (A)-(I) show the live/dead staining images of hPDLSCs on CPC scaffolds at 1 day, 7 days and 14 days (n = 5). Live cells were stained to green and dead cells were stained to red. (J) live cell density and (K) Percentages of live cells (n = 5). Values with dissimilar letters are significantly different from each other ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

showed that the typical MSC surface markers were highly expressed in these cells, and they were negative for hematopoietic and endothelial cell markers. These cells are referred to as hPDLSCs.

3.2. hPDLSC proliferation and cell viability

Fig. 2A-I show representative live/dead staining images at 1, 7 and 14 days. Live cells (stained green) appeared to have adhered to scaffolds with a good stretch and were numerous in all three groups. Dead

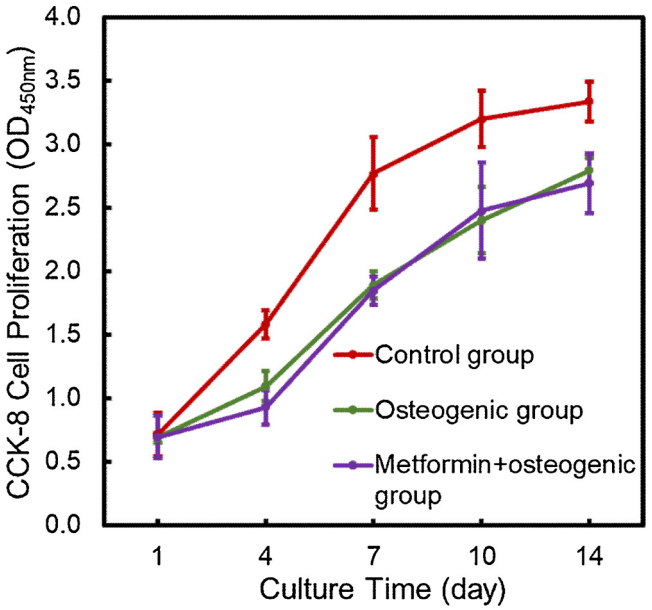

Fig. 3. CCK-8 was used to evaluate hPDLSC proliferation at 1, 4, 7, 10 and 14 days (n = 3).

cells (stained red) were relatively few. This indicates that the hPDLSCs exhibited good compatibility and viability on CPC scaffold.

Fig. 2J-K show live cell density and the percentage of live cells at 1, 7 and 14 days. In each plot, bars with dissimilar letters indicate values that are significantly different ($p < 0.05$). Live cell density (Fig. 2J) increased with time due to cell proliferation. For control group, the live cell density increased by about 5-fold from 1 to 14 days. For osteogenic group and metformin + osteogenic group, the live cell density increased by about 4-fold. Compared to control group, the live cell densities of osteogenic group and metformin + osteogenic group were slightly lower at 7 and 14 days. No significant difference was found between the live cell densities of osteogenic group and metformin + osteogenic group. Fig. 2K shows that the percentages of live cells on CPC in all three groups were around 90% and were not significantly different among three time points. These results show that the incorporation of metformin into CPC had no negative effect on cell viability as compared to that without metformin.

As shown in Fig. 3, the result of CCK-8 showed that the cell proliferation significantly increased from 1 day to 14 days. Control group showed slightly greater proliferation with a 5-fold increase from 1 to 14 days, while osteogenic group and metformin + osteogenic group exhibited a 4-fold increase from 1 to 14 days. No significant difference was found between the osteogenic group and the metformin + osteogenic group ($p < 0.05$).

3.3. hPDLSC morphology observed by SEM

Representative SEM images (Fig. 4) show the sample of metformin + osteogenic group at 14 days. Fig. 4B is a higher magnification image of the green dotted frame in Fig. 4A. The cells appeared to be well attached to the CPC scaffold and developed long cytoplasmic extensions (yellow arrows) adhering to the CPC-metformin scaffold, demonstrating that the CPC-metformin was biocompatible and supported hPDLSC attachment.

