Human Induced Pluripotent Stem Cell-Derived Mesenchymal Stem Cell Seeding on Calcium Phosphate Scaffold for Bone Regeneration

Minghui Tang, DDS, PhD,^{1,*} Wenchuan Chen, DDS, PhD,^{1,2,*} Jun Liu, DDS, PhD,^{1,2}
Michael D. Weir, PhD,¹ Linzhao Cheng, PhD,³ and Hockin H.K. Xu, PhD^{1,4-6}

Tissue engineering provides an important approach for bone regeneration. Calcium phosphate cement (CPC) can be injected to fill complex-shaped bone defects with excellent osteoconductivity. Induced pluripotent stem cells (iPSCs) are exciting for regenerative medicine due to their potential to proliferate and differentiate into cells of all three germ layers. To date, there has been no report on iPSC seeding with CPC scaffolds. The objectives of this study were to (1) obtain iPSC-derived mesenchymal stem cells (iPSC-MSCs); (2) seed iPSC-MSCs on CPC scaffold for the first time to investigate cell attachment and proliferation; and (3) investigate osteogenic differentiation of iPSC-MSCs on CPC and mineral synthesis by the cells. iPSCs were derived from adult marrow CD34+ cells that were reprogrammed by a single episomal vector pEB-C5. iPSCs were cultured to form embryoid bodies (EBs), and MSCs were migrated out of EBs. Flow cytometry indicated that iPSC-MSCs expressed typical surface antigen profile of MSCs. Mesenchymal differentiation of iPSC-MSCs demonstrated that the iPSC-MSCs had the potential to differentiate into adipocytes, chondrocytes, and osteoblasts. iPSC-MSCs had good viability when attached on CPC scaffold. iPSC-MSCs differentiated into the osteogenic lineage and synthesized bone minerals. iPSC-MSCs on CPC in osteogenic medium yielded higher gene expressions of osteogenic markers including alkaline phosphatase (ALP), osteocalcin, collagen type I, and Runt-related transcription factor 2 than those in control medium ($p < 0.05$). iPSC-MSCs on CPC in osteogenic medium had 10-fold increase in ALP protein than that in control medium ($p < 0.05$). Bone mineral synthesis by iPSC-MSCs adherent to CPC scaffold was increased with time, and mineralization in osteogenic medium was three to four fold that in control medium. In conclusion, iPSCs were derived from adult marrow CD34+ cells that were reprogrammed by a single episomal vector pEB-C5, and MSCs were generated from the EBs. iPSC-MSCs showed good viability and osteogenic differentiation on CPC scaffold for the first time; hence, the novel iPSC-MSC-CPC construct is promising to promote bone regeneration in dental, craniofacial, and orthopedic repairs.

Introduction

THE NEED FOR BONE REPAIR arises from infections, trauma, tumor resections, abnormal development, and congenital malformations. Over 500,000 bone grafts were performed to repair bone defects annually in the United States.¹ The annual health care costs plus the lost wages for people in the United States with musculoskeletal diseases reached ~\$849 billion in 2004, or 7.7% of the national gross domestic

product.² This number is predicted to dramatically increase as the population ages.³ Bone tissue engineering offers an exciting approach for bone repair and regeneration.⁴ The introduction of stem cells into the tissue engineering opens new horizons.⁵⁻¹⁰ Bone marrow-derived mesenchymal stem cells (BMSCs) are the most common cell source; however, their self-renewal and proliferative ability decreases due to aging¹¹⁻¹³ and diseases such as osteoporosis and arthritis.^{14,15} Therefore, the very patients who need bone regeneration treatments may not be able

¹Biomaterials and Tissue Engineering Division, Department of Endodontics, Prosthodontics and Operative Dentistry, University of Maryland Dental School, Baltimore, Maryland.

²State Key Laboratory of Oral Diseases, West China College of Stomatology, Sichuan University, Chengdu, China.

³Stem Cell Program in Institute for Cell Engineering and Division of Hematology, Johns Hopkins University, Baltimore, Maryland.

⁴Center for Stem Cell Biology and Regenerative Medicine, University of Maryland School of Medicine, Baltimore, Maryland.

⁵Marlene and Stewart Greenebaum Cancer Center, University of Maryland School of Medicine, Baltimore, Maryland.

⁶Department of Mechanical Engineering, University of Maryland Baltimore County, Baltimore County, Maryland.

*These two authors contributed equally.

to provide BMSCs for themselves, hence, it is critically important to explore other types of stem cells for regenerative medicine.

Recently, induced pluripotent stem cells (iPSCs) have gained wide interest in stem cells research and regenerative medicine.^{16–19} iPSCs have been established by transfecting mouse cells with the reprogramming transcription factors Oct3/4, Sox2, c-Myc, and Klf4,¹⁶ or human somatic cells with factors Oct4, Sox2, Nanog, and Lin28.¹⁷ iPSCs are believed to be very similar to natural pluripotent stem cells such as embryonic stem cells (ESCs) in many aspects, such as the expression of certain stem cell genes and proteins, doubling time, chromatin methylation patterns, embryoid body (EB) formation, teratoma formation, viable chimera formation, potency, and differentiability.^{16,18} Therefore, like their embryonic counterparts ESCs, iPSCs also have nearly unlimited potential to proliferate and differentiate into not only all derivatives of the three primary germ layers (ectoderm, endoderm, and mesoderm), but also many mature cells *in vitro*.¹⁹ In addition, iPSCs are easily and autogeneically accessible, thus removing both the ethical and immunological issues. Therefore, iPSCs represent a major breakthrough in stem cell research and provide an invaluable resource for regenerative medicine.¹⁹ Recent studies used iPSCs for regenerating cardiac myocytes,²⁰ renal lineage cells,²¹ pancreatic insulin-producing cells,²² motor neurons,²³ and other distinct tissues. However, few studies were reported on the use of iPSCs for bone regeneration.^{24,25}

Scaffolds are important for bone regeneration and serve as a template for cell function while maintaining the volume and supporting the external loading. Calcium phosphate (CaP) scaffolds are bioactive, mimic bone minerals, and can bond to neighboring bone to form a functional interface.^{7,26–28} Preformed implants require machining to fit into a bone cavity, leading to increases in bone loss, trauma, and surgical time.²⁹ In contrast, injectable scaffolds can be used in minimally-invasive procedures and intimately fit into bone defects even with irregular shapes.^{30–32} Calcium phosphate cements (CPCs) are injectable and have good bioactivity and osteoconductivity. CPC was approved in 1996 by the Food and Drug Administration for repairing craniofacial defects.^{33,34} However, there has been no report on human iPSC-derived mesenchymal stem cell (iPSC-MSC) seeding on CPC.

