IEEE Open Journal of Nanotechnology

Received 21 October 2021; revised 25 November 2021; accepted 27 November 2021. Date of publication 2 December 2021; date of current version 17 December 2021. The review of this paper was arranged by Associate Editor Pak Kin Wong.

Digital Object Identifier 10.1109/OJNANO.2021.3131653

Osteogenic Effect of Rabbit Periosteum-Derived Precursor Cells Co-Induced by Electric Stimulation and Adipose-Derived Stem Cells in a 3D Co-Culture System

ALVIN CHAO-YU CHEN[1], YUN-WEN TONG[2], CHIH-HAO CHIU[3,4], AND KIN FONG LEI[5,6] (Senior Member, IEEE)

[1] Department of Orthopedic Surgery, Bone and Joint Research Center, and Comprehensive Sports Medicine Center, Chang Gung Memorial Hospital, Linkou 333, Taiwan
[2] Department of Orthopedic Surgery, Chang Gung Memorial Hospital, Linkou 333, Taiwan
[3] Department of Orthopedic Surgery, Chang Gung Memorial Hospital, Taoyuan 333, Taiwan
[4] Bone and Joint Research Center and Comprehensive Sports Medicine Center, Chang Gung Memorial Hospital, Linkou 333, Taiwan
[5] Graduate Institute of Biomedical Engineering, Chang Gung University, Taoyuan 333, Taiwan
[6] Department of Radiation Oncology, Chang Gung Memorial Hospital, Linkou 333, Taiwan

CORRESPONDING AUTHORS: ALVIN CHAO-YU CHEN; KIN FONG LEI (e-mail: alvinchen@cgmh.org.tw; kflei@mail.cgu.edu.tw)

This work was supported in part by the Chang Gung Memorial Hospital, Linkou, Taiwan under Grant BMRP334, and in part by Ministry of Science and Technology, Taiwan under Grant MOST109-2314-B-182A-028-MY3.

ABSTRACT Periosteum-derived progenitor cells (PDPCs) are highly promising cell sources for bone fracture healing because of their stem cell-like multipotency to undergo osteogenesis and chondrogenesis. Both externally physical stimulation and internally biochemical signal were reported to enhance osteogenic differentiation of bone tissues. Electric stimulation (ES) could trigger the differentiation of stem cells, like mesenchymal stem cells (MSCs) and adipose-derived stem cells (ADSCs). But the effect is still unclear on PDPCs. In order to investigate the differentiation ability of PDPCs co-induced by ES and ADSCs, a biomimetic 3-dimensional (3D) co-culture system was developed for providing ES and co-culturing with ADSCs. Gene expression was studied after a 3-day culture course. From our results, osteogenic differentiation of PDPCs was significantly activated under the ES of 0.7 V/cm, 80 kHz, and 3 hrs/day. Moreover, co-culturing with ADSCs during the ES treatment was found to have synergistic effect of osteogenic differentiation. In addition, chondrogenic differentiation was shown when the PDPCs were cultured for a long culture course. In summary, osteogenic differentiation of PDPCs was shown to be co-induced by ES and ADSCs. This study provides significant insights of the PDPC therapy for bone tissue regeneration.

INDEX TERMS Periosteum-derived progenitor cells, adipose-derived stem cells, osteogenesis, 3D Co-culture, electric stimulation, orthopedics, rehabilitation.

I. INTRODUCTION

Periosteum tissue is a thin bilayered connective tissue that embraces bone surfaces and consists of an outer fibrous layer and a periosteum-derived progenitor cells (PDPCs)-riched inner cambium layer [1]–[3]. Many evidences showed that PDPCs possess the ability of rising osteogenesis or chondrogenesis due to the mesenchymal multipotency and mechanosensitivity under appropriate stimulation [4]–[7]. These properties make PDPCs act a promising role in bone reinvigoration and repairing.

In the past few decades, treatment of electrical stimulation (ES) was widely and successfully used in bone fracture healing [8]–[11]. The ES treatment is to generate mechanical stretching by electric potentials [12]. The electrical