3.4. Osteogenic gene expression, ALP activity, and mineralization of hPDLSCs

Osteogenic gene expressions of hPDLSCs on CPC are plotted in Fig. 5A-D: (A) ALP, (B) Runx2, (C) OCN and (D) OSX. In each plot, bars

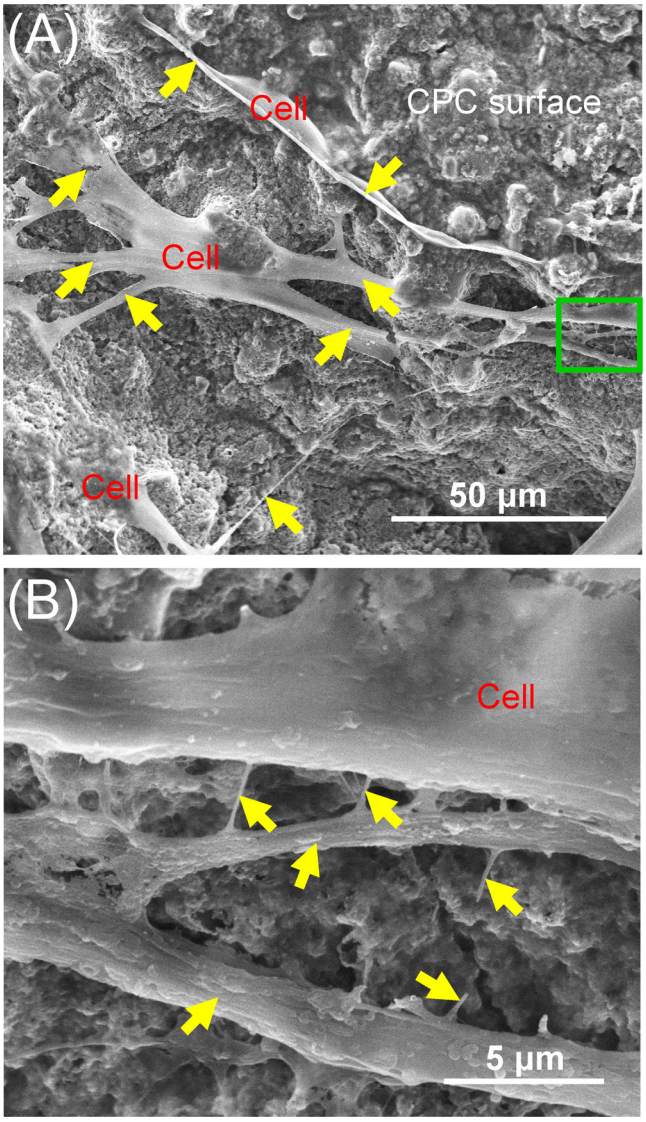

Fig. 4. Typical SEM images of CPC-metformin scaffolds cultured with hPDLSCs for 14 days (n = 5). The image in (B) is a higher magnification of the green dotted frame in (A). Yellow arrows indicate a healthy cell spreading morphology. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

with dissimilar letters indicate values that are significantly different ($p < 0.05$). For osteogenic group, all gene expressions were minimal at 1 day and increased with time, and all of them peaked at 14 days. The peak values of ALP, Runx2, OCN and OSX in the osteogenic group was 3 to 8 folds that of the control group. The gene expression levels of metformin + osteogenic group were significantly higher than those of osteogenic group at 7 days and 14 days. The peak values of metformin + osteogenic group were about 3 to 4 folds that of the osteogenic group. In contrast, the osteogenic gene expressions of the control group were much lower, and the up-regulation of osteogenic genes from 1 day to 14 days was minimal.

Fig. 6 shows the results of the ALP activity of hPDLSCs on CPC scaffolds, bars with dissimilar letters indicate values that are significantly different ($p < 0.05$). The ALP activity of the control group did not show much growth from 1 to 14 days. The ALP activity of the osteogenic group was increased by 5 folds as compared to the control group. The metformin + osteogenic group was the highest among all the tested groups. The metformin + osteogenic group had ALP activity that was 3-fold that of the osteogenic group at 14 days. The results show that