The objectives of this study were to generate human iPSC-MSCs and investigate the iPSC-MSC attachment on CPC scaffolds for proliferation and osteogenic differentiation for the first time. Two hypotheses were tested: (1) CPC scaffold will support the iPSC-MSC attachment and proliferation; (2) iPSC-MSCs adherent on CPC scaffold can successfully undergo osteogenic differentiation and synthesize bone minerals *in vitro*.

Materials and Methods

Cell culture

Human iPSCs were generated as described recently.^{35,36} iPSC BC1 line was derived from adult bone marrow CD34+ cells. Human primary mononuclear cells from a healthy adult marrow donor (code: BM2426) were isolated with a standard gradient protocol by Ficoll-Paque Plus ($p=1.077$). CD34+ cells were purified with magnetic-

activated cell sorting system and cultured for 4 days with hematopoietic cytokines before being reprogrammed by a single episomal vector pEB-C5.^{35,36} The culture of iPSCs was approved by the University of Maryland Baltimore Institutional Review Board (HP-00046649). Undifferentiated iPSCs were cultured as colonies on a feeder layer of mitotically-inactivated murine embryonic fibroblasts (MEF), which was formed by seeding 200,000 MEF cells/well on Nunclon Δ Surface six-well culture plates (Nunc). Mitotic inactivation was achieved through exposure MEF to 10 μ g/mL Mitomycin C (Sigma) for 2 h. The iPSC culture medium consisted of 80% (v/v) Dulbecco's modified Eagle medium (DMEM)/F12 (Invitrogen), 20% Knockout Serum Replacement (a serum-free formulation; Invitrogen) mixed with 50% MEF conditioned medium, 1% modified Eagle medium nonessential amino acids solution (Invitrogen), 10 ng/mL basic fibroblast growth factor (β -FGF; Invitrogen), 1 mM L-glutamine (Sigma), and 0.1 mM β -Mercaptoethanol (Sigma). For preparation of MEF conditioned medium, inactivated MEF were seeded at 90% confluent and incubated overnight. Then, the culture medium was changed to full iPSC culture medium. The supernatant of MEF culture was harvested every other day until 7 days, filtered through a 0.22 μ m pore-size filter and stored at -80°C until use. Cells were cultured at 37°C with 5% CO_2 and 100% humidity, and the medium was changed daily.

The iPSCs were induced to form EBs. Briefly, iPSC colonies were dissociated into clumps through treatment with 1 mg/mL collagenase type IV in DMEM/F12 at 37°C for 6 min, followed by mechanical scraping. The dissociated iPSC clumps were collected by sedimentation, resuspended in differentiation medium (iPSC culture medium without β -FGF), and transferred to 25 cm^2 ultra-low attachment cell culture flasks (Corning). The differentiation medium was changed every 2 days.

After 10 days, the EBs were transferred into 0.1% gelatin coated culture plates and cultured for another 10 days. At 2 days of culture, most of the EBs adhered and many cells migrated out from the edges of the EBs. Upon 70% confluence, the outgrowth of the cells was selectively isolated by using cell scrapers. Cells were subcultured in the MSC growth medium, which consisted of DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS; HyClone), 2 mM L-glutamine (Gibco), 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco). The differentiated cells which outgrew from the EBs derived from these culture conditions were termed iPSC-derived MSCs (iPSC-MSCs).

Flow cytometry of iPSC-MSCs

The expression of the cell surface antigen profile of these iPSC-MSCs was characterized using flow cytometry as described previously.³⁷ Briefly, iPSC-MSCs (passage 4) were harvested by trypsin-ethylenediaminetetraacetic acid (EDTA) and washed with cold phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA), then resuspended to $\sim 1 \times 10^6$ cells in 50 μ L of cold PBS containing 1% BSA. Cell samples were separately labeled on ice with optimal dilution of fluorescein isothiocyanate-conjugated monoclonal antibodies (mAbs; all from Invitrogen, except when indicated) against CD29, CD31, CD34 (BD), CD44, CD73 (BD), TRA-1-81 (BD), HLA-

ABC, phycoerythrin-conjugated mAbs against Oct3/4 (BD) and CD166 (BD), and Alexa Fluor 488-conjugated mAb against CD105 in the dark. After 30 min incubation, cells were washed with cold PBS containing 1% BSA. Non-specific fluorescence was determined by incubating cells with isotype-matched conjugated mAbs. At least 10,000 events were collected from each run of flow cytometry. Data were analyzed using CellQuest software (Becton Dickinson). The fluorescence histogram for each mAb was displayed alongside the control antibody. The percentages of positive cells were subtracted from the isotype control antibody of each conjugate.

Adipocytic, chondrocytic, and osteogenic differentiation of iPSC-MSCs

Adipocytic, chondrocytic, and osteogenic differentiation of iPSC-MSCs were carried out as previously described.^{38,39} Briefly, for adipocytic differentiation, iPSC-MSCs were grown by exposure to 1 mM dexamethasone, 10 μ g/mL insulin, and 0.5 mM isobutylxanthine (Sigma) in alpha MEM medium containing 10% FBS for 2–4 weeks. For chondrocytic differentiation, iPSC-MSCs was induced in pellet culture by exposure to 10 ng/mL transforming growth factor- β 3 and 200 μ M ascorbic acid (Sigma) in alpha MEM medium containing 10% FBS for 3–4 weeks. For osteogenic differentiation, iPSC-MSCs was induced in osteogenic medium, which consisted of MSC growth medium supplemented with 100 nM dexamethasone, 0.05 mM ascorbic acid, and 10 mM β -glycerophosphate and 10 nM 1 α ,25-dihydroxyvitamin (Sigma) for 3–4 weeks. Oil Red O, Alcian Blue (Sigma), and Alizarin Red S (ARS; Millipore) staining for adipocytes, chondrocytes, and osteoblasts, respectively, were performed using standard techniques.