stimulation provides electric potentials to activate cells by production of growth factors. Calcium is transported by cell-membrane via voltage-gated calcium channels to increase intracellular calcium concentration and storage, which also facilitated the storage of calcium-binding messenger protein, i.e., calmodulin. Direct current (DC), inductive coupling (IC), and capacitive coupling (CC) were categorized in the ES treatment. Some reports showed that daily DC ES treatment promotes osteogenesis of bone tissues through electrochemical reactions at the cathode, like elevated pH, low oxygen concentration, and hydrogen peroxide production [12]–[17]. The IC ES treatment usually incorporates with pulsed electromagnetic field in order to enhance the fracture healing of connective tissues, bone, and cartilage [12], [17]–[21]. The CC ES treatment has been shown to enhance osteogenesis of mesenchymal stem cells (MSCs) and adipose-derived stem cells (ADSCs) [12], [17], [22]–[25]. The ES treatment is the approach of external physical stimulation. On the other hand, internal biochemical signal between cells, e.g., growth factors, has been validated to have significant effect on osteogenic differentiation of bone tissues [26]–[30]. ADSC was recently shown to be a promising cell source for tissue regeneration, including hepatic, cardiac, neural, and bone tissues, because of the secretion of multiple growth factors, cytokines, and chemokines under certain stimulation [31]–[35]. Moreover, osteogenic differentiation was shown to be enhanced after PDPCs and osteoblasts co-culturing with MSCs [36]–[38]. However, most of the studies related to osteogenic differentiation of PDPCs were based on 2-dimensional (2D) culture model, which cells spread on a flat surface in a monolayer format. Because cells inhabiting in human body is in 3D environment which constructs with complex cell matrix instead of 2D monolayer format, *in vitro* 3D culture has been introduced to better mimic the complex *in vivo* microenvironments. Cells are encapsulated in 3-dimensional (3D) polymeric scaffold materials and it provides similar structure of natural extracellular matrix to benefit the signal transduction [7], [39]–[45]. However, few studies have attempted to investigate the osteogenic effect of PDPCs co-induced by ES and ADSCs in a 3D culture microenvironment.

In this study, osteogenic effect of PDPCs was co-induced by the combined stimulation of external ES treatment and internal biochemical signal in a dual-well 3D co-culture system. The system was composed of a polydimethylsiloxane (PDMS) culture chamber layer sandwiched by 2 indium tin oxide (ITO)-glass substrates. The culture chamber consisted of 2 sub-chambers separated by a barrier. PDPCs and ADSCs respectively encapsulated in the dextran-based hydrogel were separately cultured in the sub-chambers. The ES was applied to the cells across the ITO-glass substrates based on the preset parameters. Thus, the PDPCs were co-induced by the ES and ADSCs in a 3D culture microenvironment. Osteogenic and chondrogenic differentiation of PDPCs were then studied by examining mRNA expression, i.e., runt-related transcription factor 2 (RUNX2), osteopontin (OPN), osteopontin promoter (OPNp), osteonectin (ON) and SRY-Box transcription factor 9 (Sox9), and protein expression, i.e., RUNX2 and OPN. Results indicated that the osteogenic differentiation of PDPCs was activated by the CC ES treatment of 80 kHz and 0.7 v/cm with 3 h/day. Moreover, the osteogenic differentiation was significantly enhanced by co-culturing with ADSCs. Synergistic effect of physical and biochemical stimulations was found to induce osteogenic differentiation of PDPCs. This study provides some insights of differentiation capability of PDPCs for bone tissue regeneration.

II. MATERIALS AND METHODS

A. HARVEST AND ISOLATION OF RABBIT PDPCs

Periosteum tissue was harvested from adult female New Zealand white rabbits in a single animal group. The animal used protocol was approved by the review committee board of Chang Gung Memorial Hospital, Taiwan under the approval number of 2019121705. By using intramuscular injection of Zoletil 50 (0.5 mL/kg) and Rompun (0.5 mL/kg) mixture to anaesthetize the rabbit, a 3-cm skin incision was made along the medial tibia to collect the periosteum tissue. Then, the tissue was harvested and sectioned into pieces. The sectioned tissue pieces were washed 3 times in calcium and magnesium-free Dulbecco's phos-phate-buffered saline (DPBS; PBS001-1C; UniRegion Bio-Tech, Taiwan). After that, they were incubated for 2 h and cells were released in RPMI Medium 1640 (11875093; Gibco-BRL Life Technologies, USA) containing 0.3% collagenaseII and 1% antibiotic solution (15140122; Gibco-BRL Life Technologies, USA). The undigested tissue was removed by filtering with a 100-mm nylon sieve. The cells, i.e., PDPCs, were washed twice and collected by centrifugation at 1200 rpm for 5 min. Subsequently, the PDPCs were re-suspended in RPMI Medium 1640 supplemented with 10% fetal bovine serum (FBS; 10437 028; Gibco-RBL Life Technolo-gies, USA) and 1% antibiotic solution for following culture. The cell number was counted by an automated cell counter (Countess II FL; Invitrogen, USA).

B. DESIGN AND FABRICATION OF THE 3D CO-CULTURE SYSTEM

The 3D co-culture system was composed of a PDMS culture chamber layer sandwiched by 2 ITO-glass substrates with surface resistance of 7–10 Ω (Uni-Onward Corp., Taiwan). The culture chamber consisted of 2 sub-chambers separated by a barrier, as shown in Fig. 1(a). PDPCs and ADSCs respectively encapsulated in the dextran-based hydrogel were separately cultured in the sub-chambers. Because culture medium was applied over the barrier, biochemical interaction between PDPCs and ADSCs could be realized in the 3D co-culture system. The ITO-glass substrates were a pair of parallel plate electrodes generating ES across cell-hydrogel constructs. Thus, PDPCs could be co-induced by physical and biochemical stimulations to investigate osteogenic differentiation of PDPCs. Schematic illustration of the experimental setup is shown in Fig. 1(b). The PDMS culture chamber was replicated from poly (methyl methacrylate) (PMMA) molds