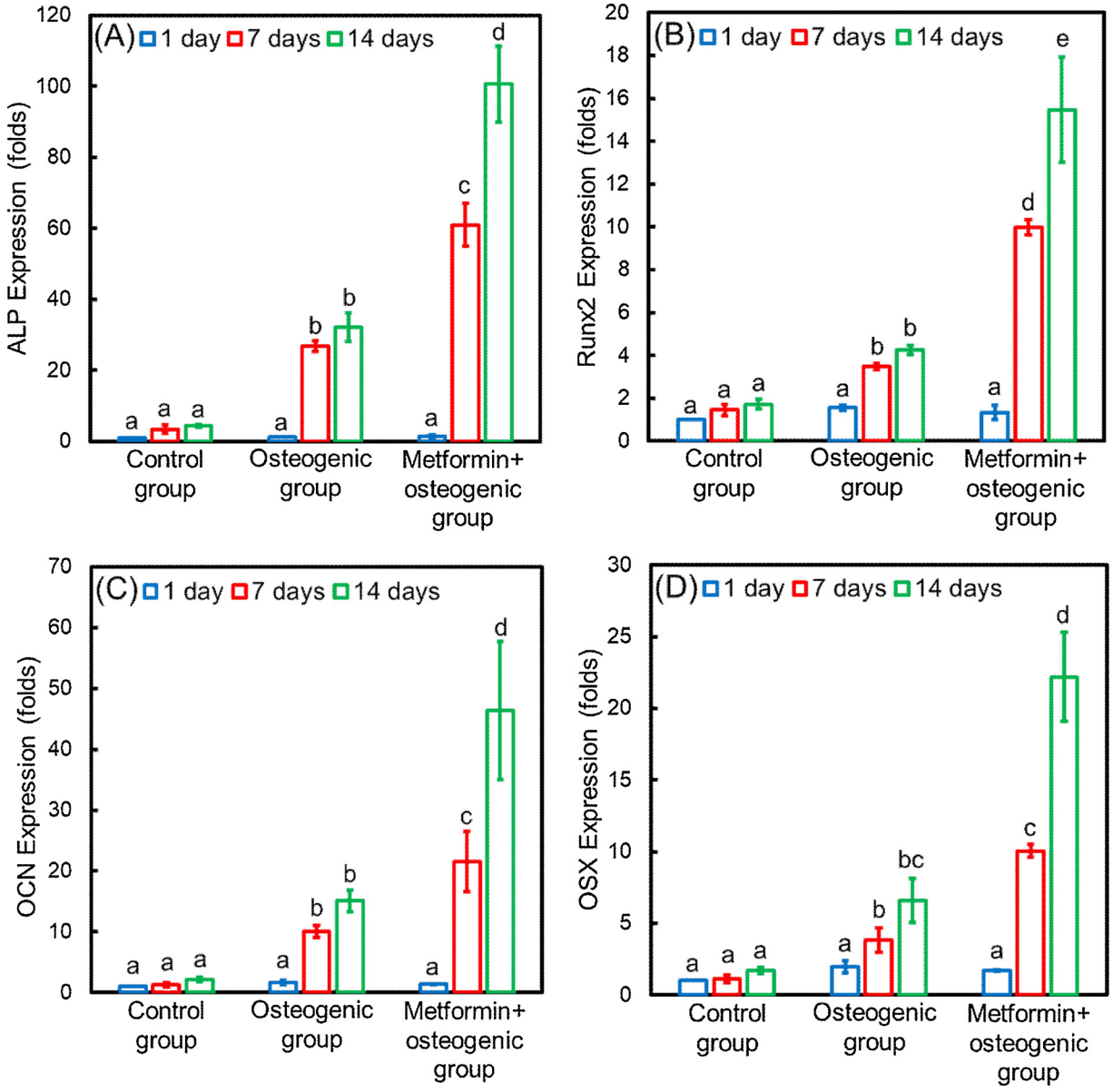

Fig. 5. qRT-PCR assay of osteogenic differentiation of hPDLSCs cultured for 1 day, 7 days and 14 days: (A) ALP, (B) Runx2, (C) OCN and (D) OSX gene expressions (n = 6). Values with dissimilar letters are significantly different from each other ($p < 0.05$).

metformin enhanced the ALP activity of hPDLSCs on CPC.