Fabrication of CPC scaffold

The CPC powder consisted of an equimolar mixture of tetracalcium phosphate ($\text{Ca}_4[\text{PO}_4]_2\text{O}$) and dicalcium phosphate anhydrous (CaHPO_4), which were prepared following a previous study.⁴⁰ CPC powder was mixed with distilled water at a powder to liquid mass ratio of 3:1 to form a flowable paste. The paste was placed into Teflon ring molds of 9 mm in diameter and 2 mm in thickness to make CPC disks. The paste in the mold was set in a humidifier with 100% relative humidity for 4 h at 37°C. The specimens were then demolded and immersed in distilled water at 37°C for 20 h. The specimens were sterilized by ethylene oxide and degassed for 7 days prior to cell culture.

Viability of iPSC-MSCs on CPC scaffold

Passage 4 iPSC-MSCs were used. Fifty thousand cells were suspended in 2 mL of either control medium (the MSC growth medium as described above) or osteogenic medium.^{37,41} They were then added to each well of a 24-well plate containing CPC disks. The medium was changed daily. After 1, 7, 14, or 21 days, the medium was removed and the CPC disks were washed twice with 2 mL of PBS. Two milliliter of PBS containing 2 μ M calcein-AM and 2 μ M ethidium homodimer-1 (Molecular Probes) was added to each well to do live/dead staining. Thirty minutes later, the disks with cells were washed with 2 mL of PBS and observed under

epifluorescence microscopy (TE2000-S; Nikon). Live cells were stained green and dead cells were stained red.

Two parameters were measured following a previous study.⁴² The first was the percentage of live cells, $P_{\text{Live}} = N_{\text{Live}} / (N_{\text{Live}} + N_{\text{Dead}})$, where N_{Live} , number of live cells, and N_{Dead} , number of dead cells, in the same image. Six specimens in each type of culture medium at each time point were tested ($n=6$). Two randomly chosen fields of view were photographed for each specimen. The second parameter was live cell density, D_{Live} , which was measured as $D_{\text{Live}} = (A_{\text{Calcein}} / A_{\text{Total}})$, where A_{Calcein} is the area covered by live cells stained green via calcein in the image, and A_{Total} is the total area of the image.

Quantitative real-time reverse transcription polymerase chain reaction measurement of osteogenic differentiation of iPSC-MSCs on CPC

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR, 7900HT; Applied Biosystems) was used to measure the osteogenic gene expression of alkaline phosphatase (ALP), osteocalcin (OC), collagen type I (Col I), and Runt-related transcription factor 2 (Runx2) in iPSC-MSCs attaching to CPC scaffolds. At 1, 7, 14 and, 21 days, the total cellular RNA on the scaffolds was extracted with TRIzol reagent and PureLink RNA Mini Kit (Invitrogen), and then reverse-transcribed into cDNA using a High-Capacity cDNA Archive Kit in a Thermal Cycler (GenAmp PCR 2720; Applied Biosystems). TaqMan gene expression assay kits, including two predesigned specific primers and probes, were used to measure the transcript levels of the proposed genes on human ALP (Hs00758162_m1), OC (Hs00609452_g1), Col I (Hs00164004), Runx2 (Hs00231692_m1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Hs99999905). Relative expression for each target gene was evaluated using the $2^{-\Delta\Delta C_t}$ method.⁴³ C_t values of target genes were normalized by the C_t values of the human housekeeping gene GAPDH to obtain the ΔC_t values. The C_t of iPSC-MSCs cultured on CPC in the control media for 1 day served as the calibrator.^{37,44}

ALP activity

The ALP activity was measured using a colorimetric *p*-nitrophenyl phosphate (pNPP) assay (Stanbio)^{3,28} to quantify the osteogenesis of iPSC-MSCs seeded on the CPC scaffolds. Fifty thousand cells were diluted into 2 mL of either control medium or osteogenic medium and then added to each well of a 24-well culture plate containing CPC disks. At 7, 14, and 21 days, cells were lysed in 0.5 mL of buffer (0.2% Triton X-100, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4), and lysates were assayed for ALP activity following the manufacturer's protocol. Normal control serum (Stanbio) with a known concentration of ALP served as a standard. ALP activity ($n=6$) was normalized by DNA content for each sample. DNA was quantified using a Quant-iT PicoGreen Kit (Invitrogen) following standard protocols.⁴⁵

Mineral synthesis by iPSC-MSCs on CPC

Mineral synthesis by iPSC-MSCs was investigated. CPC disks with iPSC-MSCs cultured in control medium or osteogenic medium for 1–21 days were fixed with 10%

formaldehyde and stained with ARS (Millipore). ARS stained calcium-rich deposits by cells into a red color.⁴⁶ An osteogenesis assay (Millipore) was used to extract the stained minerals and measure the ARS concentration, following the manufacturer's instructions. Control scaffolds with the same CPC compositions, but without cells, were measured at the same time periods. The control's ARS concentration was subtracted from that of the corresponding cell-seeded scaffold to yield the net bone mineral concentration synthesized by the cells.⁴⁶

Statistical analyses

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS 16.0). Statistical significance was assessed by using one- or two-way analyses of variance and Tukey's multiple comparison tests. A confidence level of 95% ($p < 0.05$) was considered statistically significant. All data are presented as the mean value $\pm$ standard deviation (SD).