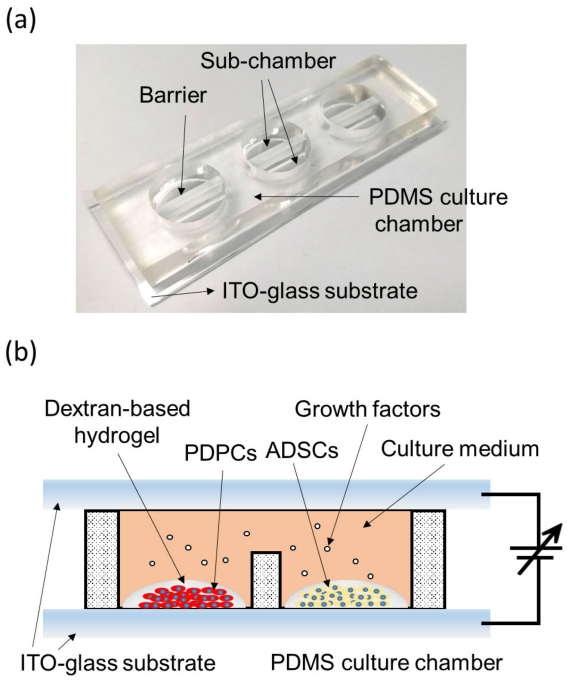

FIGURE 1. **Design and experimental setup of the investigation of osteogenic differentiation of PDPCs co-induced by physical and biochemical stimulations. (a) Photograph of the PDMS culture chamber with 2 sub-chambers separated by a barrier. (b) Schematic illustration of the experimental setup of PDPCs co-culturing with ADSCs under ES.**

containing negative structure which was designed by SolidWorks software and machined by a micro-engraving machine (EGX-400; Roland, Japan). A mixture of PDMS pre-polymer and curing agent in 10:1 (w/w) was poured to the PMMA molds and incubated at 70 °C for 1 h to obtain the solidified PDMS chamber. The dimension of the PDMS culture chamber was 15 mm in diameter and 7 mm in height and separated into 2 identical sub-chambers by a barrier of 2 mm in width and 5 mm in height, resulting in the culture area of each sub-chamber about 0.73 cm^2. Then, the PDMS chamber was carefully bonded to the ITO-glass substrates through the surface modification with an oxygen plasma machine (PDC-32G; Harrick Plasma, USA) for 5 minutes. Finally, the co-culture system was sterilized under ultraviolet light overnight for further experiments.

C. EXPERIMENTAL PROCEDURE

In order to provide a biomimetic 3D microenvironment, 2.5 $\times$ 10^4 cells were encapsulated in hydrogel using dextran-based TrueGel3D Hydrogel Kit (1641985; Sigma-Aldrich, Germany). The encapsulation protocol followed the supplier's instruction. The cell-hydrogel mixture was then loaded into the sub-chambers. After overnighting for gel gelation and cell stabilization in a cell culture incubator at 37 °C with 5% CO_2 (Thermoscientific, USA), the culture chamber was filled up by culture medium. In order to form a pair of parallel plate electrodes, another ITO-glass substrate was covered on the PDMS chamber to apply electric field across the ITO-glass substrates by a signal generator (Model: DG1022; Rigol Technologies Inc., China). Electric field was defined by the peak-to-peak voltage divided by the distance between 2 ITO-glass substrates. Different electric conditions including frequency from 70 to 100 kHz, electric field from 0.65 to 1.0 V/cm, and duration of 3, 4 and 5 h/day were applied to investigate and optimize the ES condition that significantly enhanced osteogenesis of PDPCs. After stimulation for 2 days, the ES treatment was removed and the cells were cultured for the following 1, 3 and 5 days, respectively. After the entire culture course, proliferation and differentiation of PDPCs were investigated by various bio-assays.

D. QUANTIFICATION OF CELL VIABILITY

After ES treatment, cell viability was quantified by using WST-1 cell cytotoxicity assay (Roach Applied Science, USA). Culture medium was removed from culture chamber, then dextran-based TrueGel encapsulated PDPCs were released by treating with TrueGel3D Enzymatic Cell Recovery Solution (0001636323; Sigma-Aldrich, Germany). The cells were incubated with 1:10 (v/v) diluted WST-1 reagent at 37 °C for 2 h. After that, the supernatant was transferred to a microplate. Color intensity of the supernatant was correlated to the metabolically active cell number and was analyzed by a microplate reader (ELx800; BioTek Instruments, USA) at an absorbance of 440 nm and a reference of 660 nm. The color intensity was represented by optical density (OD) value. Cell viability was defined as the

OD value of the experimental group (PDPCs after ES treatment or/and co-culturing with ADSCs) divided by the OD value of the control group (PDPCs without stimulation).