Representative ARS staining images of mineral synthesis by hPDLSCs on CPC are shown in Fig. 7A. The red staining of the synthesized bone mineral matrix became thicker and darker with the culture time increasing from 1 to 14 days. ARS staining was deeper and denser in osteogenic group than in control group while metformin + osteogenic group was darker than osteogenic group at 7 days and 14 days. Fig. 7B plots the quantitative analysis of bone matrix mineral synthesis by the hPDLSCs on CPC, bars with dissimilar letters indicate values that are significantly different ($p < 0.05$). hPDLSCs in the metformin + osteogenic group synthesized bone mineral that was 2-fold and 3-fold that of the osteogenic group, at 7 days and 14 days, respectively. Compared to osteogenic group and metformin + osteogenic group, the mineral synthesis of control group was minimal.

4. Discussion

The present study represents the first report on seeding hPDLSCs on CPC scaffold for bone tissue engineering, demonstrating that CPC supported hPDLSCs attachment and proliferation, and that CPC-metformin scaffold greatly enhanced osteogenic differentiation and bone mineral synthesis of hPDLSCs, compared to those without metformin. The hypotheses were proved that hPDLSCs seeded on CPC scaffold were able to proliferate and successfully differentiate into the osteogenic lineage, and that hPDLSCs on CPC-metformin scaffold achieved much greater osteogenic expressions, ALP activity and bone mineral synthesis than those without metformin. Therefore, the novel hPDLSC-CPC-metformin construct is promising to enhance bone repair and regeneration.

hPDLSCs have multiple advantages to serve as a promising cell source of bone tissue engineering. First, studies showed that hPDLSCs exhibited a high capacity to differentiate into the osteogenic lineage [14,15,34,35]. Second, besides bone tissue, hPDLSCs were also able to differentiate into cementum, periodontium-like connective tissues, and capillary-like structures [36–38]. Third, hPDLSCs were reported to have immunomodulatory properties, indicating that hPDLSCs had potential for achieving anti-inflammatory effects and immune-tolerance for cell transplantation [39]. Fourth, a previous study showed that hPDLSCs are more proliferative and clonogenic than hBMSCs [16]. Fifth, hPDLSCs

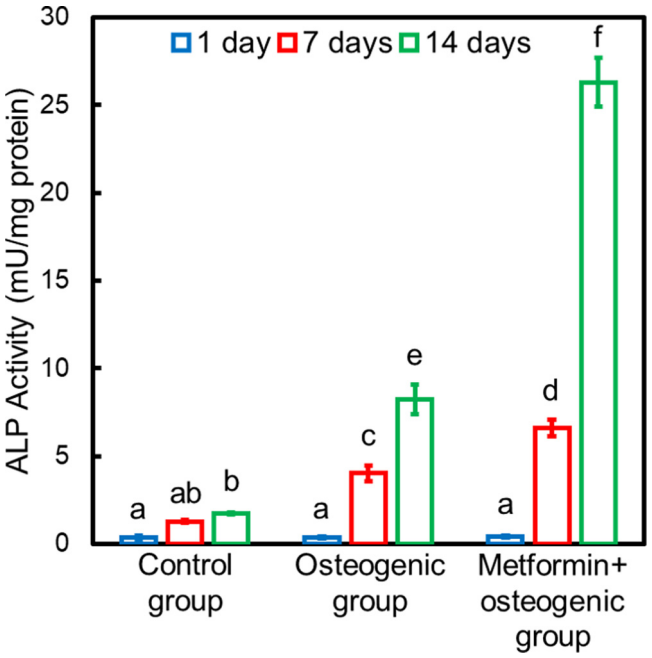

Fig. 6. An ALP activity assay was performed in hPDLSCs cultured for 1day, 7 days and 14 days (n = 3). Values with dissimilar letters are significantly different from each other ($p < 0.05$).

can be obtained from the extracted teeth due to the orthodontic extraction and the extraction of impacted tooth or supernumerary tooth, without an additional invasive procedure. This is advantageous compared to the bone marrow aspiration that would be required in obtaining hBMSCs. These advantages indicate that hPDLSCs are a promising cell source for periodontal regeneration, the reconstruction of alveolar bone defects, and other dental and orthopedic applications.