Results

By culturing on MEF feeder in the presence of β -FGF, iPSCs could undergo long-term self-renewal while retaining their pluripotency. Figure 1 shows representative phase-contrast images of iPSC culture. An iPSC colony was shown in (Fig. 1A) cultured on MEF feeder layer. Each iPSC colony showed smooth edges that were distinctive from the feeder cells. When removed from MEF feeder and placed in suspension culture, the dissociated iPSC clumps formed round EBs, with an example in (Fig. 1B). During further culture, cells with a fibroblast-like morphology were observed to migrate out of the EBs (Fig. 1C). The outgrowth of cells sprouting from EBs attached to the culture plate, which were the iPSC-derived MSCs. These iPSC-MSCs became relatively uniform in size and shape after passage 3 and had a similar morphology to fibroblasts and mesenchymal-like cells.

Flow cytometry analysis demonstrated that MSC surface markers were consistently and highly expressed in the iPSC-MSCs (Fig. 2). The MSC surface markers CD29, CD44, CD166, and CD73 were expressed to levels greater than 90% in these iPSC-MSCs. Another MSC surface marker CD105 was expressed to $>75\%$. On the other hand, the expressions of hematopoietic markers, CD31 and CD34, were $<0.6\%$ in the iPSC-MSCs, while the human ESC

(hESC) pluripotency markers, TRA-1-81 and Oct3/4, were less than 0.2%. Further, human leukocyte antigen (HLA) HLA-ABC, present on the surface of all nucleated cells and platelets, were also expressed.

Further research indicated that iPSC-MSCs possessed the potential of differentiating to three different cell lineages: adipocytes (Fig. 3A), chondrocytes (Fig. 3B) and osteoblasts (Fig. 3C). Adipocytic differentiation was efficient, with Oil Red O droplets observed in more than 80% of the iPSC-MSCs (Fig. 3A). Chondrocytic differentiation was highly efficient, with $>90\%$ of cells producing proteoglycans in extracellular matrix as detected by Alcian Blue staining (Fig. 3B). Osteogenic differentiation was also efficient, with over 80% of cells demonstrating positive staining with ARS for the detection of calcium deposition (Fig. 3C).

Representative live/dead staining images of iPSC-MSCs seeded on CPC scaffolds and cultured in control medium or osteogenic medium are shown in Figure 4A–F. Live cells (stained green) attained a normal, healthy polygonal morphology and were numerous on CPC in both media. Dead cells were stained red and were very few. In (Fig. 4G), the percentage of live cells (mean $\pm$ SD; $n = 6$) had no significant difference between control medium or osteogenic medium ($p > 0.1$). In (Fig. 4H), due to cell proliferation, the live cell density increased with time ($p < 0.05$). There was no significant difference between the two types of culture media ($p > 0.1$).

The RT-PCR results for ALP, OC, Col I, and Runx2 gene expressions are plotted in Figure 5 (mean $\pm$ SD; $n = 5$). iPSC-MSCs on CPC scaffolds in osteogenic medium showed osteogenic differentiation. The ALP expression increased with culture time; at each time period, the iPSC-MSCs on CPC in the osteogenic medium expressed significantly higher ALP than that in control medium ($p < 0.05$). The iPSC-MSCs on CPC in the osteogenic medium also expressed significantly higher OC, Col I, and Runx2 levels than that in control medium ($p < 0.05$).

The ALP activity of iPSC-MSCs on CPC scaffolds measured by a colorimetric pNPP assay is plotted in Figure 6 (mean $\pm$ SD; $n = 5$). ALP was normalized by the amount of DNA per sample [(mM pNPP/min)/(μ g DNA)]. There was no significant difference versus time from 7 to 21 days for cells cultured in control medium ($p > 0.1$). iPSC-MSCs on CPC scaffolds in osteogenic medium had higher ALP activity than those in control medium. There was a significant increase in the ALP activity for cells on CPC cultured in the

FIG. 1. Representative phase-contrast photos of human induced pluripotent stem cell (iPSC) culture. (A) Human iPSC colony cultured on mouse embryonic fibroblast (MEF) feeder layer. (B, C) Mesenchymal stem cells (MSCs) migrating out of the embryoid bodies (EBs) were harvested and passaged. Red arrows indicate examples of MSCs that migrated out of the EBs. Color images available online at www.liebertpub.com/tea

FIG. 2. Flow cytometry analysis of surface markers of iPSC-derived mesenchymal stem cells (iPSC-MSCs, passage 4 MSCs). The names of the antigens are listed inside each plot. The histogram with dotted line represents isotype controls, and the histogram with solid line represents the conjugated antibody of each antigen. The number in each plot represents the percentage of positive cells. iPSC-MSCs expressed typical surface antigen profile of MSCs. For example, MSC surface markers CD29, CD44, CD166, and CD73 were expressed to levels greater than 90%, while expressions of hematopoietic markers (CD31 and CD34) were less than 0.6%. Color images available online at www.liebertpub.com/tea

FIG. 3. When cultured in orientation medium, iPSC-MSCs successfully differentiated into (A) adipogenic lineage (Oil Red O staining); (B) chondrogenic lineage (Alcian Blue staining); and (C) osteogenic lineage (Alizarin Red S staining). Color images available online at www.liebertpub.com/tea

FIG. 4. Viability of iPSC-MSCs cultured on calcium phosphate cement (CPC) in control medium and osteogenic medium. (A–F) Representative photos of iPSC-MSCs at 1, 7, 14, and 21 days. The scale bar in (A) is the same in all images. Live cells were stained green and were numerous. Dead cells were stained red and were relatively few. (G) The percentage of live cells, P_{Live} . (H) Live cell density (number of live cells per mm^2), D_{Live} . The cell number increased with time due to proliferation. Each value is mean $\pm$ standard deviation (SD); $n=6$. Color images available online at www.liebertpub.com/tea

osteogenic medium from 7 to 14 days ($p < 0.05$). Therefore, the osteogenic medium significantly increased ALP production of the iPSC-MSCs adherent on CPC scaffolds.