E. INVESTIGATION OF OSTEOGENIC-RELATED GENE EXPRESSIONS

After releasing the PDPCs from dextran-based TrueGel, the total RNA of PDPCs was extracted by GENEzol^{TM} TriRNA Pure Kit (GZX100; Geneaid, Taiwan) following the extraction protocol recommended in supplier's instruction. Then the complementary DNA (cDNA) was synthesized from the extracted total RNA by the SuperScript^{TM} III First-Strand Synthesis SuperMix kit (18080400; Invitrogen, USA) with the T100TM Thermal Cycler (Bio-Rad, USA). To investigate osteogenesis and chondrogenesis of the PDPCs, real-time polymerase chain reaction (PCR) was performed by using iQ SYBR Green Supermix (1708882; Bio-Rad, USA) and mRNA expressions of RUNX2, OPN, OPNp, and Sox9 were examined. GAPDH expression was used as internal control. The primer sequences of RUNX2, OPN, OPNp, Sox9, and GAPDH are listed in Table 1. Relative mRNA expression level was analyzed by a CFX Connect^{TM} Real-time PCR Detection System (Bio-Rad, USA).

F. IMMUNOFLUORESCENCE ASSAY

Double immunofluorescence assay was conducted to investigate the osteogenic protein expression of RUNX2 and OPN on PDPCs after stimulation. The analytical procedure is briefly

TABLE 1 **Primer Sequences Used for Real-Time PCR**

mRNA	Primer sequence
GAPDH-F	GTGAAGGTCGGAGTGAAC
GAPDH-R	GGTGGAATCATACTGGAACA
RUNX2-F	TGATGACACTGCCACCTCTGA
RUNX2-R	GCACCTGCCTGGCTCTTCT
OPN-F	GCAGCAACCACAGTTTTCACTG
OPN-R	TACATTCAGGTGCTGAGCCACT
OPNp-F	CAGAATGCTATGTCCTCAGA
OPNp-R	CGTCCTCATCCTCATCAATA
Sox9-F	AGTACCCGCACCTGCACAAC
Sox9-R	CGCTTCTCGCTCTCGTTCAG

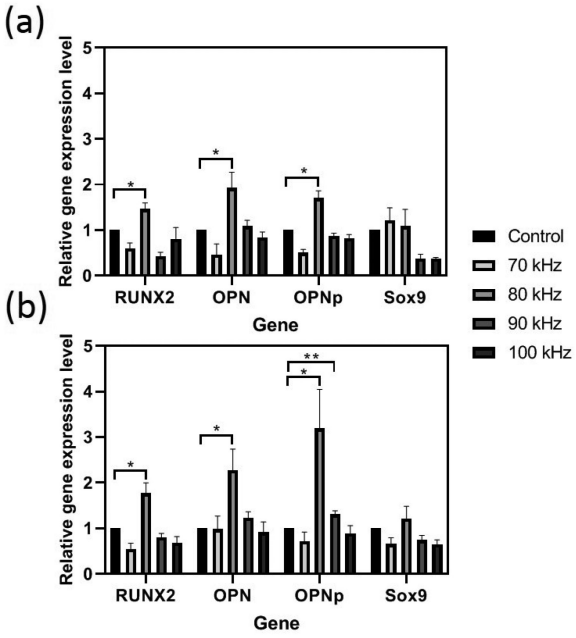

FIGURE 2. **Investigation of gene expression of the PDPCs after the ES treatment of 2+1 culture course. The ES voltage of 0.7 v/cm and duration of 3 h/day were applied for the beginning 2 days. The ES frequencies of 70, 80, 90, and 100 kHz were respectively applied in this study. The PDPCs were co-cultured (a) without / (b) with ADSCs.**

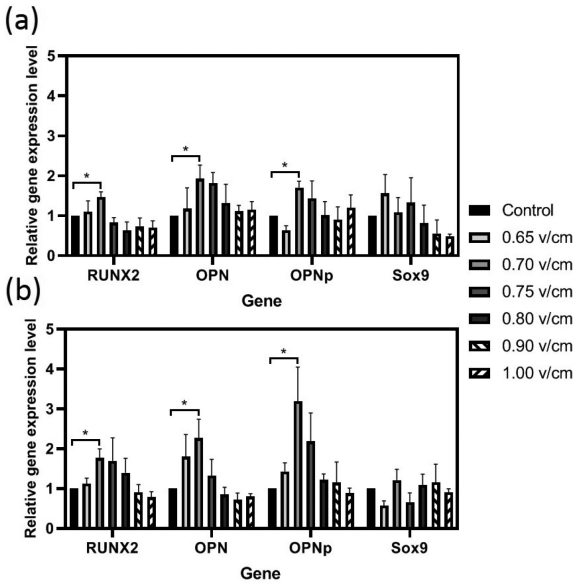

FIGURE 3. **Investigation of gene expression of the PDPCs after ES treatment of 2+1 culture course. The ES frequency of 80 kHz and duration of 3 h/day were applied for the beginning 2 days. The ES voltages of 0.65, 0.7, 0.75, 0.8, 0.9 and 1.0 v/cm were respectively applied in this study. The PDPCs were co-cultured (a) without / (b) with ADSCs.**