In previous studies, hPDLSCs were guided for osteodifferentiation on collagen scaffolds [40] and on pre-formed dicalcium/tricalcium phosphate ceramic scaffolds [41,42]. However, no publication on hPDLSC seeding on CPC was revealed in a literature search. Unlike collagen scaffolds which are not load-bearing, CPC has good mechanical strength and elastic modulus which match/exceed those of natural cancellous bone [43]. In the present study, chitosan was added into CPC, which could further increase the mechanical strength [28]. In our previous study, the flexural strength of CPC (using a liquid containing 15% chitosan) was 19.8 MPa [28]. This exceeded the strength of cancellous bone which had a tensile strength of 3.5 MPa [28], as well as and collagen scaffolds which had strength of only 0.02 MPa [44]. In addition, CPC can be readily used to deliver drug or molecules with biological activity, because these agents can be easily incorporated into the CPC paste or the CPC liquid [5], including metformin that was incorporated into CPC to investigate its effect on hPDLSCs for the first time.

Metformin, a first-line antidiabetic biguanide, is widely used by type 2 diabetic patients worldwide. As an insulin-sensitizing drug, it is considered safe and well-tolerated [19]. Metformin was first found to have an osteogenic action on osteoblasts in 2006 [20]. Previous studies suggested that metformin enhanced the osteodifferentiation and mineralization by activating the AMPK pathway, as well as inducing the expression of nitric oxide synthase (NOS) and bone morphogenetic protein-2 (BMP-2) [45]. In the present study, metformin was incorporated by adding it into the chitosan solution which would be suitable for dissolving metformin. The chitosan-metformin solution was then mixed with the CPC powder to form the CPC paste. Chitosan incorporation into CPC could increased the strength of CPC and also served as a delivery vehicle of drugs and bioactive agents [28]. Chitosan could be used to load and deliver various molecules, including low-molecular-weight drugs and macromolecules, and release pharmaceutically active agents in a controlled and sustained manner [46]. In our previous study [24], metformin was added into CPC containing 15% chitosan with 50 μg per specimen. The metformin release profile showed atypical initial burst release, reaching an accumulative release of about 505 ng/mL in the first 12 h. This was followed by a durable release which displayed a relatively sustained manner for 21 days, which would be suitable for stimulating cells to enhance the osteogenic differentiation [24]. The distribution of metformin into chitosan prolonged the release of metformin to meet the requirement of a drug carrier to sustain the release for a few weeks to stimulate osteodifferentiation, because significant ALP activity and bone mineral synthesis usually took 2–3 weeks to realize [24]. In a previous study, each CPC specimen contained 10 μg or 30 μg metformin; however, the 10 μg and 30 μg group had much less metformin release than the 50 μg group [24]. Therefore, in present study, 50 μg metformin was used in each CPC scaffold specimen, which greatly promoted the osteogenic differentiation and substantially increased the mineral synthesis of hPDLSCs for the first time.

Based on live/dead staining and SEM examinations, the hPDLSCs of all three groups in the present study had excellent viability and were well attached to the scaffolds. The hPDLSCs maintained a relatively high percentage of live cells in all groups. However, the hPDLSC live cell density and proliferation rate of the osteogenic group and the metformin + osteogenic group were slightly lower than the control group. The reason for this was likely the inverse relationship between cell proliferation and differentiation [47]. There appears to be a regulation of MSC differentiation which includes two stages [48]. In the first stage, MSCs generate precursor cells which have less self-renewal capability exhibiting a relatively slower proliferation rate [48]. Then, in the second stage, these precursor cells generate unipotent progenitor cells which eventually differentiate to the fully differentiated cells, such as osteoblasts [48]. When the precursors give rise to osteoblasts, the cell type-specific transcriptional programs are activated, while the cell cycle machinery is shut down [47]. Indeed, several essential proteins required in proliferation were found to be downregulated upon osteogenic differentiation, including the Wnt-responsive gene myc [49]. However, other studies showed that certain drugs could promote the cell differentiation without compromising the proliferation [50]. For example, von Knoch et al. [50] found that bisphosphonates enhanced both the proliferation and the osteoblastic differentiation of hBMSCs, and the mechanism was possibly through the stimulation of the basic fibroblast growth factor (b-FGF) and the upregulating of BMP-2. Both b-FGF and BMP-2 are potent growth stimulators involved in the recruitment, proliferation and differentiation of mesenchymal progenitor cells [50,51]. Therefore, the relationship between the cell differentiation and proliferation in osteogenesis is complicated, and requires further investigation. It should be noted that, in the present study, no significant difference was found between the hPDLSC viability and proliferation of the osteogenic group and the metformin + osteogenic group. This is consistent with previous studies on other types of cells [19,24]. For example, in a previous study, the iPSC-MSCs were cultured in osteogenic medium containing 10 μM metformin without using a scaffold, and no significant difference in cell viability was found between groups with or without metformin [19]. Several studies showed that metformin acted as a relatively weak mitogen to stimulate the proliferation, and this pro-proliferative effect of metformin was dose-dependent [20,26,27,52]. In these previous studies, metformin was directly added into the culture medium and kept at a relatively higher concentration. These previous studies did not use a CPC scaffold as a vehicle to deliver the metformin. In the present study, metformin was mixed into the CPC scaffold, which could be used to place into a bone defect to promote bone regeneration. In this case, the scaffold released metformin while being cultured with hPDLSCs in the medium, and with changing the medium frequently, the metformin concentration decreased with increasing culture time. That may be the reason that no significant pro-