Typical mineral staining photos of the iPSC-MSCs on CPC in control medium and osteogenic medium are shown in Figure 7A–D. ARS-staining yielded a red color for the CPC scaffold matrix because CPC consisted of hydroxyapatite. However, the red staining became much thicker

and denser with numerous granular deposits for iPSC-MSCs on CPC in osteogenic medium when the culture time was increased from 1 to 21 days. There was a layer of new mineral matrix synthesized by the cells covering the CPC disk, and the thick matrix mineralization covered not only the top surface, but also the peripheral areas at the sides of the construct. In contrast, there were little granular deposits for iPSC-MSCs on CPC in control medium,

FIG. 5. Real-time reverse transcription polymerase chain reaction results for osteogenic differentiation of iPSC-MSCs on CPC in control medium and osteogenic medium, with osteogenic gene expression of: (A) alkaline phosphatase (ALP), (B) osteocalcin (OC), (C) collagen type I (Col I), and (D) Runt-related transcription factor 2 (Runx2). Each value is mean $\pm$ SD; $n=5$. The osteogenic medium induced significant increases in the osteogenic expressions than those in control medium ($p < 0.05$). Color images available online at www.liebertpub.com/tea

FIG. 6. Colorimetric *p*-nitrophenyl phosphate (pNPP) assay of ALP protein synthesis by iPSC-MSCs on CPC in control medium and osteogenic medium (mean $\pm$ SD; $n=5$). Values with dissimilar letters are significantly different from each other ($p < 0.05$). The osteogenic medium increased the ALP production of iPSC-MSCs adherent on CPC by 10-fold than that in control medium. Color images available online at www.liebertpub.com/tea

and there was no noticeable difference in staining images when the culture time was prolonged from 1 to 21 days. Data from the osteogenesis assay are plotted in (Fig. 7E). At 14 or 21 days, the iPSC-MSCs on CPC in osteogenic medium synthesized three to fourfold more bone mineral than the counterparts in control medium (mean $\pm$ SD; $n=5$). These results demonstrate that the iPSC-MSCs seeded on CPC scaffolds were successfully differentiated into the osteogenic lineage when cultured in the osteogenic medium.

Discussion

ESCs and adult stem cells are two main types of stem cells for cell therapy in regenerative medicine. The pioneering study by Takahashi and Yamanaka has shown an alternative approach to obtaining pluripotent stem cells by reprogramming somatic cells, but without the use of embryonic materials.¹⁶ The iPSC technology enabled the generation of patient-specific or disease-specific cells of any lineage for therapeutic purposes. Although the method of reprogramming is still inefficient and many fundamental questions remain unanswered, recent developments have shown that iPSCs can be generated without using viral vectors, which further brings iPSCs a step closer to clinical application.^{47,48} iPSCs are highly promising for tissue engineering due to their ability to provide unlimited stem cells with easier cell resources, and their lack of immunologic rejection and ethical controversy. Recently, iPSCs have been shown to have the potential to differentiate into osteogenic lineage.^{49,50} However, only a few studies have

FIG. 7. Bone mineral synthesis by iPSC-MSCs on CPC in control medium or osteogenic medium: (A, B) 1 day, (C, D) 21 days. (E) Results from the osteogenesis assay (mean $\pm$ SD; $n=5$). The mineral synthesis by iPSC-MSCs adherent on CPC in osteogenic medium was three to fourfold than that in control medium at 14 and 21 days. Color images available online at www.liebertpub.com/tea

investigated iPSCs for bone tissue engineering,^{24,25} and to date there has been no report of iPSC seeding on CPC scaffolds. In the present study, iPSC-MSCs were seeded on CPC scaffolds for osteogenic differentiation and bone mineral synthesis for the first time.

The self-renewal ability of iPSCs and their capability to generate three germ layers may make them tumorigenic, and thus may hinder their clinical applications.⁵¹ The derivation of MSCs from iPSCs, referred to as iPSC-MSCs, may be a useful strategy to reduce or even eliminate the tumorigenicity of iPSCs prior to transplantation. Indeed, the biosafety of MSCs derived from iPSCs was improved when compared with the direct use of iPSCs.⁵² In the present study, iPSCs

were first induced into EBs and MSCs. Deriving MSCs from iPSCs before specific differentiation has the advantage of producing a source of multipotent progenitor cells, which then can be expanded and differentiated into specific lineages such as bone, cartilage, or fat. This strategy can potentially yield a great amount of progenitor cells to regenerate skeletal defects. After an expansion period of four passages, iPSC-MSCs exhibited a uniform fibroblast-like morphology and expressed high levels of hMSC surface markers, consistent with surface markers in previous studies on MSCs.^{53,54} The iPSC-MSCs lacked the expression of hematopoietic lineage markers,⁵⁴ hESCs pluripotency markers TRA-1-81 and Oct3/4,^{55,56} and the marker of professional antigen-presenting cells, HLA-DR.⁵⁷ In addition, the adipocytic, chondrocytic, and osteogenic differentiation of iPSC-MSCs was induced under conditions previously described for primary adult MSCs,³⁹ which are functionally characterized by their ability to differentiate into mesenchymal tissues, such as fat, cartilage, and bone. These findings confirmed that the iPSC-MSCs generated in the present study are highly comparable and similar to hMSCs.

Recent studies have shown that iPSCs have the potential to differentiate down the osteogenic lineage with a high potential for bone regeneration.²⁵ Osteogenic differentiation of iPSCs was achieved by introducing iPSC-MSCs in osteogenic media.^{24,25} Silk scaffolds were used to seed iPSC-derived cells.^{24,25} However, to date there has been no report on iPSC seeding on biomaterials with mechanical strength for moderate load-bearing bone tissue engineering applications. In the present study, iPSCs were differentiated into hMSCs and seeded on a mechanically-strong CPC. The strength and elastic modulus of CPC scaffolds were reported in previous studies to be similar to those of natural cancellous bone.^{32,58} CPC can be injected or molded to the desired shapes to achieve esthetics for dental, craniofacial, and orthopedic repairs, then set to form a scaffold that can be gradually resorbed and replaced by new bone.⁵⁹ The present study showed that CPC is biocompatible with iPSC-MSCs, and the iPSC-MSC-CPC construct is promising to cell function and enhance bone regeneration.