described. The cell-hydrogel construct was washed in PBS and fixed by 4% paraformaldehyde in 1 × PBS for 30 min at room temperature. Subsequently, the cell-hydrogel construct was washed 3 times for 5 min each in PBS and permeabilized by 0.1% Tween-20 in PBS for 2 h at room temperature. After washing, the cell-hydrogel construct was incubated with primary antibodies including diluted FITC-conjugated rabbit anti-RUNX2 polyclonal antibody (ARP36679; AVIVA SYSTEMS BIOSYSTEM, USA) and mouse anti-OPN monoclonal antibody (MBS555003; MyBioSource, USA) in PBS for 2 h at room temperature or overnight at 4 °C avoiding from the light. Afterwards, the cells were washed, cultured with the diluted secondary antibody, i.e., iFlour 594-conjugated goat anti mouse IgG (C04026-1ML; Croyez Bioscience, Taiwan), in PBS for 1 h at room temperature, washed 3 times in PBS and ddH2O, and mounted by the ProLong^{TM} Gold Antifade Mountant with DAPI (P36935; Invitrogen^{TM}, USA). The stained cell-hydrogel construct was imaged using a fluorescent microscope (IX83; Olympus, Japan) equipped with a camera (DP74; Olympus, Japan) to investigate the protein expression.

G. STATISTICAL ANALYSIS

Data are represented as means ± standard deviation (SD) of at least 3 repeated experiments. An un-paired *t*-test was used to examine the statistical significance of the differences between the data. Statistical significances are indicated as $*$ for $p < 0.05$, $**$ for $p < 0.01$, and $***$ for $p < 0.001$.

III. RESULTS AND DISCUSSION

A. DIFFERENTIATION OF PDPCs CO-INDUCED BY ES AND ADSCs IN THE 3D CO-CULTURE SYSTEM

To study the differentiation of rabbit PDPCs co-induced by ES and ADSCs, osteogenic genes (RUNX2, OPN and OPNp) and chondrogenic gene (Sox9) were examined in this study. RUNX2 was reported as a crucial factor to activate osteogenesis from mesenchymal stem cells and progenitor cells to preosteoblasts [46]–[48]. OPN and OPNp are the subsequent genes which regulate osteoblasts maturation process [49]–[52]. On the other hand, transcription factor, Sox9, was reported as a critical activator of chondrogenesis to govern the chondrocyte differentiation [53]–[55].

The dextran-based TrueGel encapsulated PDPCs were cultured with/without ADSCs in the dual-well co-culture system and simultaneously treated by ES with different frequencies, voltages and durations for 2 days. Then, the cells were cultured with ES treatment for another 1 day. After the 2+1

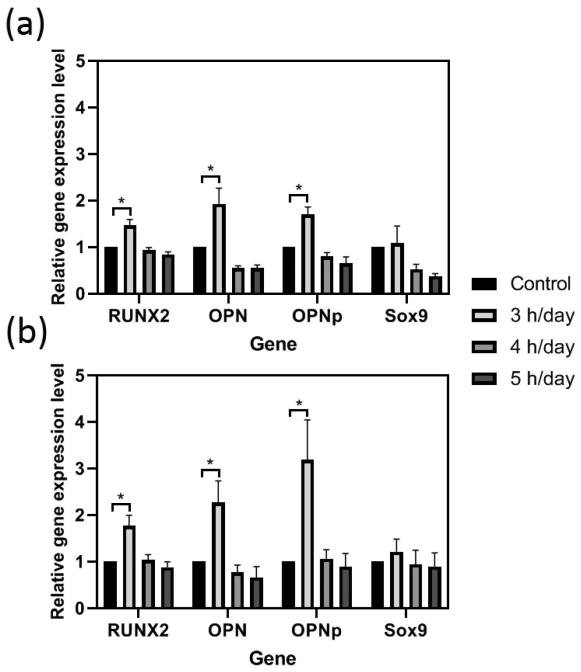

FIGURE 4. Investigation of gene expression of the PDPCs after ES treatment of 2+1 culture course. The ES voltage of 0.7 v/cm and frequency of 80 kHz were applied for the beginning 2 days. The ES durations of 3, 4, and 5 h/day were respectively applied in this study. The PDPCs were co-cultured (a) without / (b) with ADSCs.