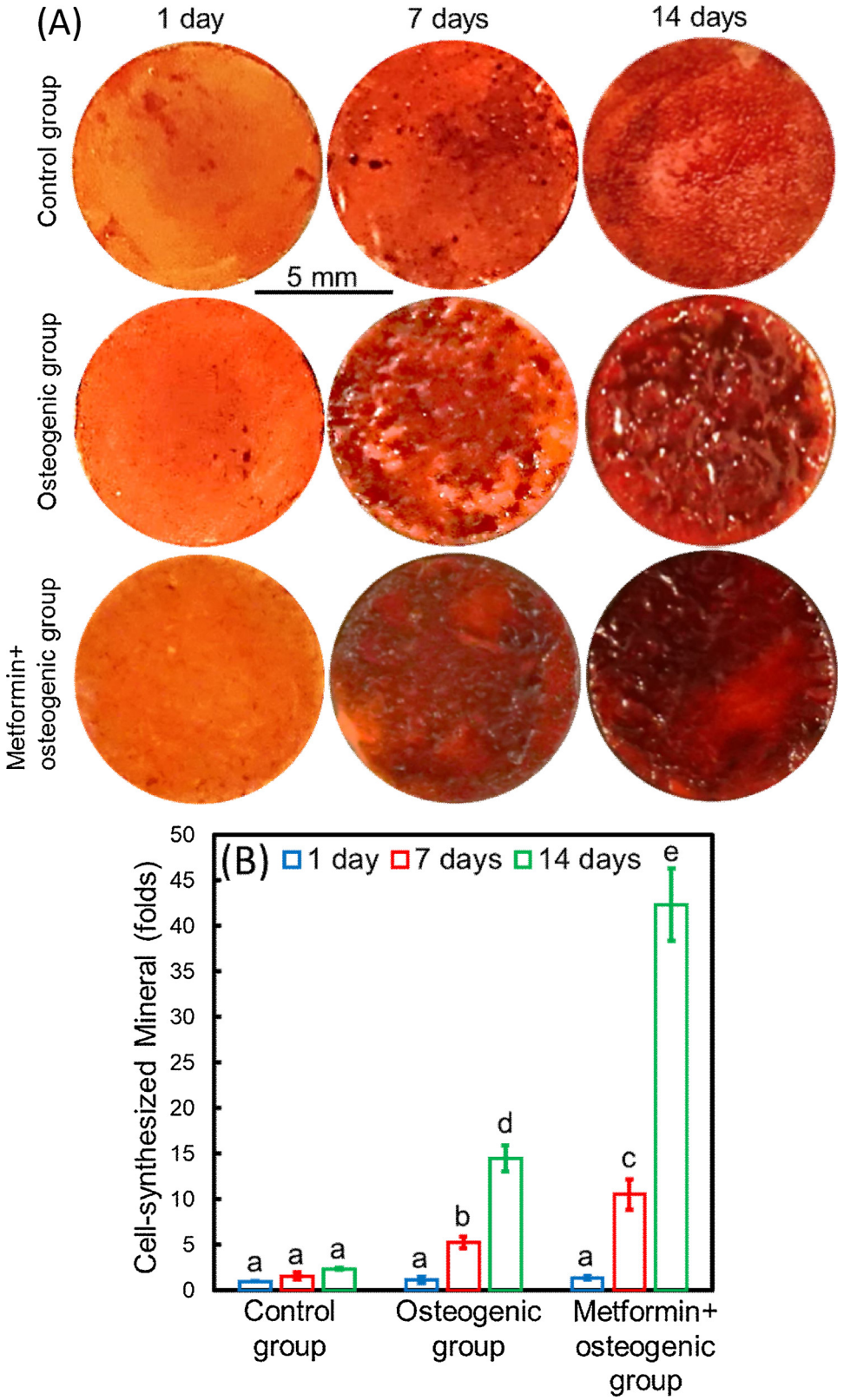

Fig. 7. (A) presents the ARS staining images of CPC scaffolds seeded with hPDLSCs at 1 day, 7 days and 14 days (n = 6). (B) shows the quantitative assay of mineral synthesis (n = 6). Values with dissimilar letters are significantly different from each other ($p < 0.05$).

proliferative effect was found for metformin in the present study. However, the metformin + osteogenic group still substantially enhanced the osteogenic differentiation of hPDLSCs, with similar cell proliferation when compared with the osteogenic group without metformin. Therefore, delivering metformin in CPC could substantially enhance the osteogenic differentiation, without compromising the cell proliferation. These results demonstrated that CPC was a suitable scaffold to deliver metformin and was a biocompatible vehicle to

support the delivery of hPDLSCs to greatly enhance the bone tissue engineering efficacy.

Several studies using BMSCs and pre-osteoblasts showed that metformin facilitated the osteogenic effects through the activation of the AMPK pathway, a conserved signaling cascade that plays the master sensor of cellular energetics [23]. AMPKα is a subunit of the AMPK complex, and AMPKα is highly expressed in bone tissues in primary osteoblasts and many osteo-lineage cells [53]. Thus, the activation of AMPKα by metformin affects bone formation, which causes a dose-dependent effect on the increases in osteogenic markers like ALP, Runx2, OCN and OSX, as well as increases in bone mineralization [19,23,53]. ALP is a key marker for osteogenesis, and its high level of expression is considered to be a prerequisite for bone matrix mineralization and the gradual maturing of bone [33]. Furthermore, Runx2, OCN and OSX also play essential roles in osteogenic differentiation [54,55]. In the present study on hPDLSCs, the ALP, Runx2, OCN and OSX of osteogenic group and metformin + osteogenic group were all highly up-regulated from 1 day to 14 days. This demonstrates that the osteogenic medium successfully induced the hPDLSCs to differentiate down the osteogenic lineage. In addition, compared to the osteogenic group, the osteogenic gene expression levels of the metformin + osteogenic group were further elevated due to the induction ability of metformin. Therefore, the excellent osteo-inductive effect of metformin on hPDLSCs was demonstrated.