The iPSC-MSCs on CPC scaffold showed successful osteogenic differentiation when cultured in osteogenic medium. Compared with the group using control medium without osteogenic supplements, iPSC-MSCs on CPC in osteogenic medium showed higher expressions of osteogenic genes ALP, OC, Col I, and Runx2, which play key roles in the osteogenic differentiation of MSCs.⁴⁴ In addition, iPSC-MSCs on CPC in osteogenic medium synthesized more ALP proteins. ALP is an enzyme expressed by cells during osteogenesis and is a well-defined marker for their differentiation.⁶⁰ Further evidence of osteogenic differentiation is presented in mineral synthesis by the cells. There were three to fourfold of more mineral formation by iPSC-MSCs on CPC in osteogenic medium than control medium. A previous study examined the substance synthesized by the cells via x-ray diffraction.⁵⁸ The x-ray pattern was similar to that of a standard hydroxyapatite, indicating that the cell-synthesized mineral was a low-crystalline apatite, similar to those in bone.⁵⁸ A chemical analysis of the cell-synthesized substance yielded a Ca/P molar ratio of 1.35,⁵⁸ which was consistent with previously-reported Ca/P ratios of 1.39–1.41 for mineralization by cells.⁶¹ Therefore, the present study

demonstrated for the first time that (1) MSCs could be successfully derived from iPSCs, which were compatible with CPC scaffolds, yielding excellent cell proliferation and differentiation; (2) iPSC-MSC-CPC construct could support bone mineral synthesis and is promising for bone tissue engineering. Further study is needed to evaluate iPSC-MSCs delivered via CPC scaffolds for bone regeneration in an animal model.

Conclusions

This study generated MSCs from human iPSCs, and investigated iPSC-MSC proliferation and osteogenic differentiation seeded on CPC scaffolds for the first time. iPSCs were derived from adult marrow CD34+ cells that were reprogrammed by a single episomal vector pEB-C5. MSCs were generated by culturing iPSC colonies and EBs. CPC scaffolds supported the iPSC-MSC attachment, yielding good cell viability, proliferation, highly elevated osteogenic marker expressions, and bone mineral synthesis. These results demonstrate the promise of iPSC for bone tissue engineering, and the potential of iPSC-MSC-CPC construct to enhance bone regeneration. The mechanically strong CPC scaffold seeded with iPSC-MSCs with excellent proliferation and differentiation may be useful for a wide range of dental, craniofacial, and orthopedic applications.

Acknowledgments

We are indebted to Bin-Kuan Chou for experimental help with iPSCs, and Dr. Ferenc Livak for help with flow cytometry, which were performed at the University of Maryland Greenbaum Cancer Center Shared Flow Cytometry Facility. We also thank Drs. Michael D. Weir and Ping Wang of the University of Maryland School of Dentistry for helpful discussions. This study was supported by NIH R01 DE14190 (H.H.K.X.), R21 DE22625 (H.H.K.X.), and R01 HL-073781 (L.C.), and the University of Maryland School of Dentistry startup fund (H.H.K.X.).

Disclosure Statement

No competing financial interests exist.

References

- Cutter, C.S., and Mehrara, B.J. Bone grafts and substitutes. *J Long Term Eff Med Implants* **16**, 249, 2006.
- United States Bone and Joint Decade. The Burden of Musculoskeletal Diseases in the United States. Rosemont, IL: American Academy of Orthopaedic Surgeons, 2008.
- Mao, J.J., Vunjak-Novakovic, G., Mikos, A.G., and Atala, A. *Translational Approaches in Tissue Engineering and Regenerative Medicine*, Chapters 1–3. Boston, MA: Artech House, 2007.
- Mikos, A.G., Herring, S.W., Ochareon, P., Elisseeff, J., Lu, H.H., Kandel, R., Schoen, F.J., Toner, M., Mooney, D., Atala, A., Van Dyke, M.E., Kaplan, D., and Vunjak-Novakovic, G. Engineering complex tissues. *Tissue Eng* **12**, 3307, 2006.
- Benoit, D.S., Nuttelman, C.R., Collins, S.D., and Anseth, K.S. Synthesis and characterization of a fluvastatin-releasing hydrogel delivery system to modulate hMSC differentiation and function for bone regeneration. *Biomaterials* **27**, 6102, 2006.
- Mao, J.J., Giannobile, W.V., Helms, J.A., Hollister, S.J., Krebsbach, P.H., Longaker, M.T., and Shi, S. Craniofacial tissue engineering by stem cells. *J Dent Res* **85**, 966, 2006.
- Reilly, G.C., Radin, S., Chen, A.T., and Ducheyne, P. Differential alkaline phosphatase responses of rat and human bone marrow derived mesenchymal stem cells to 45S5 bioactive glass. *Biomaterials* **28**, 4091, 2007.
- Johnson, P.C., Mikos, A.G., Fisher, J.P., and Jansen, J.A. Strategic directions in tissue engineering. *Tissue Eng* **13**, 2827, 2007.
- Chatterjee, K., Lin-Gibson, S., Wallace, W.E., Parekh, S.H., Lee, Y.J., Cicerone, M.T., Young, M.F., and Simon, C.G., Jr. The effect of 3D hydrogel scaffold modulus on osteoblast differentiation and mineralization revealed by combinatorial screening. *Biomaterials* **31**, 5051, 2010.
- Huebsch, N., Arany, P.R., Mao, A.S., Shvartsman, D., Ali, O.A., Bencherif, S.A., Rivera-Feliciano, J., and Mooney, D.J. Harnessing traction-mediated manipulation of the cell/matrix interface to control stem-cell fate. *Nat Mater* **9**, 518, 2010.
- Mueller, S.M., and Glowacki, J. Age-related decline in the osteogenic potential of human bone marrow cells cultured in three-dimensional collagen sponges. *J Cell Biochem* **82**, 583, 2001.
- Mendes, S.C., Tibbe, J.M., Veenhof, M., Bakker, K., Both, S., Platenburg, P.P., Oner, F.C., de Bruijn, J.D., and van Blitterswijk, C.A. Bone tissue-engineered implants using human bone marrow stromal cells: effect of culture conditions and donor age. *Tissue Eng* **8**, 911, 2002.
- Stenderup, K., Justesen, J., Clausen, C., and Kassem, M. Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. *Bone* **33**, 919, 2003.
- Rodriguez, J.P., Montecinos, L., Rios, S., Reyes, P., and Martinez, J. Mesenchymal stem cells from osteoporotic patients produce a type I collagen-deficient extracellular matrix favoring adipogenic differentiation. *J Cell Biochem* **79**, 557, 2000.
- Suzuki, Y., Kim, K.J., Kotake, S., and Itoh, T. Stromal cell activity in bone marrow from the tibia and iliac crest of patients with rheumatoid arthritis. *J Bone Miner Metab* **19**, 56, 2001.
- Takahashi, K., and Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663, 2006.
- Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R., Slukvin, I.I., and Thomson, J.A. Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**, 1917, 2007.
- Nakagawa, M., Koyanagi, M., Tanabe, K., Takahashi, K., Ichisaka, T., Aoi, T., Okita, K., Mochizuki, Y., Takizawa, N., and Yamanaka, S. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat Biotechnol* **26**, 101, 2008.
- Amabile, G., and Meissner, A. Induced pluripotent stem cells: current progress and potential for regenerative medicine. *Trends Mol Med* **15**, 59, 2009.
- Mauritz, C., Schwanke, K., Reppel, M., Neef, S., Katsirni-taki, K., Maier, L.S., Nguemo, F., Menke, S., Hausteiner, M., Hescheler, J., Hasenfuss, G., and Martin, U. Generation of