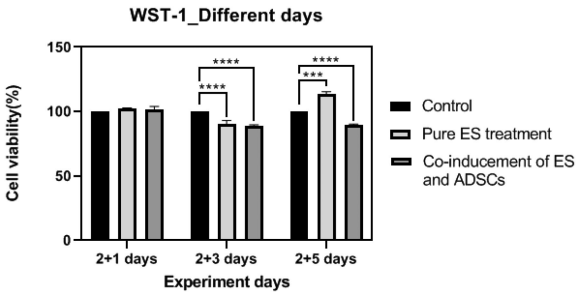

FIGURE 5. Cell viability of the PDPCs after various culture conditions. The PDPCs encapsulated in dextran-based TrueGel were received ES treatment and co-cultured with/without ADSCs for 2 days. Then, the cells were respectively cultured without ES treatment for another 1, 3, and 5 days in order to investigate the cellular response after ES treatment. The ES treatment conditions were 0.7 v/cm, 80 kHz, and 3 h/day.

culture course, the PDPCs were harvested for gene expression analysis. mRNA expressions of RUNX2, OPN, OPNp, and SOX9 were examined. The results of ES with different frequencies are shown in Fig. 2. Osteogenic genes of RUNX2, OPN, and OPNp were significantly icreased with the ES frequency of 80 kHz. On the other hand, Sox9, chondrogenic gene, did not show significance to the ES treatment. That indicated the ES at 80 kHz could significantly induce osteogenic effect of PDPCs whatever the PDPCs were cultured with/without ADSCs. The optimal frequency of 80 kHz could activate the PDPCs to undergo osteogenesis rather than chondrogenesis. Moreover, when the PDPCs were co-cultured with ADSCs, higher osteogenic expression was found and

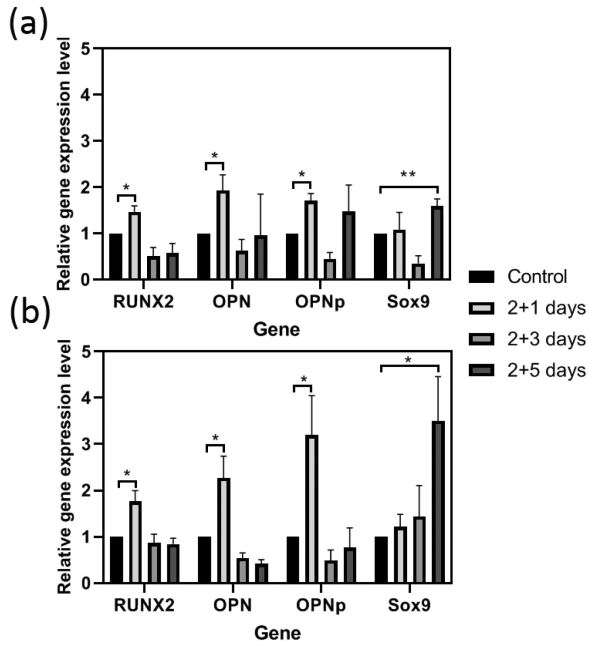

FIGURE 6. Investigation of gene expression of the PDPCs after different culture conditions. The PDPCs encapsulated in dextran-based TrueGel were received ES treatment and co-cultured (a) without / (b) with ADSCs for 2 days. Then, the cells were respectively cultured without ES treatment for another 1, 3, and 5 days. The ES treatment conditions were 0.7 v/cm, 80 kHz, and 3 h/day.

the result is shown in Fig. 2(b). Synergistic effect of external ES treatment and internal biochemical signal could promote the PDPCs undergoing osteogenesis with higher effectiveness. Next, ES with different voltages and durations was investigated and the results are respectively shown in Figs. 3 and Fig. 4. When the ES voltage of 0.7 v/cm was applied, osteogenic gene expressions were significantly up-regulated, as shown in Fig. 3(a). The optimal voltage of 0.7 v/cm was found for inducing the highest osteogenic gene expression. The ES durations of 3, 4, and 5 h/day were respectively applied for the beginning 2 days of the 2+1 culture course. The result indicated that 3 h/day could significantly induce the osteogenic gene expressions of PDPCs, as shown in Fig. 4(a). Similarly, co-culturing ADSCs could promote the osteogenesis of the PDPCs, as shown in Figs. 3(b) and 4(b). The above investigations confirmed that there are optimal conditions of voltage, frequency and duration for the ES treatment to induce osteogenesis of the PDPCs. Moreover, the ADSCs can be an assistive agency to promote higher osteogenic effect.

B. CELLULAR RESPONSE AFTER ES TREATMENT

The PDPCs encapsulated in dextran-based TrueGel were received ES treatment and co-cultured with/without ADSCs for 2 days. Then, the cells were respectively cultured without ES treatment for another 1, 3, and 5 days in order to investigate the cellular response after ES treatment. The ES treatment conditions were 0.7 v/cm, 80 kHz, and 3 h/day. Cell viability was quantified and compared with the control group (the cells