Bone-related protein levels are closely related to the bone regeneration. A previous study showed that hPDLSCs cultured in medium supplemented with 50 μM metformin had significantly higher protein expression level of ALP and Runx2 [26]. In our previous study, Ping et al. [19] proved that metformin treatment (10 μM in the medium) significantly upregulated the Runx2 and OSX protein expression of iPSC-MSCs. Indeed, in the present study, the ALP protein synthesis by hPDLSCs (Fig. 6) were greatly increased *via* metformin delivery in CPC scaffold, compared to that without metformin. These results demonstrated the strong osteoindutive effect of metformin on the cells. Further study is needed to evaluate the protein synthesis of Runx2, OCN and OSX by hPDLSCs attached on CPC delivering metformin.

ALP activity is an important marker of osteogenic differentiation and plays a key role in cell mineralization [56,57]. The results of ALP activity assay indicated that, compared to the hPDLSCs incubated in the growth medium, the ALP activity of cells induced with osteogenic medium was markedly increased. Furthermore, the ALP activity was further enhanced with the simulation of metformin. Houshmand et al. [58] found that metformin did not increase the ALP activity of hDPSCs in a non-osteogenic medium, and it was thought that metformin did not initially stimulate the osteogenic differentiation, but rather, metformin increased the osteodifferentiation once it was initiated by the osteogenic medium. This is consistent with other previous studies showing the osteo-inductive effect of metformin on stem cells by culturing in an osteogenic medium [19,21,24,59]. Furthermore, in the present study, the ARS staining quantification proved that the hPDLSCs of the metformin + osteogenic group synthesized the most amount of bone minerals among the three groups. These results confirmed that the osteogenic medium successfully induced the osteodifferentiation and mineral synthesis of hPDLSCs seeded on CPC, and the metformin delivery in CPC further substantially enhanced the osteogenesis and mineralization of hPDLSCs.