- functional murine cardiac myocytes from induced pluripotent stem cells. *Circulation* **118**, 507, 2008.
21. Morizane, R., Monkawa, T., and Itoh, H. Differentiation of murine embryonic stem and induced pluripotent stem cells to renal lineage *in vitro*. *Biochem Biophys Res Commun* **390**, 1334, 2009.
 22. Zhang, D., Jiang, W., Liu, M., Sui, X., Yin, X., Chen, S., Shi, Y., and Deng, H. Highly efficient differentiation of human ES cells and iPS cells into mature pancreatic insulin-producing cells. *Cell Res* **19**, 429, 2009.
 23. Dimos, J.T., Rodolfa, K.T., Niakan, K.K., Weisenthal, L.M., Mitsumoto, H., Chung, W., Croft, G.F., Saphier, G., Leibel, R., Goland, R., Wichterle, H., Henderson, C.E., and Eggan, K. Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. *Science* **321**, 1218, 2008.
 24. Duan, X., Tu, Q., Zhang, J., Ye, J., Sommer, C., Mostoslavsky, G., Kaplan, D., Yang, P., and Chen, J. Application of induced pluripotent stem (iPS) cells in periodontal tissue regeneration. *J Cell Physiol* **226**, 150, 2011.
 25. Ye, J.H., Xu, Y.J., Gao, J., Yan, S.G., Zhao, J., Tu, Q., Zhang, J., Duan, X.J., Sommer, C.A., Mostoslavsky, G., Kaplan, D.L., Wu, Y.N., Zhang, C.P., Wang, L., and Chen, J. Critical-size calvarial bone defects healing in a mouse model with silk scaffolds and SATB2-modified iPSCs. *Biomaterials* **32**, 5065, 2011.
 26. Ducheyne, P., and Qiu, Q. Bioactive ceramics: the effect of surface reactivity on bone formation and bone cell function. *Biomaterials* **20**, 2287, 1999.
 27. Deville, S., Saiz, E., Nalla, R.K., and Tomsia, A.P. Freezing as a path to build complex composites. *Science* **311**, 515, 2006.
 28. Leach, J.K., Kaigler, D., Wang, Z., Krebsbach, P.H., and Mooney, D.J. Coating of VEGF-releasing scaffolds with bioactive glass for angiogenesis and bone regeneration. *Biomaterials* **27**, 3249, 2006.
 29. Laurencin, C.T., Ambrosio, A.M., Borden, M.D., and Cooper, J.A., Jr. Tissue engineering: orthopedic applications. *Annu Rev Biomed Eng* **1**, 19, 1999.
 30. Barralet, J.E., Gaunt, T., Wright, A.J., Gibson, I.R., and Knowles, J.C. Effect of porosity reduction by compaction on compressive strength and microstructure of calcium phosphate cement. *J Biomed Mater Res* **63**, 1, 2002.
 31. Ginebra, M.P., Driessens, F.C., and Planell, J.A. Effect of the particle size on the micro and nanostructural features of a calcium phosphate cement: a kinetic analysis. *Biomaterials* **25**, 3453, 2004.
 32. Bohner, M. Design of ceramic-based cements and putties for bone graft substitution. *Eur Cell Mater* **20**, 1, 2010.
 33. Brown, W.E., and Chow, L.C. A new calcium phosphate water setting cement. In: Brown, P.W., ed. *Cements Research Progress*. Westerville, OH: American Ceramic Society, 1986, pp. 352.
 34. Friedman, C.D., Costantino, P.D., Takagi, S., and Chow, L.C. Bone source hydroxyapatite cement: a novel biomaterial for craniofacial skeletal tissue engineering and reconstruction. *J Biomed Mater Res* **43**, 428, 1998.
 35. Chou, B.K., Mali, P., Huang, X., Ye, Z., Dowe, S.N., Resar, L.M., Zou, C., Zhang, Y.A., Tong, J., and Cheng, L. Efficient human iPS cell derivation by a non-integrating plasmid from blood cells with unique epigenetic and gene expression signatures. *Cell Res* **21**, 518, 2011.
 36. Cheng, L., Hansen, N.F., Zhao, L., Du, Y., Zou, C., Donovan, F.X., Chou, B.K., Zhou, G., Li, S., Dowe, S.N., Ye, Z., Chandrasekharappa, S.C., Yang, H., Mullikin, J.C., and Liu, P.P. Low incidence of DNA sequence variation in human induced pluripotent stem cells generated by non-integrating plasmid expression. *Cell Stem Cell* **10**, 337, 2012.
 37. Tang, M.H., Chen, W.C., Weir, M.D., Thein-Han, W., and Xu, H.K.K. Human embryonic stem cell encapsulation in alginate microbeads in macroporous calcium phosphate cement for bone tissue engineering. *Acta Biomater* **8**, 3436, 2012.
 38. Barberi, T., Willis, L.M., Socci, N.D., and Studer, L. Derivation of multipotent mesenchymal precursors from human embryonic stem cells. *PLoS Med* **2**, e161, 2005.
 39. Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143, 1999.
 40. Zhao, L., Burguera, E.F., Xu, H.K.K., Amin, N., Ryou, H., and Arola, D.D. Fatigue and human umbilical cord stem cell seeding characteristics of calcium phosphate-chitosan-biodegradable fiber scaffolds. *Biomaterials* **31**, 840, 2010.
 41. Hwang, N.S., Varghese, S., Lee, H.J., Zhang, Z., Ye, Z., Bae, J., Cheng, L., and Elisseeff, J. *In vivo* commitment and functional tissue regeneration using human embryonic stem cell-derived mesenchymal cells. *Proc Natl Acad Sci U S A* **105**, 20641, 2008.
 42. Weir, M.D., and Xu, H.K.K. Culture human mesenchymal stem cells with calcium phosphate cement scaffolds for bone repair. *J Biomed Mater Res B Appl Biomater* **93**, 93, 2010.
 43. Livak, K.J., and Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25**, 402, 2001.
 44. Kim, K., Dean, D., Mikos, A.G., and Fisher, J.P. Effect of initial cell seeding density on early osteogenic signal expression of rat bone marrow stromal cells cultured on cross-linked poly(propylene fumarate) disks. *Biomacromolecules* **10**, 1810, 2009.
 45. Zhao, L., Weir, M.D., and Xu, H.K.K. An injectable calcium phosphate-alginate hydrogel-umbilical cord mesenchymal stem cell paste for bone tissue engineering. *Biomaterials* **31**, 6502, 2010.
 46. Thein-Han, W., and Xu, H.K.K. Collagen-calcium phosphate cement scaffolds seeded with umbilical cord stem cells for bone tissue engineering. *Tissue Eng Part A* **17**, 2943, 2011.
 47. Kaji, K., Norrby, K., Paca, A., Mileikovsky, M., Mohseni, P., and Woltjen, K. Virus-free induction of pluripotency and subsequent excision of reprogramming factors. *Nature* **458**, 771, 2009.
 48. Zhou, H., Wu, S., Joo, J.Y., Zhu, S., Han, D.W., Lin, T., Trauger, S., Bien, G., Yao, S., Zhu, Y., Siuzdak, G., Scholer, H.R., Duan, L., and Ding, S. Generation of induced pluripotent stem cells using recombinant proteins. *Cell Stem Cell* **4**, 381, 2009.
 49. Tashiro, K., Inamura, M., Kawabata, K., Sakurai, F., Yamanishi, K., Hayakawa, T., and Mizuguchi, H. Efficient adipocyte and osteoblast differentiation from mouse induced pluripotent stem cells by adenoviral transduction. *Stem Cells* **27**, 1802, 2009.
 50. Kao, C.L., Tai, L.K., Chiou, S.H., Chen, Y.J., Lee, K.H., Chou, S.J., Chang, Y.L., Chang, C.M., Chen, S.J., Ku, H.H., and Li, H.Y. Resveratrol promotes osteogenic dif-