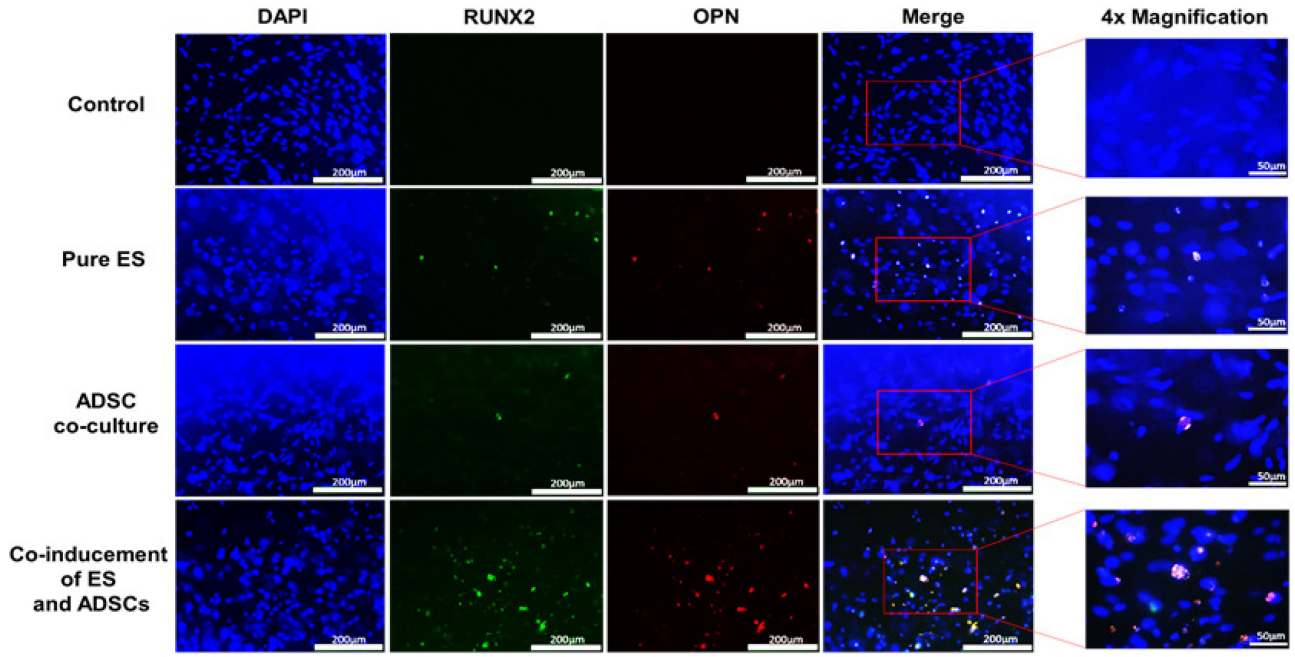

FIGURE 7. **Double immunofluorescence staining showing protein expressions of the PDPCs after receiving ES treatment and co-culturing with ADSCs for 2 days. The ES treatment conditions were 0.7 v/cm, 80 kHz, and 3 h/day. The cell nuclei was stained by DPAI fluorescence (blue). The RUNX2 protein was stained by FITC fluorescence (green). The OPN protein was stained by iFlour 594 fluorescence (red).**

cultured in the hydrogel without stimulation). The result is shown in Fig. 5. In the culture condition of 2+1 days, there was no significant difference in cell viability between control, pure ES treatment, and co-inducement of ES and ADSCs. On the other hand, cell viability was significantly varied in the culture conditions of 2+3 and 2+5 days. That indicated the cells required at least 1 day to respond to the ES treatment. Moreover, gene expression was respectively analyzed in the culture conditions of 2+1, 2+3, and 2+5 days. Result of gene expression after ES treatment without co-culturing ADSCs is shown in Fig. 6(a). Osteogenic genes of RUNX2, OPN, and OPNp were significantly up-regulated in the condition of 2+1 days and did not show significant difference in other conditions. In contrast, chondrogenic gene of Sox9 showed significant increase in the condition of 2+5 days. Osteogenesis could be stimulated right after the ES treatment and chondrogenesis were induced after 5 days of the ES treatment. Similarly, when the PDPCs were co-induced by ES and ADSCs, synergistic effect was found to show significant enhancement of gene expression, as shown in Fig. 6(b).

C. PROTEIN EXPRESSION OF THE PDPCs CO-INDUCED BY ES AND ADSCs

To verify the osteogenesis of the PDPCs after receiving ES treatment and co-culturing with ADSCs for 2 days, osteogenic proteins of RUNX2 and OPN were examined by double immunofluorescence staining. DAPI marker was to stain cell nuclei in order to show the cell location. The RUNX2 and OPN markers were to analyze the early osteogenic stage. That indicated that osteogenesis was activated from progenitor cells to preosteoblasts. The PDPCs were respectively cultured under pure ES, ADSC co-culture, and co-inducement of ES and ADSCs for 2 days. Control group was to culture the PDPCs for 2 days without any stimulation. The images of double immunofluorescence staining of RUNX2 and OPN are shown in Fig. 7. The results revealed that pure ES or ADSC co-culture could slightly promote the protein expression of RUNX2 (green) and OPN (red). Moreover, co-inducement of ES and ADSCs significantly enhanced the protein expressions. The results further confirmed the osteogenic proteins were regulated and facilitated when the PDPCs were stimulated by ES and ADSCs under 3D microenvironment.