Several *in vivo* studies showed that hPDLSCs were a highly potent population of progenitor cells. When used as seed cells, hPDLSCs demonstrated an excellent survival rate and differentiated into specialized lineages to form periodontal fiber ligament structures and mineralized tissues as well as vasculatures *in vivo* [16,60]. CPC has been tested in animal models in our previous studies, showing excellent biocompability, osteoconductivity, osteoinductivity and biodegradability [30,33,61]. After implanting CPC into bone defect for 12 weeks, new bone was observed around the peripheral sides of the CPC scaffold, as well as new bone inside the interior of the CPC scaffold, indicating the CPC was being resorbed and replaced by new bone [30,33,61]. In addition, normal bone features including osteoid with osteocytes, blood vessels, and numerous osteoblasts lining the new bone front, were established in the bone defects [30,33,61]. Furthermore, these previous studies showed that, compared to CPC scaffold control without cells, there was much more new bone formation in the groups with seed cells, including human embryonic stem cells, hiPSCs, hBMSCs and hUCMSCs [30,33,61]. Based on these previous studies, and based on the *in vitro* results in the present study showing for the first time that CPC-metformin substantially increased the hPDLSC differentiation, ALP activity and bone mineral synthesis, it is expected that hPDLSC seeding would produce much more new bone than that without hPDLSCs. However, further study is needed to investigate the effects of the novel hPDLSC-CPC-metformin construct on new bone formation and vascular generation in an animal model.

CPC has been shown to have the ability to biodegrade and be replaced by new bone *in vivo* [33,62,63]. The biodegradation of CPC takes place by solution-driven extracellular liquid dissolution and cell-mediated resorption processes [64]. A previous study showed that, at 3 months after operation, the CPC mass in the defects was partially replaced by new bone. At 6 months post-operation, most of the CPC mass had been replaced by new bone [62]. In our previous studies, macropores were introduced to CPC scaffolds to further increase the degradation [33,63]. A study showed that the remaining CPC material in the bone defect was approximately 6.9%–26.5% after 12 weeks [63]. Further study is needed to investigate how much the delivery of metformin and hPDLSC seeding would increase the CPC resorption rate *in vivo*, compared to that of CPC control without metformin and without hPDLSCs.

Therefore, the novel hPDLSC-CPC-metformin construct is promising for bone tissue engineering in dental, craniofacial and orthopedic applications. Further animal studies are needed to investigate the bone regeneration efficacy of the novel hPDLSC-CPC-metformin construct *in vivo*.

5. Conclusions

This study investigated a novel hPDLSC-CPC-metformin construct for bone tissue engineering, and showed that CPC scaffold supported hPDLSCs, and that metformin delivered by CPC substantially promoted the osteodifferentiation and mineral synthesis of hPDLSCs for the first time. hPDLSCs were harvested from extracted human teeth and showed excellent attachment and proliferation on CPC. hPDLSCs seeded on CPC were able to differentiate into the osteogenic lineage, and their osteogenesis was greatly enhanced *via* metformin delivery in the scaffold. The osteogenic group had 3–8 folds, 5 folds and 6 folds of increases in osteogenic gene expressions, ALP activity and mineral synthesis, compared to control group. Furthermore, the metformin + osteogenic group had 3–4 folds of increases over these properties of the osteogenic group. These results support the use of hPDLSCs as a readily-harvestable, autologous and low-cost alternative to the gold-standard hBMSCs which require an invasive procedure to harvest. Therefore, the CPC-chitosan scaffold is a suitable vehicle to deliver metformin, and the hPDLSC-CPC-metformin construct is highly promising to increase the bone repair and regeneration efficacy in dental, craniofacial and orthopedic applications.

Declaration of Competing Interest

The authors declare no conflict of interest.

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