- ferentiation and protects against dexamethasone damage in murine induced pluripotent stem cells. *Stem Cells Dev* **19**, 247, 2010.
51. Ben-David, U., and Benvenisty, N. The tumorigenicity of human embryonic and induced pluripotent stem cells. *Nat Rev Cancer* **11**, 268, 2011.
52. Jung, Y., Bauer, G., and Nolte, J.A. Concise review: induced pluripotent stem cell-derived mesenchymal stem cells: progress toward safe clinical products. *Stem Cells* **30**, 42, 2012.
53. Barry, F.P., and Murphy, J.M. Mesenchymal stem cells: clinical applications and biological characterization. *Int J Biochem Cell Biol* **36**, 568, 2004.
54. Tocci, A., and Forte, L. Mesenchymal stem cell: use and perspectives. *Hematol J* **4**, 92, 2003.
55. Gruenloh, W., Kambal, A., Sondergaard, C., McGee, J., Nacey, C., Kalomoiris, S., Pepper, K., Olson, S., Fierro, F., and Nolte, J.A. Characterization and *in vivo* testing of mesenchymal stem cells derived from human embryonic stem cells. *Tissue Eng Part A* **17**, 1517, 2011.
56. Niwa, H., Miyazaki, J., and Smith, A.G. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet* **24**, 372, 2000.
57. Le, B.K., Tammik, C., Rosendahl, K., Zetterberg, E., and Ringden, O. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. *Exp Hematol* **31**, 890, 2003.
58. Xu, H.K.K., Zhao, L., and Weir, M.D. Stem cell-calcium phosphate constructs for bone engineering. *J Dent Res* **89**, 1482, 2010.
59. Bohner, M., Gbureck, U., and Barralet, J.E. Technological issues for the development of more efficient calcium phosphate bone cements: a critical assessment. *Biomaterials* **26**, 6423, 2005.
60. Wang, J., de Boer, J., and de Groot, K. Proliferation and differentiation of MC3T3-E1 cells on calcium phosphate/chitosan coatings. *J Dent Res* **87**, 650, 2008.
61. Nakamura, H., Saruwatari, L., Aita, H., Takeuchi, K., and Ogawa, T. Molecular and biomechanical characterization of mineralized tissue by dental pulp cells on titanium. *J Dent Res* **84**, 515, 2005.

Address correspondence to:

Hockin H.K. Xu, PhD

Biomaterials and Tissue Engineering Division

Department of Endodontics, Prosthodontics

and Operative Dentistry

University of Maryland Dental School

Baltimore, MD 21201

E-mail: hxu@umaryland.edu

Received: April 2, 2013

Accepted: November 12, 2013

Online Publication Date: January 7, 2014