IV. CONCLUSION

A 3D co-culture system was developed and exploited for the investigation of osteogenesis of PDPCs under co-inducement of ES and ADSCs in 3D microenvironment. The PDPCs and ADSCs were separately cultured in the co-culture system and interacted with the interlinked medium to exchange the growth factors secreted from each other. Simultaneously, ES was applied to the PDPCs by parallel plate electrodes installed in the co-culture system. Osteogenic mRNA and protein expressions were respectively examined by real-time PCR and immunofluorescence assay. The results showed the optimal ES conditions were 0.7 v/cm, 80 kHz, and 3 h/day for activating osteogenic process of the PDPCs encapsulated in hydrogel. Synergistic effect was shown to significantly enhance the osteogenesis when the PDPCs were co-induced by the ADSCs. Moreover, chondrogenic differentiation was observed when the PDPCs were cultured for a long culture course. In summary, osteogenic differentiation of PDPCs co-induced

by ES and ADSCs was investigated to provide significant insights of bone regeneration therapy.

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ALVIN CHAO-YU CHEN is currently an Attending Staff and Associate Professor with the Department of Orthopaedic Surgery, Chang Gung Memorial Hospital, Taiwan. Since 2016, he has been the Section Chief of Orthopedic Sports Medicine. From 2010 to 2012, he was the President of the Taiwan Society for Surgery of Hand and has made significant contribution in Taiwan continuing medical education and international interaction in the field of hand surgery. Form 2012 to 2018, he was the Taiwan Research Convener of AOTrauma Asian Pacific Board and Country Council. With more than 100 of journal publications since 1996, he has committed to the subspecialty of orthopedic traumatology, joint reconstruction and arthroscopic surgery, and focused in clinically oriented research regarding periosteal regeneration and secondary bone healing through a tacit cooperation with Professor Kin Fong Lei.

YUN WEN TONG received the B.S. degree from the Department of Biomedical Science, Chang Gung University, Taoyuan City, Taiwan in 2017. She is currently an Assistant with the Department of Orthopedic Surgery, Linkou Chang Gung Memorial Hospital. She has work in stem cell therapy for bone fracture healing for more than three years. She has also studied kinds of mechanical stimulation to promote cell osteogenesis.

CHIH-HAO CHIU received the Doctor of Medicine degree and the Ph.D. degree in biomedical engineering from Chang Gung University, Taoyuan City, Taiwan, in 2005 and 2018, respectively. He is currently an Associate Professor and Medical Doctor with Chang Gung Memorial Hospital, who majors in shoulder and knee arthroscopies and arthroplasties. He had a board certificate from Taiwan Orthopedic Association, Taiwan Hand Surgery Association, Taiwan Sports Medicine Association, and was previously the secretary of Taiwan Shoulder and Elbow Society. He is also a Member of the Chang Gung Sports Committee, which is responsible for national and professional athletics care in Taiwan. Dr. Chiu also takes part in practicing and training young orthopedic doctors in the shoulder ultrasonography exam of my Department and had published the benefit of an office-based shoulder ultrasound exam. He had several grants from the Taiwan Minister of Science and technology and Chang Gung Memorial Hospital.

KIN FONG LEI (Senior Member, IEEE) received the B.S. degree in mechanical engineering from National Tsing-Hua University, Hsinchu,Taiwan, in 1998, and Ph.D. degree in mechanical engineering from The Chinese University of Hong Kong, Hong Kong in 2005. He is currently a Professor of biomedical engineering with Chang Gung University (CGU), Taoyuan City, Taiwan. Prior to joining CGU, he was a Lecturer with The Hong Kong Polytechnic University, Hong Kong during 2007–2010. In 2006, he was a Postdoctoral Fellow with the University of Western, London, ON, Canada. Dr. Lei has made significant original contributions to research in bio-microfluidics, bio-sensing, and molecular diagnostics. He has authored or coauthored more than 100 academic peer-reviewed articles and was invited to contribute in eight book or book chapters. Dr. Lei is a Fellow of Royal Society of Chemistry (RSC) and Institute of Physics (IOP). He serves as the Chair of IEEE-EMBS Technical Committee on Bionanotechnology and BioMEMS (BNM) during 2020–2021 and an Associate Editor at EMBS Conference Editorial Board during 2020–2021. In 2021, he is also the Chair of IEEE-NTC Technical Committee on Nanorobotics and Nanomanufacturing. Dr. Lei participated in organizing committees for many IEEE conferences for MEMS/microfluidics researchers. He was the General Chair of The 15th IEEE International Conference on Nano/Molecular Medicine & Engineering (IEEE-NANOMED 2021). He is an Associate Editor for the IEEE ACCESS and IEEE TRANSACTIONS ON NANOBIOSCIENCE, and an Editorial Board Member of *Scientific Reports*.